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Early experiences affect the vulnerability to adolescent-onset depression and determine antidepressant efficacy

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AIM OF THE STUDY

The general goal of the thesis work is to unravel the potential biological factors underlying vulnerability to depression in adolescence, paying attention to sex differences, and then to identify improved therapeutic strategies based on a drug by environment interaction. To achieve this goal, I have performed three preclinical studies.

Study 1. We investigated the neurobiological bases of depression-like behavior in adolescence. Since the immune system and neural plasticity have been recently implicated in depression, we assessed their contribution to the onset of the psychopathology in the two sexes. To this aim, we isolated mice during adolescence and phenotyped their behavioral responses and inflammatory and plasticity markers.

Study 2. We explored how to improve selective serotonin reuptake inhibitor (SSRI) treatment efficacy for adolescent-onset depression, reportedly associated with treatment resistance. We exploited a *drug x environment* interaction strategy as an increasing number of studies indicate that SSRIs do not univocally lead to recovery but amplify the influence of the environment on mood. Our hypothesis was that (1) for an effective recovery, a favorable environment is key since it regulates behavior, improving the depression-like phenotype, (2) fluoxetine treatment, by enhancing plasticity, amplifies the beneficial effects of the favorable environment, and (3) the effects of fluoxetine are environment-dependent. To test a such hypothesis, we treated depressed-like mice with the SSRI fluoxetine in either a standard or an enriched environment and measured behavioral and hormonal endpoints reportedly affected in depression.

Study 3. Since the quality of the living environment does not depend only on its objective features, but also on the subjective experience of it, we investigated whether the latter is key in determining SSRI treatment outcome. To explore such a hypothesis, we generated two groups of mice showing distinct experiences of the environment, according to their response to social and emotional stimuli, and assessed whether fluoxetine outcome differs according to their subjective experiences.

GENERAL ABSTRACT

Adolescence is a critical developmental phase during which adverse experiences, such as a lack of appropriate social interactions, increase the vulnerability to major depression and raise the risk of antidepressant treatment-resistance in adulthood. In addition, adolescent adverse experiences have been shown to have a gender-dependent effect, with girls being more susceptible to mental illness than boys. However, the biological processes underlying both the onset of psychopathology, including the sex-dependent vulnerability, and the lack of response to antidepressant treatments have not been identified yet. To investigate such biological processes, we have performed three independent studies.

Study 1. We aimed at identifying the neural bases of adolescent-onset depression and sex-dependent vulnerability. Special attention was paid to neural plasticity and immune function that recently have been critically involved in this psychopathology. To this aim, we exposed C57BL/6 male and female mice either to standard laboratory housing (i.e., controls) or to social isolation during adolescence and phenotyped their behavioral and hormonal responses. Our results show that isolation leads to depressive- and anxiety-like phenotype and reduced neural plasticity and inflammatory markers in both sexes, though affecting different behavioral domains: females showed more marked impairments in the emotional domain, while males displayed alterations in the cognitive domain and stress response. These findings suggest that social experiences in adolescence represent a key factor in the neurodevelopment of both sexes and that the sex-differences in vulnerability to depression could be, at least in part, ascribed to biological factors and not to cultural and social influences.

The aim of **Study 2** was to explore the role of the interplay between the environment and SSRIs in the recovery from adolescent-onset depression-like phenotype. According to recent findings, we hypothesized that a supportive environment is critical for recovery and antidepressant treatment can further enhance such beneficial action. To test this hypothesis, C57BL/6 male mice have

been exposed to social isolation from weaning to adulthood to induce a depressive-like phenotype. We then compared the efficacy of the exposure to an enriched environment, the administration of selective serotonin reuptake inhibitor (SSRI) fluoxetine, and their combination to treat depression-like adult mice. The control group consisted of mice not exposed to social isolation during adolescence and socially housed. We assessed the effects of the different treatment strategies on the cognitive and emotional domains and stress hormone levels. Our results showed that environmental enrichment alone or in combination with fluoxetine, but not fluoxetine alone, counterbalanced the detrimental effects of isolation on the depression-like phenotype. Our findings outline the relevance of environmental interventions alone or in combination with SSRI to treat adolescence-onset depression and confirm that SSRIs do not univocally lead to recovery but amplify the influence of the environment on the emotional response.

With **Study 3**, we explored the influence of the subjective experience of the environment as a determinant of SSRI treatment outcome. Since the quality of the living environment determines SSRI outcome and depends not only on its objective features but also on the subjective experience of it, we hypothesized that the latter plays a key role in determining antidepressant efficacy. To test this hypothesis, we generated two experimental groups of CD-1 male mice that reportedly show different subjective views of the environment, as they differently reacted at emotional and social levels to the same environmental stimuli. These distinct socioemotional profiles were generated by rearing mice either in standard laboratory conditions (SN) or in a communal nest (CN). Twenty-one days of fluoxetine produced different effects in the two groups, increasing offensive and anxious responses in SN while producing opposite effects in CN mice. BDNF regulation was modified accordingly. These results indicate that the subjective experience of the environment determines fluoxetine outcome. In a translational perspective, our findings point out the need to consider not only the objective quality but also the subjective experience of the patient's living environment to develop effective personalized therapeutic approaches in psychiatry.

GENERAL INTRODUCTION

1. Adolescent-onset depression

Adolescence is a critical phase of development

Adolescence is the transitory stage between childhood and adulthood (from 10 up to 24 years) and represents a critical phase of individuals' physical and psychosocial development (Christie and Viner, 2005). During this period, adolescents experience not only biological and physical changes (Sawyer et al., 2018), but they go through relevant modifications in their social environment, such as the transition from primary to secondary school associated with an increase in the number of peers, and in the time spent with them (Andrews et al., 2021; Blakemore, 2019). These modifications in their psychosocial environment are accompanied by the structural and functional maturation of brain regions involved in social and emotional regulation that continues till adulthood (Andrews et al., 2021). It is increasingly evident that this important transition phase is associated with vulnerability to psychopathology. Youths and young adults are highly susceptible to major depression episodes, consistent with recent literature reporting that rates of depression increased by 52 percent between 2005 and 2017 among adolescents and 63 percent between 2009 and 2017 in young adults (Twenge et al., 2019; Fig.1).

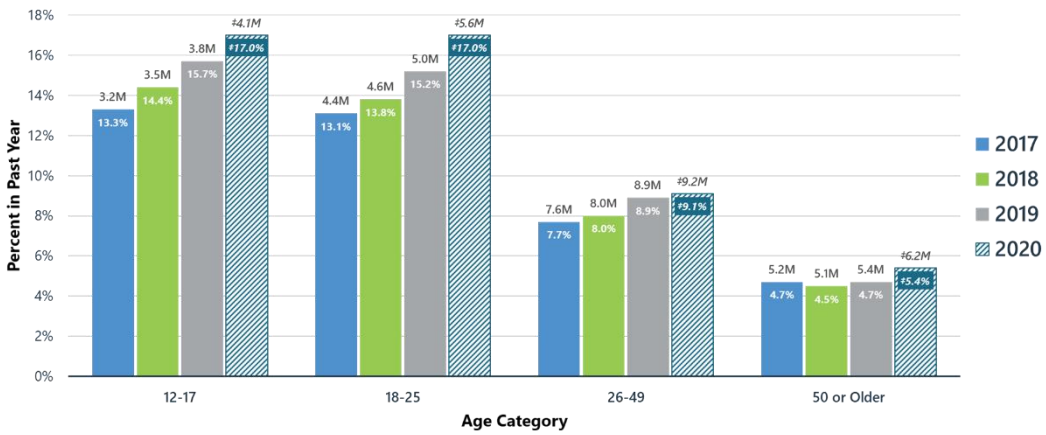

Figure 1. Major depressive episodes in the past year among people aged 12+. Youths and young adults reports rising and quick rates of major depressive episodes. Results from the National Survey on Drug Use and Health 2020 (NSDUH;SAMSHA, 2020).

Clinical features

Depression often has an onset in adolescence (Timbremont et al., 2004) and represents a leading public health problem in this age phase with a prevalence of 3-5% in mid to late adolescence (Byrne et al., 2015). Symptoms and criteria for diagnosing this psychopathology are reported in the Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-5), published by the American Psychiatric Association, and by the World Health Organization (WHO). The DSM-5 criteria for the major depressive disorder include a 2-week period where at least five out of nine symptoms are present most of the day, almost every day. These symptoms can be divided into the following three groups:

- Core symptoms, such as depressed mood, loss of interest or pleasure, irritability
- Cognitive symptoms, such as excessive guilt, loss of concentration, and suicidal thoughts
- Vegetative symptoms, such as a change in appetite and body weight, insomnia or hypersomnia, psychomotor agitation or retardation, loss of energy

Vegetative symptoms appear more common in depressed adolescents than in depressed adults (Rice et al., 2019), with concentration problems, feelings of worthlessness/guilt, and sleep disturbance associated with mild depression, while psychomotor agitation/retardation, weight, and appetite change, and suicidal ideation or attempts associated with severe depression (Cole et al., 2011). In addition, adolescent depression is characterized by changes in several social behaviors and features such as loss of interest in peer play or school activities, school refusal, frequent absences, and disengagement from peer play (Bhatia and Bhatia, 2007), showing that the socio-emotional development is key during this period. Adolescents with mental health conditions are particularly vulnerable to social exclusion, discrimination, educational difficulties, risk-taking behaviors, physical ill-health, and human rights violations (WHO, 2021). Indeed, depression in adolescence represents the major risk factor for somatic disease, premature death, and suicide among teenagers (Byrne et al., 2015; Leone et al., 2021). About 90% of adolescents who commit suicide have been diagnosed with

a psychiatric disorder. The single most common diagnosis is major depression, which presents in 35% of suicidal adolescents (Wilkinson et al., 2011; Fig.2).

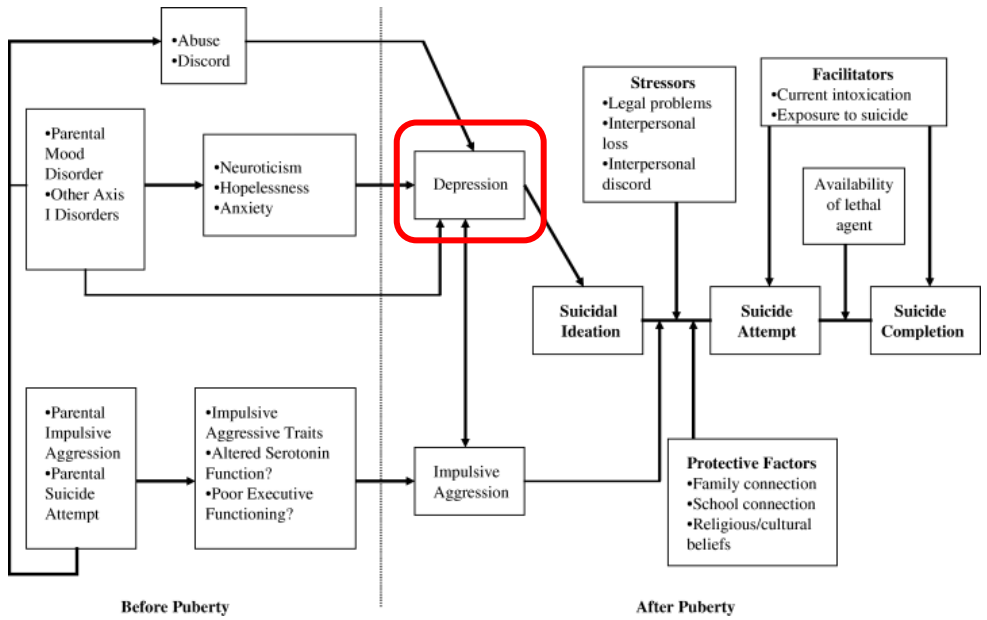

Figure 2. Developmental-transactional model of youth suicidal behavior. This model suggests that suicidal behavior may arise through an interaction of two sets of vulnerabilities – major psychiatric disorder, most commonly mood disorder, and a tendency to impulsive aggression, which in turn may have neurobiological correlates such as impaired executive functioning and altered serotonin metabolism in the ventral prefrontal cortex (modified by Bridge et al., 2006).

Sex-related difference

Females are approximately twice as likely as males to be depressed, a phenomenon that emerges during puberty and remains stable in adulthood (Salk et al., 2017; Fig.3). In particular, depressed females are more likely to display higher and more severe symptoms compared to males (Marcus et al., 2005). Sex also influences comorbidities in individuals with a primary diagnosis of depression: females are more likely than males to develop anxiety (Schuch et al., 2014), while males show higher comorbidity with substance use disorders (Marcus et al., 2005; Schuch et al., 2014). Several studies show that male and female subjects have a similar risk for depression during childhood, but female subjects begin to have a higher risk when they reach their early teens (Angold and Costello, 2000; Cohen et al., 1993; Hankin et al., 1998; Thapar et al., 2012).

Sex hormones may play a key role in this sex-related difference, given their increased influences on brain and behavior development during puberty (Naninck et al., 2011). However, Breslau and collaborators suggest that the sex difference in depression originates during childhood and grows in magnitude during adolescence (Breslau et al., 2017). Therefore, further studies to validate this hypothesis are still warranted.

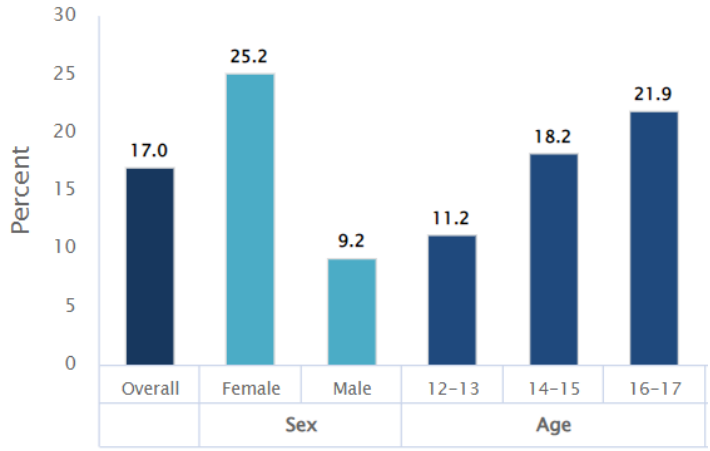

Figure 3. The prevalence of major depressive episodes among United States (U.S.) adolescents in 2020. An estimated 4.1 million adolescents aged 12 to 17 in the United States had at least one major depressive episode. This number represented 17.0% of the U.S. population aged 12 to 17. The prevalence of major depressive episodes was higher among adolescent females (25.2%) compared to males (9.2%, modified by SAMSHA, 2020).

Brain-immune crosstalk in the onset of adolescent depression

The relative contribution of biological and cultural factors to adolescent-onset depression and to the sex-dependent vulnerability to depression is not yet clear (Conley and Rudolph, 2009). An increasing number of preclinical and clinical findings implicate the brain-immune crosstalk as a key determinant in the onset and treatment of psychiatric disorders – from depression to schizophrenia (Branchi, 2011; Branchi et al., 2021; Bullmore, 2018; Milaneschi et al., 2020; Pariante, 2016; Thibaut, 2018; Viglione et al., 2019). In particular, the expression of inflammatory cytokines in the brain has been reported to be involved in the pathogenesis of and susceptibility to depression, indicating adolescence as a

unique stage of immune development (Granata et al., 2022; Mariani et al., 2021). The link between disturbances in the immuno-inflammatory system and the etiology and pathophysiology of several psychiatric disorders has been specifically explored, making the field of *immunopsychiatry* rise (Dantzer et al., 2008; Leboyer et al., 2016a; Leboyer et al., 2016b; Pariante, 2017). Some authors have hypothesized that an increase in inflammatory markers occurs before the onset of depressive symptoms, suggesting that immune system activation precedes psychopathology (Gimeno et al., 2009; Liu et al., 2019; Pasco et al., 2010). Others proposed an opposite temporal relationship, suggesting that psychiatric disorders cause an impairment in immunocompetence, leading to an increased vulnerability to infectious diseases (Copeland et al., 2012; Dantzer, 2012) or reported a lack of correlation (Copeland et al., 2012; Dantzer, 2012; Levine et al., 1999). In addition, only 30% of depressed patients show high inflammatory levels (Raison et al., 2006) and, in turn, depression does not always follow an immune activation (Raison and Miller, 2011). Since stress increases both immune response and the risk of psychiatric disorders, exposure to stressful conditions, including childhood adversity, has been hypothesized to represent the common factor triggering the increase in both inflammatory markers and the likelihood of depression (Benros et al., 2013; Garcia-Bueno et al., 2008; Muller et al., 2019). Adverse childhood events occurring in late childhood and adolescence, but not earlier in life, were recently reported to be associated with persistent immune abnormalities (Iob et al., 2022), suggesting immune signaling may be particularly influential during the adolescent period and that it may play a crucial role in the onset of psychopathologies.

Adolescent depression and the risk of relapse in adulthood

Adolescent depression increases the risk for subsequent depression and anxiety disorders in adulthood (Hill et al., 2014; Johnson et al., 2018; Fig.4). Several clinical studies have shown that depressed adolescents are more likely than their non-depressed peers to subsequently have depression, as well as non-mood disorders (Fergusson and Woodward, 2002; Harrington et al., 1990; Lewinsohn et al., 1999; Rao et al., 1995). The persistence of adolescent

depressive symptoms is predictive of subsequent mental health. A forty-year follow-up of the 1946 British birth cohort showed that adolescents with internalizing disorders at both ages 13 and 15 were more likely than healthy adolescents to have adult mental disorders and to be treated for mental disorders (Colman et al., 2007). In addition, a long duration of depression in adolescence, a period of rapid mental development and acquisition of knowledge and social skills, might be particularly devastating to future mental health. Thus, a better comprehension of the social and biological processes underlying the onset of psychopathology represents an urgent need.

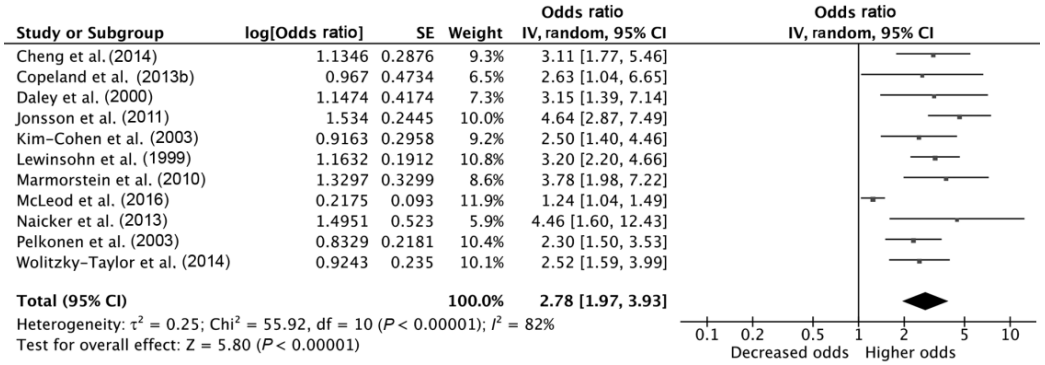

Figure 4. Meta-analysis of the association between adolescent depression and adult depression. The pooled estimate from 11 articles showed that adolescent depression increased the odds for adult depression by a factor of 2.78 suggesting a positive association between adolescent depression and adult psychopathology. CI, confidence interval; df, degrees of freedom; IV, independent variable (Johnson et al., 2018).

2. The role of early experiences and adversity in adolescent-onset depression: the lack of life skills

Early adversities and the lack of appropriate social experiences can trigger psychopathologies

During adolescence, individuals negotiate puberty and the completion of growth, take on sexually dimorphic body shape, develop new cognitive skills (including abstract thinking capacities), a clearer sense of personal and sexual identity, and a degree of emotional, personal, and financial independence from their parents (Christie and Viner, 2005). Internal physical and psychological changes interact with external or social changes and, although puberty is largely biologically determined, the greater part of psychological and social development depends on environmental and sociocultural influences (Christie and Viner, 2005). Adolescents redefine themselves in relation to others, experience emotional separation from their parents, strong peer identifications and sexual peer interest, and increased exploratory behavior. The modifications in the adolescent psychosocial environment are accompanied by the structural and functional maturation of brain regions involved in social and emotional regulation that continues till adulthood (Andrews et al., 2021). These brain changes are experience-dependent and without proper social stimuli result in neural and behavioral maladaptive outcomes (Dow-Edwards et al., 2019; Fig. 5).

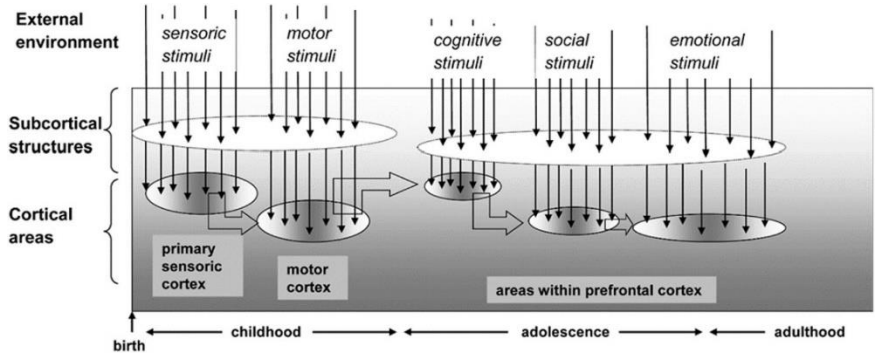

Figure 5. Impact of environmental stimuli on adolescent brain maturation. After birth, external stimuli trigger, via subcortical structures, the final maturation of specific cortical areas during critical and sensitive periods (Dow-Edwards et al., 2019).

In such a challenging period, early negative experiences or adversities could affect adolescent development. Adolescents exposed to life stressors (i.e. parental divorce, death of a parent, or other family adversities) or physical adversities (i.e. undergo maltreatment in early life) are three times more likely to develop depression or to attempt suicide than individuals without a history of stressful life events (Flament et al., 2001; Lewinsohn et al., 2001). Several studies show how adverse early life events, associated with a dysfunctional socioemotional environment, predict the onset of major depressive disorder in adolescence and its recurrence (Lewinsohn et al., 2001; Lewinsohn et al., 1999). Adolescent interpersonal relationships and peer interaction have been linked to anxiety and depression till adulthood. In particular, Jacobson and colleagues showed that adolescent perceptions of both close (e.g., “best friendships”) and group relationships (e.g., classroom peer groups) significantly mediated the relationship between adolescent anxiety and adult levels of depression. Adolescent perceptions of not feeling loved or accepted in close relationships may be a mechanism by which heightened anxiety in adolescence leads to clinical depression in adulthood (Jacobson and Newman, 2016). Therefore, adversities involving the lack of appropriate emotional and social experiences during adolescence jeopardize individual development and can trigger psychopathologies, such as major depressive disorder (Almeida et al., 2021; Viduani et al., 2021). Indeed, in adolescents and young adults, social isolation has been associated with poor mental and physical health (Christiansen et al., 2021). Based on these evidences, McCrory and collaborators reviewed a neurocognitive social transactional model of psychiatric vulnerability focused on the neuroprotective role of a supportive social architecture (McCrory et al., 2022). In particular, they show how a child who has experienced maltreatment (repeated exposure to abuse or neglect, or both) and subsequently exposed to further risk factors and a lack of protective influences such as social thinning, has an increased risk of psychiatric disorder (Fig.6). On the contrary, a child who has experienced maltreatment and is subsequently exposed to a range of protective influences that help build and maintain their social relationships has a more

adaptive social architecture which contributes to a decreased risk of psychiatric disorder (McCrory et al., 2022; Fig.7).

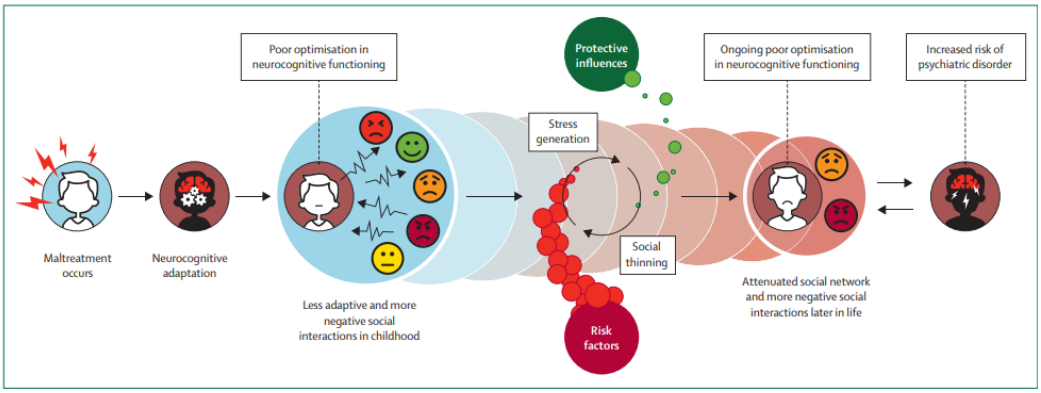

Figure 6. A schematic illustration of the neurocognitive social transactional model. This scenario depicts a child who has experienced maltreatment (repeated exposure to abuse or neglect, or both) and subsequently experiences further risk factors and a paucity of protective influences. In this context, increased stress generation and social thinning lead to an impoverished social architecture, contributing ultimately to an increased risk of psychiatric disorders (McCrory et al., 2022).

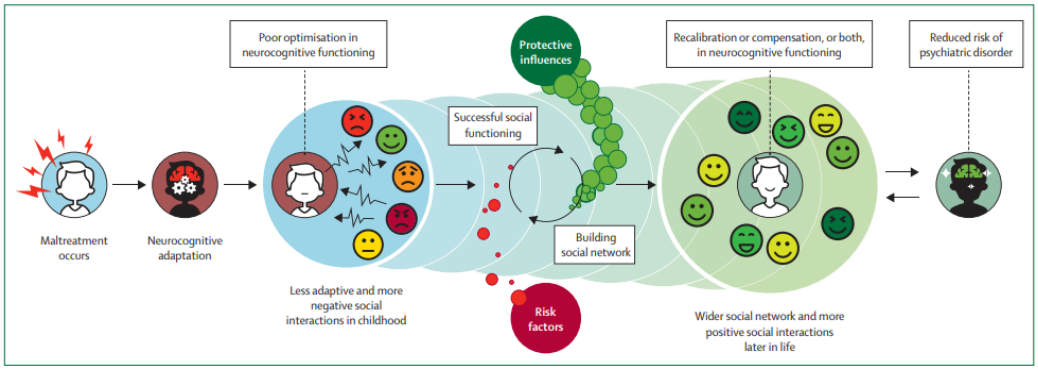

Figure 7. A second version of the neurocognitive social transactional model. This scenario depicts a child who has experienced maltreatment (repeated exposure to abuse or neglect, or both) and subsequently experiences a range of protective influences that help build and maintain their social relationships, and relatively few risk factors. In this context, less stress generation and social thinning lead to a richer, more adaptive social architecture, contributing ultimately to a decreased risk of psychiatric disorders (McCrory et al., 2022).

Therefore, experiences during childhood and adolescence could represent both a protective and a risk factor for psychopathologies. Children could adapt

well to change and acquire security and a balanced social position, with consequent positive results for both physical and psychological health, or they could be overwhelmed by these changes with harmful and difficult-to-repair consequences (Andrews et al., 2021). In this framework, the social architecture specifically consists of both family and peers. Indeed, the family context has a relevant influence in preventing the onset of mental stress, and depression in adolescents. It is widely known that a positive family context, very cohesive, welcoming, and with little conflict, improve the condition of adolescents and the tools to cope with difficult ones (Rice et al., 2006). A negative family context, with conflicts between parents, parent and child, and parental substance abuse, leads teenagers to internalize their difficulties, and symptoms associated with depression (Mason et al., 2009). Other studies underline the key role of social networking in adolescent life and the onset of disease. For instance, adolescents who attend social groups experiencing one or more substance abuse, have a higher risk of depressive onset than adolescents do not expose to drug abuse in their social networks (Mason et al., 2019). On the contrary, extracurricular activities are considered positive tools of protection against mental stress and depression (Mason et al., 2009). Longitudinal studies on social networks have shown that these risk behaviors are associated with belonging to a negative group and the behavior of the individual changes over time in relation to how friends behave (Andrews et al., 2021). These findings suggest the critical role of social stimuli and environment in children and adolescents' development and in the prevention and treatment of psychopathologies.

Increased rate of psychiatric disorders after forced isolation due to COVID-19 pandemic

In the last three years, the COVID-19 pandemic has dramatically impacted the social life of adolescents worldwide (Branje and Morris, 2021). Many governments have implemented preventive measures to contain the SARS-CoV-2 spread, including prolonged school closures and home confinement that forced children and adolescents to experience a prolonged state of psychophysical isolation from their peers, teachers, and community networks, radically reducing

the opportunities for them to engage in face-to-face activities (Loades et al., 2020). This condition has increased the rates of psychiatric disorders during and after the enforced isolation ended (Loades et al., 2020; Orben et al., 2020); Fig.8). The global prevalence of child and adolescent depression and anxiety has doubled pre-pandemic estimates (Racine et al., 2021) during the first year of the COVID-19 pandemic, reaching 25.2% and 20.5%, respectively, and being more common in girls than boys (Liu et al., 2022). Therefore, a better comprehension of the mechanisms involved in depression may promote mental health in adolescence and represents an urgent need to face the consequences of social deprivation before and after COVID-19.

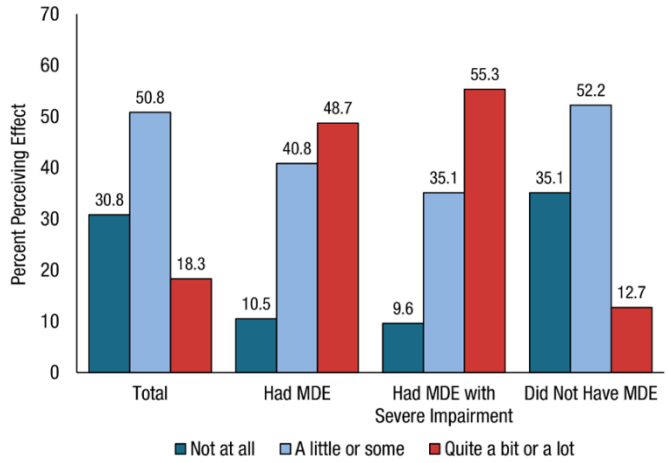

Figure 8. Perceived COVID-19 pandemic negative effect on emotional or mental health among youths aged 12 to 17; by past year major depressive episode (MDE) status. In Quarter 4 of 2020 (October to December), adolescents aged 12 to 17 who had a past year MDE were more likely than those who did not have a past year MDE to perceive that the coronavirus disease 2019 (COVID-19) pandemic negatively affected their mental health “quite a bit or a lot” (SAMSHA, 2020).

3. The antidepressant outcome depends on the environment and the personal capability to face the environmental challenges

Antidepressant treatment

The main therapies to treat major depression are drugs, psychotherapy, and Electroconvulsive Therapy, but international trends indicate an increase in the use of antidepressant drugs, particularly after the introduction of selective serotonin reuptake inhibitors (SSRIs) in the early 1990s (Whooley and Simon, 2000). These drugs increase the extracellular level of the neurotransmitter serotonin by inhibiting its reuptake into the presynaptic cell. Consequently, SSRIs increase serotonin levels in the synaptic cleft available to bind postsynaptic receptors (Hiemke and Hartter, 2000). The increased levels of serotonin have been widely associated with brain plasticity and potentiality for modification. Indeed, serotonin shapes neuronal networks during development and modulates a wide spectrum of essential brain functions ranging from perception and cognitive appraisal to emotional responses in the mature brain (Lesch and Waider, 2012).

The observation that the increase in the synaptic concentrations of monoamines leads to the improvement of depressive symptoms, based on the *ex iuvantibus* principle, drove to the adoption of the monoamine hypothesis of depression. It posits that decreased function of monoamine, such as norepinephrine and/or serotonin, caused depression, and the re-establishment of their balance leads to recovery (Belmaker and Agam, 2008; Heninger et al., 1996; Krishnan and Nestler, 2008; Lesch, 2007; Wong and Licinio, 2001). Although monoamine imbalance might be involved in the pathogenesis of the disorder (Lambert et al., 2000; Meyer et al., 2006), depression affects the brain in a complex and sophisticated manner and its causes appear to be different from the decrease of monoamine concentration in the brain (Moncrieff et al., 2022). Indeed, it has been reported the absence of changes in monoamine levels in depressed patients, while the depletion of monoamines does not alter mood in healthy controls (Ruhe et al., 2007). SSRIs are no more efficacious or have a rapid onset of action than the older antidepressants drugs, like tricyclic

antidepressant, but their improved safety and tolerability in clinical use has been them the most widely used of all antidepressants (Beasley et al., 1993; Sternbach, 1991). Moreover, to prevent the recurrence of depressive symptoms, antidepressants are taken on a long-term basis and studies have reported no evidence of long-term safety problems with the SSRI treatment (Doogan and Caillard, 1992; Janicak et al., 2001).

The use of antidepressants in youth increased substantially from 1.5% in 1996 to 2.5% in 2005 but has remained stable in the past 10 years, with about 2.6% in 2012 of 6–17-year-olds receiving an antidepressant medication annually for any indication (i.e., not only for depression). Use is much greater among youths with more severe mental health impairment (13.4%) than among those with mild or no mental health impairment (1.4%; Olfson et al., 2014; Olfson et al., 2015). It is worrying to know that more than 70% of adolescents affected by depressive disorders do not receive the correct diagnosis and therefore the right treatment (Bhatia and Bhatia, 2007).

Controversial results of serotoninergic antidepressant treatment

A large number of studies show that treatment with SSRIs achieves mixed results, and patient success in achieving remission depends on several factors. The pivotal Treatment of Adolescent Depression Study (TADS) compared the SSRI fluoxetine, Cognitive Behavioral Therapy (CBT), fluoxetine + CBT (combination treatment), and medication placebo in 439 adolescents with moderate-to-severe depression (March et al., 2004). At the end of the 12-week acute phase, combination treatment was superior to fluoxetine monotherapy and CBT alone. The rates of response were 71% for combined treatment, 61% for fluoxetine monotherapy, 43% for CBT alone, and 35% for placebo (March et al., 2004; Mullen, 2018).

Conversely, two recent meta-analyses have noted a small therapeutic effect for all antidepressants with fluoxetine being the only antidepressant to have a statistically significant effect over placebo on efficacy for the treatment of depression (Cipriani et al., 2016; Mullen, 2018; Vitiello and Ordonez, 2016). The Adolescent Depression Antidepressant and Psychotherapy Trial assessed

subjects 11-17 years of age with moderate-to-severe depression who did not respond to brief initial psychosocial intervention. At the end of 12 and 28 weeks, SSRIs plus CBT did not provide any additional benefit over SSRIs alone and 21% of participants did not respond by week 28 to either treatment, SSRI or SSRI plus CBT (Goodyer et al., 2007). In double-blind, randomized controlled trials testing the efficacy of antidepressants, SSRIs demonstrate small yet statistically significant advantages over placebo but do not substantially improve patient symptoms (Le Noury et al., 2015; Fig.9). In particular, placebo response rates range from 30% to 50%; this high percentage occurs partly because some patients show a spontaneous remission from depression during the 6-8 weeks of the drug trial, and partly because of the power of the placebo effect, which is particularly noticeable in the management of psychiatric illness. Indeed, the efficacy of the placebo is exceptionally large, at least as that attributable to the antidepressants and this means that it is hard to obtain a statistically significant difference between the drug and placebo (Fournier et al., 2010; Kirsch et al., 2008).

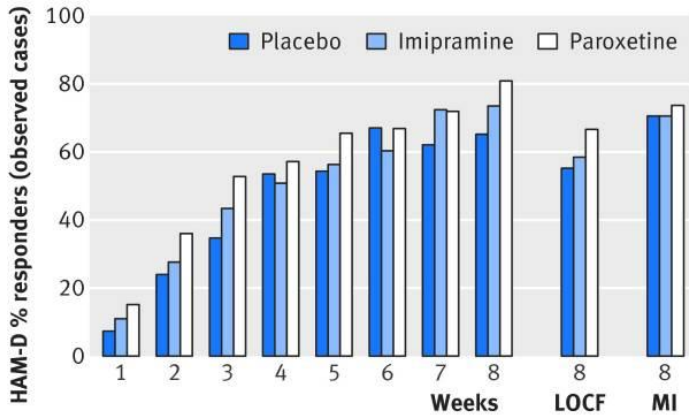

Figure 9. Differences in Hamilton depression scale (HAM-D) % responders in the study of efficacy and harms of paroxetine and imipramine in treatment of major depression in adolescence. LOCF=last observation carried forward, MI=multiple imputation. (Le Noury et al., 2015).

Furthermore, in 2004, the Food and Drug Administration added a *black box warning* to all antidepressants about the increased risk of suicidal thoughts and behaviors for children, adolescents, and young adults. Following the issuance of

the warning but despite the continued diagnosis of depression, the prescribing of SSRIs in adolescents decreased, particularly for youth 14 years of age and younger (Gibbons et al., 2007). A meta-analysis of 24 studies revealed an increased risk of suicidal behavior or ideation for those receiving medication compared with placebo (4% vs 2%; Hammad et al., 2006). Therefore, it is becoming increasingly necessary to find more efficient and less variable antidepressant treatments.

"Undirected susceptibility to change" hypothesis

To explain the incomplete efficacy of SSRIs, in 2011 a novel view of the role of SSRI action named the "undirected susceptibility to change" hypothesis (USC) has been proposed (Branchi, 2011). According to such a view, the relationship between SSRIs administration, the consequent change in serotonin levels, and the recovery from depression is not a direct one. In particular, increasing serotonin levels *per se* does not univocally lead to recovery from depression, but may even worsen the symptomatology. The reason is that high serotonin levels increase brain plasticity and thus enhance biological sensitivity to context, that is, susceptibility to the quality of the environment. Consequently, an individual treated with SSRI is more affected by environmental stimuli, both favorable and adverse, compared to an untreated individual. SSRI treatment is effective because it enhances individual's reactivity to the environment, allowing, in the case of a favorable environment, to be beneficially affected. This hypothesis claims thus that increasing serotonin levels is not the direct cause of recovery from depression but a permissive factor (Fig.10). The USC hypothesis has been recently demonstrated at preclinical and clinical levels. In particular, it has been shown in a mouse model of depression that the administration of the SSRI fluoxetine in an enriched environment improves the depressive-like phenotype while, in stressful conditions, it has no effect or even leads to a worsening (Alboni et al., 2017; Bashiri et al., 2021; Branchi et al., 2013; Karpova et al., 2011; Lima et al., 2019; Poggini et al., 2021). Clinical studies demonstrated that the administration of SSRI Citalopram amplifies the influence of living conditions on mood, leading to an improvement of symptoms according to the individual's

socio-economic status (Chiarotti et al., 2017; Viglione et al., 2019). It appears thus necessary to modify the quality of the living environment and its influence on SSRI action to improve the efficacy of treatments.

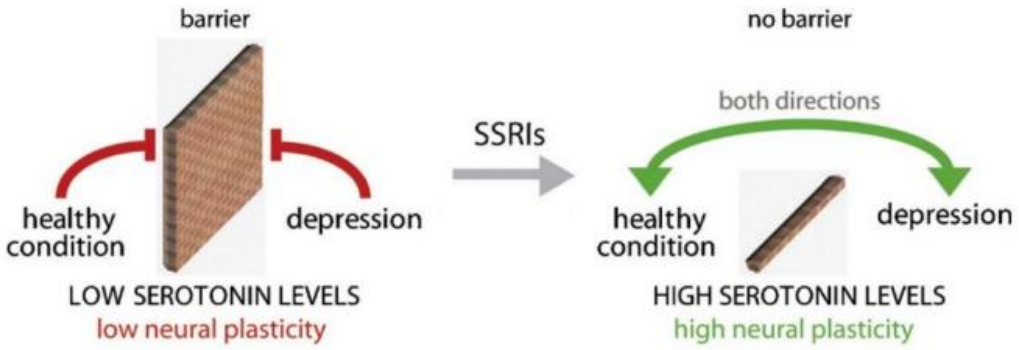

Figure 10. Schematic description of the undirected change susceptibility hypothesis. Serotonin levels determine vulnerability to depression and the capacity to recover from it. Low serotonin levels are associated with reduced neural plasticity. This condition does not allow it to shift from a healthy condition to a depression condition (i.e., vulnerability) and vice versa (i.e., capacity to recover). By contrast, when serotonin levels are high, the increased levels of neural plasticity allow shifting from one condition to the other, increasing both vulnerability and capacity to recover. The change in neural plasticity levels can be induced by SSRI administration increases (modified by Branchi, 2011).

Lack of life skills makes the environment adverse and determines the antidepressant outcome

The environment, i.e. physical surroundings, social relationships, and cultural milieus within which defined groups of people function and interact (Barnett and Casper, 2001), plays a fundamental role in adolescent and adolescent-onset depression and offers opportunities for intervention strategies aimed to prevent and/or treat this pathology. The innovative view offered by the USC hypothesis opens new perspectives on how to improve SSRI efficacy by controlling the environment. Indeed, it states that SSRI treatment makes the individual able to change but does not determine the direction of such change. In the literature, several studies support this model. For instance, individuals bearing the s/s allele of the serotonin-transporter-linked promoter region (5-HTTLPR), which leads to higher levels of brain serotonin, if exposed to childhood

maltreatment or other stressful life events, show increased neuroticism (Schinka et al., 2004) and incidence of depression (Caspi et al., 2003). However, s/s individuals were first more susceptible to the environment but also showed higher capabilities to change and faster recovery from depressive symptoms. In addition, different types of therapy, such as psychological (e.g., cognitive and other) and physical (e.g., light), if associated with a positive environment, exert beneficial effects on depression symptoms and rate of recovery (DeRubeis et al., 2008; Wirz-Justice et al., 2005). Also, in this case, serotonin levels modulate these effects: either cognitive or light therapy when combined with antidepressant administration leads to a greater improvement rate than either treatment alone (Benedetti et al., 1997; Cuijpers et al., 2009; Kaufman et al., 2004; Pampallona et al., 2004; Scott, 2000). Thus, according to the USC model, psychological and drug therapies should not be considered as alternative treatments, but as mutually potentiating tools whose combination is the key factor for the cure of major depression. The USC hypothesis provides a potential explanation of the inconsistent results about the low percentage of depressed patients who benefit from antidepressants and the relatively high percentage who benefit from placebo. Indeed, we can infer that not considering the influence of the environment limits the identification of the conditions permitting beneficial outcomes. In addition, mood modifications can occur only because of the environment and in the absence of treatment, though at a slower rate. In particular, the beneficial environment, associated with the expectancy created by placebo, may lead to recovery also without drug administration. These findings appear particularly relevant considering that epidemiological studies have indicated that SSRI treatment, which should reduce the risk of suicide, may cause suicidal feelings, self-harm, and suicide in some susceptible individuals, particularly in adolescents (Barbui et al., 2009; Fergusson et al., 2005; Gunnell et al., 2005; Hall and Lucke, 2006).

The role of the subjective view of the environment

Recent studies aimed at investigating the effects of SSRI in healthy subjects further confirmed the context dependence of the SSRI effects showing that, in

clinical practice, these drugs enhance neuroplasticity rather than directly improving psychiatric symptoms (Klobl et al., 2022; Reed et al., 2022). Thus, the quality of the living environment is key in determining SSRI treatment outcomes. It is important to note that the quality of the environment, such as the living conditions, does not depend only on its objective features, but also on the subjective experience of it (Kempermann, 2019; Fig.11). For instance, trust impressions are highly dependent on individuality (Sutherland et al., 2020) and individuals show important differences in their response to fear and threat (LeDoux and Brown, 2017). In addition, twin studies demonstrate that subjective perceptions of social status correlate with indicators of well-being (Rivenbark et al., 2020). Therefore, exploring the individual's subjective view of the environment appears key to improving SSRIs treatment efficacy.

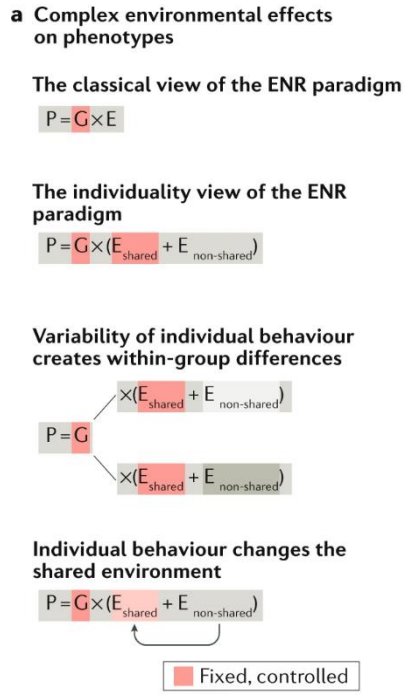

Figure 11. The enriched environment (ENR) paradigm and the quality of the living environment. The ENR is traditionally seen as an experimental strategy to address the effects of the environment (E) on phenotypes (P) when the genetic background (G) is fixed. However, individual animals within the ENR cage also behave and form their own environment, as do the other animals. This 'non-shared' environment has a substantial impact on the phenotype and prevents the E part of the equation from being fully controlled, even if in the experimental situation the nominal environment for the subjects is identical. Behaving animals change the environment of their cage-mates, for example, by altering the social structure (modified by Kempermann, 2019).

References

(SAMSHA), S.A.a.M.H.S.A. (2020). The National Survey on Drug Use and Health 2020 (NSDUH).

(WHO), W.H.O. (2021). Adolescent mental health.

Alboni, S., van Dijk, R.M., Poggini, S., Milior, G., Perrotta, M., Drenth, T., Brunello, N., Wolfer, D.P., Limatola, C., Amrein, I.*, et al.* (2017). Fluoxetine effects on molecular, cellular and behavioral endophenotypes of depression are driven by the living environment. Molecular psychiatry *22*, 552-561.

Almeida, I.L.L., Rego, J.F., Teixeira, A.C.G., and Moreira, M.R. (2021). Social isolation and its impact on child and adolescent development: a systematic review. Revista paulista de pediatria : orgao oficial da Sociedade de Pediatria de Sao Paulo *40*, e2020385.

Andrews, J.L., Ahmed, S.P., and Blakemore, S.J. (2021). Navigating the Social Environment in Adolescence: The Role of Social Brain Development. Biological psychiatry *89*, 109-118.

Angold, A., and Costello, E.J. (2000). The Child and Adolescent Psychiatric Assessment (CAPA). Journal of the American Academy of Child and Adolescent Psychiatry *39*, 39-48.

Barbui, C., Esposito, E., and Cipriani, A. (2009). Selective serotonin reuptake inhibitors and risk of suicide: a systematic review of observational studies. CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne *180*, 291-297.

Barnett, E., and Casper, M. (2001). A definition of "social environment". American journal of public health *91*, 465.

Bashiri, H., Houwing, D.J., Homberg, J.R., and Salari, A.A. (2021). The combination of fluoxetine and environmental enrichment reduces postpartum stress-related behaviors through the oxytocinergic system and HPA axis in mice. Scientific reports *11*, 8518.

Beasley, C.M., Jr., Sayler, M.E., and Potvin, J.H. (1993). Fluoxetine versus amitriptyline in the treatment of major depression: a multicenter trial. International clinical psychopharmacology *8*, 143-149.

Belmaker, R.H., and Agam, G. (2008). Major depressive disorder. The New England journal of medicine *358*, 55-68.

Benedetti, F., Barbini, B., Lucca, A., Campori, E., Colombo, C., and Smeraldi, E. (1997). Sleep deprivation hastens the antidepressant action of fluoxetine. European archives of psychiatry and clinical neuroscience *247*, 100-103.

Benros, M.E., Waltoft, B.L., Nordentoft, M., Ostergaard, S.D., Eaton, W.W., Krogh, J., and Mortensen, P.B. (2013). Autoimmune diseases and severe infections as risk factors for mood disorders: a nationwide study. JAMA psychiatry *70*, 812-820.

Bhatia, S.K., and Bhatia, S.C. (2007). Childhood and adolescent depression. American family physician *75*, 73-80.

Blakemore, S.J. (2019). Adolescence and mental health. Lancet *393*, 2030-2031.

Branchi, I. (2011). The double edged sword of neural plasticity: increasing serotonin levels leads to both greater vulnerability to depression and improved capacity to recover. Psychoneuroendocrinology *36*, 339-351.

Branchi, I., Poggini, S., Capuron, L., Benedetti, F., Poletti, S., Tamouza, R., Drexhage, H.A., Penninx, B., Pariante, C.M., European College of Neuropsychopharmacology ImmunoNeuroPsychiatry Thematic Working, G.*, et al.* (2021). Brain-immune crosstalk in the treatment of major depressive disorder. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology *45*, 89-107.

Branchi, I., Santarelli, S., Capoccia, S., Poggini, S., D'Andrea, I., Cirulli, F., and Alleva, E. (2013). Antidepressant treatment outcome depends on the quality of the living environment: a pre-clinical investigation in mice. PloS one *8*, e62226.

Branje, S., and Morris, A.S. (2021). The Impact of the COVID-19 Pandemic on Adolescent Emotional, Social, and Academic Adjustment. Journal of research on adolescence : the official journal of the Society for Research on Adolescence *31*, 486-499.

Breslau, J., Gilman, S.E., Stein, B.D., Ruder, T., Gmelin, T., and Miller, E. (2017). Sex differences in recent first-onset depression in an epidemiological sample of adolescents. Translational psychiatry *7*, e1139.

Bridge, J.A., Goldstein, T.R., and Brent, D.A. (2006). Adolescent suicide and suicidal behavior. Journal of child psychology and psychiatry, and allied disciplines *47*, 372-394.

Bullmore, E. (2018). The art of medicine: Inflamed depression. Lancet *392*, 1189-1190.

Byrne, M.L., O'Brien-Simpson, N.M., Mitchell, S.A., and Allen, N.B. (2015). Adolescent-Onset Depression: Are Obesity and Inflammation Developmental Mechanisms or Outcomes? Child psychiatry and human development *46*, 839-850.

Caspi, A., Sugden, K., Moffitt, T.E., Taylor, A., Craig, I.W., Harrington, H., McClay, J., Mill, J., Martin, J., Braithwaite, A.*, et al.* (2003). Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science *301*, 386-389.

Chiarotti, F., Viglione, A., Giuliani, A., and Branchi, I. (2017). Citalopram amplifies the influence of living conditions on mood in depressed patients enrolled in the STAR*D study. Translational psychiatry *7*, e1066.

Christiansen, J., Qualter, P., Friis, K., Pedersen, S.S., Lund, R., Andersen, C.M., Bekker-Jeppesen, M., and Lasgaard, M. (2021). Associations of loneliness and social isolation with physical and mental health among adolescents and young adults. Perspectives in public health *141*, 226-236.

Christie, D., and Viner, R. (2005). Adolescent development. Bmj *330*, 301-304.

Cipriani, A., Zhou, X., Del Giovane, C., Hetrick, S.E., Qin, B., Whittington, C., Coghill, D., Zhang, Y., Hazell, P., Leucht, S.*, et al.* (2016). Comparative efficacy and tolerability of antidepressants for major depressive disorder in children and adolescents: a network meta-analysis. Lancet *388*, 881-890.

Cohen, P., Cohen, J., Kasen, S., Velez, C.N., Hartmark, C., Johnson, J., Rojas, M., Brook, J., and Streuning, E.L. (1993). An epidemiological study of disorders in late childhood and adolescence--I. Age- and gender-specific prevalence. Journal of child psychology and psychiatry, and allied disciplines *34*, 851-867.

Cole, D.A., Cai, L., Martin, N.C., Findling, R.L., Youngstrom, E.A., Garber, J., Curry, J.F., Hyde, J.S., Essex, M.J., Compas, B.E.*, et al.* (2011). Structure

and measurement of depression in youths: applying item response theory to clinical data. Psychological assessment *23*, 819-833.

Colman, I., Wadsworth, M.E., Croudace, T.J., and Jones, P.B. (2007). Forty-year psychiatric outcomes following assessment for internalizing disorder in adolescence. The American journal of psychiatry *164*, 126-133.

Conley, C.S., and Rudolph, K.D. (2009). The emerging sex difference in adolescent depression: interacting contributions of puberty and peer stress. Development and psychopathology *21*, 593-620.

Copeland, W.E., Shanahan, L., Worthman, C., Angold, A., and Costello, E.J. (2012). Cumulative depression episodes predict later C-reactive protein levels: a prospective analysis. Biological psychiatry *71*, 15-21.

Cuijpers, P., Dekker, J., Hollon, S.D., and Andersson, G. (2009). Adding psychotherapy to pharmacotherapy in the treatment of depressive disorders in adults: a meta-analysis. The Journal of clinical psychiatry *70*, 1219-1229.

Dantzer, R. (2012). Depression and inflammation: an intricate relationship. Biological psychiatry *71*, 4-5.

Dantzer, R., O'Connor, J.C., Freund, G.G., Johnson, R.W., and Kelley, K.W. (2008). From inflammation to sickness and depression: when the immune system subjugates the brain. Nature reviews Neuroscience *9*, 46-56.

DeRubeis, R.J., Siegle, G.J., and Hollon, S.D. (2008). Cognitive therapy versus medication for depression: treatment outcomes and neural mechanisms. Nature reviews Neuroscience *9*, 788-796.

Doogan, D.P., and Caillard, V. (1992). Sertraline in the prevention of depression. The British journal of psychiatry : the journal of mental science *160*, 217-222.

Dow-Edwards, D., MacMaster, F.P., Peterson, B.S., Niesink, R., Andersen, S., and Braams, B.R. (2019). Experience during adolescence shapes brain development: From synapses and networks to normal and pathological behavior. Neurotoxicology and teratology *76*, 106834.

Fergusson, D., Doucette, S., Glass, K.C., Shapiro, S., Healy, D., Hebert, P., and Hutton, B. (2005). Association between suicide attempts and selective serotonin reuptake inhibitors: systematic review of randomised controlled trials. Bmj *330*, 396.

Fergusson, D.M., and Woodward, L.J. (2002). Mental health, educational, and social role outcomes of adolescents with depression. Archives of general psychiatry *59*, 225-231.

Flament, M.F., Cohen, D., Choquet, M., Jeammet, P., and Ledoux, S. (2001). Phenomenology, psychosocial correlates, and treatment seeking in major depression and dysthymia of adolescence. Journal of the American Academy of Child and Adolescent Psychiatry *40*, 1070-1078.

Fournier, J.C., DeRubeis, R.J., Hollon, S.D., Dimidjian, S., Amsterdam, J.D., Shelton, R.C., and Fawcett, J. (2010). Antidepressant drug effects and depression severity: a patient-level meta-analysis. Jama *303*, 47-53.

Garcia-Bueno, B., Caso, J.R., and Leza, J.C. (2008). Stress as a neuroinflammatory condition in brain: damaging and protective mechanisms. Neuroscience and biobehavioral reviews *32*, 1136-1151.

Gibbons, R.D., Brown, C.H., Hur, K., Marcus, S.M., Bhaumik, D.K., Erkens, J.A., Herings, R.M., and Mann, J.J. (2007). Early evidence on the effects of regulators' suicidality warnings on SSRI prescriptions and suicide in children and adolescents. The American journal of psychiatry *164*, 1356-1363.

Gimeno, D., Kivimaki, M., Brunner, E.J., Elovainio, M., De Vogli, R., Steptoe, A., Kumari, M., Lowe, G.D., Rumley, A., Marmot, M.G.*, et al.* (2009). Associations of C-reactive protein and interleukin-6 with cognitive symptoms of depression: 12-year follow-up of the Whitehall II study. Psychological medicine *39*, 413-423.

Goodyer, I., Dubicka, B., Wilkinson, P., Kelvin, R., Roberts, C., Byford, S., Breen, S., Ford, C., Barrett, B., Leech, A.*, et al.* (2007). Selective serotonin reuptake inhibitors (SSRIs) and routine specialist care with and without cognitive behaviour therapy in adolescents with major depression: randomised controlled trial. Bmj *335*, 142.

Granata, L., Gildawie, K.R., Ismail, N., Brenhouse, H.C., and Kopec, A.M. (2022). Immune signaling as a node of interaction between systems that sex-specifically develop during puberty and adolescence. Developmental cognitive neuroscience *57*, 101143.

Gunnell, D., Saperia, J., and Ashby, D. (2005). Selective serotonin reuptake inhibitors (SSRIs) and suicide in adults: meta-analysis of drug company data from placebo controlled, randomised controlled trials submitted to the MHRA's safety review. Bmj *330*, 385.

Hall, W.D., and Lucke, J. (2006). How have the selective serotonin reuptake inhibitor antidepressants affected suicide mortality? The Australian and New Zealand journal of psychiatry *40*, 941-950.

Hammad, T.A., Laughren, T., and Racoosin, J. (2006). Suicidality in pediatric patients treated with antidepressant drugs. Archives of general psychiatry *63*, 332-339.

Hankin, B.L., Abramson, L.Y., Moffitt, T.E., Silva, P.A., McGee, R., and Angell, K.E. (1998). Development of depression from preadolescence to young adulthood: emerging gender differences in a 10-year longitudinal study. Journal of abnormal psychology *107*, 128-140.

Harrington, R., Fudge, H., Rutter, M., Pickles, A., and Hill, J. (1990). Adult outcomes of childhood and adolescent depression. I. Psychiatric status. Archives of general psychiatry *47*, 465-473.

Heninger, G.R., Delgado, P.L., and Charney, D.S. (1996). The revised monoamine theory of depression: a modulatory role for monoamines, based on new findings from monoamine depletion experiments in humans. Pharmacopsychiatry *29*, 2-11.

Hiemke, C., and Hartter, S. (2000). Pharmacokinetics of selective serotonin reuptake inhibitors. Pharmacology & therapeutics *85*, 11-28.

Hill, R.M., Pettit, J.W., Lewinsohn, P.M., Seeley, J.R., and Klein, D.N. (2014). Escalation to Major Depressive Disorder among adolescents with subthreshold depressive symptoms: evidence of distinct subgroups at risk. Journal of affective disorders *158*, 133-138.

Iob, E., Lacey, R., Giunchiglia, V., and Steptoe, A. (2022). Adverse childhood experiences and severity levels of inflammation and depression from childhood to young adulthood: a longitudinal cohort study. Molecular psychiatry *27*, 2255-2263.

Jacobson, N.C., and Newman, M.G. (2016). Perceptions of Close and Group Relationships Mediate the Relationship between Anxiety and Depression over a Decade Later. Depression and anxiety *33*, 66-74.

Janicak, P.G., Keck, P.E., Jr., Davis, J.M., Kasckow, J.W., Tugrul, K., Dowd, S.M., Strong, J., Sharma, R.P., and Strakowski, S.M. (2001). A double-blind, randomized, prospective evaluation of the efficacy and safety of risperidone versus haloperidol in the treatment of schizoaffective disorder. Journal of clinical psychopharmacology *21*, 360-368.

Johnson, D., Dupuis, G., Piche, J., Clayborne, Z., and Colman, I. (2018). Adult mental health outcomes of adolescent depression: A systematic review. Depression and anxiety *35*, 700-716.

Karpova, N.N., Pickenhagen, A., Lindholm, J., Tiraboschi, E., Kulesskaya, N., Agustsdottir, A., Antila, H., Popova, D., Akamine, Y., Bahi, A.*, et al.* (2011). Fear erasure in mice requires synergy between antidepressant drugs and extinction training. Science *334*, 1731-1734.

Kaufman, J., Yang, B.Z., Douglas-Palumberi, H., Houshyar, S., Lipschitz, D., Krystal, J.H., and Gelernter, J. (2004). Social supports and serotonin transporter gene moderate depression in maltreated children. Proceedings of the National Academy of Sciences of the United States of America *101*, 17316-17321.

Kempermann, G. (2019). Environmental enrichment, new neurons and the neurobiology of individuality. Nature reviews Neuroscience *20*, 235-245.

Kirsch, I., Deacon, B.J., Huedo-Medina, T.B., Scoboria, A., Moore, T.J., and Johnson, B.T. (2008). Initial severity and antidepressant benefits: a meta-analysis of data submitted to the Food and Drug Administration. PLoS medicine *5*, e45.

Klobl, M., Seiger, R., Vanicek, T., Handschuh, P., Reed, M.B., Spurny-Dworak, B., Ritter, V., Godbersen, G.M., Gryglewski, G., Kraus, C.*, et al.* (2022). Escitalopram modulates learning content-specific neuroplasticity of functional brain networks. NeuroImage *247*, 118829.

Krishnan, V., and Nestler, E.J. (2008). The molecular neurobiology of depression. Nature *455*, 894-902.

Lambert, G., Johansson, M., Agren, H., and Friberg, P. (2000). Reduced brain norepinephrine and dopamine release in treatment-refractory depressive illness: evidence in support of the catecholamine hypothesis of mood disorders. Archives of general psychiatry *57*, 787-793.

Le Noury, J., Nardo, J.M., Healy, D., Jureidini, J., Raven, M., Tufanaru, C., and Abi-Jaoude, E. (2015). Restoring Study 329: efficacy and harms of paroxetine and imipramine in treatment of major depression in adolescence. Bmj *351*, h4320.

Leboyer, M., Berk, M., Yolken, R.H., Tamouza, R., Kupfer, D., and Groc, L. (2016a). Immuno-psychiatry: an agenda for clinical practice and innovative research. BMC medicine *14*, 173.

Leboyer, M., Oliveira, J., Tamouza, R., and Groc, L. (2016b). Is it time for immunopsychiatry in psychotic disorders? Psychopharmacology *233*, 1651-1660.

LeDoux, J.E., and Brown, R. (2017). A higher-order theory of emotional consciousness. Proceedings of the National Academy of Sciences of the United States of America *114*, E2016-E2025.

Leone, M., Kuja-Halkola, R., Leval, A., D'Onofrio, B.M., Larsson, H., Lichtenstein, P., and Bergen, S.E. (2021). Association of Youth Depression With Subsequent Somatic Diseases and Premature Death. JAMA psychiatry *78*, 302-310.

Lesch, K.P. (2007). Linking emotion to the social brain. The role of the serotonin transporter in human social behaviour. EMBO reports *8 Spec No*, S24-29.

Lesch, K.P., and Waider, J. (2012). Serotonin in the modulation of neural plasticity and networks: implications for neurodevelopmental disorders. Neuron *76*, 175-191.

Levine, J., Barak, Y., Chengappa, K.N., Rapoport, A., Rebey, M., and Barak, V. (1999). Cerebrospinal cytokine levels in patients with acute depression. Neuropsychobiology *40*, 171-176.

Lewinsohn, P.M., Joiner, T.E., Jr., and Rohde, P. (2001). Evaluation of cognitive diathesis-stress models in predicting major depressive disorder in adolescents. Journal of abnormal psychology *110*, 203-215.

Lewinsohn, P.M., Rohde, P., Klein, D.N., and Seeley, J.R. (1999). Natural course of adolescent major depressive disorder: I. Continuity into young adulthood. Journal of the American Academy of Child and Adolescent Psychiatry *38*, 56-63.

Lima, J., Sharp, T., Bannerman, D.M., and McHugh, S.B. (2019). Enhanced discriminative aversive learning and amygdala responsivity in 5-HT transporter mutant mice. Translational psychiatry *9*, 139.

Liu, C.H., Zhang, G.Z., Li, B., Li, M., Woelfer, M., Walter, M., and Wang, L. (2019). Role of inflammation in depression relapse. Journal of neuroinflammation *16*, 90.

Liu, S.R., Davis, E.P., Palma, A.M., Sandman, C.A., and Glynn, L.M. (2022). The acute and persisting impact of COVID-19 on trajectories of adolescent depression: Sex differences and social connectedness. Journal of affective disorders *299*, 246-255.

Loades, M.E., Chatburn, E., Higson-Sweeney, N., Reynolds, S., Shafran, R., Brigden, A., Linney, C., McManus, M.N., Borwick, C., and Crawley, E. (2020). Rapid Systematic Review: The Impact of Social Isolation and Loneliness on the Mental Health of Children and Adolescents in the Context of COVID-19. Journal of the American Academy of Child and Adolescent Psychiatry *59*, 1218-1239 e1213.

March, J., Silva, S., Petrycki, S., Curry, J., Wells, K., Fairbank, J., Burns, B., Domino, M., McNulty, S., Vitiello, B.*, et al.* (2004). Fluoxetine, cognitive-behavioral therapy, and their combination for adolescents with depression: Treatment for Adolescents With Depression Study (TADS) randomized controlled trial. Jama *292*, 807-820.

Marcus, S.M., Young, E.A., Kerber, K.B., Kornstein, S., Farabaugh, A.H., Mitchell, J., Wisniewski, S.R., Balasubramani, G.K., Trivedi, M.H., and Rush, A.J. (2005). Gender differences in depression: findings from the STAR*D study. Journal of affective disorders *87*, 141-150.

Mariani, N., Cattane, N., Pariante, C., and Cattaneo, A. (2021). Gene expression studies in Depression development and treatment: an overview of the

underlying molecular mechanisms and biological processes to identify biomarkers. Translational psychiatry *11*, 354.

Mason, M., Mennis, J., Russell, M., Moore, M., and Brown, A. (2019). Adolescent Depression and Substance Use: the Protective Role of Prosocial Peer Behavior. Journal of abnormal child psychology *47*, 1065-1074.

Mason, M.J., Schmidt, C., Abraham, A., Walker, L., and Tercyak, K. (2009). Adolescents' social environment and depression: social networks, extracurricular activity, and family relationship influences. Journal of clinical psychology in medical settings *16*, 346-354.

McCrory, E., Foulkes, L., and Viding, E. (2022). Social thinning and stress generation after childhood maltreatment: a neurocognitive social transactional model of psychiatric vulnerability. The lancet Psychiatry *9*, 828-837.

Meyer, J.H., Ginovart, N., Boovariwala, A., Sagrati, S., Hussey, D., Garcia, A., Young, T., Praschak-Rieder, N., Wilson, A.A., and Houle, S. (2006). Elevated monoamine oxidase a levels in the brain: an explanation for the monoamine imbalance of major depression. Archives of general psychiatry *63*, 1209-1216.

Milaneschi, Y., Lamers, F., Berk, M., and Penninx, B. (2020). Depression Heterogeneity and Its Biological Underpinnings: Toward Immunometabolic Depression. Biological psychiatry *88*, 369-380.

Moncrieff, J., Cooper, R.E., Stockmann, T., Amendola, S., Hengartner, M.P., and Horowitz, M.A. (2022). The serotonin theory of depression: a systematic umbrella review of the evidence. Molecular psychiatry.

Mullen, S. (2018). Major depressive disorder in children and adolescents. The mental health clinician *8*, 275-283.

Muller, N., Krause, D., Barth, R., Myint, A.M., Weidinger, E., Stettinger, W., Zill, P., Drexhage, H., and Schwarz, M.J. (2019). Childhood Adversity and Current Stress are related to Pro- and Anti-inflammatory Cytokines in Major Depression. Journal of affective disorders *253*, 270-276.

Naninck, E.F., Lucassen, P.J., and Bakker, J. (2011). Sex differences in adolescent depression: do sex hormones determine vulnerability? Journal of neuroendocrinology *23*, 383-392.

Olfson, M., Blanco, C., Wang, S., Laje, G., and Correll, C.U. (2014). National trends in the mental health care of children, adolescents, and adults by office-based physicians. JAMA psychiatry *71*, 81-90.

Olfson, M., Druss, B.G., and Marcus, S.C. (2015). Trends in mental health care among children and adolescents. The New England journal of medicine *372*, 2029-2038.

Orben, A., Tomova, L., and Blakemore, S.J. (2020). The effects of social deprivation on adolescent development and mental health. The Lancet Child & adolescent health *4*, 634-640.

Pampallona, S., Bollini, P., Tibaldi, G., Kupelnick, B., and Munizza, C. (2004). Combined pharmacotherapy and psychological treatment for depression: a systematic review. Archives of general psychiatry *61*, 714-719.

Pariante, C.M. (2016). Neuroscience, mental health and the immune system: overcoming the brain-mind-body trichotomy. Epidemiology and psychiatric sciences *25*, 101-105.

Pariante, C.M. (2017). Why are depressed patients inflamed? A reflection on 20 years of research on depression, glucocorticoid resistance and inflammation. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology *27*, 554-559.

Pasco, J.A., Nicholson, G.C., Williams, L.J., Jacka, F.N., Henry, M.J., Kotowicz, M.A., Schneider, H.G., Leonard, B.E., and Berk, M. (2010). Association of high-sensitivity C-reactive protein with de novo major depression. The British journal of psychiatry : the journal of mental science *197*, 372-377.

Poggini, S., Matte Bon, G., Golia, M.T., Ciano Albanese, N., Viglione, A., Poleggi, A., Limatola, C., Maggi, L., and Branchi, I. (2021). Selecting antidepressants according to a drug-by-environment interaction: A comparison of fluoxetine and minocycline effects in mice living either in enriched or stressful conditions. Behavioural brain research *408*, 113256.

Racine, N., McArthur, B.A., Cooke, J.E., Eirich, R., Zhu, J., and Madigan, S. (2021). Global Prevalence of Depressive and Anxiety Symptoms in Children and Adolescents During COVID-19: A Meta-analysis. JAMA pediatrics *175*, 1142-1150.

Raison, C.L., Capuron, L., and Miller, A.H. (2006). Cytokines sing the blues: inflammation and the pathogenesis of depression. Trends in immunology *27*, 24-31.

Raison, C.L., and Miller, A.H. (2011). Is depression an inflammatory disorder? Current psychiatry reports *13*, 467-475.

Rao, U., Ryan, N.D., Birmaher, B., Dahl, R.E., Williamson, D.E., Kaufman, J., Rao, R., and Nelson, B. (1995). Unipolar depression in adolescents: clinical outcome in adulthood. Journal of the American Academy of Child and Adolescent Psychiatry *34*, 566-578.

Reed, M.B., Klobl, M., Godbersen, G.M., Handschuh, P.A., Ritter, V., Spurny-Dworak, B., Unterholzner, J., Kraus, C., Gryglewski, G., Winkler, D*., et al.* (2022). Serotonergic modulation of effective connectivity in an associative relearning network during task and rest. NeuroImage *249*, 118887.

Rice, F., Harold, G.T., Shelton, K.H., and Thapar, A. (2006). Family conflict interacts with genetic liability in predicting childhood and adolescent depression. Journal of the American Academy of Child and Adolescent Psychiatry *45*, 841-848.

Rice, F., Riglin, L., Lomax, T., Souter, E., Potter, R., Smith, D.J., Thapar, A.K., and Thapar, A. (2019). Adolescent and adult differences in major depression symptom profiles. Journal of affective disorders *243*, 175-181.

Rivenbark, J., Arseneault, L., Caspi, A., Danese, A., Fisher, H.L., Moffitt, T.E., Rasmussen, L.J.H., Russell, M.A., and Odgers, C.L. (2020). Adolescents' perceptions of family social status correlate with health and life chances: A twin difference longitudinal cohort study. Proceedings of the National Academy of Sciences of the United States of America *117*, 23323-23328.

Ruhe, H.G., Mason, N.S., and Schene, A.H. (2007). Mood is indirectly related to serotonin, norepinephrine and dopamine levels in humans: a meta-analysis of monoamine depletion studies. Molecular psychiatry *12*, 331-359.

Salk, R.H., Hyde, J.S., and Abramson, L.Y. (2017). Gender differences in depression in representative national samples: Meta-analyses of diagnoses and symptoms. Psychological bulletin *143*, 783-822.

Sawyer, S.M., Azzopardi, P.S., Wickremarathne, D., and Patton, G.C. (2018). The age of adolescence. The Lancet Child & adolescent health *2*, 223-228.

Schinka, J.A., Busch, R.M., and Robichaux-Keene, N. (2004). A meta-analysis of the association between the serotonin transporter gene polymorphism (5-HTTLPR) and trait anxiety. Molecular psychiatry *9*, 197-202.

Schuch, J.J., Roest, A.M., Nolen, W.A., Penninx, B.W., and de Jonge, P. (2014). Gender differences in major depressive disorder: results from the Netherlands study of depression and anxiety. Journal of affective disorders *156*, 156-163.

Scott, J. (2000). Treatment of chronic depression. The New England journal of medicine *342*, 1518-1520.

Sternbach, H. (1991). The serotonin syndrome. The American journal of psychiatry *148*, 705-713.

Sutherland, C.A.M., Burton, N.S., Wilmer, J.B., Blokland, G.A.M., Germine, L., Palermo, R., Collova, J.R., and Rhodes, G. (2020). Individual differences in trust evaluations are shaped mostly by environments, not genes. Proceedings of the National Academy of Sciences of the United States of America *117*, 10218-10224.

Thapar, A., Collishaw, S., Pine, D.S., and Thapar, A.K. (2012). Depression in adolescence. Lancet *379*, 1056-1067.

Thibaut, F. (2018). The mind-body Cartesian dualism and psychiatry. Dialogues in clinical neuroscience *20*, 3.

Timbremont, B., Braet, C., and Dreessen, L. (2004). Assessing depression in youth: relation between the Children's Depression Inventory and a structured interview. Journal of clinical child and adolescent psychology : the official journal for the Society of Clinical Child and Adolescent Psychology, American Psychological Association, Division 53 *33*, 149-157.

Twenge, J.M., Cooper, A.B., Joiner, T.E., Duffy, M.E., and Binau, S.G. (2019). Age, period, and cohort trends in mood disorder indicators and suicide-

related outcomes in a nationally representative dataset, 2005-2017. Journal of abnormal psychology *128*, 185-199.

Viduani, A., Benetti, S., Martini, T., Buchweitz, C., Ottman, K., Wahid, S.S., Fisher, H.L., Mondelli, V., Kohrt, B.A., and Kieling, C. (2021). Social isolation as a core feature of adolescent depression: a qualitative study in Porto Alegre, Brazil. International journal of qualitative studies on health and well-being *16*, 1978374.

Viglione, A., Chiarotti, F., Poggini, S., Giuliani, A., and Branchi, I. (2019). Predicting antidepressant treatment outcome based on socioeconomic status and citalopram dose. The pharmacogenomics journal *19*, 538-546.

Vitiello, B., and Ordonez, A.E. (2016). Pharmacological treatment of children and adolescents with depression. Expert opinion on pharmacotherapy *17*, 2273-2279.

Whooley, M.A., and Simon, G.E. (2000). Managing depression in medical outpatients. The New England journal of medicine *343*, 1942-1950.

Wilkinson, P., Kelvin, R., Roberts, C., Dubicka, B., and Goodyer, I. (2011). Clinical and psychosocial predictors of suicide attempts and nonsuicidal self-injury in the Adolescent Depression Antidepressants and Psychotherapy Trial (ADAPT). The American journal of psychiatry *168*, 495-501.

Wirz-Justice, A., Benedetti, F., Berger, M., Lam, R.W., Martiny, K., Terman, M., and Wu, J.C. (2005). Chronotherapeutics (light and wake therapy) in affective disorders. Psychological medicine *35*, 939-944.

Wong, M.L., and Licinio, J. (2001). Research and treatment approaches to depression. Nature reviews Neuroscience *2*, 343-351.

EXPERIMENTAL SECTION

STUDY 1

Social isolation in adolescence leads to sex-dependent emotional and cognitive dysfunction, reduced BDNF and altered immune activation: a preclinical study

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In preparation

Abstract

Adolescence is a critical period of both physiological and socio-emotional development. Adversities involving the lack of appropriate emotional and social experiences during adolescence jeopardize individual development and can trigger psychopathologies, such as major depressive disorder, which represents the major risk factor for suicide among teenagers. Girls appear to be more susceptible to depression than boys over their adolescent years. However, the relative contribution of biological and cultural factors to sex differences in the vulnerability to depression is not yet clear. To unravel the neural bases of adolescent-onset depression and sex-dependent vulnerability, we exposed C57BL/6 adolescent male and female mice first to social isolation and then to behavioral phenotyping, and to several neural plasticity and inflammatory biomarkers. Our results show that adolescent social isolation leads to depressive- and anxiety-like phenotype and reduced neural plasticity in both sexes. However, the lack of social experiences in adolescence produced different effects in the two sexes: females showed more marked impairments in the emotional domain, while males displayed alterations in the cognitive domain and the stress response. Moreover, in both sexes, adolescent isolation decreased inflammatory markers. These findings suggest that social experiences in adolescence represent a key factor in the neurodevelopment of both sexes and the sex-differences in vulnerability to depression could be, at least in part, ascribed to biological factors and not to cultural and social influences.

Keywords

Adolescent-onset depression, social experience, sex-dependent vulnerability, BDNF, inflammation

Introduction

Adolescence is the transitional phase of growth and development from childhood to adulthood (from 10 up to 24 years; Andersen, 2016; Sawyer et al., 2018). During this period, adolescents experience not only biological and physical changes (Sawyer et al., 2018), but they go through relevant modifications in their social environment, such as the transition from primary to secondary school associated with an increase in the number of peers, and in the time spent with them (Andrews et al., 2021; Blakemore, 2019). These modifications in their psychosocial environment are accompanied by the structural and functional maturation of brain regions involved in social and emotional regulation that continues till adulthood (Andrews et al., 2021). These brain changes are experience-dependent and without proper social stimuli result in neural and behavioral maladaptive outcomes (Dow-Edwards et al., 2019). Indeed, adversities involving the lack of appropriate emotional and social experiences during adolescence jeopardize individual development and can trigger psychopathologies, such as major depressive disorder (Almeida et al., 2021; Viduani et al., 2021). In fact, in adolescents and young adults, social isolation has been associated with poor mental and physical health (Christiansen et al., 2021). Depression in adolescence represents the major risk factor for somatic disease, premature death, and suicide among teenagers (Byrne et al., 2015; Leone et al., 2021).

In the last three years, the COVID-19 pandemic has dramatically impacted the social life of adolescents worldwide (Branje and Morris, 2021). Many governments have implemented preventive measures to contain the SARS-CoV-2 spread, including prolonged school closures and home confinement that forced children and adolescents to experience a prolonged state of psychophysical isolation from their peers, teachers, and community networks, radically reducing the opportunities for them to engage in face-to-face activities (Loades et al., 2020). This condition has increased the rates of psychiatric disorders during and after the enforced isolation ended (Loades et al., 2020; Orben et al., 2020). The global prevalence of child and adolescent depression and anxiety has doubled pre-pandemic estimates (Racine et al., 2021) during the first year of the COVID-

19 pandemic, reaching 25.2% and 20.5%, respectively, and being more common in girls than boys (Liu et al., 2022; Racine et al., 2021). This gender-dependent vulnerability during COVID-19 is in line with pre-pandemic data showing that girls are approximately twice as likely as boys to be depressed, a difference emerging in puberty and lasting into adulthood (Salk et al., 2017). Therefore, a better comprehension of the mechanisms involved in this different vulnerability to depression may promote mental health in adolescence and represents an urgent need to face the consequences of social deprivation before and after COVID-19.

Despite the large impact of adolescent depression, the relative contribution of biological and cultural factors to sex differences in the vulnerability to depression is not yet clear (Conley and Rudolph, 2009). The expression of inflammatory cytokines in the brain has been reported to be involved in the pathogenesis of and susceptibility to depression, indicating adolescence as a unique stage of immune development (Granata et al., 2022; Mariani et al., 2021). Moreover, several studies have linked the impairment of neuroplasticity and adolescent depression, focusing on the role of neurotrophins as Brain-Derived Neurotrophic Factor (BDNF; Levy et al., 2018; Medendorp et al., 2018; Pandey et al., 2010). However, the mechanisms involved have not been clearly identified yet (Mills et al., 2013; Nomoto et al., 2015).

To unravel the neural bases of adolescent-onset depression and sex-dependent vulnerability, we exposed male and female mice, to social isolation in addition to several neural plasticity and inflammatory biomarkers. We hypothesized that the lack of appropriate social experiences during adolescence can lead to psychiatric disorders in both sexes but in different ways, impairing the development of social and emotional skills of experimental subjects and affecting neural plasticity processes and immune system regulation. To explore such a hypothesis, we exposed C57BL/6 adolescent male and female mice (n=12-15) either to standard laboratory housing conditions (i.e. controls) or to a social isolation period [from postnatal day (PND) 21 to 60]. From PND 46 to 60, we phenotyped the behavioral response in the emotional and cognitive domains through a battery of tests, including saccharin preference, open field, elevated plus maze, novel object recognition, and fear conditioning test. In addition, we

assessed the activation of the hypothalamus-pituitary-adrenal (HPA) axis by measuring plasma levels of corticosterone in response to a psychophysical stressful challenge. Finally, to explore the potential role of immunity and neural plasticity mechanisms, we measured the expression levels of selected markers, including BDNF, Postsynaptic Density Protein 95 (PSD95), and both pro- and anti-inflammatory cytokines at the end of the behavioral tests period (PND60). We expect that isolated mice displayed depressive and anxiety profiles and impaired cognitive abilities in a sex-dependent manner and these behavioral abnormalities are associated with decreased neural plasticity and dysregulated inflammatory levels.

Materials and methods

Ethical standards

All procedures were carried out in accordance with the European and Italian legislation on animal experimentation (respectively European Directive 2010/63/UE and Decreto Legislativo 26/2014). Animals were examined for signs of discomfort as indicated by the animal care and use guidelines [National Academy of Sciences. Guide for the care and use of laboratory animals, 1998, ‘‘Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research” (National Research Council 2003)]. The Italian Ministry of Health approved the protocol with permit number D9997.111.

Animals and breeding procedure

13 male and 26 female C57BL/6 mice weighing 16-21 g (5 weeks old) were purchased from Charles River Laboratories placed in Calco (Lecco, Italy). Animals were housed under reversed 12 light-dark cycle (07.00-19.00) in an air-conditioned room (temperature 21±1 °C, relative humidity 60±10%). Males and females were housed in same-sex groups of 4-5 individuals in 42.5 x 27.6 x 15.3 Plexiglas cages with a metal top and sawdust as bedding, with pellet food (Enriched standard diet, Riper, Vendoies, BZ, Italy) and tap water *ad libitum*. Each cage was provided with tissue paper (300 x 200 mm). When animals were 9 weeks old, two females and one male were mated for 8 consecutive days. Afterward, males were removed from the breeding cages, and each female was singly housed for the entire duration of pregnancy until the pups’ weaning, postnatal day (PND) 21. On PND-1 litters were culled to 3 male and 3 female pups and a cross-fostering procedure was performed to limit the number of mates needed (Fig.1).

The paradigm of chronic stress: post-weaning social isolation protocol

On PND 21, mice were assigned to the two experimental groups per sex isolated or group-housed. Isolated mice were singly housed in a cage of 29 x 22 x 14 cm. Grouped mice were housed 4/cage with a same-sex conspecific (42.5

x 27.6 x 15.3 cm). To identify each subject, we marked the animals with an ear punch. Following 3 weeks of social isolation (PND 41), we performed both behavioral and molecular assessments, maintaining the same housing conditions (Fig.1). In particular, 52 mice were addressed to the behavioral tests (28 isolated and 24 group-housed mice, balanced for sex), and 36 mice were addressed to the quantification of peripheric corticosterone levels (9 individuals per group per sex). To monitor animals' well-being over the experimental phases, every week we measured their body weight (See Supplementary materials).

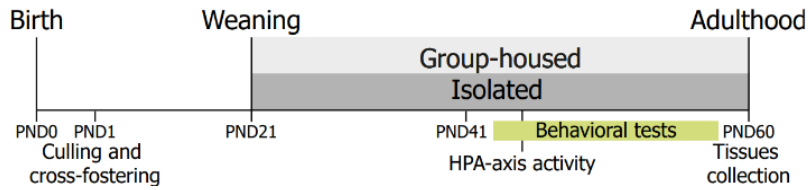

Figure 1. Experimental design. After weaning (PND 21), mice were exposed to a post-weaning isolation protocol to induce a depressive-like phenotype. Therefore, mice were assigned to the two experimental groups per sex isolated or group-housed. Isolated mice were singly housed. Grouped mice were housed 4/cage with a same-sex conspecific. Following 3 weeks of social isolation (PND 41), we performed both behavioral and molecular assessments, maintaining the same housing conditions.

Behavioral tests

We assessed the depressive-like profile of the animals, through the saccharine preference, while the anxiety-like profile and the spontaneous exploration through the open field and elevated plus-maze test. Novel object recognition and fear conditioning tests were selected to evaluate cognitive abilities. All tests were carried out during the dark period (between 09.30 and 16.00 h). Animals were transferred to the experimental room at least 30 min before the test to let them acclimatize to the new environment. All the tests were video-recorded using a digital video camera (CANON LEGRIA HFR86). The behavioral scoring was carried out using the commercial software Observer XT (Noldus, Wageningen, The Netherlands) and AnyMaze software (Stoelting Europe).

Liking-type anhedonia - Saccharin preference

First, mice were exposed to the saccharin solution only for 72 hours to make them used to the saccharin flavor (0.1%, Sigma Aldrich Co, St Louis, MO, USA) After habituation, at PND46 and PND47 a two-bottle choice procedure was used to test saccharin preference and determined as follows (saccharin solution consumed/ saccharin solution consumed+water consumed) x100. Water and saccharin solution consumed was estimated by weighing the bottles once a day. The position of water and saccharin in each cage was counterbalanced across the experimental group. Saccharin preference was expressed as the average daily intake of water and saccharin (in grams; Pierre et al., 2019).

Elevated plus-maze

To assess anxiety-related and explorative behavior, at PND 51 we performed the elevated plus-maze test. The elevated plus-maze comprises two open arms (30 x 5 cm) and two closed arms (30 x 5 x 15 cm) that extended from a common central platform (5 x 5 cm). The apparatus, made of Plexiglas (gray floor, clear walls), was elevated to a height of 60 cm above the floor level. Mice were individually placed on the central platform facing an open arm and allowed to explore the maze for 5 minutes under indirect dim light (10 lux). Behavioral parameters observed were the percentage of *time spent in the open arms* [(open/total) ×100] and latency of *the first open arm entry* (arm entry = all four paws into an arm).

Open field test

At PND 46 we performed the open field test. Mice were allowed to explore the apparatus, consisting of an empty black box (40 x 40 x 50 cm) for 15 minutes under indirect dim light (10 lux). The central area was defined as a central 20 cm × 20 cm square, and the other region was defined as the peripheral area. Mice were placed in the periphery with the snout facing the wall, and we scored the percentage of *time spent in the center* calculated as follows [(center/total) ×100] and latency of *first entry in the center* (entry in center = all four paws into the center). Furthermore, we scored the duration of *the stretched attend postures*

(s.a.p.), an exploratory posture in which the body is stretched forward and then retracted to the original position without any forward locomotion, considered an important component of the risk-assessment repertoire of mouse defensive behavior.

Novel object recognition test

At PND 47 we performed the novel object recognition test. Mice were tested individually in an empty black box (40 x 40 x 50 cm) under indirect dim light (10 lux). The test consisted of two trials, each of 10 minutes, with an inter-trial interval of 2 hours. Twenty-four hours before testing, subjects were familiarized with the arena during a 15-minutes pre-exposure session. During the first trial, the familiarization phase, a single animal is placed in the open-field arena containing two identical sample objects [glass (G) or lego (L)], for 10 minutes, as the following objects scheme: G-G or L-L. Plastic or glass objects of identical volume and different shapes constituted the stimuli. Objects and object locations were counterbalanced to avoid a potential object and/or location bias. To prevent coercion to explore the objects, rodents were released against the center of the opposite wall with their back to the objects. During the second trial, corresponding to the test phase, the animal is exposed to two objects, one is identical to the sample and the other is novel (i.e. G-L). During both the familiarization and the test phases, objects were located in opposite and symmetrical corners of the center of the arena, and the location of novel versus familiar objects is counterbalanced. A *discrimination index*, calculated as [(A-B)/(A+B)] in which *A* represents the time exploring the novel object and *B* is the time exploring the familiar one, was used to measure the novel object exploration. This index can vary between +1 and −1, where a positive score indicates more time spent with the novel object, a negative score indicates more time spent with the familiar object, and a zero score indicates a null preference. Exploration of an object was defined as directing the nose towards an object at a distance of less than 1 cm and/or touching the object with the nose and/or paws. Sitting on the objects was not considered exploratory behavior. Alcohol solution (70%) was used to remove odors in the novel object recognition apparatus between all sessions.

Fear conditioning test

To assess emotional learning and memory, on PND 55 we performed the fear conditioning test. The test is based on the association between an unconditioned stimulus (US) and a conditioned stimulus (CS) causing an unconditioned response (UR). In particular, the CS corresponds to a neutral tone, paired with a brief electric foot-shock (US). This produces a typical rodents UR, called *freezing behavior*, corresponding to the total absence of all movement except respiration. The experimental apparatus corresponds to a training chamber (30×35×20 cm) with four opaque sidewalls and a removable foot-shocking grid floor used for the first two phases of the task, training, and context test. The third phase, the tone test, requires a different context to verify the mice's association between CS and US. The fear conditioning test was performed on two days. In the training session, mice learn to associate a neutral tone (1000 Hz, 90 dB) to the foot-shock (0.23 mA average intensity) for three trials, 60 seconds each. Before the first trial, was performed 60 seconds of observation to assess the baseline activity of the animals. Each trial consisted of a tone phase of 30 seconds with two seconds of foot-shock starting at 28 seconds, the last 30 seconds of trial were without tone (intertrial). In this phase, we assess emotional response and learning by scoring the percentage of *freezing time behavior*. After 24 hours we performed the context test, mice are exposed to the same training chamber for 120 seconds without neutral tone and foot-shock. We assess memory by scoring the percentage of *freezing time behavior*. During the last session, the tone test, mice are observed in a new environment consisting of a chamber (25×28×16.5 cm) with four white walls, black visual cues, and red light. 90 minutes after the context test, mice are exposed to the same neutral tone in the new test chamber without the final foot-shock for three trials of 60 seconds, divided into 30 seconds of tone and 30 seconds of intertrial. The association between CS and US is scored by the percentage of *freezing time*.

Corticosterone levels

Following the three weeks of social isolation (PND44), the activation of the hypothalamus-pituitary-adrenal (HPA) axis was assessed by measuring plasma

levels of corticosterone in response to a psychophysical stressful challenge. All subjects underwent acute restraint stress (30 min) and blood samples were collected by a tail nick at different time points, i.e., immediately before (0 min) and after (30 and 180 min) the exposure to stress. The restraint procedure consisted in placing animals in 50ml-plastic falcon tubes with a few holes to keep airflow with white light during the stress. This procedure took place at 02:30 p.m. when the levels of free corticosterone were far from the circadian peak (Kitchener et al., 2004). Blood samples were collected individually in potassium–ethylenediaminetetraacetic acid (EDTA) coated 10 ml tubes (1.6 mg EDTA/ml blood: Sarstedt). All samples were kept on ice and later centrifuged at 3000 rpm for 15 min at 4 ◦C. Blood plasma was transferred to Eppendorf tubes for CORT determination and stored at − 80 ◦C until analysis. Plasma corticosterone levels were quantified by enzyme immunoassay according to the manufacturer's recommendations (Corticosterone Elisa kit, Enzo Life Sciences). Samples were diluted 1:40 before analysis, and data were analyzed by sigmoidal 4-parameter logistic curve fit using Prism 6 (Graphpad). The sensitivity of the assay was 27.0 pg/ml and the intra-assay percent coefficients of variations were between 3.35% and 3.82%. Because of the great variability, the logarithmic transformation will be implemented.

RNA extraction and RT-PCR on the entire hippocampus

We assessed the expression levels of genes related to inflammation [Interleukin (IL)-1β, IL-10, IL-6, and Tumor Necrosis Factor-α (TNF-α)] and neuroplasticity (BDNF and PSD95) by RT-PCR tests and according to published protocol (Poggini et al., 2021). Briefly, total RNA was extracted, at the end of the behavioral tests period (PND60), with Purelink RNA mini kit (Thermo Fisher) from the entire mice hippocampus and according to the manufacturer protocol. Any residual genomic DNA contamination was eliminated by the use of the Pure Link DNase set (Thermo Fisher). The purified total RNA was eluted in 50 µl RNase-Free water and immediately stored in aliquots (of 10 µl each) at -80 ° C until use. The amount and quality of total RNA extracted were assessed by Nanodrop 2000 (Thermo Fisher) readings (average concentration = 168.2 ng / µl; mean

A260/280 = 2.1). Purified RNA (400 ng in total) was used in 20 µl of reverse transcription according to the High Capacity RNA to cDNA kit protocol (Thermo Fisher). Negative retrotranscription controls (without retrotranscriptase enzyme) were also performed. The resulting cDNA samples were diluted 1:10 with RNase-free water and processed by the real-time PCR protocol using PowerUp SYBR GREEN Master mix (Thermo Fisher). Negative (no cDNA) and genomic contamination (negative retrotranscription control) controls were included in all experiments. The pairs of primers used in this study were designed (with the help of online software) in such a way as to be as specific as possible for the regions being investigated and are presented in table 1 in the Supplementary. For RT-PCR each well of a 96 wells plate (Euroclone) was loaded with 10 µl of the solution containing 1 µl of nuclease-free water, 5 µl of SYBR Green mastermix 2X (Thermo Scientific), 1 µl of primers pair (10 µM each) and 3 µl of cDNA. Amplification was carried out in an ABI 7500 (Applied Biosystem) thermocycler with the following conditions: 50°C for 2 min, 95°C for 2 min, followed by 40 cycles (95°C for 15 s and 58°C for 1 min). After the amplification, a melting curve was obtained to analyze specificity. The amplification curves were analyzed using the ABI7500 instrument software both to determine the Ct and for the analysis of melting curves. The same fluorescence threshold of 0.1 was kept for all plates. Each sample was done in triplicates. All assays presenting multiple melting temperatures or temperatures different from expected were omitted from the analysis. Data were then extracted in excel files for further statistical analyses and calculations. In order to perform appropriate normalization in our experiments, we used two housekeeping genes (B2M and bACT) as references (Supplementary Tab.1). Amplification curves with Ct >35 were considered negative (not expressed) except for the IL-10 gene on which we carried out an explorative analysis even if the Ct values were above the threshold (Average Ct = 37.3). The 2-DCt method was used for gene expression determination.

Statistical methods

All data were analyzed with one-way ANOVA with the Statistical software Statview II (Abacus Concepts, CA, USA), to compare isolated animals versus

group-housed animals, independently for the two sexes. Time (minutes or days) was considered a repeated measure within subjects. Latency data, which had no normal distribution, were analyzed with the Mann-Whitney nonparametric test. *Post-hoc* comparisons were performed using Tukey's test. All mean differences were considered statistically significant when $p < 0.05$.

Results

Emotional response

Depressive-like behavior: Liking-type anhedonia

Following 3 weeks of social isolation, both male and female mice showed higher liking-type anhedonia, displaying significantly lower saccharin preference compared to respective group-housed mice (male: $F_{1,26} = 5.649$, $p = 0.0251$; female: $F_{1,24} = 6.913$, $p = 0.0147$; Fig.2).

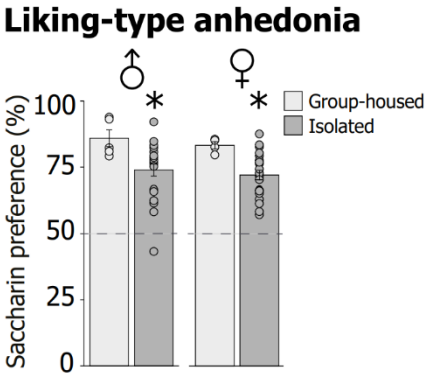

Figure 2. Depressive-like behavior. Following 3 weeks of social isolation, both male and female mice showed higher liking-type anhedonia, displaying significantly lower saccharin preference compared to group-housed mice. * $p<0.05$ vs. group-housed mice, data are shown as mean ± S.E.M.

Anxiety-like behavior- Elevated plus-maze and Open field test

In the elevated plus maze, isolated male mice showed an enhanced anxiety-like profile, displaying higher latency to enter the open arm compared to group-housed mice ($U = 39.500$, $p = 0.0256$), although no difference in time spent in open arms emerged ($F_{1,25} = 1.126$, $p = 0.2988$). Isolated female mice did not show significant differences compared to group-housed mice in both latency to enter ($U = 71.500$, $p = 0.9770$) and time spent in open arms ($F_{1,22} = 1.612$, $p = 0.2174$; Fig.3A-B).

Interestingly, in the open field test, the anxiety-like profile of the two sexes appeared opposite in comparison with the performance in the elevated plus-maze test. In male mice, no difference has been observed in time spent in the center of the arena ($F_{1,25} = 2.063$, $p = 0.1633$), while isolated female mice spent

lower time in the center compared to group-housed mice all over the duration of the test ($F_{1,23}$ = 13.450, p = 0.0013; Fig.3C). Moreover, both isolated male and female mice displayed significantly more s.a.p. than their control groups all over the duration of the test (respectively, $F_{1,23}$ = 12.137, p = 0.0020 and $F_{1,25}$ = 4.731, p = 0.0393; Fig.3D).

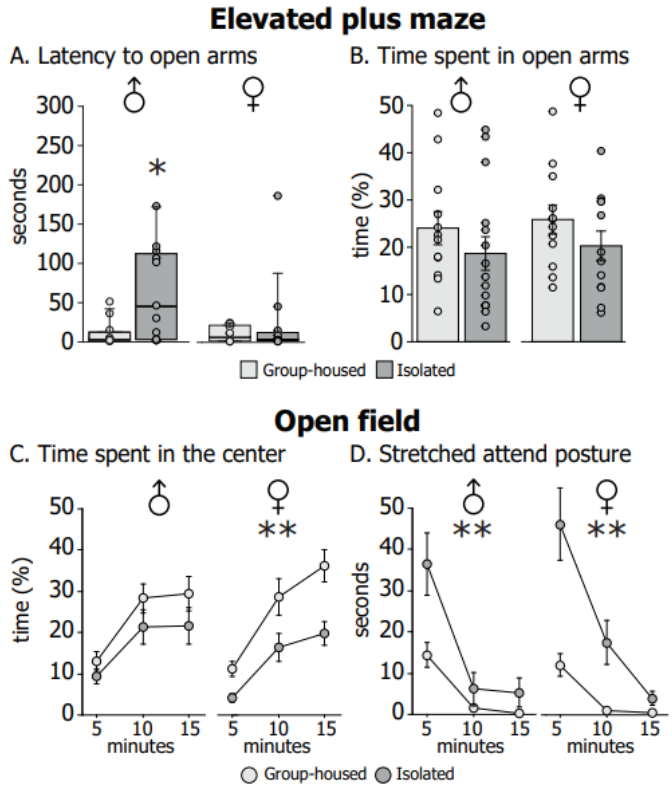

Figure 3. Anxiety-related behavior. **A**) Isolated male mice displayed higher latency to enter the open arm, (**B**) but no difference in time spent in open arms emerged compared to group-housed mice. **A-B**) Isolated female mice did not show significant differences in both latency to enter and time spent in open arms compared to group-housed mice. **C**) In the open field test, in male mice, no difference has been observed in time spent in the center of the arena, while isolated female mice spent lower time in the center compared to group-housed female mice. **D**) Both isolated male and female mice display significantly more stretched attend posture than their control groups. * p<0.05 and ** p<0.01 vs. group-housed mice, data shown as mean ± S.E.M.

Cognitive abilities

Learning and memory: Novel object recognition test

Adolescent social isolation affects cognitive abilities only in male subjects. Indeed, during the test phase, isolated male mice showed a significantly lower discrimination index compared to group-housed mice ($F_{1,12} = 4.975$, $p = 0.0456$). We did not observe a significant difference in female mice ($F_{1,14} = 1.979$, $p = 0.1813$; Fig.4A).

Learning and emotional memory: Fear conditioning test

In the first phase of the fear conditioning test, isolated male mice showed a greater percentage of freezing behavior compared to group-housed mice ($F_{1,25} = 5.985$, $p = 0.0218$), while we did not observe a significant difference in females ($F_{1,22} = 1.578$, $p = 0.2222$; Fig.4B). Concerning memory retrieval, no difference has been found between isolated and group-housed males (context test: $F_{1,25} = 0.2980$, $p = 0.5902$; tone test: $F_{1,25} = 1.195$, $p = 0.2847$) and females (context test: $F_{1,22} = 1.048$, $p = 0.3171$; tone test: $F_{1,22} = 1.339$, $p = 0.2596$; Fig.4C-D).

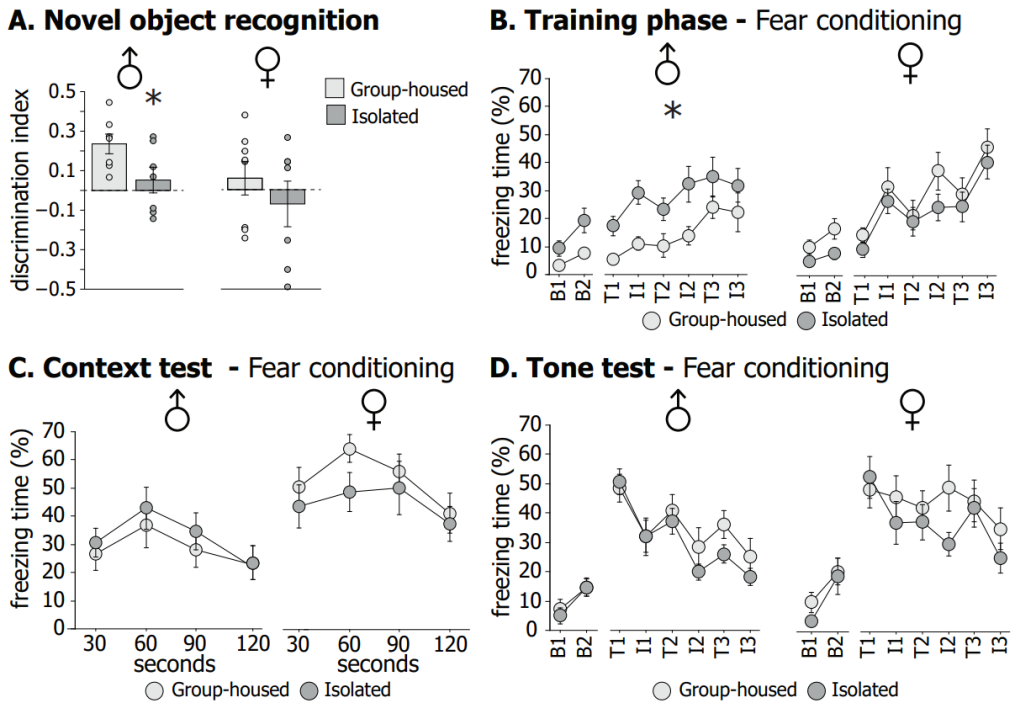

Figure 4. Cognitive domain. **A**) During the test phase of the novel object recognition test, isolated male mice showed a significantly lower discrimination index compared to group-housed mice. We did not observe a significant difference in female mice. **B**) In the first phase of the fear conditioning test, isolated male mice showed a greater percentage of freezing behavior compared to group-housed mice, while we did not observe a significant difference between isolated and group-housed females. **C-D**) Concerning memory retrieval, no difference has been found between isolated and group-housed males and females. B1: Baseline 30 seconds; B2: Baseline 60 seconds; T: Tone; I: interatrial. * p<0.05 vs. group-housed mice, data are shown as mean ± S.E.M.

HPA-axis activity: corticosterone levels

HPA-axis activity has been affected by adolescent social isolation only in male subjects, with isolated mice showing increased levels of corticosterone levels compared to group-housed mice ($F_{1,12}$ = 4.730, p = 0.0504). By contrast, no difference has been found between isolated and group-housed female mice ($F_{1,12}$ = 3.985, p=0.0691; Fig.5).

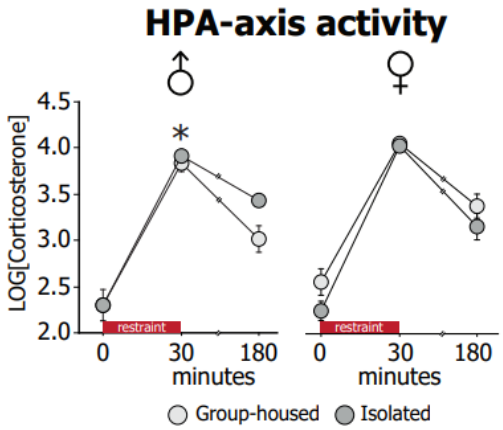

Figure 5. Corticosterone levels. HPA-axis activity has been affected by adolescent social isolation only in male subjects, with isolated mice showing increased levels of corticosterone levels compared to group-housed mice. By contrast, no difference has been found between isolated and group-housed female mice. *p=0.05 vs. group-housed mice, data are shown as mean ± S.E.M.

Hippocampal expression levels of neural plasticity and inflammatory markers

Pooled data of both males and females revealed that adolescent social isolation impaired neural plasticity, isolated mice were characterized by significantly lower expression levels of BDNF compared to group-housed mice ($F_{1,47}$ = 4.055, p = 0.0498). Data split by sex missed to reach the statistical significance (male: $F_{1,23}$ = 2.256, p = 0.1467; female; $F_{1,22}$ = 1.474, p = 0.2376).

By contrast, no difference was found in the expression levels of PSD95 (pooled data: $F_{1,45} = 0.376$, $p = 0.5428$; male: $F_{1,23} = 0.865$, $p = 0.3619$; female; $F_{1,22} = 1.514$, $p = 0.2315$; Fig.6A). Regarding the inflammatory profile, we found a significant decrease of the pro-inflammatory cytokine IL-1β expression levels in both males and females (male: $F_{1,23} = 4.096$, $p = 0.0547$; female; $F_{1,20} = 7.680$, $p = 0.0118$). In addition, isolated male mice showed increased levels of the anti-inflammatory cytokine IL-10 (male: $F_{1,11} = 5.837$, $p = 0.0343$; female: $F_{1,9} = 0.137$, $p = 0.7195$). Finally, no difference emerged concerning hippocampal expression levels of IL-6 (male: $F_{1,23} = 0.134$, $p = 0.7180$; female: $F_{1,21} = 1.590$, $p = 0.2212$) and TNF-α (male: $F_{1,23} = 0.415$, $p = 0.5227$; female; $F_{1,21} = 0.006$, $p = 0.9398$; Fig.6B).

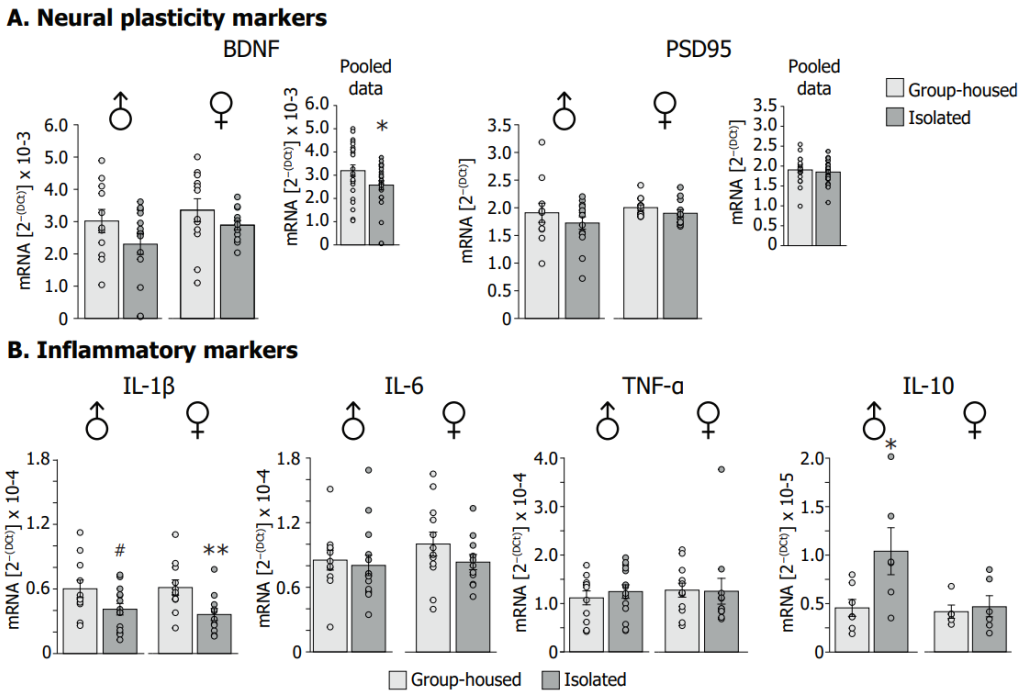

Figure 6. Neural plasticity and inflammatory markers. **A**) Pooled data of both males and females revealed that adolescent social isolation impaired neural plasticity, isolated mice were characterized by significantly lower expression levels of BDNF compared to group-housed mice. Data split by sex missed reaching the statistical significance. By contrast, no difference was found in the expression levels of PSD95. **B**) Regarding the inflammatory profile, we found a significant decrease in the pro-inflammatory cytokine IL-1β expression levels both in males and females. In addition, isolated male mice showed increased levels of the anti-inflammatory cytokine IL-10. no difference emerged concerning hippocampal expression levels of IL-6 and TNF-α. * $p<0.05$ and ** $p<0.01$ vs. group-housed mice, data are shown as mean ± S.E.M.

Discussion

The present findings show that adolescent social isolation leads to depressive- and anxiety-like phenotype and reduced neural plasticity in both sexes. However, the lack of social experiences in adolescence produced different effects in the two sexes: females showed more marked impairments in the emotional domain, while males displayed alterations in the cognitive domain and the stress response. In addition, in both sexes, adolescent isolation led to a reduction of inflammatory markers.

Adolescent social isolation increased anhedonic response in both male and female subjects compared to not-isolated mice. The result concerning male subjects is in line with the literature (Amiri et al., 2015; Burstein and Doron, 2018; Haj-Mirzaian et al., 2015; Zhang et al., 2017). Whereas this is one of the first studies demonstrating that both sexes are equally vulnerable to social isolation in adolescence concerning anhedonic behavior. Indeed, the comparison between male and female rodent models of depression yielded contrasting results (Kokras and Dalla, 2014), with some studies reporting lower, higher, or no difference in depression-like behaviors in females compared to males (Pitzer et al., 2022). In addition, Eltokhi and collaborators showed that baseline depression-like behaviors of adolescent mice are age but not sex-dependent (Eltokhi et al., 2021). By contrast, concerning the anxious profile of the animals after social isolation, we found an increased anxiety-like behavior in both sexes but in a test-dependent manner. Isolated male mice displayed an increased anxiety-like profile in the elevated plus maze, while isolated female mice showed a pronounced anxiety-like behavior in the open field test. These findings confirm that social deprivation affects the anxious profile of male subjects in the elevated plus maze as reported in the literature (Hellemans et al., 2004; Maisonnette et al., 1993; Workman et al., 2011) and that females appear spontaneously less anxious in this test (Knight et al., 2021). For instance, McCormick and colleagues showed that isolation stress in female rats during mid-adolescence resulted in a decreased anxiety-related behavior in the elevated plus maze test (McCormick et al., 2008), while Huang and collaborators displayed no differences between group-housed and isolated females (Huang et al., 2017). Conversely, in the open

field test, an overlapping increased anxious profile between the two sexes has been found, but it reaches statistical significance only for the isolated females, who spend less time in the center of the arena. Indeed, decreased time in the arena center represents a key measure of the anxiety-related behavior of mice (Kraeuter et al., 2019). This anxious profile is confirmed in both sexes by increased time spent in the stretched attend posture behavior, an important component of the “risk-assessment” repertoire of defensive behavior in rodents (Grewal et al., 1997). Our findings suggest that isolated mice have higher anxiety-like behavior related to a novel environment compared with group-housed mice and that social isolation affects mostly the anxious profile of females.

We found a clear sex-dependent effect considering the cognitive abilities of experimental subjects, only isolated male mice show a memory impairment in the novel object recognition. These findings are in line with the dysregulation of the HPA-axis activity displayed only by our isolated male mice, which is reported to affect the cognitive abilities of subjects including memory and selective attention (Bremner et al., 2004; Herbert et al., 2006; Jameison and Dinan, 2001). However, several studies exploring cognitive abilities after social isolation revealed contrasting data in both male and female adolescent subjects, although memory impairment after social isolation is well characterized in adulthood in males (Liu et al., 2020; Pais et al., 2019). For instance, H. Koike and colleagues confirmed our results concerning adolescent male mice (Koike et al., 2009), while Rivera-Irizarry and colleagues showed that all subjects display a preference for a novel object over a familiar one, independently of the adolescent social isolation, and this preference was greater in males than females (Rivera-Irizarry et al., 2020). Therefore, the interaction between higher corticosterone levels and the intrinsically higher preference for the novel object of male rodents than females at mid-adolescence (Cyrenne and Brown, 2011) could explain how adolescent social isolation affects the cognitive profile exclusively of male subjects. A sex-dependent effect appears also in the training phase of fear conditioning test in which only isolated male mice show an increased fear response and anxiety-related behavior after foot shock compared to group-housed, but no memory retrieval deficit. By contrast, no difference appears in the

fear response and memory of female subjects although females display a greater percentage of freezing behavior compared to males as reported in the literature (Rivera-Irizarry et al., 2020). Different studies reported how adolescent social isolation does not affect fear memory in rodents after 24 hours from the training phase of fear conditioning (Drummond et al., 2021; Rivera-Irizarry et al., 2020). Drummond and colleagues show how adolescent social isolation in rats impaired the extinction of fear in isolated males and females after 72 hours of the test (Drummond et al., 2021), and Liu and collaborators prolonged this time point and show how adolescent social isolation strengthens the retention of fear memories increasing the percentage of freezing after 14 and 28 days from the training phase compared to controls (Liu et al., 2015). These evidences suggest that the missing effect on fear memory retrieval could be ascribed to the timing of the test and that adolescent social isolation would have long-lasting effects on fear memory and retention.

Adolescent social isolation affected the HPA-axis activity only in male subjects, with isolated mice showing increased levels of corticosterone compared to group-housed mice, while no difference was found between isolated and group-housed female mice. Although the effect of stress on corticosterone levels is clear (Ulrich-Lai and Herman, 2009), there is several different stress-susceptibility between males and females (Handa and Weiser, 2014). Males' HPA-axis activity is in line with the literature (Ros-Simo and Valverde, 2012) reporting hyperstimulation of the axis that leads to a slower decrease of corticosterone levels after acute stress in the isolated group. By contrast, isolated females' HPA-axis activity is not affected by social isolation and these results confirm those of Lopez and colleagues who do not find any differences between group-housed and isolated female mice (Lopez and Laber, 2015). Interestingly, Pyter and collaborators showed that social isolation reduced basal circulating corticosterone concentration in female mice compared to group-housed (Pyter et al., 2014). This effect could be ascribed to increased glucocorticoid negative feedback sensitivity via increases in mineralocorticoid and/or glucocorticoid receptors (Elakovic et al., 2011) and to diminished responsiveness of the HPA-axis after the exposure to prolonged stress (Rivier and Vale, 1987).

Adolescent social isolation impaired neural plasticity reducing expression levels of BDNF. It has been reported the link between BDNF decreased levels and depressive disorder in adolescence (Chen et al., 2012; Lee et al., 2020). Since the key role played by BDNF on neural plasticity and early development of the brain (Antal et al., 2010; Yang et al., 2020), it could be related to the pathophysiology of depression in both males and females, in particular concerning depressive- and anxiety-like symptoms displayed equally by both sexes in our work. However, there are still few studies on adolescent depression and BDNF levels in both sexes and it is necessary to better understand the mechanisms underlying the development of adolescent depression (Lee et al., 2022).

Adolescent isolation leads also to inflammatory profile alterations in terms of hippocampal expression levels of IL-1β and IL-10. These findings are in line with the studies of Donovan and collaborators showing that post-weaning social isolation reduces IL-1β in male and female prairie voles (Donovan et al., 2021), and Berry et collaborators, showing lower mRNA levels of Il-1β in the dorsal hippocampus of rats exposed to prenatal stress (Berry et al., 2022). In addition, Panetta and colleagues correlate the lower levels of hippocampal IL-1β to increased anxiety-related behavior in the elevated plus maze and impaired neural plasticity in terms of decreased BDNF levels (Panetta et al., 2017). It is important to note that cytokines are also constitutively released in the healthy brain by resident myeloid cells to keep proper synaptic plasticity, as the modulation of LTP and synaptic scaling (a form of homeostatic plasticity) (Rizzo et al., 2018; Salim et al., 2012). Thus, both immune activation and suppression impair synaptic plasticity and could lead to psychopathologies in mice (Alboni et al., 2016; Alboni et al., 2017; Golia et al., 2019). This mechanism could explain the link between the behavioral abnormalities and the significant reduction of pro-inflammatory cytokine IL-1β and increased levels of anti-inflammatory cytokine IL-10 after adolescent social stress. Lopizzo and colleagues show how the alterations in ‘inflammatory’ pathways in the rat prefrontal cortex and hippocampus could be a predictor of the negative consequences of early life stress exposure, in particular pathways related to IL-10, neuroplasticity, and

neurogenesis (Lopizzo et al., 2021). Finally, since TNF-α, IL-1β and IL-6 are the main activators of the HPA axis and are in turn modulated (inhibited) by glucocorticoid hormones (Besedovsky and del Rey, 2000), the increased levels of corticosterone reached by isolated mice could induce a reduction in cytokines levels through this feedback mechanism, in particular in male subjects.

In conclusion, social experiences in adolescence represent a key factor in the neurodevelopment of individuals, and the lack of social interaction triggers psychopathologies in both sexes. The different vulnerability to depression observed in individuals of the two sexes could be ascribed not to cultural and social influences but to their different responses to the same environmental stimuli leading to distinct stress response activation, but not to different neural plasticity and inflammatory pattern activation between sexes.

References

Alboni, S., Poggini, S., Garofalo, S., Milior, G., El Hajj, H., Lecours, C., Girard, I., Gagnon, S., Boisjoly-Villeneuve, S., Brunello, N.*, et al.* (2016). Fluoxetine treatment affects the inflammatory response and microglial function according to the quality of the living environment. Brain, behavior, and immunity *58*, 261-271.

Alboni, S., van Dijk, R.M., Poggini, S., Milior, G., Perrotta, M., Drenth, T., Brunello, N., Wolfer, D.P., Limatola, C., Amrein, I.*, et al.* (2017). Fluoxetine effects on molecular, cellular and behavioral endophenotypes of depression are driven by the living environment. Molecular psychiatry *22*, 552-561.

Almeida, I.L.L., Rego, J.F., Teixeira, A.C.G., and Moreira, M.R. (2021). Social isolation and its impact on child and adolescent development: a systematic review. Revista paulista de pediatria : orgao oficial da Sociedade de Pediatria de Sao Paulo *40*, e2020385.

Amiri, S., Haj-Mirzaian, A., Rahimi-Balaei, M., Razmi, A., Kordjazy, N., Shirzadian, A., Ejtemaei Mehr, S., Sianati, H., and Dehpour, A.R. (2015). Co-occurrence of anxiety and depressive-like behaviors following adolescent social isolation in male mice; possible role of nitrergic system. Physiology & behavior *145*, 38-44.

Andersen, S.L. (2016). Commentary on the special issue on the adolescent brain: Adolescence, trajectories, and the importance of prevention. Neuroscience and biobehavioral reviews *70*, 329-333.

Andrews, J.L., Ahmed, S.P., and Blakemore, S.J. (2021). Navigating the Social Environment in Adolescence: The Role of Social Brain Development. Biological psychiatry *89*, 109-118.

Antal, A., Chaieb, L., Moliadze, V., Monte-Silva, K., Poreisz, C., Thirugnanasambandam, N., Nitsche, M.A., Shoukier, M., Ludwig, H., and Paulus, W. (2010). Brain-derived neurotrophic factor (BDNF) gene polymorphisms shape cortical plasticity in humans. Brain stimulation *3*, 230-237.

Berry, A., Mazzelli, M., Musillo, C., Riva, M.A., Cattaneo, A., and Cirulli, F. (2022). High-fat diet during adulthood interacts with prenatal stress, affecting both

brain inflammatory and neuroendocrine markers in male rats. The European journal of neuroscience *55*, 2326-2340.

Besedovsky, H.O., and del Rey, A. (2000). The cytokine-HPA axis feed-back circuit. Zeitschrift fur Rheumatologie *59 Suppl 2*, II/26-30.

Blakemore, S.J. (2019). Adolescence and mental health. Lancet *393*, 2030-2031.

Branje, S., and Morris, A.S. (2021). The Impact of the COVID-19 Pandemic on Adolescent Emotional, Social, and Academic Adjustment. Journal of research on adolescence : the official journal of the Society for Research on Adolescence *31*, 486-499.

Bremner, J.D., Vythilingam, M., Vermetten, E., Anderson, G., Newcomer, J.W., and Charney, D.S. (2004). Effects of glucocorticoids on declarative memory function in major depression. Biological psychiatry *55*, 811-815.

Burstein, O., and Doron, R. (2018). The Unpredictable Chronic Mild Stress Protocol for Inducing Anhedonia in Mice. Journal of visualized experiments : JoVE.

Byrne, M.L., O'Brien-Simpson, N.M., Mitchell, S.A., and Allen, N.B. (2015). Adolescent-Onset Depression: Are Obesity and Inflammation Developmental Mechanisms or Outcomes? Child psychiatry and human development *46*, 839-850.

Chen, J., Li, X., and McGue, M. (2012). Interacting effect of BDNF Val66Met polymorphism and stressful life events on adolescent depression. Genes, brain, and behavior *11*, 958-965.

Christiansen, J., Qualter, P., Friis, K., Pedersen, S.S., Lund, R., Andersen, C.M., Bekker-Jeppesen, M., and Lasgaard, M. (2021). Associations of loneliness and social isolation with physical and mental health among adolescents and young adults. Perspectives in public health *141*, 226-236.

Conley, C.S., and Rudolph, K.D. (2009). The emerging sex difference in adolescent depression: interacting contributions of puberty and peer stress. Development and psychopathology *21*, 593-620.

Cyrenne, D.L., and Brown, G.R. (2011). Ontogeny of sex differences in response to novel objects from adolescence to adulthood in lister-hooded rats. Developmental psychobiology *53*, 670-676.

Donovan, M.L., Chun, E.K., Liu, Y., and Wang, Z. (2021). Post-weaning Social Isolation in Male and Female Prairie Voles: Impacts on Central and Peripheral Immune System. Frontiers in behavioral neuroscience *15*, 802569.

Dow-Edwards, D., MacMaster, F.P., Peterson, B.S., Niesink, R., Andersen, S., and Braams, B.R. (2019). Experience during adolescence shapes brain development: From synapses and networks to normal and pathological behavior. Neurotoxicology and teratology *76*, 106834.

Drummond, K.D., Waring, M.L., Faulkner, G.J., Blewitt, M.E., Perry, C.J., and Kim, J.H. (2021). Hippocampal neurogenesis mediates sex-specific effects of social isolation and exercise on fear extinction in adolescence. Neurobiology of stress *15*, 100367.

Elakovic, I., Djordjevic, A., Adzic, M., Djordjevic, J., Radojcic, M., and Matic, G. (2011). Gender-specific response of brain corticosteroid receptors to stress and fluoxetine. Brain research *1384*, 61-68.

Eltokhi, A., Kurpiers, B., and Pitzer, C. (2021). Baseline Depression-Like Behaviors in Wild-Type Adolescent Mice Are Strain and Age but Not Sex Dependent. Frontiers in behavioral neuroscience *15*, 759574.

Golia, M.T., Poggini, S., Alboni, S., Garofalo, S., Ciano Albanese, N., Viglione, A., Ajmone-Cat, M.A., St-Pierre, A., Brunello, N., Limatola, C*., et al.* (2019). Interplay between inflammation and neural plasticity: Both immune activation and suppression impair LTP and BDNF expression. Brain, behavior, and immunity *81*, 484-494.

Granata, L., Gildawie, K.R., Ismail, N., Brenhouse, H.C., and Kopec, A.M. (2022). Immune signaling as a node of interaction between systems that sex-specifically develop during puberty and adolescence. Developmental cognitive neuroscience *57*, 101143.

Grewal, S.S., Shepherd, J.K., Bill, D.J., Fletcher, A., and Dourish, C.T. (1997). Behavioural and pharmacological characterisation of the canopy stretched attend posture test as a model of anxiety in mice and rats. Psychopharmacology *133*, 29-38.

Haj-Mirzaian, A., Amiri, S., Kordjazy, N., Rahimi-Balaei, M., Haj-Mirzaian, A., Marzban, H., Aminzadeh, A., Dehpour, A.R., and Mehr, S.E. (2015). Blockade of NMDA receptors reverses the depressant, but not anxiogenic effect of adolescence social isolation in mice. European journal of pharmacology *750*, 160-166.

Handa, R.J., and Weiser, M.J. (2014). Gonadal steroid hormones and the hypothalamo-pituitary-adrenal axis. Frontiers in neuroendocrinology *35*, 197-220.

Hellemans, K.G., Benge, L.C., and Olmstead, M.C. (2004). Adolescent enrichment partially reverses the social isolation syndrome. Brain research Developmental brain research *150*, 103-115.

Herbert, J., Goodyer, I.M., Grossman, A.B., Hastings, M.H., de Kloet, E.R., Lightman, S.L., Lupien, S.J., Roozendaal, B., and Seckl, J.R. (2006). Do corticosteroids damage the brain? Journal of neuroendocrinology *18*, 393-411.

Huang, Q., Zhou, Y., and Liu, L.Y. (2017). Effect of post-weaning isolation on anxiety- and depressive-like behaviors of C57BL/6J mice. Experimental brain research *235*, 2893-2899.

Jameison, K., and Dinan, T.G. (2001). Glucocorticoids and cognitive function: from physiology to pathophysiology. Human psychopharmacology *16*, 293-302.

Kitchener, P., Di Blasi, F., Borrelli, E., and Piazza, P.V. (2004). Differences between brain structures in nuclear translocation and DNA binding of the glucocorticoid receptor during stress and the circadian cycle. The European journal of neuroscience *19*, 1837-1846.

Knight, P., Chellian, R., Wilson, R., Behnood-Rod, A., Panunzio, S., and Bruijnzeel, A.W. (2021). Sex differences in the elevated plus-maze test and large open field test in adult Wistar rats. Pharmacology, biochemistry, and behavior *204*, 173168.

Koike, H., Ibi, D., Mizoguchi, H., Nagai, T., Nitta, A., Takuma, K., Nabeshima, T., Yoneda, Y., and Yamada, K. (2009). Behavioral abnormality and

pharmacologic response in social isolation-reared mice. Behavioural brain research *202*, 114-121.

Kokras, N., and Dalla, C. (2014). Sex differences in animal models of psychiatric disorders. British journal of pharmacology *171*, 4595-4619.

Kraeuter, A.K., Guest, P.C., and Sarnyai, Z. (2019). The Open Field Test for Measuring Locomotor Activity and Anxiety-Like Behavior. Methods in molecular biology *1916*, 99-103.

Lee, B., Shin, E., Song, I., and Chang, B. (2022). Depression in Adolescence and Brain-Derived Neurotrophic Factor. Frontiers in molecular neuroscience *15*, 947192.

Lee, J., Lee, K.H., Kim, S.H., Han, J.Y., Hong, S.B., Cho, S.C., Kim, J.W., and Brent, D. (2020). Early changes of serum BDNF and SSRI response in adolescents with major depressive disorder. Journal of affective disorders *265*, 325-332.

Leone, M., Kuja-Halkola, R., Leval, A., D'Onofrio, B.M., Larsson, H., Lichtenstein, P., and Bergen, S.E. (2021). Association of Youth Depression With Subsequent Somatic Diseases and Premature Death. JAMA psychiatry *78*, 302-310.

Levy, M.J.F., Boulle, F., Steinbusch, H.W., van den Hove, D.L.A., Kenis, G., and Lanfumey, L. (2018). Neurotrophic factors and neuroplasticity pathways in the pathophysiology and treatment of depression. Psychopharmacology *235*, 2195-2220.

Liu, J.H., You, Q.L., Wei, M.D., Wang, Q., Luo, Z.Y., Lin, S., Huang, L., Li, S.J., Li, X.W., and Gao, T.M. (2015). Social Isolation During Adolescence Strengthens Retention of Fear Memories and Facilitates Induction of Late-Phase Long-Term Potentiation. Molecular neurobiology *52*, 1421-1429.

Liu, N., Wang, Y., An, A.Y., Banker, C., Qian, Y.H., and O'Donnell, J.M. (2020). Single housing-induced effects on cognitive impairment and depression-like behavior in male and female mice involve neuroplasticity-related signaling. The European journal of neuroscience *52*, 2694-2704.

Liu, S.R., Davis, E.P., Palma, A.M., Sandman, C.A., and Glynn, L.M. (2022). The acute and persisting impact of COVID-19 on trajectories of adolescent

depression: Sex differences and social connectedness. Journal of affective disorders *299*, 246-255.

Loades, M.E., Chatburn, E., Higson-Sweeney, N., Reynolds, S., Shafran, R., Brigden, A., Linney, C., McManus, M.N., Borwick, C., and Crawley, E. (2020). Rapid Systematic Review: The Impact of Social Isolation and Loneliness on the Mental Health of Children and Adolescents in the Context of COVID-19. Journal of the American Academy of Child and Adolescent Psychiatry *59*, 1218-1239 e1213.

Lopez, M.F., and Laber, K. (2015). Impact of social isolation and enriched environment during adolescence on voluntary ethanol intake and anxiety in C57BL/6J mice. Physiology & behavior *148*, 151-156.

Lopizzo, N., Mazzelli, M., Zonca, V., Begni, V., D'Aprile, I., Cattane, N., Pariante, C.M., Riva, M.A., and Cattaneo, A. (2021). Alterations in 'inflammatory' pathways in the rat prefrontal cortex as early biological predictors of the long-term negative consequences of exposure to stress early in life. Psychoneuroendocrinology *124*, 104794.

Maisonnette, S., Morato, S., and Brandao, M.L. (1993). Role of resocialization and of 5-HT1A receptor activation on the anxiogenic effects induced by isolation in the elevated plus-maze test. Physiology & behavior *54*, 753-758.

Mariani, N., Cattane, N., Pariante, C., and Cattaneo, A. (2021). Gene expression studies in Depression development and treatment: an overview of the underlying molecular mechanisms and biological processes to identify biomarkers. Translational psychiatry *11*, 354.

McCormick, C.M., Smith, C., and Mathews, I.Z. (2008). Effects of chronic social stress in adolescence on anxiety and neuroendocrine response to mild stress in male and female rats. Behavioural brain research *187*, 228-238.

Medendorp, W.E., Petersen, E.D., Pal, A., Wagner, L.M., Myers, A.R., Hochgeschwender, U., and Jenrow, K.A. (2018). Altered Behavior in Mice Socially Isolated During Adolescence Corresponds With Immature Dendritic Spine Morphology and Impaired Plasticity in the Prefrontal Cortex. Frontiers in behavioral neuroscience *12*, 87.

Mills, N.T., Scott, J.G., Wray, N.R., Cohen-Woods, S., and Baune, B.T. (2013). Research review: the role of cytokines in depression in adolescents: a systematic review. Journal of child psychology and psychiatry, and allied disciplines *54*, 816-835.

Nomoto, H., Baba, H., Satomura, E., Maeshima, H., Takebayashi, N., Namekawa, Y., Suzuki, T., and Arai, H. (2015). Serum brain-derived neurotrophic factor levels and personality traits in patients with major depression. BMC psychiatry *15*, 33.

Orben, A., Tomova, L., and Blakemore, S.J. (2020). The effects of social deprivation on adolescent development and mental health. The Lancet Child & adolescent health *4*, 634-640.

Pais, A.B., Pais, A.C., Elmisurati, G., Park, S.H., Miles, M.F., and Wolstenholme, J.T. (2019). A Novel Neighbor Housing Environment Enhances Social Interaction and Rescues Cognitive Deficits from Social Isolation in Adolescence. Brain sciences *9*.

Pandey, G.N., Dwivedi, Y., Rizavi, H.S., Ren, X., Zhang, H., and Pavuluri, M.N. (2010). Brain-derived neurotrophic factor gene and protein expression in pediatric and adult depressed subjects. Progress in neuro-psychopharmacology & biological psychiatry *34*, 645-651.

Panetta, P., Berry, A., Bellisario, V., Capoccia, S., Raggi, C., Luoni, A., Longo, L., Riva, M.A., and Cirulli, F. (2017). Long-Term Sex-Dependent Vulnerability to Metabolic challenges in Prenatally Stressed Rats. Frontiers in behavioral neuroscience *11*, 113.

Pierre, A., Regin, Y., Van Schuerbeek, A., Fritz, E.M., Muylle, K., Beckers, T., Smolders, I.J., Singewald, N., and De Bundel, D. (2019). Effects of disrupted ghrelin receptor function on fear processing, anxiety and saccharin preference in mice. Psychoneuroendocrinology *110*, 104430.

Pitzer, C., Kurpiers, B., and Eltokhi, A. (2022). Sex Differences in Depression-Like Behaviors in Adult Mice Depend on Endophenotype and Strain. Frontiers in behavioral neuroscience *16*, 838122.

Poggini, S., Matte Bon, G., Golia, M.T., Ciano Albanese, N., Viglione, A., Poleggi, A., Limatola, C., Maggi, L., and Branchi, I. (2021). Selecting antidepressants

according to a drug-by-environment interaction: A comparison of fluoxetine and minocycline effects in mice living either in enriched or stressful conditions. Behavioural brain research *408*, 113256.

Pyter, L.M., Yang, L., da Rocha, J.M., and Engeland, C.G. (2014). The effects of social isolation on wound healing mechanisms in female mice. Physiology & behavior *127*, 64-70.

Racine, N., McArthur, B.A., Cooke, J.E., Eirich, R., Zhu, J., and Madigan, S. (2021). Global Prevalence of Depressive and Anxiety Symptoms in Children and Adolescents During COVID-19: A Meta-analysis. JAMA pediatrics *175*, 1142-1150.

Rivera-Irizarry, J.K., Skelly, M.J., and Pleil, K.E. (2020). Social Isolation Stress in Adolescence, but not Adulthood, Produces Hypersocial Behavior in Adult Male and Female C57BL/6J Mice. Frontiers in behavioral neuroscience *14*, 129.

Rivier, C., and Vale, W. (1987). Diminished responsiveness of the hypothalamic-pituitary-adrenal axis of the rat during exposure to prolonged stress: a pituitary-mediated mechanism. Endocrinology *121*, 1320-1328.

Rizzo, F.R., Musella, A., De Vito, F., Fresegna, D., Bullitta, S., Vanni, V., Guadalupi, L., Stampanoni Bassi, M., Buttari, F., Mandolesi, G.*, et al.* (2018). Tumor Necrosis Factor and Interleukin-1beta Modulate Synaptic Plasticity during Neuroinflammation. Neural plasticity *2018*, 8430123.

Ros-Simo, C., and Valverde, O. (2012). Early-life social experiences in mice affect emotional behaviour and hypothalamic-pituitary-adrenal axis function. Pharmacology, biochemistry, and behavior *102*, 434-441.

Salim, S., Chugh, G., and Asghar, M. (2012). Inflammation in anxiety. Advances in protein chemistry and structural biology *88*, 1-25.

Salk, R.H., Hyde, J.S., and Abramson, L.Y. (2017). Gender differences in depression in representative national samples: Meta-analyses of diagnoses and symptoms. Psychological bulletin *143*, 783-822.

Sawyer, S.M., Azzopardi, P.S., Wickremarathne, D., and Patton, G.C. (2018). The age of adolescence. The Lancet Child & adolescent health *2*, 223-228.

Ulrich-Lai, Y.M., and Herman, J.P. (2009). Neural regulation of endocrine and autonomic stress responses. Nature reviews Neuroscience *10*, 397-409.

Viduani, A., Benetti, S., Martini, T., Buchweitz, C., Ottman, K., Wahid, S.S., Fisher, H.L., Mondelli, V., Kohrt, B.A., and Kieling, C. (2021). Social isolation as a core feature of adolescent depression: a qualitative study in Porto Alegre, Brazil. International journal of qualitative studies on health and well-being *16*, 1978374.

Workman, J.L., Fonken, L.K., Gusfa, J., Kassouf, K.M., and Nelson, R.J. (2011). Post-weaning environmental enrichment alters affective responses and interacts with behavioral testing to alter nNOS immunoreactivity. Pharmacology, biochemistry, and behavior *100*, 25-32.

Yang, T., Nie, Z., Shu, H., Kuang, Y., Chen, X., Cheng, J., Yu, S., and Liu, H. (2020). The Role of BDNF on Neural Plasticity in Depression. Frontiers in cellular neuroscience *14*, 82.

Zhang, M., Liu, Y., Zhao, M., Tang, W., Wang, X., Dong, Z., and Yu, S. (2017). Depression and anxiety behaviour in a rat model of chronic migraine. The journal of headache and pain *18*, 27.

Supplementary materials

Body weight

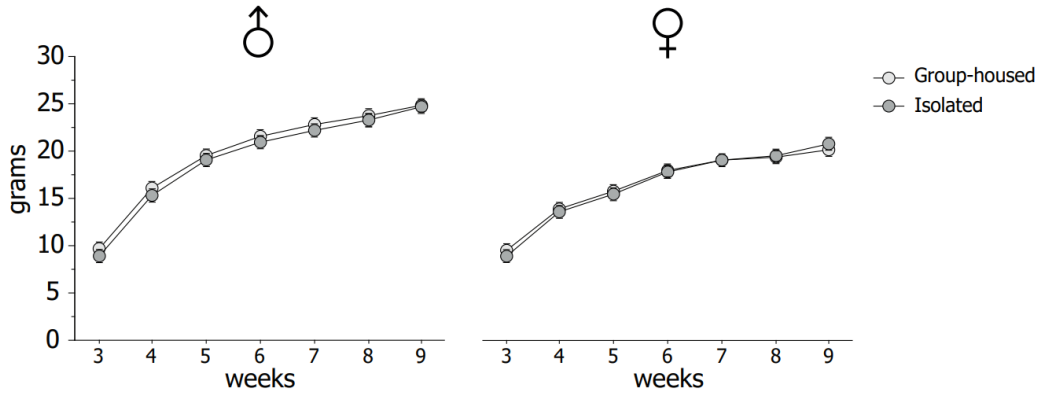

Figure S1. Body weight. *No difference emerged concerning body weight between isolated and group-housed mice both in males* (main effect, $F_{1,23} = 1.391$, $p = 0.2503$) *and females* (main effect, $F_{1,23} = 0.033$, $p = 0.8584$). *Data are shown as mean* ± S.E.M.

Supplementary table 1

Primers used for Real Time RT-PCR analysis

Gene	Forward	Reverse
IL-1β	GCAGCTACCTGTGTCTTTCC	TGTCCATTGAGGTGGAGAGC
IL-6	TCCCTACTTCACAAGTCCGG	CCAGGTAGCTATGGTACTCC
IL-10	TGACTGGCATGAGGATCAGC	TTCTGGGCCATGCTTCTCTG
TNF-α	AAACCACCAAGTGGAGGAGC	GATAGCAAATCGGCTGACGG
BDNF	TTACCTTCCTGCATCTGTTGG	GTCATCACTCTTCTCACCTGG
PSD95	TCAGGGTCAACGACAGCATC	TGCGATGCTGAAGCCAAGTC
ACT β	TGACGTTGACATCCGTAAAG	GAGGAGCAATGATCTTGATC
B2M	GGGAAGCCGAACATACTG	TGCTTAACTCTGCAGGCG

Table 1. Primers used for Real Time RT-PCR analysis. *The pairs of primers used in this study were designed (with the help of online software) in such a way as to be as specific as possible for the regions being investigated.*

STUDY 2

The interaction between antidepressants and environment determines treatment outcome in a preclinical model of adolescence-onset depression

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In preparation

Abstract

Adolescence is a sensitive phase where adverse experiences, such as a lack of appropriate social interactions, increase the vulnerability to depressive episodes and to the onset of treatment-resistant major depressive disorder in adulthood. The first-line treatment for depression is selective serotonin reuptake inhibitors (SSRIs), but their efficacy is incomplete. Recently, to explain such variable effects, it has been proposed that SSRIs do not affect mood per se, but by enhancing neural plasticity, amplify the influences of the living environment on mood. Therefore, we hypothesized that a supportive environment is critical for recovery and antidepressant treatment can further enhance such beneficial action. To test this hypothesis, we compared the efficacy of the exposure to an enriched environment, the SSRI fluoxetine administration, and their combination in depressed-like adult mice. From weaning to adulthood, C57BL/6 male mice have been exposed to either standard social housing or social isolation to induce a depressive-like phenotype. In adulthood, socially housed mice received vehicle while exposed to an enriched environment (Control group). By contrast, isolated subjects received either fluoxetine or vehicle while exposed to a standard or enriched environment. We assessed the effects of the different treatment strategies on the cognitive and emotional domains, and stress hormone levels. Our results showed that environmental enrichment alone or in combination with fluoxetine, but not fluoxetine alone, counterbalanced the detrimental effects of isolation on the depression-like phenotype. Our findings outline the relevance of environmental interventions alone or in combination with SSRI to treat adolescence-onset depression.

Keywords

Major depression disorder, adolescence, treatment-resistant, SSRI, social experience, environment

Introduction

Major depressive disorder in adolescents constitutes a huge medical and social issue and represents the second leading cause of death among teenagers (Costello et al., 2005; Windfuhr et al., 2008). In addition, the onset of depression in adolescence is associated with later depressive episodes and treatment-resistance in adulthood (Hill et al., 2014; Johnson et al., 2018; Qualter et al., 2010). Indeed, several clinical studies have shown that depressed adolescents are more likely than their non-depressed peers to subsequently have depression, as well as non-mood disorders (Fergusson and Woodward, 2002; Harrington et al., 1990; Lewinsohn et al., 1999; Rao et al., 1995).

Adverse early life events, associated with a dysfunctional socioemotional environment, predict the onset of major depressive disorder in adolescence and its recurrence (Lewinsohn et al., 2001; Lewinsohn et al., 1999). For instance, adolescent interpersonal relationships and peer interaction have been linked to anxiety and depression till adulthood (Jacobson and Newman, 2016). Therefore, adversities involving the lack of appropriate emotional and social experiences during adolescence jeopardize individual development and can trigger psychopathologies, such as major depressive disorder (Almeida et al., 2021; Viduani et al., 2021).

The first-line treatment for depression is selective serotonin reuptake inhibitors (SSRIs), but their efficacy is variable and incomplete (Trivedi et al., 2006). In double-blind, randomized controlled trials testing the efficacy of antidepressants, SSRIs demonstrate small yet statistically significant advantages over placebo but do not substantially improve patient symptoms. In particular, placebo response rates range from 30% to 50%; this high percentage occurs partly because some patients show a spontaneous remission from depression during the 6-8 weeks of the drug trial, and partly because of the power of the placebo effect, which is particularly noticeable in the management of psychiatric illness (Le Noury et al., 2015). Recently, two meta-analyses confirmed this evidence showing a small therapeutic effect for all antidepressants with fluoxetine being the only antidepressant to have a statistically significant effect over placebo on efficacy for the treatment of depression (Cipriani et al.,

2016; Mullen, 2018; Vitiello and Ordonez, 2016). Therefore, it is becoming increasingly necessary to find more efficient and less variable antidepressant treatments.

To explain such variable and incomplete efficacy, a novel hypothesis – the *undirected susceptibility to change hypothesis* – has been recently proposed. It posits that the increase in serotonin levels induced by SSRIs does not affect mood *per se* but, by enhancing neural plasticity, amplifies the influence of the environment on mood. Thus, SSRI outcome depends on the quality of the living environment (Branchi, 2011; Branchi and Giuliani, 2021). This hypothesis has been recently demonstrated both at preclinical and clinical levels. In a mouse model of depression, treatment with the SSRI fluoxetine led to an improvement of depressive phenotype in a favorable environment, while having a limited efficacy or even leading to a worsening in a stressful environment (Alboni et al., 2016; Branchi et al., 2013; Poggini et al., 2019). In patients, the SSRI citalopram has been demonstrated to amplify the influence of the living conditions on mood, improving depressive symptoms according to socioeconomic status (Chiarotti et al., 2017; Viglione et al., 2019).

Because of the environment-dependent outcome of SSRIs, here we hypothesized that treatment with serotoninergic antidepressants for adolescence-onset depression has to be administered in a supportive environment to have the highest efficacy. To test this hypothesis, we compared the efficacy of the SSRI fluoxetine, the exposure to an enriched environment, and their combination in adult mice displaying a depressive-like phenotype induced via social isolation during adolescence. From weaning to adulthood, C57BL/6 male mice have been exposed to either standard social housing or social isolation to induce a depressive-like phenotype. In adulthood, socially housed mice received vehicle while exposed to an enriched environment (Controls). By contrast, isolated subjects received either fluoxetine or vehicle while exposed to a standard or enriched environment. To assess the effects of the different treatment strategies, we phenotyped the behavioral response in the emotional and cognitive domains through a battery of tests, including open field, elevated plus maze, forced swim test, novel object recognition, and fear conditioning test.

In addition, as a physiological endpoint of the stress response, we assessed the circulating levels of corticosterone. We expected first that adolescent isolation induces both depressive- and anxiety-like behavior, then that fluoxetine effects are environment-dependent and the combination of both environmental enrichment and fluoxetine, but not fluoxetine alone, counterbalanced the detrimental effects of isolation.

Materials and methods

Ethical standards

All procedures were carried out in accordance with the European and Italian legislation on animal experimentation (respectively European Directive 2010/63/UE and Decreto Legislativo 26/2014). Animals were examined for signs of discomfort as indicated by the animal care and use guidelines [National Academy of Sciences. Guide for the care and use of laboratory animals, 1998, ‘‘Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research” (National Research Council 2003)]. The Italian Ministry of Health approved the protocol with permit number D9997.111.

Animals and breeding procedure

13 male and 36 female C57BL/6 mice weighing 16-21 g (5 weeks old) were purchased from Charles River Laboratories placed in Calco (Lecco, Italy). Animals were housed under reversed 12 light-dark cycle (07.00-19.00) in an air-conditioned room (temperature 21±1 °C, relative humidity 60±10%). Males and females were housed in same-sex groups of 4-5 individuals in 42.5 x 27.6 x 15.3 Plexiglas cages with a metal top and sawdust as bedding, with pellet food (Enriched standard diet, Riper, Vendoies, BZ, Italy) and tap water *ad libitum*. Each cage was provided with tissue paper (300 x 200 mm). When animals were 9 weeks old, two females and one male were mated for 8 consecutive days. Afterward, males were removed from the breeding cages, and each female was singly housed for the entire duration of pregnancy until the pups’ weaning, postnatal day (PND) 21. On PND-1 litters were culled to 3 male and 3 female pups and a cross-fostering procedure was performed to limit the number of mates needed.

The paradigm of chronic stress: post-weaning social isolation protocol

On PND 21, male mice were selected and assigned to the two experimental groups isolated or group-housed. Isolated mice were singly housed in a cage of 29 x 22 x 14 cm. Grouped mice were housed 4/cage with a same-sex conspecific

(42.5 x 27.6 x 15.3 cm). To identify each subject, we marked the animals with an ear punch. After 6 weeks of social isolation (PND 60), the isolated mice were subjected to resocialization in group-housed conditions (four *per* cage), while group-housed mice (i.e. Controls) maintained the conditions mentioned above. During behavioral analysis, the previous housing conditions were maintained.

Experimental design

From weaning (PND 21) up to adulthood (PND 60) mice underwent social isolation to induce a depressive- and anxiety-like phenotype or group-housing. At the end of the isolation period, mice were exposed to treatments till PND 120. In particular, a total of 67 male mice were selected, 55 of them were subjected to the social isolation protocol, while 12 were exposed to an enriched environment and treated with vehicles representing the Control group. The group of mice socially isolated during adolescence was randomly divided into 4 experimental groups, exposed to either standard or enriched environment in combination with fluoxetine or vehicle treatment: 12 mice treated with fluoxetine and enriched environment, 14 mice in an enriched environment treated with vehicle, 13 mice in standard-housed condition and treated with fluoxetine, 9 mice in standard-housed condition and treated with vehicle. Behavioral tests started after 14 days of fluoxetine treatment (Fig.1A).

Environmental conditions

Standard environment

In the standard condition, mice were housed in groups of 4 Plexiglas cages with a metal top and sawdust as bedding, with pellet food and tap water, and saccharine *ad libitum*. Each cage was provided with tissue paper.

Enriched environment

In the enriched condition, in addition to the food, water, saccharine *ad libitum,* and tissue papers, mice were given several objects with which mice could interact, such as paper tubes, nesting material, a rearrangeable set of plastic tubes, paper rolls, plastic shelters, rubber rings, and bells and balls (Fig.1B). To

maintain a stimulating effect of environmental enrichment, except for paper shelter and tissue, the objects were changed twice a week.

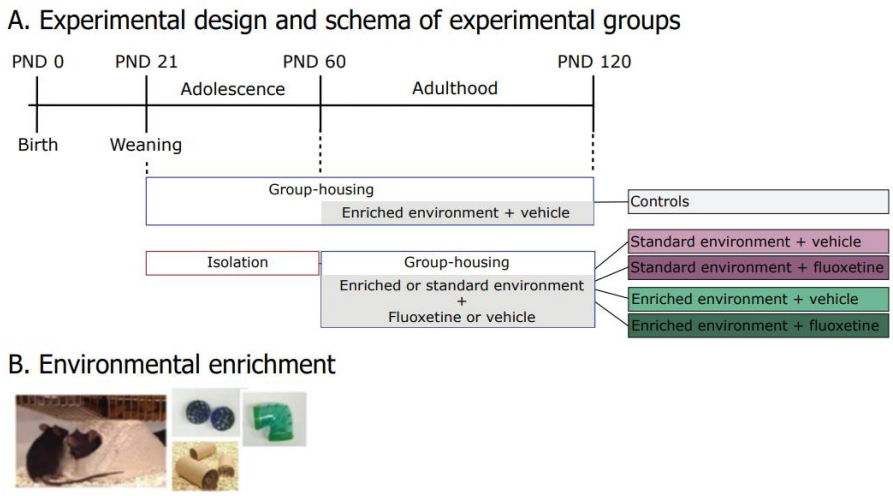

Figure 5. Experimental design, schema of experimental groups, and environmental enrichment. (**A**) From weaning (PND 21) to adulthood (PND 60), male mice were exposed either to a group-housing condition (4 individuals per cage) or to a social isolation protocol, to induce a depressive-like phenotype. Therefore, Controls have been exposed to an enriched environment while treated with vehicle. By contrast, isolated mice in adulthood have been group-housed while exposed either to a standard or enriched environment and treated with the SSRI fluoxetine or vehicle, in order to obtain four experimental groups: Standard+vehicle, Standard+fluoxetine, Enrichment+vehicle, and Enrichment+fluoxetine. We evaluated the effects of the interventions on both emotional and cognitive domains from PND 90 to PND120, then the animals have been sacrificed, and central and peripheral tissues collected. (**B**) Environmental enrichment. Mice were exposed to several objects such as paper tubes, nesting material, a rearrangeable set of plastic tubes, paper rolls, plastic shelters, rubber rings, and bells and balls. The objects were changed twice a week.

Fluoxetine treatment

After the first 6 weeks of social isolation aimed at inducing a depression-like phenotype, mice received for 60 days fluoxetine or vehicle while they were exposed either to an enriched or standard environment. To avoid the stress due to the manipulation, fluoxetine (Fluoxetine HCl, SantaCruz, USA) was dissolved in water and saccharin solution avoiding that saccharin preference could affect the amount of drug received. The solutions were prepared according to the average weight and daily water consumption previously assessed of the mice, to provide an average daily intake of 10 mg/kg. Bottles were wrapped in tin foil to

protect the substance from light. Fluoxetine dose has been selected according to the results previously obtained with the same pharmaceutical form and route of administration in C57BL/6 mice (Branchi et al., 2013; Dulawa et al., 2004; Iniguez et al., 2014a).

Behavioral tests

Starting from 14 days of treatment, we assessed the depressive-like profile of the animals, through the forced swim test, while the anxiety-like profile and the spontaneous exploration through the open field and elevated plus-maze test. Novel object recognition and fear conditioning tests were selected to evaluate cognitive abilities. All tests were carried out during the dark period (between 09.30 and 16.00 h). Animals were transferred to the experimental room at least 30 min before the test to let them acclimatize to the new environment. All the tests were video-recorded using a digital video camera (CANON LEGRIA HFR86). The behavioral scoring was carried out using the commercial software Observer XT (Noldus, Wageningen, The Netherlands) and AnyMaze software (Stoelting Europe).

Open field test

At PND 83 we performed the open field test. Mice were allowed to explore the apparatus, consisting of an empty black box (40 x 40 x 50 cm) for 15 minutes under indirect dim light (10 lux). The central area was defined as a central 20 cm × 20 cm square, and the other region was defined as the peripheral area. Mice were placed in the periphery with the snout facing the wall, and we scored the *total distance traveled* in the arena, the percentage of *time spent in the periphery* calculated as follows [(periphery/total) ×100], and the latency of *first entry in the center* (entry in center = all four paws into the center).

Novel object recognition test

At PND 84 we performed the novel object recognition test. Mice were tested individually in an empty black box (40 x 40 x 50 cm) under indirect dim light (10

lux). The test consisted of two trials, each of 10 minutes, with an inter-trial interval of 2 hours. Twenty-four hours before testing, subjects were familiarized with the arena during a 15-minutes pre-exposure session. During the first trial, the familiarization phase, a single animal is placed in the open-field arena containing two identical sample objects [glass (G) or lego (L)], for 10 minutes, as the following objects scheme: G-G or L-L. Plastic or glass objects of identical volume and different shapes constituted the stimuli. Objects and object locations were counterbalanced to avoid a potential object and/or location bias. To prevent coercion to explore the objects, rodents were released against the center of the opposite wall with their back to the objects. During the second trial, corresponding to the test phase, the animal is exposed to two objects, one is identical to the sample and the other is novel (i.e. G-L). During both the familiarization and the test phases, objects were located in opposite and symmetrical corners of the center of the arena, and the location of novel versus familiar objects is counterbalanced. A *discrimination index*, calculated as [(A-B)/(A+B)] in which *A* represents the time exploring the novel object and *B* is the time exploring the familiar one, was used to measure the novel object exploration. This index can vary between +1 and −1, where a positive score indicates more time spent with the novel object, a negative score indicates more time spent with the familiar object, and a zero score indicates a null preference. Exploration of an object was defined as directing the nose towards an object at a distance of less than 1 cm and/or touching the object with the nose and/or paws. Sitting on the objects was not considered exploratory behavior. Alcohol solution (70%) was used to remove odors in the novel object recognition apparatus between all sessions.

Elevated plus-maze

To assess anxiety-related and explorative behavior, at PND 89 we performed the elevated plus-maze test. The elevated plus-maze comprises two open arms (30 x 5 cm) and two closed arms (30 x 5 x 15 cm) that extended from a common central platform (5 x 5 cm). The apparatus, made of Plexiglas (gray floor, clear walls), was elevated to a height of 60 cm above the floor level. Mice were individually placed on the central platform facing an open arm and allowed

to explore the maze for 5 minutes under indirect dim light (10 lux). Behavioral parameters observed were the percentage of *time spent in the open arms* [(open/total) ×100] and latency of *the first open arm entry* (arm entry = all four paws into an arm). Furthermore, we scored the percentage of *time spent performing the stretched attend postures* (s.a.p.), an exploratory posture in which the body is stretched forward and then retracted to the original position without any forward locomotion, and the percentage of *time spent performing the head-dipping behavior*, an exploratory posture in which mice scanning their head over the sides of the maze towards the floor, both considered two important components of the risk-assessment repertoire of mouse defensive behavior (Fernandez Espejo, 1997).

Forced swim test

At PND 95 mice were tested according to the Porsolt's procedure (Porsolt et al., 1977). Each experimental subject was gently placed into a cylindrical glass container (20 cm diameter, 40 cm height), filled with 25 cm of water at a temperature of 26 ± 1 °C for 6 minutes with a dim light illumination (3-5 lux). When removed from the water, the mouse was allowed to dry for 5 min under red light. The following behavioral responses were scored: the percentage of *time spent performing struggle*, vigorous attempts at climbing the walls of the cylinder, and the percentage of *time spent performing floating*, total absence of movement.

Fear conditioning test

To assess emotional learning and memory, on PND 99 we performed the fear conditioning test. The test is based on the association between an unconditioned stimulus (US) and a conditioned stimulus (CS) causing an unconditioned response (UR). In particular, the CS corresponds to a neutral tone, paired with a brief electric foot-shock (US). This produces a typical rodents UR, called *freezing behavior*, corresponding to the total absence of all movement except respiration. The experimental apparatus corresponds to a training chamber (30×35×20 cm) with four opaque sidewalls and a removable foot-shocking grid floor used for the first two phases of the task, training, and context

test. The third phase, the tone test, requires a different context to verify the mice's association between CS and US. The fear conditioning test was performed on two days. In the training session, mice learn to associate a neutral tone (1000 Hz, 90 dB) to the foot-shock (0.23 mA average intensity) for three trials, 60 seconds each. Before the first trial, was performed 60 seconds of observation to assess the baseline activity of the animals. Each trial consisted of a tone phase of 30 seconds with two seconds of foot-shock starting at 28 seconds, the last 30 seconds of trial were without tone (intertrial). In this phase, we assess emotional response and learning by scoring the percentage of *freezing time behavior*. After 24 hours we performed the context test, mice are exposed to the same training chamber for 120 seconds without neutral tone and foot-shock. We assess memory by scoring the percentage of *freezing time behavior*. During the last session, the tone test, mice are observed in a new environment consisting of a chamber (25×28×16.5 cm) with four white walls, black visual cues, and red light. 90 minutes after the context test, mice are exposed to the same neutral tone in the new test chamber without the final foot-shock for three trials of 60 seconds, divided into 30 seconds of tone and 30 seconds of intertrial. The association between CS and US is scored by the percentage of *freezing time*.

Corticosterone levels

At the end of the behavioral test phase (PND117), the activation of the hypothalamus-pituitary-adrenal (HPA) axis was assessed by measuring plasma levels of corticosterone in response to a psychophysical stressful challenge. All subjects underwent acute restraint stress (30 min) and blood samples were collected by a tail nick at different time points, i.e., immediately before (0 min) and after (30 and 180 min) the exposure to stress. The restraint procedure consisted in placing animals in 50ml-plastic falcon tubes with a few holes to keep airflow with white light during the stress. This procedure took place at 02:30 p.m. when the levels of free corticosterone were far from the circadian peak (Kitchener et al., 2004). Blood samples were collected individually in potassium–ethylenediaminetetraacetic acid (EDTA) coated 10 ml tubes (1.6 mg EDTA/ml blood: Sarstedt). All samples were kept on ice and later centrifuged at 3000 rpm

for 15 min at 4 ◦C. Blood plasma was transferred to Eppendorf tubes for CORT determination and stored at − 80 ◦C until analysis. Plasma corticosterone levels were quantified by enzyme immunoassay according to the manufacturer's recommendations (Corticosterone Elisa kit, Enzo Life Sciences). Samples were diluted 1:40 before analysis, and data were analyzed by sigmoidal 4-parameter logistic curve fit using Prism 6 (Graphpad). The sensitivity of the assay was 27.0 pg/ml and the intra-assay percent coefficients of variations were between 3.35% and 3.82%. Because of the great variability, the logarithmic transformation will be implemented.

Statistical methods

All data were analyzed with one-way ANOVA with the Statistical software Statview II (Abacus Concepts, CA, USA), to compare Controls versus Standard+vehicle, Standard+ fluoxetine, Enrichment+vehicle, and Enrichment+fluoxetine. Time (minutes or days) was considered a repeated measure within subjects. Latency data, which had no normal distribution, were analyzed with the Mann-Whitney nonparametric test. *Post-hoc* comparisons were performed using Tukey's test. All mean differences were considered statistically significant when $p < 0.05$.

Results

Emotional response

Locomotor activity and anxiety-like behavior- Open field test

The analysis of the behavior in the open field test revealed a statistically significant main effect of the intervention on the total distance traveled in the arena ($F_{4,55}$ = 2.722, p = 0.0386). *Post-hoc* analysis revealed that Enrichment+vehicle mice displayed higher locomotor activity compared to Standard+fluoxetine mice (p < 0.05; Fig.2A). Concerning the anxiety-like behavior, no differences between the experimental groups were found on both the percentage of time spent in the periphery ($F_{4,53}$ = 0.818, p = 0.5197; Fig.2B) and the latency of first entry in the center (U = 2.324, p = 0.6764; Fig.2C).

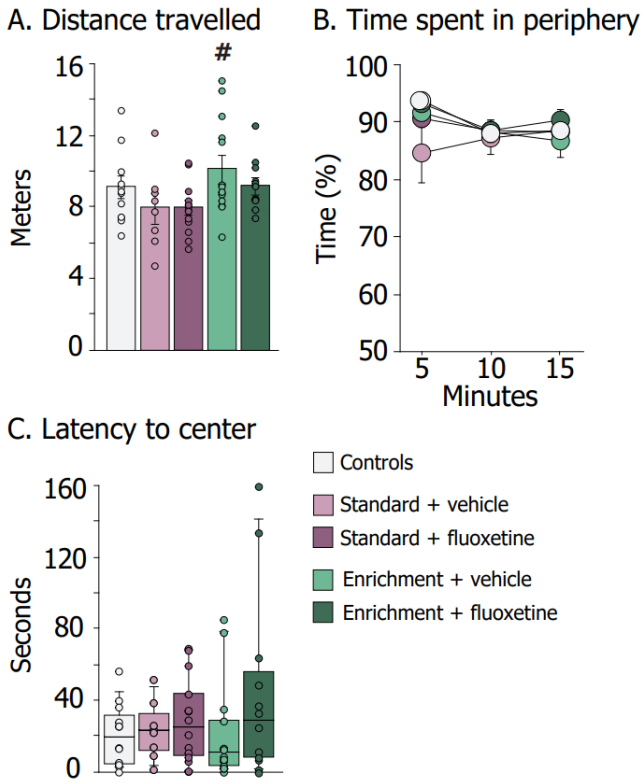

Figure 2. The exposure to the enriched environment increased locomotor activity in the open field test. (**A**) The analysis of the behavior in the open field test revealed a statistically significant main effect of the intervention on the total distance traveled in the arena. (**B**) Concerning the anxiety-like behavior, no differences between the experimental groups were found on both the

percentage of time spent in the periphery and (**C**) the latency of the first entry in the center. # $p < 0.05$ vs Standard+fluoxetine, n = 8-12. Data are means ± S.E.M.

Anxiety-like behavior- Elevated plus-maze

In the elevated plus maze, we found a statistically significant main effect of the intervention on the percentage of time spent in the open arms ($F_{4,54} = 3.347$, $p = 0.0161$): the exposure to the standard environment enhanced anxiety-like profile, and fluoxetine treatment produced opposite effects in the two environmental conditions (Fig.3A). *Post-hoc* analysis revealed that mice exposed to the standard environment and treated with fluoxetine significantly decreased the percentage of time spent in the open arms compared with the Controls ($p < 0.05$), while mice treated with fluoxetine in the enriched environment increased the percentage compared both to Standard+vehicle and Standard+fluoxetine mice ($p < 0.05$). However, no significant differences were found concerning both the latency to enter in the open arms ($U = 6.926$, $p = 0.1398$; Fig.3B) and the risk-assessment behavior as stretched attend posture ($F_{4,55} = 1.449$, $p = 0.2304$; Fig.3C) and head-dipping behavior ($F_{4,53} = 1.876$, $p = 0.1282$; Fig.3D).

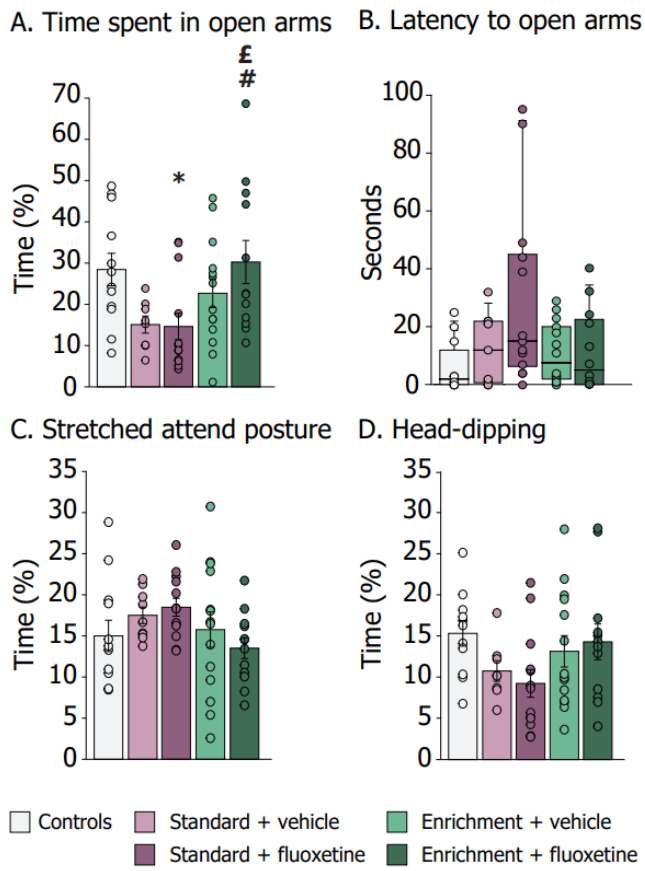

Figure 3. The combination of environmental enrichment and fluoxetine treatment decreased the anxiety-like behavior in the Elevated plus-maze. (**A**) Standard +fluoxetine mice significantly decreased the percentage of time spent in the open arms, while Enrichment+fluoxetine mice increased their percentage compared to both Standard+vehicle and Standard+fluoxetine mice ($p<0.05$). (**B**) No significant differences were found concerning latency to enter in the open arms, and risk-assessment behavior as (**C**) stretched attend posture, and (**D**) head-dipping behavior. * $p < 0.05$ vs Controls, £ $p < 0.05$ vs Standard+vehicle, # $p < 0.05$ vs Standard+fluoxetine, n = 8-12. Data are means ± S.E.M.

Despair-like behavior- Forced swim test

We observed that social isolation from adolescence till adulthood tent to increase the despair-like behavior of those mice which have been subsequently exposed to the standard environment and that the exposure to the enriched environment alone could reverse such a tendency. In particular, we found a statistically significant main effect of the intervention on the percentage of floating time ($F_{4,55}$ = 3.592, p = 0.0113), and *post-hoc* analysis revealed that both

Standard+vehicle and Standard+fluoxetine mice spent a higher percentage of time floating compared to the Enrichment+vehicle mice (respectively $p < 0.05$ and $p < 0.01$; Fig.4).

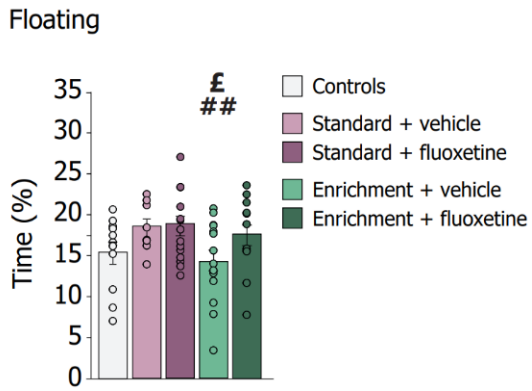

Figure 6. The exposure to the enriched environment, alone or in combination with fluoxetine, decreased the despair-like behavior. The intervention affected the floating behavior during the forced swim test, Enrichment+vehicle mice significantly decreased the percentage of time floating compared to both Standard+vehicle mice (£ $p < 0.05$) and Standard+fluoxetine mice (## $p < 0.01$), n = 8-12. Data are means ± S.E.M.

Cognitive abilities

Learning and memory: Novel object recognition test

The evaluation of the discrimination index during the novel object recognition test did not reveal a significant difference between experimental groups ($F_{4,55}$ = 0.326, p = 0.8590; Fig.5A).

Learning and emotional memory: Fear conditioning test

In the first phase of the fear conditioning test, we did not observe a significant difference between groups in terms of the percentage of freezing behavior ($F_{4,49}$ = 2.380, p = 0.0643; Fig.5B). Concerning memory retrieval, no differences between experimental groups have been found during the context test ($F_{4,53}$ = 1.008, p = 0.4118; Fig.5C), while statistically significant main effect of the intervention on the percentage of freezing behavior has been observed in the tone test ($F_{4,53}$ = 3.712, p = 0.0098). *Post-hoc* analysis revealed a decreased

freezing response of the Enrichment+fluoxetine mice compared to the experimental subjects exposed to Enrichment+vehicle (Fig.5D).

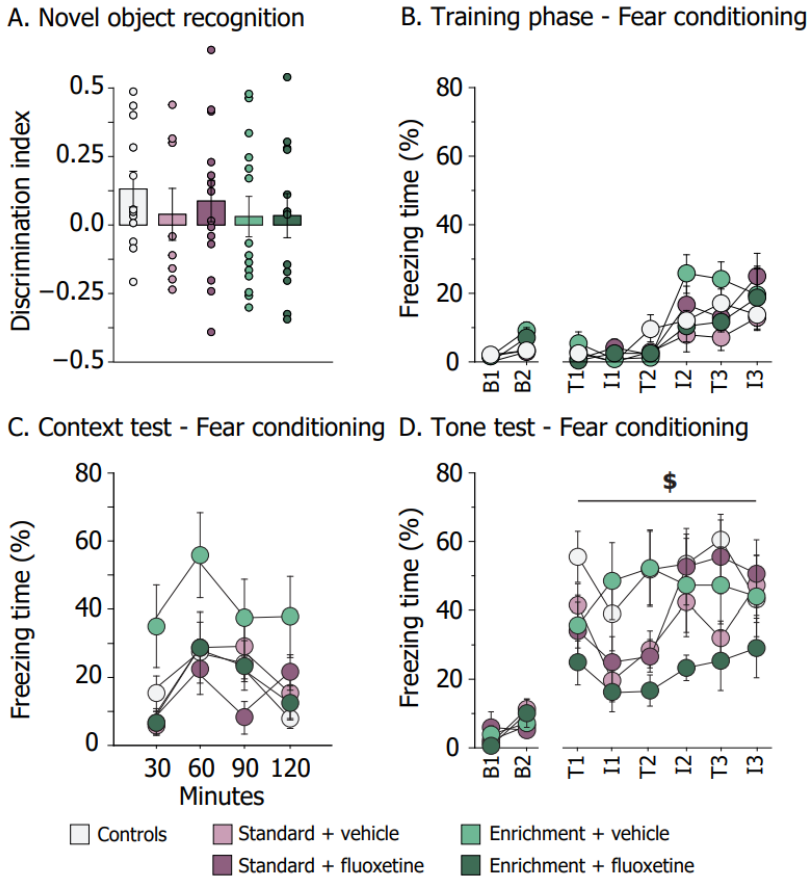

Figure 7. Learning and memory functions assessed through Novel object recognition test and Fear conditioning test. (**A**) No differences emerged concerning the discrimination capability of the animals during the novel object recognition test. (**B-C**) In the training and memory retrieval phases of the fear conditioning test, we did not observe a significant difference between groups in terms of the percentage of freezing behavior. (**D**) During the tone test, Enrichment+fluoxetine mice decreased their percentage of freezing compared to Enrichment+vehicle mice, $ $p < 0.05$, n = 8-12. Data are means ± S.E.M.

HPA-axis activity: corticosterone levels

Fluoxetine treatment in combination with the standard environment led to a hyperactivation of the HPA-axis and consequently to an increased stress response, by contrast in the enriched environment the treatment led to an opposite effect. Comparing the corticosterone levels after the exposure to the restraint with the basal levels, we found a statistically significant main effect of the intervention ($F_{4,28}$ = 7.817, p = 0.0002). *Post-hoc* analysis revealed that after the acute stress (30 minutes) Standard+fluoxetine mice showed an increased stress response compared to both Standard+vehicle and Enriched+fluoxetine (p < 0.01). Following 180 minutes, Standard+fluoxetine mice showed increased levels compared with Enriched+fluoxetine mice (p < 0.01; Fig.6).

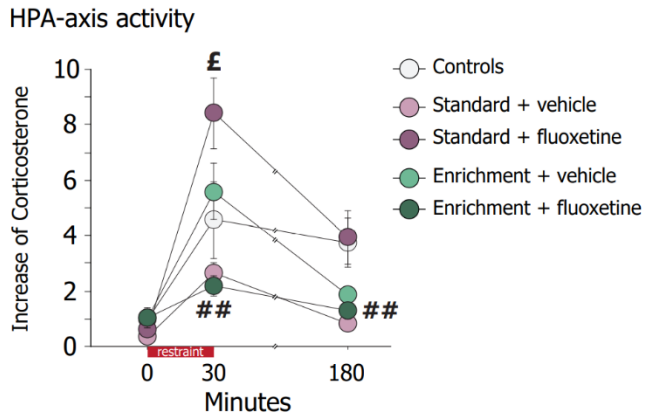

Figure 8. HPA-axis activity. HPA-axis activity has been assessed by measuring plasma levels of corticosterone in response to acute stress. In particular, we calculated the ratio between the baseline levels and the levels of corticosterone following both 30 and 180 minutes of restraint. Standard+fluoxetine mice showed increased corticosterone levels compared to both Standard+vehicle and Enriched+fluoxetine immediately after the restraint protocol. Following 180 minutes, Standard+fluoxetine mice showed increased levels compared with Enriched+fluoxetine animals. £ p < 0.05 ; ## p < 0.01, n = 4-9. Data are means ± S.E.M.

Discussion

Our results confirm that adolescent isolation induced both depressive- and anxiety-like behavior and show that environmental enrichment alone or in combination with fluoxetine, but not fluoxetine alone, improves the depressive-like phenotype. The combination of drug treatment with favorable enrichment is specifically effective to counteract anxiety-like behavior. Our findings outline the relevance of environmental conditions alone or in combination with antidepressant drugs in the treatment of adolescence-onset depression.

The environment is key in recovering from depressive-like behavior because it provides the skills needed by the individual isolated during adolescence to face the environmental challenges at adulthood, as indicated by the behaviors in the forced swim and the open field test. Indeed, in the forced swim test the Enriched+vehicle mice showed a decreased time of floating behavior compared to both Standard+vehicle and Standard+fluoxetine mice, a result indicating that enriched environment could counteract the increased despair-like behavior induced by prolonged social deprivation during adolescence (Bogdanova et al., 2013; Guarnieri et al., 2020; Porsolt et al., 1977). In addition, experimental subjects exposed to the enriched environment and treated with vehicle displayed increased exploratory activity in the open field test compared to animals exposed to the standard environment and treated with fluoxetine. These results are in line with the literature reporting the beneficial effects of a supportive environment on exploratory activity (Guarnieri et al., 2020; Korholz et al., 2018; Krupina et al., 2020) and are supported by several studies indicating that abnormalities in the exploratory behavior of rodents it has been linked to their general health (Fahlstrom et al., 2012) and anxiety-like profile (Crawley, 1985).

The lack of social experiences during adolescence affects the emotional profile of experimental subjects in the elevated plus maze and fluoxetine treatment, by enhancing plasticity, amplifies the beneficial effect of the favorable environment. In line with the literature (Hu et al., 2017; Iniguez et al., 2014b; Wang et al., 2011), mice exposed to the standard environment showed an increased anxiety-like behavior, in particular in the Standard+fluoxetine compared to Control mice. The interaction between enriched environment and

fluoxetine treatment counterbalances the anxiolytic effect of social isolation as indicated by the increased time spent in the open arms of Enriched+fluoxetine compared to both Standard+vehicle and Standard+fluoxetine mice. An increased anxiety-like profile and the beneficial effects of the combination of enriched environment and fluoxetine treatment were suggested by the latency to open arms and the behaviors related to the risk assessment profile of the animals, such as stretched attend posture and head-dipping, although these parameters missed to rich the statistical significance. These results are in line with previous studies showing the higher efficacy of fluoxetine treatment administered in a supportive environment compared to a not-beneficial one, underlying the key role of the environment in determining the SSRI outcome (Branchi and Giuliani, 2021; Forsyth et al., 2015; Poggini et al., 2019; Poggini et al., 2021; Viglione et al., 2019; Wilkinson et al., 2019). According to this evidence, the opposite results displayed by Standard+fluoxetine and Enriched+fluoxetine mice in the elevated plus suggest also that the effects of fluoxetine are environment dependent. The results concerning the HPA-axis activation corroborated this hypothesis. Mice exposed to the standard environment and treated with fluoxetine showed significantly increased levels of corticosterone compared to mice exposed to the enriched environment and treated with fluoxetine, which confirmed the environment-dependent effect of fluoxetine treatment. Moreover, these findings are in line with the literature showing that an enriched environment and fluoxetine tend to decrease corticosterone levels (Baker et al., 2018; Lopez and Laber, 2015; Mesa-Gresa et al., 2016).

Finally, our findings confirm that the lack of social experiences affects more the emotional profile than the cognitive profile of the experimental subjects in adulthood, concerning both depressive- and anxiety-like responses and hormonal reactivity to stress. Indeed, in line with the literature (Guarnieri et al., 2020; Rivera-Irizarry et al., 2020), we did not observe differences in the animals' cognitive abilities in both novel object recognition and fear conditioning test. At the clinical level, several studies focused their attention on the emotional profile of individuals and their social environment, reporting that adverse early life events, associated with a dysfunctional socioemotional environment, predict the

onset of major depressive disorder in adolescence and its recurrence (Lewinsohn et al., 2001; Lewinsohn et al., 1999). Calati and colleagues show that social environment was deeply associated with emotional dysfunction leading to suicidal thoughts and attempts in both adolescents and young adults. The main construct was represented by the living conditions, i.e. being single, divorced, widowed, or living alone. In addition, both the objective condition (e.g., living alone) and the subjective feeling of being alone (i.e., loneliness) were strongly associated with suicidal outcomes, suggesting that both objective social isolation and the subjective feeling of loneliness should be incorporated into the risk assessment of mental disorders and suicide (Calati et al., 2019).

Overall, these findings underly that (1) the environment is key in recovering from depressive-like behavior, (2) fluoxetine treatment, by enhancing plasticity, amplifies the beneficial effect of the favorable environment, and (3) the effects of fluoxetine are environment dependent. In a translational perspective, by controlling the subjects living conditions has been possible to improve therapeutic intervention and predict treatment outcomes, as suggested by the study of Viglione and collaborators. Indeed, they show that citalopram, a common SSRI, produces a dose-dependent amplification of the influence of the environment on mood, making symptom severity dependent on the level of socioeconomic status. As a consequence, they are able to effectively predict the treatment outcome based on the patient's socioeconomic status in a dose-dependent fashion. Patients who received the high, but not the low, dose of citalopram could be effectively identified at baseline as patients who will show or not show remission following 12 weeks of treatment (Viglione et al., 2019).

In conclusion, our findings confirm and further show the key role of the living environment in psychiatric treatment, in particular for those compounds leveraging on the enhancement of neural and behavioral plasticity such as SSRIs (Alboni et al., 2017; Branchi, 2011; Poggini et al., 2019; Wilkinson et al., 2019). We thus underpin the relevance of the patient's living conditions in the selection of the most effective pharmacological treatment and advocate for considering the key role of the environment in the precision medicine paradigm in the psychiatric field.

References

Alboni, S., Poggini, S., Garofalo, S., Milior, G., El Hajj, H., Lecours, C., Girard, I., Gagnon, S., Boisjoly-Villeneuve, S., Brunello, N.*, et al.* (2016). Fluoxetine treatment affects the inflammatory response and microglial function according to the quality of the living environment. Brain, behavior, and immunity *58*, 261-271.

Alboni, S., van Dijk, R.M., Poggini, S., Milior, G., Perrotta, M., Drenth, T., Brunello, N., Wolfer, D.P., Limatola, C., Amrein, I.*, et al.* (2017). Fluoxetine effects on molecular, cellular and behavioral endophenotypes of depression are driven by the living environment. Molecular psychiatry *22*, 552-561.

Almeida, I.L.L., Rego, J.F., Teixeira, A.C.G., and Moreira, M.R. (2021). Social isolation and its impact on child and adolescent development: a systematic review. Revista paulista de pediatria : orgao oficial da Sociedade de Pediatria de Sao Paulo *40*, e2020385.

Baker, E.P., Magnuson, E.C., Dahly, A.M., and Siegel, J.A. (2018). The effects of enriched environment on the behavioral and corticosterone response to methamphetamine in adolescent and adult mice. Developmental psychobiology *60*, 664-673.

Bogdanova, O.V., Kanekar, S., D'Anci, K.E., and Renshaw, P.F. (2013). Factors influencing behavior in the forced swim test. Physiology & behavior *118*, 227-239.

Branchi, I. (2011). The double edged sword of neural plasticity: increasing serotonin levels leads to both greater vulnerability to depression and improved capacity to recover. Psychoneuroendocrinology *36*, 339-351.

Branchi, I., and Giuliani, A. (2021). Shaping therapeutic trajectories in mental health: Instructive vs. permissive causality. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology *43*, 1-9.

Branchi, I., Santarelli, S., Capoccia, S., Poggini, S., D'Andrea, I., Cirulli, F., and Alleva, E. (2013). Antidepressant treatment outcome depends on the quality of the living environment: a pre-clinical investigation in mice. PloS one *8*, e62226.

Calati, R., Ferrari, C., Brittner, M., Oasi, O., Olie, E., Carvalho, A.F., and Courtet, P. (2019). Suicidal thoughts and behaviors and social isolation: A narrative review of the literature. Journal of affective disorders *245*, 653-667.

Chiarotti, F., Viglione, A., Giuliani, A., and Branchi, I. (2017). Citalopram amplifies the influence of living conditions on mood in depressed patients enrolled in the STAR*D study. Translational psychiatry *7*, e1066.

Cipriani, A., Zhou, X., Del Giovane, C., Hetrick, S.E., Qin, B., Whittington, C., Coghill, D., Zhang, Y., Hazell, P., Leucht, S.*, et al.* (2016). Comparative efficacy and tolerability of antidepressants for major depressive disorder in children and adolescents: a network meta-analysis. Lancet *388*, 881-890.

Costello, E.J., Egger, H., and Angold, A. (2005). 10-year research update review: the epidemiology of child and adolescent psychiatric disorders: I. Methods and public health burden. Journal of the American Academy of Child and Adolescent Psychiatry *44*, 972-986.

Crawley, J.N. (1985). Exploratory behavior models of anxiety in mice. Neuroscience and biobehavioral reviews *9*, 37-44.

Dulawa, S.C., Holick, K.A., Gundersen, B., and Hen, R. (2004). Effects of chronic fluoxetine in animal models of anxiety and depression. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *29*, 1321-1330.

Fahlstrom, A., Zeberg, H., and Ulfhake, B. (2012). Changes in behaviors of male C57BL/6J mice across adult life span and effects of dietary restriction. Age *34*, 1435-1452.

Fergusson, D.M., and Woodward, L.J. (2002). Mental health, educational, and social role outcomes of adolescents with depression. Archives of general psychiatry *59*, 225-231.

Fernandez Espejo, E. (1997). Structure of the mouse behaviour on the elevated plus-maze test of anxiety. Behavioural brain research *86*, 105-112.

Forsyth, J.K., Bachman, P., Mathalon, D.H., Roach, B.J., and Asarnow, R.F. (2015). Augmenting NMDA receptor signaling boosts experience-dependent neuroplasticity in the adult human brain. Proceedings of the National Academy of Sciences of the United States of America *112*, 15331-15336.

Guarnieri, L.O., Pereira-Caixeta, A.R., Medeiros, D.C., Aquino, N.S.S., Szawka, R.E., Mendes, E., Moraes, M.F.D., and Pereira, G.S. (2020). Pro-neurogenic effect of fluoxetine in the olfactory bulb is concomitant to improvements in social memory and depressive-like behavior of socially isolated mice. Translational psychiatry *10*, 33.

Harrington, R., Fudge, H., Rutter, M., Pickles, A., and Hill, J. (1990). Adult outcomes of childhood and adolescent depression. I. Psychiatric status. Archives of general psychiatry *47*, 465-473.

Hill, R.M., Pettit, J.W., Lewinsohn, P.M., Seeley, J.R., and Klein, D.N. (2014). Escalation to Major Depressive Disorder among adolescents with subthreshold depressive symptoms: evidence of distinct subgroups at risk. Journal of affective disorders *158*, 133-138.

Hu, C., Luo, Y., Wang, H., Kuang, S., Liang, G., Yang, Y., Mai, S., and Yang, J. (2017). Re-evaluation of the interrelationships among the behavioral tests in rats exposed to chronic unpredictable mild stress. PloS one *12*, e0185129.

Iniguez, S.D., Alcantara, L.F., Warren, B.L., Riggs, L.M., Parise, E.M., Vialou, V., Wright, K.N., Dayrit, G., Nieto, S.J., Wilkinson, M.B.*, et al.* (2014a). Fluoxetine exposure during adolescence alters responses to aversive stimuli in adulthood. The Journal of neuroscience : the official journal of the Society for Neuroscience *34*, 1007-1021.

Iniguez, S.D., Riggs, L.M., Nieto, S.J., Dayrit, G., Zamora, N.N., Shawhan, K.L., Cruz, B., and Warren, B.L. (2014b). Social defeat stress induces a depression-like phenotype in adolescent male c57BL/6 mice. Stress *17*, 247-255.

Jacobson, N.C., and Newman, M.G. (2016). Perceptions of Close and Group Relationships Mediate the Relationship between Anxiety and Depression over a Decade Later. Depression and anxiety *33*, 66-74.

Johnson, D., Dupuis, G., Piche, J., Clayborne, Z., and Colman, I. (2018). Adult mental health outcomes of adolescent depression: A systematic review. Depression and anxiety *35*, 700-716.

Kitchener, P., Di Blasi, F., Borrelli, E., and Piazza, P.V. (2004). Differences between brain structures in nuclear translocation and DNA binding of the

glucocorticoid receptor during stress and the circadian cycle. The European journal of neuroscience *19*, 1837-1846.

Korholz, J.C., Zocher, S., Grzyb, A.N., Morisse, B., Poetzsch, A., Ehret, F., Schmied, C., and Kempermann, G. (2018). Selective increases in inter-individual variability in response to environmental enrichment in female mice. eLife *7*.

Krupina, N.A., Shirenova, S.D., and Khlebnikova, N.N. (2020). Prolonged Social Isolation, Started Early in Life, Impairs Cognitive Abilities in Rats Depending on Sex. Brain sciences *10*.

Le Noury, J., Nardo, J.M., Healy, D., Jureidini, J., Raven, M., Tufanaru, C., and Abi-Jaoude, E. (2015). Restoring Study 329: efficacy and harms of paroxetine and imipramine in treatment of major depression in adolescence. Bmj *351*, h4320.

Lewinsohn, P.M., Joiner, T.E., Jr., and Rohde, P. (2001). Evaluation of cognitive diathesis-stress models in predicting major depressive disorder in adolescents. Journal of abnormal psychology *110*, 203-215.

Lewinsohn, P.M., Rohde, P., Klein, D.N., and Seeley, J.R. (1999). Natural course of adolescent major depressive disorder: I. Continuity into young adulthood. Journal of the American Academy of Child and Adolescent Psychiatry *38*, 56-63.

Lopez, M.F., and Laber, K. (2015). Impact of social isolation and enriched environment during adolescence on voluntary ethanol intake and anxiety in C57BL/6J mice. Physiology & behavior *148*, 151-156.

Mesa-Gresa, P., Ramos-Campos, M., and Redolat, R. (2016). Corticosterone levels and behavioral changes induced by simultaneous exposure to chronic social stress and enriched environments in NMRI male mice. Physiology & behavior *158*, 6-17.

Mullen, S. (2018). Major depressive disorder in children and adolescents. The mental health clinician *8*, 275-283.

Poggini, S., Golia, M.T., Alboni, S., Milior, G., Sciarria, L.P., Viglione, A., Matte Bon, G., Brunello, N., Puglisi-Allegra, S., Limatola, C.*, et al.* (2019). Combined Fluoxetine and Metformin Treatment Potentiates Antidepressant

Efficacy Increasing IGF2 Expression in the Dorsal Hippocampus. Neural plasticity *2019*, 4651031.

Poggini, S., Matte Bon, G., Golia, M.T., Ciano Albanese, N., Viglione, A., Poleggi, A., Limatola, C., Maggi, L., and Branchi, I. (2021). Selecting antidepressants according to a drug-by-environment interaction: A comparison of fluoxetine and minocycline effects in mice living either in enriched or stressful conditions. Behavioural brain research *408*, 113256.

Porsolt, R.D., Le Pichon, M., and Jalfre, M. (1977). Depression: a new animal model sensitive to antidepressant treatments. Nature *266*, 730-732.

Qualter, P., Brown, S.L., Munn, P., and Rotenberg, K.J. (2010). Childhood loneliness as a predictor of adolescent depressive symptoms: an 8-year longitudinal study. European child & adolescent psychiatry *19*, 493-501.

Rao, U., Ryan, N.D., Birmaher, B., Dahl, R.E., Williamson, D.E., Kaufman, J., Rao, R., and Nelson, B. (1995). Unipolar depression in adolescents: clinical outcome in adulthood. Journal of the American Academy of Child and Adolescent Psychiatry *34*, 566-578.

Rivera-Irizarry, J.K., Skelly, M.J., and Pleil, K.E. (2020). Social Isolation Stress in Adolescence, but not Adulthood, Produces Hypersocial Behavior in Adult Male and Female C57BL/6J Mice. Frontiers in behavioral neuroscience *14*, 129.

Trivedi, M.H., Rush, A.J., Wisniewski, S.R., Nierenberg, A.A., Warden, D., Ritz, L., Norquist, G., Howland, R.H., Lebowitz, B., McGrath, P.J.*, et al.* (2006). Evaluation of outcomes with citalopram for depression using measurement-based care in STAR*D: implications for clinical practice. The American journal of psychiatry *163*, 28-40.

Viduani, A., Benetti, S., Martini, T., Buchweitz, C., Ottman, K., Wahid, S.S., Fisher, H.L., Mondelli, V., Kohrt, B.A., and Kieling, C. (2021). Social isolation as a core feature of adolescent depression: a qualitative study in Porto Alegre, Brazil. International journal of qualitative studies on health and well-being *16*, 1978374.

Viglione, A., Chiarotti, F., Poggini, S., Giuliani, A., and Branchi, I. (2019). Predicting antidepressant treatment outcome based on socioeconomic status and citalopram dose. The pharmacogenomics journal *19*, 538-546.

Vitiello, B., and Ordonez, A.E. (2016). Pharmacological treatment of children and adolescents with depression. Expert opinion on pharmacotherapy *17*, 2273-2279.

Wang, L., Jiao, J., and Dulawa, S.C. (2011). Infant maternal separation impairs adult cognitive performance in BALB/cJ mice. Psychopharmacology *216*, 207-218.

Wilkinson, S.T., Holtzheimer, P.E., Gao, S., Kirwin, D.S., and Price, R.B. (2019). Leveraging Neuroplasticity to Enhance Adaptive Learning: The Potential for Synergistic Somatic-Behavioral Treatment Combinations to Improve Clinical Outcomes in Depression. Biological psychiatry *85*, 454-465.

Windfuhr, K., While, D., Hunt, I., Turnbull, P., Lowe, R., Burns, J., Swinson, N., Shaw, J., Appleby, L., Kapur, N.*, et al.* (2008). Suicide in juveniles and adolescents in the United Kingdom. Journal of child psychology and psychiatry, and allied disciplines *49*, 1155-1165.

STUDY 3

Individuality in experiencing the environment determines serotoninergic antidepressant treatment outcome: a preclinical study

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Submitted

Abstract

The efficacy of selective serotonin reuptake inhibitors (SSRIs), the first-line antidepressant treatment, is variable. To explain such variability, it has been recently proposed that SSRIs act, at least in part, by amplifying the influence of the living environment on mood. Since the quality of the living environment depends not only on its objective features but also on the subjective experience of it, we hypothesized that the latter plays a key role in determining SSRI treatment outcome. To test this hypothesis, we used two groups of CD-1 male mice that reportedly show different subjective experiences of the social and emotional environment because of different rearing conditions: standard nesting (SN) mice, reared in standard laboratory conditions, and communal nesting (CN) mice, reared in a nest where three dams breed together their offspring and share caregiving behavior. At adulthood, CN mice experienced the same test environment as more socially challenging and potentially dangerous compared to SN mice. We then treated them with either the SSRI fluoxetine or vehicle for 21 days. Fluoxetine increased offensive and anxious response in SN, while producing opposite effects in CN mice. BDNF regulation was modified by the treatment accordingly. These results indicate that the subjective experience of the environment determines fluoxetine outcome. In a translational perspective, our findings point out the need to consider not only the objective quality but also the subjective experience of the patient's living environment to develop effective personalized therapeutic approaches in psychiatry.

Keywords
Major depression disorder, SSRI, individual experience, subjective, environment, context

Introduction

Selective serotonin reuptake inhibitors (SSRIs) are the first-line treatment for depression. Nevertheless, their efficacy is variable and incomplete: 60-70% of patients do not achieve remission and 30-40% do not show a clinical response (Trivedi et al., 2006). To explain such variability, a novel hypothesis, named *undirected susceptibility to change*, has been recently proposed (Branchi, 2011, 2022). It posits that SSRIs do not affect mood *per se* but, by boosting the serotonergic tone, they enhance neural plasticity and thus amplify the influence of the environment on mood. This hypothesis has been validated in both preclinical and clinical studies (Branchi, 2022; Branchi and Giuliani, 2021). In mouse models, the administration of the SSRI fluoxetine in an enriched environment improves the depressive-like phenotype while, in stressful conditions, it has no effect or even leads to a worsening (Alboni et al., 2017; Bashiri et al., 2021; Branchi et al., 2013b; Karpova et al., 2011; Lima et al., 2019; Poggini et al., 2021). Clinical studies demonstrated that the administration of SSRI Citalopram amplifies the influence of living conditions on mood, leading to an improvement of symptoms according to the individual's socioeconomic status (Chiarotti et al., 2017; Viglione et al., 2019). Accordingly, recent studies aimed at investigating the effects of SSRI in healthy subjects further confirmed the context dependence of the SSRI effects showing that, in clinical practice, these drugs enhance neuroplasticity rather than directly improving psychiatric symptoms (Klobl et al., 2022; Reed et al., 2022). Thus, the quality of the living environment is key in determining SSRI treatment outcome.

The quality of the environment, such as the living conditions, does not depend only on its objective features, but also on the subjective experience of it (Kempermann, 2019). For instance, trust impressions are highly dependent on individuality (Sutherland et al., 2020) and individuals show important differences in their response to fear and threat (LeDoux and Brown, 2017). In addition, twin studies demonstrate that subjective perceptions of social status correlate with indicators of well-being (Rivenbark et al., 2020).

Given the role of the environment in driving SSRI efficacy and the relevance of the subjective experience in experiencing the environment, we hypothesized

that the latter is key in determining SSRI treatment outcome. To explore such a hypothesis, we generated two groups of mice showing distinct experiences of the social and emotional environment. These mice are indistinguishable at the genetic level but were reared in two different reading conditions: standard laboratory (Standard Nesting -- SN) and Communal Nesting (CN) conditions. CN mice received caregiving behavior by multiple dams and interacted with a larger number of genetic and non-genetic peers during the first four weeks of life (Branchi, 2009; Branchi et al., 2011a). At adulthood, CN mice reportedly experience the environment as more socially challenging and potentially dangerous compared to SN mice (Branchi, 2009; Branchi and Alleva, 2006). To assess whether the subjective experience of the environment affects SSRI treatment outcome, we administered either vehicle or fluoxetine for 21 days and then phenotyped their response to the same test environment. We measured emotional and social behavior endpoints that are reportedly targeted by SSRIs (Cirulli et al., 2000; Mikics et al., 2018; Molendijk et al., 2012) and analyzed brain derived neurotrophic factor (BDNF) epigenetic regulation and protein levels because this is considered one of the most reliable molecular proxies of antidepressant action (Branchi et al., 2011b; Casarotto et al., 2021; Tsankova et al., 2006). We predicted that SSRI treatment produces different effects in SN and CN because of their different subjective experience of the context.

Methods and Materials

Animals and Breeding Procedure

All procedures were performed according to European Communities guidelines (European Directive 2010/63/UE) and the Italian legislation on animal experimentation (Decreto L.vo 26/2014).

32 male and 64 female mice of an outbred CD-1 Swiss-derived strain (ICR) weighing 25-27 g (3-4 weeks old) were purchased from Envigo (San Pietro al Natisone (UD), Italy). Animals were housed under reversed 12 light-dark cycle (08.00-20.00) in an air-conditioned room (temperature 21±1 °C, relative humidity 60±10%). Males and females were housed in same-sex groups of 6 individuals in 42 x 17 x 14 cm Plexiglas cages with a metal top and sawdust as bedding, with pellet food (Enriched standard diet, Riper, Vendoies, BZ, Italy) and tap water *ad libitum*. After one week of acclimatization, animals were used to form breeding groups, made up of 1 male and 2 females, housed in 33 x 13 x 14 cm Plexiglas cages. Vaginal plugs were checked twice a day (at 09.00 h and 19.00 h) from the first day after breeding group formation. The male was removed from the breeding cage around gestational day 12 and females were assigned to one of the two experimental groups: standard nesting (SN) or communal nesting (CN). For the SN condition, one female was housed in a 33 x 13 x 14 cm Plexiglas cage. In the CN condition, according to the expected day of delivery (calculated basing on the day of vaginal plug detection), the females were housed in trios in 42 x 17 x 14 cm Plexiglas cages, five days before the delivery. In the CN group, females of the same trio gave birth on the same day. Each litter was culled on the day after birth, postnatal day (PND) 1 (birth = PND 0) to 6 males and 2 females for the SN group, and to 18 males and 6 females for the CN group. Pups were weaned on their own PND 25 and males of each litter were housed in groups of 4 animals in 42 x 17 x 14 cm Plexiglas cages until PND 120. From PND 120 male mice were housed in groups of 2 animals (according to their experimental group and treatment) and tested at PND 141 (Fig.1A). Female offspring were not studied in this work.

Treatment

Fluoxetine (Biomol International, LP, USA) was dissolved in water and delivered *ad libitum* in the drinking bottles for 21 days. The solution was prepared every day in different concentrations for SN and CN mice (respectively, 0.07 and 0.08 mg/ml), according to the average weight and daily water consumption previously assessed, to provide an average daily intake of 10 mg/kg. Bottles were wrapped in tin foil to protect the substance from light and weighed every day to monitor consumption. Compared to administration by injection, this method allows avoiding the stress due to the manipulation. Behavioral tests and neurochemical analyses were performed in different batches of animals (Fig.1B).

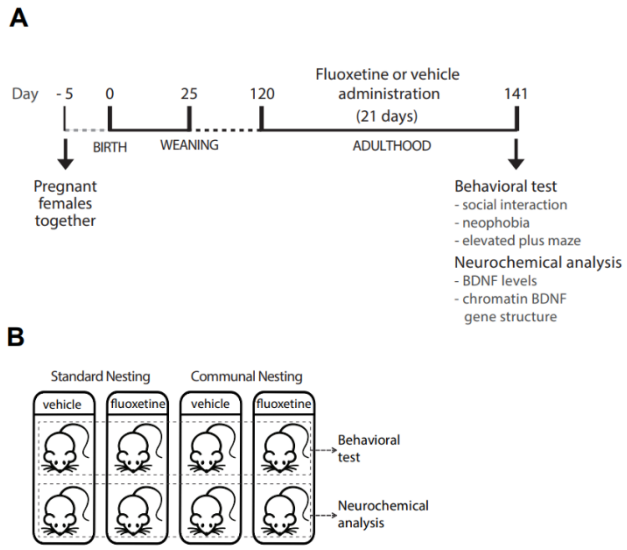

Figure 1. Experimental design and schema of experimental groups. (**A**) Timeline of communal nest procedure, fluoxetine or vehicle administration, and behavioral and neurochemical analyses. (**B**) In each couple of SN and CN mice, treated with fluoxetine or vehicle, one subject was randomly assigned to behavioral tests, while the other one was addressed to neurochemical analysis.

Behavioral tests

All tests were carried out during the dark period (between 09.30 and 15.30 h). Animals were transferred to the experimental room at least 45 min before the test to let them acclimatize to the new environment. Behavior was video-recorded using a digital videocamera (TR 7000E, Sony, Tokyo). The behavioral scoring

was carried out using the commercial software Observer 3.0 (Noldus, Wageningen, The Netherlands).

Social interaction test

Experimental subjects were placed in a novel cage, identical to the home cage, with a standard opponent of the same weight. The maximum length of social encounters was 20 min.

Behavioral categories and elements scored for both frequency and duration were:

- agonistic behaviors: *attack*, fighting episode when a mouse belonging to the experimental group approaches the opponent and bites it; *aggressive grooming*, violent grooming of the animal on the back of the partner; *offensive upright posture*, the animal stands on its hindlimbs facing the opponent aggressively; *defensive upright posture*, the animal stands on its hindlimbs and pushes the aggressive opponent with its forepaws; *crouched posture*, the animal lies on its ventrum, the head flat on the cage floor (this posture is often observed as an answer to aggressive grooming performed by the partner); *submissive upright posture*, the animal stands on its hindlimbs, the head pulled far back and its body rigid; *tail rattling*, fast tail vibration often observed in a distance ambivalence situation.

- affiliative behaviors: *anogenital sniff*, sniffing the anogenital area of the partner; *nose sniff*, sniffing the head and the snout region of the partner; *allogrooming*, grooming the partner; *body sniff*, sniffing any other area of the body of the partner.

- non-social activities: *wall-rearing*; *bar holding*, animal grasps the metal top of the cage holding itself above the level of the ground.; *self-grooming*; *digging*, animal digging the sawdust with the forelimbs, often kicking it away with the hindlimbs.

To discriminate the experimental subject from the standard opponent during data collection, standard opponents were marked by a blue, scentless and nontoxic felt pen 30 min before testing.

Novelty Suppressed Feeding

A highly palatable food, i.e. a butter cookie, was placed into a cage contained in an unfamiliar cylindrical transparent cup. Mice were free to explore the cup containing food for 10 minutes. The behavioral parameters scored were latency, frequency, and duration of *object exploration* and *food contact.* The use of high-palatable food allows for performing the test without food deprivation.

Elevated plus maze

The elevated plus-maze comprises two open arms (30 x 5 cm) and two closed arms (30 x 5 x 15 cm) that extended from a common central platform (5 x 5 cm). The apparatus, made of Plexiglas (gray floor, clear walls), was elevated to a height of 60 cm above the floor level. Mice were individually placed on the central platform facing a closed arm and allowed to freely explore the maze for 5 min. Behavioral parameters observed were frequencies of *total, open* and *closed entries* (arm entry = all four paws into an arm), percentage of *open entries* ((open/total)×100], and *time spent in open* and *closed* arms and in the *central part* of the platform. Furthermore, we scored frequency, duration and latency of *head dipping* (exploratory movement of head and shoulders over the edge of the maze) and *stretched attend postures* (s.a.p.; exploratory posture in which the body is stretched forward and then retracted to the original position without any forward locomotion).

Brain derived neurotrophic factor determination

BDNF protein levels were measured in the hippocampus following the procedure suggested by the manufacturer (Emaxtm ImmunoAssay System number G6891 by Promega, Madison, WI, USA). A monoclonal anti-mouse-BDNF antibody was used. BDNF concentration was determined from the regression line for the BDNF standard curve (ranging from 7.8 to 500 pg/ml-purified mouse BDNF) incubated under similar conditions in each assay. The sensitivity of the assay is about 15 pg/ml of BDNF, and the cross-reactivity with other related neurotrophic factors (NGF, NT-3, and NT-4) is considered nil.

Chromatin immunoprecipitation (ChIP) assay

The chromatin from the whole hippocampus was subjected to immunoprecipitation using antibodies against trimethyl-Histone H3 (K27) and acetyl-Histone H3 (K9 + K14) (Upstate, Millipore, Temecula, CA, USA). The specificity of these antibodies in the ChIP assay has been established previously (Kumar et al. 2005). To control for the specificity of antibody binding in our conditions, non-immune rabbit IgG (Chemicon, Millipore, Temecula, CA, USA) and no-antibody immunoprecipitations were performed for each chromatin sample. The following primers were used to selectively amplify a portion of BDNF:

- promoter I (5'-GTGCCTCTCGCCTAGTCATC–3' and 5'-GGGAACAACTGCGTGAATC-3')
- promoter II (5'-GGGCATATAATTGACATCCGCAA-3' and 5'-TCCACCACTATCCTCACCTAAACTCT–3')
- promoter III (5'-GAATCAGAGGATGGAGGGAGTGTG–3' and 5'-GGAAAGAGAGGGAGGCAGGGATA-3')
- promoter IV (5'-TGCGCGGAATTCTGATTCTGGTAAT–3' and 5'-AGTCCACGAGAGGGCTCCACGCT-3')
- promoter V (5'-CAAGGAAAAGGCGCGTCGTC–3' and 5'-TCTTCGGTTGAGCTTCGATT-3')
- promoter VI (5'-GGGGAAATGGACAGAAGCC–3' and 5'-CGCCACCGATACCCATT-3')
- promoter VII (5'-GTGTAGTCCGAGAATGGGTCTTGG-3' and 5'-ACCTTGGACCTGTAAGTAAGCTTTG-3')

Control PCR was performed with the primers for a promoter of a housekeeping gene β-actin (5'-AAAATGCTGCACTGTGCGGCGA–3' and 5'-GGACGCGACTCGACAGTGGCTG –3') to ensure the validity of the ChIP assay. DNA amplification reactions were run in triplicate at least two independent times in the presence of SYBR-Green (LightCycler 480 SYBR Green 1 Master mix, Roche Diagnostics GmbH, Mannheim, Germany). Ct values from each sample were obtained using the LightCycler 480 software (Roche Diagnostics GmbH,

Mannheim, Germany). The real-time PCR ChIP data were normalized to the 'input'.

Statistical analysis

All data were analyzed by analyses of variance (ANOVA), using nesting and treatment (respectively: standard vs. communal and vehicle vs. fluoxetine) as between-subject factors and the experimental subject as a random factor nested within nesting and treatment. Latency data, which had no normal distribution, were analyzed with the Mann-Whitney nonparametric test. *Post-hoc* comparisons were performed using the Tukey test. Analyses were carried out using the software Statview II (Abacus Concepts, CA, USA).

Results

Social interaction test

SN and CN mice differently modified their offensive behaviors according to treatment, as shown by the statistically significant effect of experimental group x treatment interaction for frequency and duration of attacks ($F_{1,26}$ = 24.235 and $F_{1,27}$ = 24.050, respectively for frequency and duration and $p < 0.001$ for both) and offensive upright postures ($Fs_{1,25}$ = 20.611 and 27.416, respectively for frequency and duration and $p < 0.001$ for both). In line with previous studies (Branchi, 2009; Branchi et al., 2013a), CN mice receiving vehicle showed higher attacks and offensive upright postures in both frequency and duration compared to SN mice receiving vehicle (*ps* < 0.01). In addition, *post-hoc* analysis revealed that fluoxetine treatment increased the number and the duration of attacks and upright offensive postures in SN mice (*ps* < 0.05) and decreased the same endpoints in CN mice (*ps* < 0.01). Moreover, fluoxetine decreased the number of upright offensive postures in CN compared to SN mice (*ps* < 0.01; Fig.2A). No statistically significant effects were found for defensive behaviors (Fig. 2B). For social investigation, main effects of fluoxetine treatment ($Fs_{1,28}$ = 19.780 and 7.080, $p = 0.0001$ and p = 0.0128, respectively for frequency and duration) and experimental group ($Fs_{1,28}$ = 20.597 and 14.787, *ps* < 0.001 and < 0.01, respectively for frequency and duration) were found, all mice markedly increasing such social investigation following fluoxetine administration and CN showing more and longer interactions with a conspecific compared to SN mice (Fig.3).

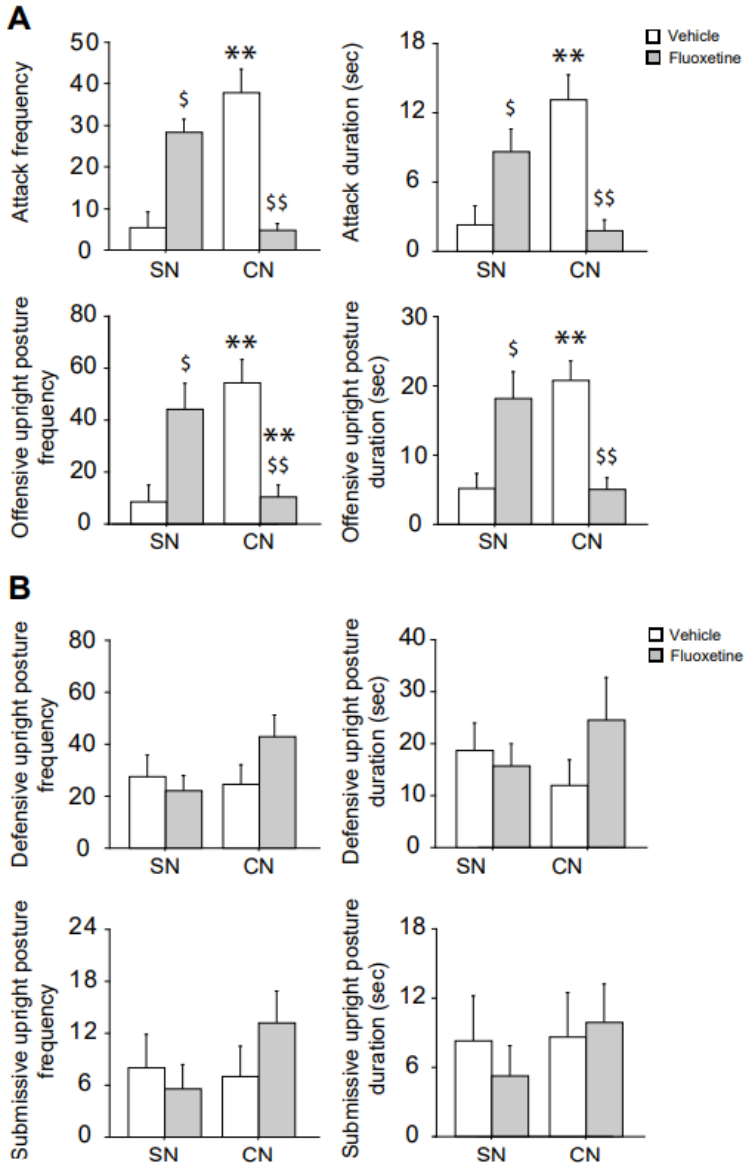

Figure 2. Agonistic behavior in adult mice. *(**A**) CN vehicle mice showed more attacks and offensive upright postures than SN of the same treatment group. Fluoxetine acted in an opposite direction in the two groups, reducing offensive behaviors in CN mice and increasing the same parameters in SN. (**B**) Defensive behaviors were not significantly different between groups. ** p < 0.01, CN vs. SN of the same treatment; $ and $$ p < 0.05 and < 0.01 fluoxetine vs. vehicle of the same experimental group, n = 8. Data are means ± S.E.M.*

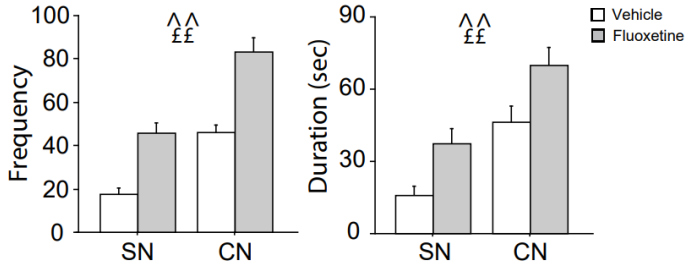

Figure 3. Social investigation behavior. CN mice showed a higher propensity to interact with a conspecific compared to SN (main effect of the experimental group). Fluoxetine increased social investigation behavior in both CN and SN mice (main effect of treatment). ^^ $p < 0.01$, CN vs. SN mice; ££ $p < 0.01$ fluoxetine vs. vehicle, n = 8. Data are means ± S.E.M.

Novelty suppressed feeding

The analysis of behavior in the novelty suppressed test, revealed a statistically significant experimental group x treatment interaction for frequency and duration of object exploration ($F_{1,35} = 20.169$ and $F_{1,36} = 20.540$, respectively for frequency and duration and *ps* < 0.0001 for both) and food contact ($F_{1,34} = 25.399$ and $F_{1,35} = 16.276$, *ps* < 0.0001 and < 0.001, respectively for frequency and duration). *Post-hoc* analysis revealed that CN fluoxetine mice spent more time exploring the novel object ($p < 0.01$) and performed a higher number of explorations ($p < 0.01$; Fig.4A). Moreover, fluoxetine increased the number and the duration of food contact in CN mice (*ps* < 0.01; Fig.4B). Latency data confirmed such profile showing that fluoxetine led to shorter latencies in both object exploration ($U = 31.500$ and $P < 0.001$) and food contact ($U = 81.000$ and $p < 0.001$; Fig.4C) in CN mice.

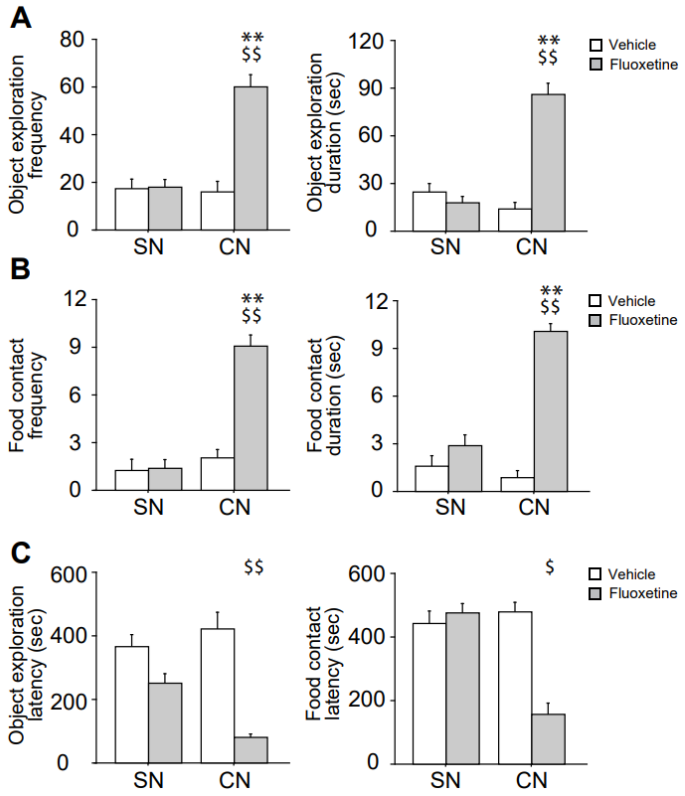

Figure 4. The emotional response in the Novelty Suppressed Feeding test. Fluoxetine treatment affected emotional response only in CN mice, increasing both (**A**) object exploration (**B**) and food contact (C as well as latency to the first contact. ** p < 0.01, CN vs. SN mice of the same treatment; $ and $$ p < 0.05 and < 0.01 fluoxetine vs. vehicle of the same experimental group, n = 8. Data are means ± S.E.M.

Elevated plus maze

We found a statistically significant experimental group x treatment interaction for the percentage of open entries ($F_{1,35}$ = 15.760 and p < 0.01), time in open arms ($F_{1,36}$ = 10.355 and p < 0.01), and the number of open entries ($F_{1,35}$ = 13.022 and p < 0.001), showing that fluoxetine treatment produced opposite effects in CN and SN mice. *Post-hoc* analysis revealed that fluoxetine increased the percentage of open entries, time in open and the number of open entries in CN (p < 0.05 for all parameters) while reducing them in SN mice (p < 0.05 for the percentage of open entries and time in open, and p < 0.01 for the number of open entries). Furthermore, CN mice receiving vehicle spent less time and displayed

lower entries in the open arms compared to SN mice receiving vehicle ($p < 0.05$). Finally, CN mice receiving fluoxetine displayed a significantly higher percentage of open entries compared to SN mice receiving fluoxetine ($p < 0.05$; Fig.5A). For locomotor activity, measured as the number of total entries in the maze arms, a statistically significant effect of experimental group x treatment interaction was found ($F_{1,35} = 25.674$ and $p < 0.0001$). Fluoxetine increased the number of total entries in CN ($p < 0.05$) while reducing it in SN mice ($p < 0.01$), compared to the relative vehicle groups. In addition, fluoxetine increased the number of total entries in CN compared to SN mice ($p < 0.05$; Fig.5A). No significant effects were found for the number of closed entries (data not shown). Concerning other behavioral parameters, CN mice showed higher frequency and duration of stretched attend posture (main effect of experimental group: $Fs_{1,36} = 36.641$ and 23.467, respectively for frequency and duration and $ps < 0.0001$ for both) and lower head-dipping frequency and duration (main effect of experimental group: $Fs_{1,36} = 12.193$ and 10.743, $ps < 0.001$ and < 0.01, respectively for frequency and duration) compared to SN mice (Fig.5B).

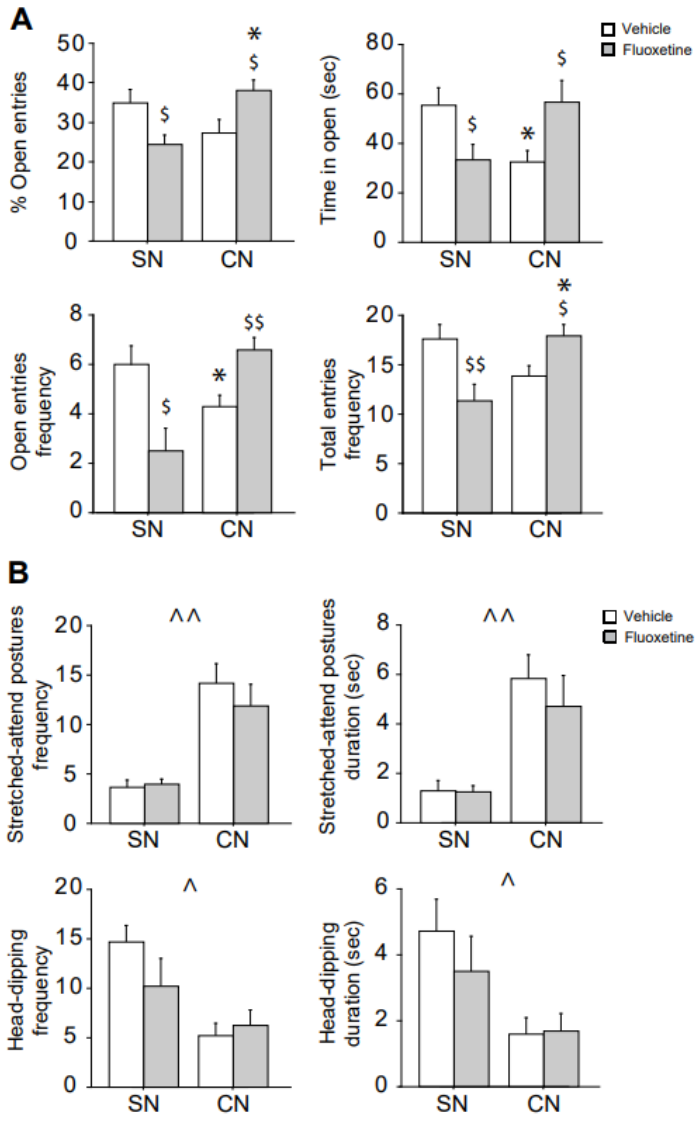

Figure 5. Anxiety-like behavior and activity levels in the elevated plus maze test. (**A**) CN vehicle mice spent less time in the open arms compared to SN mice of the same group. Fluoxetine administration reduced emotionality in CN while increasing it in SN. (**B**) CN mice performed high stretched attend postures and less head-dipping compared to SN (main effect of the experimental group). * and ** $p < 0.05$ and < 0.01, CN vs. SN of the same treatment; $ and $$ $p < 0.05$ and < 0.01 fluoxetine vs. vehicle of the same experimental group; ^ and ^^ $p < 0.05$ and <0.01 CN vs. SN mice, n = 10. Data are means ± S.E.M.

Brain derived neurotrophic factor levels

We found a statistically significant effect of experimental group x treatment interaction ($F_{1,28} = 7.213$ $p < 0.05$) for hippocampal BDNF, showing that fluoxetine differently affected neurotrophin levels in CN and SN mice. *Post-hoc* comparisons revealed that fluoxetine treatment increased BDNF in SN ($p < 0.05$) and reduced it in CN mice ($p < 0.05$). In addition, CN mice receiving vehicle showed higher levels of BDNF compared to SN mice receiving vehicle ($p < 0.05$; Fig.6).

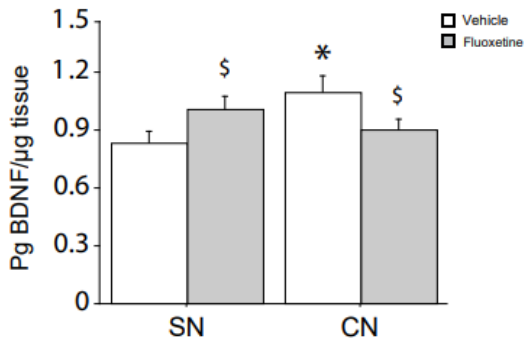

Figure 6. Hippocampal BDNF protein levels. CN vehicle mice had higher hippocampal BDNF levels compared to SN at baseline. Fluoxetine increased BDNF levels in SN mice and reduced it in CN. * p < 0.05, CN vs. SN of the same treatment group; $ p < 0.05 fluoxetine vs. vehicle of the same experimental group, n = 8. Data are means ± S.E.M.

Epigenetic structure at the promoter IV of BDNF

The ChIP analysis revealed a statistically significant experimental group x treatment interaction. *Post-hoc* analysis showed that fluoxetine treatment led to a trend toward an increase in the levels of the repressive mark H3K27me3 in CN mice (p = 0.052; Fig.7A). CN mice displayed an overall increase in the levels of acetylation of the histone H3 compared to SN mice (p = 0.006; Fig.7B). *Post-hoc* analysis revealed that fluoxetine treatment led to a trend toward an increase of the acetylation levels in SN mice (p = 0.056; Fig.7B).

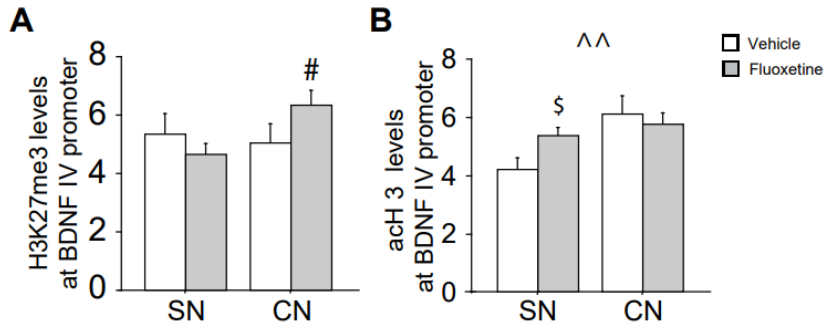

Figure 7. Chromatin remodeling at BDNF IV promoter in the hippocampus of adult mice. (**A**) ChIP analysis revealed a clear trend toward an increase in the levels of the repressive mark H3K27me3 in CN mice. (**B**) CN mice showed higher levels of the acetylated form of histone H3 at the BDNF promoter IV compared to SN mice. In addition, fluoxetine administration led to a trend toward an increased acetylation levels in SN mice. # $p = 0.052$ vs. SN, same treatment; $ $p = 0.056$, fluoxetine vs. vehicle within the same experimental group, ^^ $p < 0.01$, CN vs. SN mice; $n = 8$. Data are means ± S.E.M.

Discussion

Adult SN and CN mice demonstrated different subjective experiences of the environment as indicated by their distinct social and emotional responses in facing the same test environment. Accordingly, chronic fluoxetine treatment produced significantly different effects in the two experimental groups both at behavioral and BDNF regulation levels.

In line with the literature (Branchi, 2009; Branchi and Alleva, 2006; Branchi et al., 2013a), the two experimental groups confirmed that they differently experience the environment. Overall, CN experienced it as more socially challenging and potentially dangerous showing higher levels of offensive and anxiety-like behavior compared to SN mice. We thus administered fluoxetine to the experimental subjects to assess whether treatment outcome depends on their subjective experience of the environment. As predicted, the drug produced different effects in the two groups. In the social interaction test, the treatment reduced offensive behavior in CN, while increasing it in SN mice. In the elevated plus maze, fluoxetine increased the time spent in the open arms and the number of entries in CN, while reducing it in SN mice. Our results are in line with previous preclinical studies showing that fluoxetine effects depend on the behavioral profile of the individual (Mitchell, 2005). For instance, SSRIs are reported to be more likely to produce anti-aggressive effects in conditions of escalated agonistic behavior (Caldwell and Miczek, 2008; Mitchell, 2005) and have effects that are dependent on social status, reducing anxiety-like response only in dominants (Murlanova et al., 2021). In addition, other factors that affect the behavioral profile, such as sex and genetic background, have been reported to modulate the effects of SSRIs as well (Cervo et al., 2005; Gunther et al., 2011; Lifschytz et al., 2006; Lopez-Rubalcava and Lucki, 2000). The role of the subjective experience of the environment in determining fluoxetine outcome may explain, at least partially, the reported variable drug effects and may reconcile previous discordant findings on the effects of SSRIs on aggressive and emotional responses. Indeed, many studies found that SSRIs exert an anti-aggressive action (Fuller, 1996), reducing offensive behaviors in mouse models of aggression (Mamiya et al., 2017; Wagner et al., 1993) and following chronic

stress (Laugeray et al., 2016), and produce anxiolytic effects (Kurt et al., 2000; Rogoz and Skuza, 2011). However, other studies found no (Silva and Brandao, 2000) or opposite results (Caldwell and Miczek, 2008; Drapier et al., 2007; Kurt et al., 2000; Mitchell, 2005; Oh et al., 2009; Robert et al., 2011). It is worth noting that the different effects of fluoxetine in SN and CN appear not concerning all behavioral domains, as fluoxetine equally increased the frequency and the duration of affiliative behavior, such as allosniffing and allogrooming, in both experimental groups.

To investigate the action of fluoxetine effect at molecular level, we assessed BDNF levels and epigenetic structure as this neurotrophin is considered to be one of the prominent proxy of SSRI action (Castren and Monteggia, 2021; Duman et al., 2021). In addition, the epigenetic regulation of the BDNF promoter IV has been involved in the effects of antidepressants on the emotional response and antidepressant treatments have been found to reverse its downregulation in animals exposed to chronic stress (Branchi et al., 2011b; Tsankova et al., 2006). We showed, on the one hand, that CN had significantly higher hippocampal BDNF levels compared to SN mice at baseline, as previously described (Branchi et al., 2006a; Branchi et al., 2006b). On the other, in line with the behavioral results, fluoxetine effects on BDNF regulation differed in the two experimental groups: neurotrophin levels were significantly reduced in CN, while increased in SN mice. In addition, the treatment modified methylation and acetylation levels of exon promoter IV the BDNF gene accordingly. As for the social and emotional responses, viewing the subjective experience of the environment as determining treatment effects provides a potential explanation for the discordant results concerning the effects of SSRIs on BDNF expression (Bessa et al., 2009; Goekint et al., 2011; Jacobsen and Mork, 2004; Nibuya et al., 1996; Torrisi et al., 2021).

The present results are in line with the hypothesis that the amplification of the influence of the environment on mood produced by SSRIs concerns, not only the objective features (Branchi, 2011; Branchi and Giuliani, 2021), but also the subjective experience of the environment. Indeed, as the objective features have been kept the same for SN and CN mice, the distinct fluoxetine effects in the two groups can be ascribed to their different subjective experiences. Since SN mice

are relatively naïve and with a history of limited social interactions and CN mice are socially and emotionally more experienced, fluoxetine enhanced the experience of a challenging environment in SN, increasing their aggressive and anxiety-like behavior, while producing an opposite effect in CN mice. In accordance, as BDNF is produced in response to a stimulating environment in order to promote neural and behavioral adaptive changes (Branchi et al., 2004; Clayton et al., 2020; Rossi et al., 2006; Sale et al., 2014; Yoshii and Constantine-Paton, 2010), we found its levels to be increased by fluoxetine treatment in SN but reduced in CN mice. The BDNF epigenetic regulation changes show an overlapping profile confirming that epigenetic modifications mediate the effect of the environment on brain function (Branchi et al., 2004; Clayton et al., 2020; Rossi et al., 2006; Sale et al., 2014; Yoshii and Constantine-Paton, 2010).

The view that the subjective experience of the environment affects the response to psychiatric treatments modulating plasticity, such as antidepressants, is rapidly emerging in clinical studies (Bottemanne et al., 2022; Murphy et al., 2021; Reed et al., 2022; Wardenaar et al., 2014). In line with our hypothesis positing that SSRIs amplify the influence of the subjective experience of the living environment on mood, an increasing number of studies is elegantly showing that antidepressant outcome can be predicted according to patient's affective bias (Godlewska et al., 2016; Shiroma et al., 2014). A positive bias in perceiving positive facial expressions has been found to be associated with subsequent improvement in depression severity (Tranter et al., 2009). On the basis of these results, the PReDicT study has developed an algorithm to predict the clinical effectiveness of the antidepressant treatment (Browning et al., 2021; Kingslake et al., 2017) managing to improve the functional outcome as well as the anxiety symptoms in patients (Browning et al., 2021). Further evidence supporting our results concerns personality studies. Differences in personality, which significantly affect the experience of the environment and stressful conditions (Kendler et al., 2003), are associated with different efficacies of antidepressant treatments: patients with a more positive attitude toward the living environment, show lower levels of neuroticism and higher levels of extraversion,

agreeableness, and conscientiousness, recover faster (Kim et al., 2016; Wardenaar et al., 2014).

Our study suffers from some limitations. First, though SN and CN mice are virtually genetically undistinguishable and come from the same batch of animals, the subjective view of the environment may not be the only explanation of the present results as the different rearing conditions may have produced distinct adult neural organizations potentially accounting for the differences in treatment outcome. However, as neural substrates and behavioral profiles are highly related, the subjective behavioral response to the environment can be considered a sensitive proxy of brain functioning and thus a factor predicting therapeutic efficacy. Second, we focused on hippocampal BDNF but other brain regions and molecular markers could have also been considered. However, BDNF regulation in the hippocampus is considered the gold standard reference to assess the action and effects of serotoninergic antidepressants (Castren and Monteggia, 2021; Duman et al., 2021). Finally, as sexes show neural differences due to both genetic and hormonal factors, studying both male and female subjects would have improved the translational power of the present findings.

Overall, we have demonstrated the role of subjective view in experiencing the environment as key determinant of SSRI outcome at the preclinical level. Indeed, the latter depends not only on the objective features of the living environment, as previously shown (Branchi, 2011; Viglione et al., 2019), but also on the subjective experience of it. Translating these findings to clinical practice, we suggest that psychiatric treatment increasing plasticity and susceptibility to change behavioral outcome according to context -- such as SSRIs, psychedelics, and ketamine (Carhart-Harris et al., 2018; Casarotto et al., 2021; Price et al., 2022) -- should consider individuality in facing environmental challenges to designing tailored therapeutic strategies with high efficacy. Moreover, such a personalized approach will allow improving also our ability to predict treatment outcome.

References

Alboni, S., van Dijk, R.M., Poggini, S., Milior, G., Perrotta, M., Drenth, T., Brunello, N., Wolfer, D.P., Limatola, C., Amrein, I.*, et al.* (2017). Fluoxetine effects on molecular, cellular and behavioral endophenotypes of depression are driven by the living environment. Molecular psychiatry *22*, 552-561.

Bashiri, H., Houwing, D.J., Homberg, J.R., and Salari, A.A. (2021). The combination of fluoxetine and environmental enrichment reduces postpartum stress-related behaviors through the oxytocinergic system and HPA axis in mice. Scientific reports *11*, 8518.

Bessa, J.M., Ferreira, D., Melo, I., Marques, F., Cerqueira, J.J., Palha, J.A., Almeida, O.F., and Sousa, N. (2009). The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. Molecular psychiatry *14*, 764-773, 739.

Bottemanne, H., Morlaas, O., Claret, A., Sharot, T., Fossati, P., and Schmidt, L. (2022). Evaluation of Early Ketamine Effects on Belief-Updating Biases in Patients With Treatment-Resistant Depression. JAMA psychiatry.

Branchi, I. (2009). The mouse communal nest: investigating the epigenetic influences of the early social environment on brain and behavior development. Neuroscience and biobehavioral reviews *33*, 551-559.

Branchi, I. (2011). The double edged sword of neural plasticity: increasing serotonin levels leads to both greater vulnerability to depression and improved capacity to recover. Psychoneuroendocrinology *36*, 339-351.

Branchi, I. (2022). Plasticity in mental health: A network theory. Neuroscience and biobehavioral reviews *138*, 104691.

Branchi, I., and Alleva, E. (2006). Communal nesting, an early social enrichment, increases the adult anxiety-like response and shapes the role of social context in modulating the emotional behavior. Behavioural brain research *172*, 299-306.

Branchi, I., Curley, J.P., D'Andrea, I., Cirulli, F., Champagne, F.A., and Alleva, E. (2013a). Early interactions with mother and peers independently build adult social skills and shape BDNF and oxytocin receptor brain levels. Psychoneuroendocrinology *38*, 522-532.

Branchi, I., D'Andrea, I., Fiore, M., Di Fausto, V., Aloe, L., and Alleva, E. (2006a). Early social enrichment shapes social behavior and nerve growth factor and brain-derived neurotrophic factor levels in the adult mouse brain. Biological psychiatry *60*, 690-696.

Branchi, I., D'Andrea, I., Santarelli, S., Bonsignore, L.T., and Alleva, E. (2011a). The richness of social stimuli shapes developmental trajectories: Are laboratory mouse pups impoverished? Progress in neuro-psychopharmacology & biological psychiatry *35*, 1452-1460.

Branchi, I., D'Andrea, I., Sietzema, J., Fiore, M., Di Fausto, V., Aloe, L., and Alleva, E. (2006b). Early social enrichment augments adult hippocampal BDNF levels and survival of BrdU-positive cells while increasing anxiety- and "depression"-like behavior. Journal of neuroscience research *83*, 965-973.

Branchi, I., Francia, N., and Alleva, E. (2004). Epigenetic control of neurobehavioural plasticity: the role of neurotrophins. Behavioural pharmacology *15*, 353-362.

Branchi, I., and Giuliani, A. (2021). Shaping therapeutic trajectories in mental health: Instructive vs. permissive causality. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology *43*, 1-9.

Branchi, I., Karpova, N.N., D'Andrea, I., Castren, E., and Alleva, E. (2011b). Epigenetic modifications induced by early enrichment are associated with changes in timing of induction of BDNF expression. Neuroscience letters *495*, 168-172.

Branchi, I., Santarelli, S., Capoccia, S., Poggini, S., D'Andrea, I., Cirulli, F., and Alleva, E. (2013b). Antidepressant treatment outcome depends on the quality of the living environment: a pre-clinical investigation in mice. PloS one *8*, e62226.

Browning, M., Bilderbeck, A.C., Dias, R., Dourish, C.T., Kingslake, J., Deckert, J., Goodwin, G.M., Gorwood, P., Guo, B., Harmer, C.J.*, et al.* (2021). The clinical effectiveness of using a predictive algorithm to guide antidepressant treatment in primary care (PReDicT): an open-label, randomised controlled

trial. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *46*, 1307-1314.

Caldwell, E.E., and Miczek, K.A. (2008). Long-term citalopram maintenance in mice: selective reduction of alcohol-heightened aggression. Psychopharmacology *196*, 407-416.

Carhart-Harris, R.L., Roseman, L., Haijen, E., Erritzoe, D., Watts, R., Branchi, I., and Kaelen, M. (2018). Psychedelics and the essential importance of context. Journal of psychopharmacology *32*, 725-731.

Casarotto, P.C., Girych, M., Fred, S.M., Kovaleva, V., Moliner, R., Enkavi, G., Biojone, C., Cannarozzo, C., Sahu, M.P., Kaurinkoski, K.*, et al.* (2021). Antidepressant drugs act by directly binding to TRKB neurotrophin receptors. Cell *184*, 1299-1313 e1219.

Castren, E., and Monteggia, L.M. (2021). Brain-Derived Neurotrophic Factor Signaling in Depression and Antidepressant Action. Biological psychiatry *90*, 128-136.

Cervo, L., Canetta, A., Calcagno, E., Burbassi, S., Sacchetti, G., Caccia, S., Fracasso, C., Albani, D., Forloni, G., and Invernizzi, R.W. (2005). Genotype-dependent activity of tryptophan hydroxylase-2 determines the response to citalopram in a mouse model of depression. The Journal of neuroscience : the official journal of the Society for Neuroscience *25*, 8165-8172.

Chiarotti, F., Viglione, A., Giuliani, A., and Branchi, I. (2017). Citalopram amplifies the influence of living conditions on mood in depressed patients enrolled in the STAR*D study. Translational psychiatry *7*, e1066.

Cirulli, F., Berry, A., and Alleva, E. (2000). Intracerebroventricular administration of brain-derived neurotrophic factor in adult rats affects analgesia and spontaneous behaviour but not memory retention in a Morris Water Maze task. Neuroscience letters *287*, 207-210.

Clayton, D.F., Anreiter, I., Aristizabal, M., Frankland, P.W., Binder, E.B., and Citri, A. (2020). The role of the genome in experience-dependent plasticity: Extending the analogy of the genomic action potential. Proceedings of the National Academy of Sciences of the United States of America *117*, 23252-23260.

Drapier, D., Bentue-Ferrer, D., Laviolle, B., Millet, B., Allain, H., Bourin, M., and Reymann, J.M. (2007). Effects of acute fluoxetine, paroxetine and desipramine on rats tested on the elevated plus-maze. Behavioural brain research *176*, 202-209.

Duman, R.S., Deyama, S., and Fogaca, M.V. (2021). Role of BDNF in the pathophysiology and treatment of depression: Activity-dependent effects distinguish rapid-acting antidepressants. The European journal of neuroscience *53*, 126-139.

Fuller, R.W. (1996). The Influence of Fluoxetine on Aggressive Behavior. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *14*, 77-81.

Godlewska, B.R., Browning, M., Norbury, R., Cowen, P.J., and Harmer, C.J. (2016). Early changes in emotional processing as a marker of clinical response to SSRI treatment in depression. Translational psychiatry *6*, e957.

Goekint, M., Roelands, B., Heyman, E., Njemini, R., and Meeusen, R. (2011). Influence of citalopram and environmental temperature on exercise-induced changes in BDNF. Neuroscience letters *494*, 150-154.

Gunther, L., Rothe, J., Rex, A., Voigt, J.P., Millan, M.J., Fink, H., and Bert, B. (2011). 5-HT(1A)-receptor over-expressing mice: genotype and sex dependent responses to antidepressants in the forced swim-test. Neuropharmacology *61*, 433-441.

Jacobsen, J.P., and Mork, A. (2004). The effect of escitalopram, desipramine, electroconvulsive seizures and lithium on brain-derived neurotrophic factor mRNA and protein expression in the rat brain and the correlation to 5-HT and 5-HIAA levels. Brain research *1024*, 183-192.

Karpova, N.N., Pickenhagen, A., Lindholm, J., Tiraboschi, E., Kulesskaya, N., Agustsdottir, A., Antila, H., Popova, D., Akamine, Y., Bahi, A.*, et al.* (2011). Fear erasure in mice requires synergy between antidepressant drugs and extinction training. Science *334*, 1731-1734.

Kempermann, G. (2019). Environmental enrichment, new neurons and the neurobiology of individuality. Nature reviews Neuroscience *20*, 235-245.

Kendler, K.S., Gardner, C.O., and Prescott, C.A. (2003). Personality and the experience of environmental adversity. Psychological medicine *33*, 1193-1202.

Kim, S.Y., Stewart, R., Bae, K.Y., Kim, S.W., Shin, I.S., Hong, Y.J., Ahn, Y., Jeong, M.H., Yoon, J.S., and Kim, J.M. (2016). Influences of the Big Five personality traits on the treatment response and longitudinal course of depression in patients with acute coronary syndrome: A randomised controlled trial. Journal of affective disorders *203*, 38-45.

Kingslake, J., Dias, R., Dawson, G.R., Simon, J., Goodwin, G.M., Harmer, C.J., Morriss, R., Brown, S., Guo, B., Dourish, C.T.*, et al.* (2017). The effects of using the PReDicT Test to guide the antidepressant treatment of depressed patients: study protocol for a randomised controlled trial. Trials *18*, 558.

Klobl, M., Seiger, R., Vanicek, T., Handschuh, P., Reed, M.B., Spurny-Dworak, B., Ritter, V., Godbersen, G.M., Gryglewski, G., Kraus, C.*, et al.* (2022). Escitalopram modulates learning content-specific neuroplasticity of functional brain networks. NeuroImage *247*, 118829.

Kurt, M., Arik, A.C., and Celik, S. (2000). The effects of sertraline and fluoxetine on anxiety in the elevated plus-maze test in mice. Journal of basic and clinical physiology and pharmacology *11*, 173-180.

Laugeray, A., Launay, J.M., Callebert, J., Mutlu, O., Guillemin, G.J., Belzung, C., and Barone, P.R. (2016). Chronic Treatment with the IDO1 Inhibitor 1-Methyl-D-Tryptophan Minimizes the Behavioural and Biochemical Abnormalities Induced by Unpredictable Chronic Mild Stress in Mice - Comparison with Fluoxetine. PloS one *11*, e0164337.

LeDoux, J.E., and Brown, R. (2017). A higher-order theory of emotional consciousness. Proceedings of the National Academy of Sciences of the United States of America *114*, E2016-E2025.

Lifschytz, T., Shalom, G., Lerer, B., and Newman, M.E. (2006). Sex-dependent effects of fluoxetine and triiodothyronine in the forced swim test in rats. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology *16*, 115-121.

Lima, J., Sharp, T., Bannerman, D.M., and McHugh, S.B. (2019). Enhanced discriminative aversive learning and amygdala responsivity in 5-HT transporter mutant mice. Translational psychiatry *9*, 139.

Lopez-Rubalcava, C., and Lucki, I. (2000). Strain differences in the behavioral effects of antidepressant drugs in the rat forced swimming test. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *22*, 191-199.

Mamiya, P.C., Matray-Devoti, J., Fisher, H., and Wagner, G.C. (2017). Mice increased target biting behaviors 24h after co-administration of alcohol and fluoxetine. Brain research *1662*, 110-115.

Mikics, E., Guirado, R., Umemori, J., Toth, M., Biro, L., Miskolczi, C., Balazsfi, D., Zelena, D., Castren, E., Haller, J.*, et al.* (2018). Social Learning Requires Plasticity Enhanced by Fluoxetine Through Prefrontal Bdnf-TrkB Signaling to Limit Aggression Induced by Post-Weaning Social Isolation. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *43*, 235-245.

Mitchell, P.J. (2005). Antidepressant treatment and rodent aggressive behaviour. European journal of pharmacology *526*, 147-162.

Molendijk, M.L., van Tol, M.J., Penninx, B.W., van der Wee, N.J., Aleman, A., Veltman, D.J., Spinhoven, P., and Elzinga, B.M. (2012). BDNF val66met affects hippocampal volume and emotion-related hippocampal memory activity. Translational psychiatry *2*, e74.

Murlanova, K., Michaelevski, I., Kreinin, A., Terrillion, C., Pletnikov, M., and Pinhasov, A. (2021). Link between temperament traits, brain neurochemistry and response to SSRI: insights from animal model of social behavior. Journal of affective disorders *282*, 1055-1066.

Murphy, S.E., Capitao, L.P., Giles, S.L.C., Cowen, P.J., Stringaris, A., and Harmer, C.J. (2021). The knowns and unknowns of SSRI treatment in young people with depression and anxiety: efficacy, predictors, and mechanisms of action. The lancet Psychiatry *8*, 824-835.

Nibuya, M., Nestler, E.J., and Duman, R.S. (1996). Chronic antidepressant administration increases the expression of cAMP response element binding

protein (CREB) in rat hippocampus. The Journal of neuroscience : the official journal of the Society for Neuroscience *16*, 2365-2372.

Oh, J.E., Zupan, B., Gross, S., and Toth, M. (2009). Paradoxical anxiogenic response of juvenile mice to fluoxetine. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *34*, 2197-2207.

Poggini, S., Matte Bon, G., Golia, M.T., Ciano Albanese, N., Viglione, A., Poleggi, A., Limatola, C., Maggi, L., and Branchi, I. (2021). Selecting antidepressants according to a drug-by-environment interaction: A comparison of fluoxetine and minocycline effects in mice living either in enriched or stressful conditions. Behavioural brain research *408*, 113256.

Price, R.B., Spotts, C., Panny, B., Griffo, A., Degutis, M., Cruz, N., Bell, E., Do-Nguyen, K., Wallace, M.L., Mathew, S.J.*, et al.* (2022). A Novel, Brief, Fully Automated Intervention to Extend the Antidepressant Effect of a Single Ketamine Infusion: A Randomized Clinical Trial. The American journal of psychiatry, appiajp20220216.

Reed, M.B., Klobl, M., Godbersen, G.M., Handschuh, P.A., Ritter, V., Spurny-Dworak, B., Unterholzner, J., Kraus, C., Gryglewski, G., Winkler, D.*, et al.* (2022). Serotonergic modulation of effective connectivity in an associative relearning network during task and rest. NeuroImage *249*, 118887.

Rivenbark, J., Arseneault, L., Caspi, A., Danese, A., Fisher, H.L., Moffitt, T.E., Rasmussen, L.J.H., Russell, M.A., and Odgers, C.L. (2020). Adolescents' perceptions of family social status correlate with health and life chances: A twin difference longitudinal cohort study. Proceedings of the National Academy of Sciences of the United States of America *117*, 23323-23328.

Robert, G., Drapier, D., Bentue-Ferrer, D., Renault, A., and Reymann, J.M. (2011). Acute and chronic anxiogenic-like response to fluoxetine in rats in the elevated plus-maze: modulation by stressful handling. Behavioural brain research *220*, 344-348.

Rogoz, Z., and Skuza, G. (2011). Anxiolytic-like effects of olanzapine, risperidone and fluoxetine in the elevated plus-maze test in rats. Pharmacological reports : PR *63*, 1547-1552.

Rossi, C., Angelucci, A., Costantin, L., Braschi, C., Mazzantini, M., Babbini, F., Fabbri, M.E., Tessarollo, L., Maffei, L., Berardi, N.*, et al.* (2006). Brain-derived neurotrophic factor (BDNF) is required for the enhancement of hippocampal neurogenesis following environmental enrichment. The European journal of neuroscience *24*, 1850-1856.

Sale, A., Berardi, N., and Maffei, L. (2014). Environment and brain plasticity: towards an endogenous pharmacotherapy. Physiological reviews *94*, 189-234.

Shiroma, P.R., Thuras, P., Johns, B., and Lim, K.O. (2014). Emotion recognition processing as early predictor of response to 8-week citalopram treatment in late-life depression. International journal of geriatric psychiatry *29*, 1132-1139.

Silva, R.C., and Brandao, M.L. (2000). Acute and chronic effects of gepirone and fluoxetine in rats tested in the elevated plus-maze: an ethological analysis. Pharmacology, biochemistry, and behavior *65*, 209-216.

Sutherland, C.A.M., Burton, N.S., Wilmer, J.B., Blokland, G.A.M., Germine, L., Palermo, R., Collova, J.R., and Rhodes, G. (2020). Individual differences in trust evaluations are shaped mostly by environments, not genes. Proceedings of the National Academy of Sciences of the United States of America *117*, 10218-10224.

Torrisi, S.A., Lavanco, G., Maurel, O.M., Gulisano, W., Laudani, S., Geraci, F., Grasso, M., Barbagallo, C., Caraci, F., Bucolo, C.*, et al.* (2021). A novel arousal-based individual screening reveals susceptibility and resilience to PTSD-like phenotypes in mice. Neurobiology of stress *14*, 100286.

Tranter, R., Bell, D., Gutting, P., Harmer, C., Healy, D., and Anderson, I.M. (2009). The effect of serotonergic and noradrenergic antidepressants on face emotion processing in depressed patients. Journal of affective disorders *118*, 87-93.

Trivedi, M.H., Rush, A.J., Wisniewski, S.R., Nierenberg, A.A., Warden, D., Ritz, L., Norquist, G., Howland, R.H., Lebowitz, B., McGrath, P.J.*, et al.* (2006). Evaluation of outcomes with citalopram for depression using measurement-

based care in STAR*D: implications for clinical practice. The American journal of psychiatry *163*, 28-40.

Tsankova, N.M., Berton, O., Renthal, W., Kumar, A., Neve, R.L., and Nestler, E.J. (2006). Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. Nature neuroscience *9*, 519-525.

Viglione, A., Chiarotti, F., Poggini, S., Giuliani, A., and Branchi, I. (2019). Predicting antidepressant treatment outcome based on socioeconomic status and citalopram dose. The pharmacogenomics journal.

Wagner, G.C., Fisher, H., Pole, N., Borve, T., and Johnson, S.K. (1993). Effects of monoaminergic agonists on alcohol-induced increases in mouse aggression. Journal of studies on alcohol Supplement *11*, 185-191.

Wardenaar, K.J., Conradi, H.J., Bos, E.H., and de Jonge, P. (2014). Personality modulates the efficacy of treatment in patients with major depressive disorder. The Journal of clinical psychiatry *75*, e916-923.

Yoshii, A., and Constantine-Paton, M. (2010). Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease. Developmental neurobiology *70*, 304-322.

GENERAL DISCUSSION AND CONCLUSIONS

General discussion

The lack of life skills induced by social deprivation affects both males and females at behavioral and molecular levels

Adolescent social isolation leads to depressive- and anxiety-like phenotype and reduced neural plasticity in both sexes. However, the lack of social experiences in adolescence altered different behavioral domains of the two sexes: females showed more marked impairments in the emotional domain, while males displayed alterations in the cognitive domain and the stress response. Since the key role played by BDNF on neural plasticity and early development of the brain (Antal et al., 2010; Chen et al., 2012; Lee et al., 2020; Yang et al., 2020), it could be related to the pathophysiology of depression in both males and females, in particular concerning depressive- and anxiety-like symptoms displayed equally by both sexes in our work. However, there are still few studies on adolescent depression and BDNF levels in the two sexes and it is necessary to better understand the mechanisms underlying the development of adolescent depression (Lee et al., 2022). In addition, in both sexes, adolescent isolation led to a reduction of inflammatory markers. These findings are in line with the studies of Donovan and collaborators showing that post-weaning social isolation reduces IL-1β in male and female prairie voles (Donovan et al., 2021), and with the work conducted by Berry and collaborators, showing lower mRNA levels of Il-1β in the dorsal hippocampus of rats exposed to prenatal stress (Berry et al., 2022). In addition, Panetta and colleagues correlate the lower levels of hippocampal IL-1β to increased anxiety-related behavior in the elevated plus maze and impaired neural plasticity in terms of decreased BDNF levels (Panetta et al., 2017). It is important to note that cytokines are also constitutively released in the healthy brain by resident myeloid cells to keep proper synaptic plasticity, as the modulation of LTP and synaptic scaling, a form of homeostatic plasticity (Rizzo et al., 2018; Salim et al., 2012). Thus, both immune activation and suppression impair synaptic plasticity and could lead to emotional and cognitive dysfunctions in mice (Alboni et al., 2016; Alboni et al., 2017; Golia et al., 2019). This mechanism could explain the link between the behavioral abnormalities and

the significant reduction of pro-inflammatory cytokine IL-1β and increased levels of anti-inflammatory cytokine IL-10 after adolescent social stress.

These findings indicate that social deprivation deeply affects the physiological development of both sexes and that the different vulnerability to mental disorders could be ascribed to their different responses to the same environmental stimuli and different stress responses. Indeed, the HPA-axis reactivity after social isolation was different between males and females suggesting different glucocorticoid negative feedback sensitivity and HPA-axis responsiveness.

Overall, in a translational perspective, these findings point out the essential role of social experiences in the development of skills and the capability to face environmental challenges in boys and girls. Indeed, life skills are defined as adaptive and positive behaviors that enable individuals to deal effectively with the demands and challenges of everyday life (UNICEF, 2012). Since many healthy behaviors that contribute to morbidity and mortality are established during adolescence (Asarnow and Miranda, 2014; Campo, 2012; Kieling et al., 2011), this period is recognized as a critical time to introduce and sustain critical health-related knowledge and skills, in particular to mental health (Pandey et al., 2018). Evidence supports multicomponent interventions which aim to strengthen life skills including emotional regulation, self-efficacy, and conflict resolution that are typically delivered through a classroom-based curriculum (Durlak et al., 2011; Pandey et al., 2018; UNICEF, 2012; Weare and Nind, 2011). Singla and collaborators show the beneficial effects of programs improving life skills targeting one or more mental health outcomes and co-occurring risk factors in school and community settings. In particular, comprehensive programs focusing on multiple life skills related to the individual, his or her social environment and parent-child interactions appear more effective to improve individuals' mental health (Singla et al., 2020).

The interaction between antidepressants, environment, and individuality in experiencing the environment determines the treatment outcome

Our findings showed that adolescent isolation induces both depressive- and anxiety-like behavior and that environmental enrichment alone or in combination with fluoxetine, but not fluoxetine alone, counterbalanced the detrimental effects of isolation on the emotional domain. In particular, we showed that (1) the environment is key in recovering from the depressive-like behavior because it provides the skills needed by the individual isolated during adolescence to face the environmental challenges at adulthood, (2) fluoxetine treatment, by enhancing plasticity, amplifies the beneficial effect of the favorable environment, and (3) the effects of fluoxetine are environment dependent.

These findings are in line with previous studies showing the higher efficacy of fluoxetine treatment administered in a supportive environment compared to a not-beneficial one, underlying the key role of the environment in determining the SSRI outcome (Branchi and Giuliani, 2021; Forsyth et al., 2015; Poggini et al., 2019; Poggini et al., 2021; Viglione et al., 2019; Wilkinson et al., 2019). According to this evidence, the opposite results displayed by Standard+fluoxetine and Enriched+fluoxetine mice in the elevated plus maze in Study 2 displayed also the environment-dependent effects of fluoxetine. The results concerning the HPA-axis activation corroborated this hypothesis. Mice exposed to the standard environment and treated with fluoxetine showed significantly increased levels of corticosterone compared to mice exposed to the enriched environment and treated with fluoxetine.

The influence of the environment on the onset of mental disorders has been reported at the clinical level, but further studies should investigate the interaction between the environment and the progression of psychopathologies. Several studies focused their attention on the emotional profile of individuals and their social environment, reporting that adverse early life events, associated with a dysfunctional socioemotional environment, predict the onset of major depressive disorder in adolescence and its recurrence (Lewinsohn et al., 2001; Lewinsohn et al., 1999). Calati and colleagues show that social environment was deeply associated with emotional dysfunction leading to suicidal thoughts and attempts

in both adolescents and young adults. The main construct was represented by the living conditions, i.e., being single, divorced, widowed, or living alone. In addition, both the objective condition (e.g., living alone) and the subjective feeling of being alone (i.e., loneliness) were strongly associated with suicidal outcomes, suggesting that both objective social isolation and the subjective feeling of loneliness should be incorporated into the risk assessment of mental disorders and suicide (Calati et al., 2019).

Given the role of the individual's subjective feeling in the onset of mental illness, we demonstrated the role of the subjective experience of the environment as a further key determinant of SSRI outcome. As the objective features have been kept the same for SN and CN mice, the distinct fluoxetine effects in the two groups can be ascribed to their different subjective experiences. In line with the literature (Branchi, 2009; Branchi and Alleva, 2006; Branchi et al., 2013), the two experimental groups confirmed that they differently experience the environment. Since SN mice are relatively naïve and with a history of limited social interactions and CN mice are socially and emotionally more experienced, fluoxetine enhanced the experience of a challenging environment in SN, increasing their aggressive and anxiety-like behavior, while producing an opposite effect in CN mice. In accordance, since BDNF is produced in response to a stimulating environment in order to promote neural and behavioral adaptive changes (Branchi et al., 2004; Clayton et al., 2020; Rossi et al., 2006; Sale et al., 2014; Yoshii and Constantine-Paton, 2010), we found its levels to be increased by fluoxetine treatment in SN but reduced in CN mice. The view that the subjective experience of the environment affects the response to psychiatric treatments modulating plasticity, such as antidepressants, is rapidly emerging in clinical studies (Bottemanne et al., 2022; Murphy et al., 2021; Reed et al., 2022; Wardenaar et al., 2014). Our findings suggest that the quality of the environment depends not only on the objective features of the living environment, as previously shown (Branchi, 2011; Viglione et al., 2019), but also on the subjective experience of it, thus such a personalized approach will allow improving also our ability to predict treatment outcome.

Limits

The limitations of the present work include, first, the lack of data concerning the estrous cycle in female subjects. However, vaginal cell sampling imposes stress on the experimental subjects, and previous studies have reported that the effect of the estrous cycle in several behavioral tests, such as open field or fear conditioning, is limited: behavioral parameters were not affected across all four estrous phases (Hiroi and Neumaier, 2006; Lovick and Zangrossi, 2021; Meziane et al., 2007).

A further limitation concerning the study exploring the immune system activation after social isolation, is the assessment of inflammatory markers only in the central nervous system, though in clinical settings peripheral levels are routinely measured. Therefore, future studies assessing both central and peripheral markers will have a high translational value and will help illustrate the link between central and peripheral inflammation.

Finally, in the study aimed at assessing the role of subjective appraisal in determining the antidepressant outcome, we focused mainly on hippocampal BDNF, though other brain regions and molecular markers could have also been considered. However, BDNF regulation in the hippocampus is considered the gold standard reference to assess the action and effects of serotoninergic antidepressants (Castren and Monteggia, 2021; Duman et al., 2021).

General conclusions and future perspectives

Our findings confirm and further describe the key role of context in adolescent neurodevelopment in both sexes and in driving the outcome of psychoactive compounds, especially those leveraging on the enhancement of neural and behavioral plasticity such as SSRIs (Alboni et al., 2017; Branchi, 2011; Poggini et al., 2019; Wilkinson et al., 2019).

In a translational perspective, modifications in the living conditions/lifestyle and/or of the subjective appraisal should be considered to improve therapeutic intervention and predict treatment outcomes in both adolescence and adulthood, as previously suggested by the study of Viglione and collaborators (Viglione et al., 2019). We thus underpin the relevance of the patient's living conditions in the selection of the most effective pharmacological treatment and advocate for considering the key role of context in the precision medicine paradigm in the psychiatric field.

The availability of several longitudinal studies such as The Resilience, Ethnicity and AdolesCent Mental Health (REACH) Study, The National Community Mental Health Care Database (NCMHCD), Adolescent Brain Cognitive Development (ABCD) Study, the Covid-Mind Network, The Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study and the Combining medications to enhance depression outcomes (CO-MED) study, will allow to further explore the interaction between environmental conditions, subjective appraisal, and their interaction at a clinical level to setup novel and effective therapeutic strategies.

References

Alboni, S., Poggini, S., Garofalo, S., Milior, G., El Hajj, H., Lecours, C., Girard, I., Gagnon, S., Boisjoly-Villeneuve, S., Brunello, N.*, et al.* (2016). Fluoxetine treatment affects the inflammatory response and microglial function according to the quality of the living environment. Brain, behavior, and immunity *58*, 261-271.

Alboni, S., van Dijk, R.M., Poggini, S., Milior, G., Perrotta, M., Drenth, T., Brunello, N., Wolfer, D.P., Limatola, C., Amrein, I.*, et al.* (2017). Fluoxetine effects on molecular, cellular and behavioral endophenotypes of depression are driven by the living environment. Molecular psychiatry *22*, 552-561.

Antal, A., Chaieb, L., Moliadze, V., Monte-Silva, K., Poreisz, C., Thirugnanasambandam, N., Nitsche, M.A., Shoukier, M., Ludwig, H., and Paulus, W. (2010). Brain-derived neurotrophic factor (BDNF) gene polymorphisms shape cortical plasticity in humans. Brain stimulation *3*, 230-237.

Asarnow, J.R., and Miranda, J. (2014). Improving care for depression and suicide risk in adolescents: innovative strategies for bringing treatments to community settings. Annual review of clinical psychology *10*, 275-303.

Berry, A., Mazzelli, M., Musillo, C., Riva, M.A., Cattaneo, A., and Cirulli, F. (2022). High-fat diet during adulthood interacts with prenatal stress, affecting both brain inflammatory and neuroendocrine markers in male rats. The European journal of neuroscience *55*, 2326-2340.

Bottemanne, H., Morlaas, O., Claret, A., Sharot, T., Fossati, P., and Schmidt, L. (2022). Evaluation of Early Ketamine Effects on Belief-Updating Biases in Patients With Treatment-Resistant Depression. JAMA psychiatry.

Branchi, I. (2009). The mouse communal nest: investigating the epigenetic influences of the early social environment on brain and behavior development. Neuroscience and biobehavioral reviews *33*, 551-559.

Branchi, I. (2011). The double edged sword of neural plasticity: increasing serotonin levels leads to both greater vulnerability to depression and improved capacity to recover. Psychoneuroendocrinology *36*, 339-351.

Branchi, I., and Alleva, E. (2006). Communal nesting, an early social enrichment, increases the adult anxiety-like response and shapes the role of social context in modulating the emotional behavior. Behavioural brain research *172*, 299-306.

Branchi, I., Curley, J.P., D'Andrea, I., Cirulli, F., Champagne, F.A., and Alleva, E. (2013). Early interactions with mother and peers independently build adult social skills and shape BDNF and oxytocin receptor brain levels. Psychoneuroendocrinology *38*, 522-532.

Branchi, I., Francia, N., and Alleva, E. (2004). Epigenetic control of neurobehavioural plasticity: the role of neurotrophins. Behavioural pharmacology *15*, 353-362.

Branchi, I., and Giuliani, A. (2021). Shaping therapeutic trajectories in mental health: Instructive vs. permissive causality. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology *43*, 1-9.

Calati, R., Ferrari, C., Brittner, M., Oasi, O., Olie, E., Carvalho, A.F., and Courtet, P. (2019). Suicidal thoughts and behaviors and social isolation: A narrative review of the literature. Journal of affective disorders *245*, 653-667.

Campo, J.V. (2012). Annual research review: functional somatic symptoms and associated anxiety and depression--developmental psychopathology in pediatric practice. Journal of child psychology and psychiatry, and allied disciplines *53*, 575-592.

Castren, E., and Monteggia, L.M. (2021). Brain-Derived Neurotrophic Factor Signaling in Depression and Antidepressant Action. Biological psychiatry *90*, 128-136.

Chen, J., Li, X., and McGue, M. (2012). Interacting effect of BDNF Val66Met polymorphism and stressful life events on adolescent depression. Genes, brain, and behavior *11*, 958-965.

Clayton, D.F., Anreiter, I., Aristizabal, M., Frankland, P.W., Binder, E.B., and Citri, A. (2020). The role of the genome in experience-dependent plasticity: Extending the analogy of the genomic action potential. Proceedings of the

National Academy of Sciences of the United States of America *117*, 23252-23260.

Donovan, M.L., Chun, E.K., Liu, Y., and Wang, Z. (2021). Post-weaning Social Isolation in Male and Female Prairie Voles: Impacts on Central and Peripheral Immune System. Frontiers in behavioral neuroscience *15*, 802569.

Duman, R.S., Deyama, S., and Fogaca, M.V. (2021). Role of BDNF in the pathophysiology and treatment of depression: Activity-dependent effects distinguish rapid-acting antidepressants. The European journal of neuroscience *53*, 126-139.

Durlak, J.A., Weissberg, R.P., Dymnicki, A.B., Taylor, R.D., and Schellinger, K.B. (2011). The impact of enhancing students' social and emotional learning: a meta-analysis of school-based universal interventions. Child development *82*, 405-432.

Forsyth, J.K., Bachman, P., Mathalon, D.H., Roach, B.J., and Asarnow, R.F. (2015). Augmenting NMDA receptor signaling boosts experience-dependent neuroplasticity in the adult human brain. Proceedings of the National Academy of Sciences of the United States of America *112*, 15331-15336.

Golia, M.T., Poggini, S., Alboni, S., Garofalo, S., Ciano Albanese, N., Viglione, A., Ajmone-Cat, M.A., St-Pierre, A., Brunello, N., Limatola, C*., et al.* (2019). Interplay between inflammation and neural plasticity: Both immune activation and suppression impair LTP and BDNF expression. Brain, behavior, and immunity *81*, 484-494.

Hiroi, R., and Neumaier, J.F. (2006). Differential effects of ovarian steroids on anxiety versus fear as measured by open field test and fear-potentiated startle. Behavioural brain research *166*, 93-100.

Kieling, C., Baker-Henningham, H., Belfer, M., Conti, G., Ertem, I., Omigbodun, O., Rohde, L.A., Srinath, S., Ulkuer, N., and Rahman, A. (2011). Child and adolescent mental health worldwide: evidence for action. Lancet *378*, 1515-1525.

Lee, B., Shin, E., Song, I., and Chang, B. (2022). Depression in Adolescence and Brain-Derived Neurotrophic Factor. Frontiers in molecular neuroscience *15*, 947192.

Lee, J., Lee, K.H., Kim, S.H., Han, J.Y., Hong, S.B., Cho, S.C., Kim, J.W., and Brent, D. (2020). Early changes of serum BDNF and SSRI response in adolescents with major depressive disorder. Journal of affective disorders *265*, 325-332.

Lewinsohn, P.M., Joiner, T.E., Jr., and Rohde, P. (2001). Evaluation of cognitive diathesis-stress models in predicting major depressive disorder in adolescents. Journal of abnormal psychology *110*, 203-215.

Lewinsohn, P.M., Rohde, P., Klein, D.N., and Seeley, J.R. (1999). Natural course of adolescent major depressive disorder: I. Continuity into young adulthood. Journal of the American Academy of Child and Adolescent Psychiatry *38*, 56-63.

Lovick, T.A., and Zangrossi, H., Jr. (2021). Effect of Estrous Cycle on Behavior of Females in Rodent Tests of Anxiety. Frontiers in psychiatry *12*, 711065.

Meziane, H., Ouagazzal, A.M., Aubert, L., Wietrzych, M., and Krezel, W. (2007). Estrous cycle effects on behavior of C57BL/6J and BALB/cByJ female mice: implications for phenotyping strategies. Genes, brain, and behavior *6*, 192-200.

Murphy, S.E., Capitao, L.P., Giles, S.L.C., Cowen, P.J., Stringaris, A., and Harmer, C.J. (2021). The knowns and unknowns of SSRI treatment in young people with depression and anxiety: efficacy, predictors, and mechanisms of action. The lancet Psychiatry *8*, 824-835.

Pandey, A., Hale, D., Das, S., Goddings, A.L., Blakemore, S.J., and Viner, R.M. (2018). Effectiveness of Universal Self-regulation-Based Interventions in Children and Adolescents: A Systematic Review and Meta-analysis. JAMA pediatrics *172*, 566-575.

Panetta, P., Berry, A., Bellisario, V., Capoccia, S., Raggi, C., Luoni, A., Longo, L., Riva, M.A., and Cirulli, F. (2017). Long-Term Sex-Dependent Vulnerability to Metabolic challenges in Prenatally Stressed Rats. Frontiers in behavioral neuroscience *11*, 113.

Poggini, S., Golia, M.T., Alboni, S., Milior, G., Sciarria, L.P., Viglione, A., Matte Bon, G., Brunello, N., Puglisi-Allegra, S., Limatola, C.*, et al.* (2019). Combined Fluoxetine and Metformin Treatment Potentiates Antidepressant Efficacy Increasing IGF2 Expression in the Dorsal Hippocampus. Neural plasticity *2019*, 4651031.

Poggini, S., Matte Bon, G., Golia, M.T., Ciano Albanese, N., Viglione, A., Poleggi, A., Limatola, C., Maggi, L., and Branchi, I. (2021). Selecting antidepressants according to a drug-by-environment interaction: A comparison of fluoxetine and minocycline effects in mice living either in enriched or stressful conditions. Behavioural brain research *408*, 113256.

Reed, M.B., Klobl, M., Godbersen, G.M., Handschuh, P.A., Ritter, V., Spurny-Dworak, B., Unterholzner, J., Kraus, C., Gryglewski, G., Winkler, D.*, et al.* (2022). Serotonergic modulation of effective connectivity in an associative relearning network during task and rest. NeuroImage *249*, 118887.

Rizzo, F.R., Musella, A., De Vito, F., Fresegna, D., Bullitta, S., Vanni, V., Guadalupi, L., Stampanoni Bassi, M., Buttari, F., Mandolesi, G.*, et al.* (2018). Tumor Necrosis Factor and Interleukin-1beta Modulate Synaptic Plasticity during Neuroinflammation. Neural plasticity *2018*, 8430123.

Rossi, C., Angelucci, A., Costantin, L., Braschi, C., Mazzantini, M., Babbini, F., Fabbri, M.E., Tessarollo, L., Maffei, L., Berardi, N.*, et al.* (2006). Brain-derived neurotrophic factor (BDNF) is required for the enhancement of hippocampal neurogenesis following environmental enrichment. The European journal of neuroscience *24*, 1850-1856.

Sale, A., Berardi, N., and Maffei, L. (2014). Environment and brain plasticity: towards an endogenous pharmacotherapy. Physiological reviews *94*, 189-234.

Salim, S., Chugh, G., and Asghar, M. (2012). Inflammation in anxiety. Advances in protein chemistry and structural biology *88*, 1-25.

Singla, D.R., Waqas, A., Hamdani, S.U., Suleman, N., Zafar, S.W., Zill, E.H., Saeed, K., Servili, C., and Rahman, A. (2020). Implementation and effectiveness of adolescent life skills programs in low- and middle-income

countries: A critical review and meta-analysis. Behaviour research and therapy *130*, 103402.

UNICEF (2012). Global evaluation of life skills education programs.

Viglione, A., Chiarotti, F., Poggini, S., Giuliani, A., and Branchi, I. (2019). Predicting antidepressant treatment outcome based on socioeconomic status and citalopram dose. The pharmacogenomics journal *19*, 538-546.

Wardenaar, K.J., Conradi, H.J., Bos, E.H., and de Jonge, P. (2014). Personality modulates the efficacy of treatment in patients with major depressive disorder. The Journal of clinical psychiatry *75*, e916-923.

Weare, K., and Nind, M. (2011). Mental health promotion and problem prevention in schools: what does the evidence say? Health promotion international *26 Suppl 1*, i29-69.

Wilkinson, S.T., Holtzheimer, P.E., Gao, S., Kirwin, D.S., and Price, R.B. (2019). Leveraging Neuroplasticity to Enhance Adaptive Learning: The Potential for Synergistic Somatic-Behavioral Treatment Combinations to Improve Clinical Outcomes in Depression. Biological psychiatry *85*, 454-465.

Yang, T., Nie, Z., Shu, H., Kuang, Y., Chen, X., Cheng, J., Yu, S., and Liu, H. (2020). The Role of BDNF on Neural Plasticity in Depression. Frontiers in cellular neuroscience *14*, 82.

Yoshii, A., and Constantine-Paton, M. (2010). Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease. Developmental neurobiology *70*, 304-322.

APPENDIX

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Selecting antidepressants according to a drug-by-environment interaction: A comparison of fluoxetine and minocycline effects in mice living either in enriched or stressful conditions

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ABSTRACT

Selective serotonin reuptake inhibitors (SSRIs) are the first-line treatment for major depressive disorder. It has been recently proposed that these drugs, by enhancing neural plasticity, amplify the influences of the living conditions on mood. Consequently, SSRI outcome depends on the quality of the environment, improving symptomatology mainly in individuals living in favorable conditions. In adverse conditions, drugs with a different mechanism of action might have higher efficacy. The antibiotic minocycline, with neuroprotective and anti-inflammatory properties, has been recently proposed as a novel potential antidepressant treatment. To explore the drug-by-environment interaction, we compared the effects on depressive-like behavior and neural plasticity of the SSRI fluoxetine and minocycline in enriched and stressful conditions. We first exposed C57BL/6 adult female mice to 14 days of chronic unpredictable mild stress to induce a depressive-like profile. Afterward, mice received vehicle, fluoxetine, or minocycline for 21 days, while exposed to either enriched or stressful conditions. During the first five days, fluoxetine led to an improvement in enrichment but not in stress. By contrast, minocycline led to an improvement in both conditions. After 21 days, all groups showed a significant improvement in enrichment while fluoxetine worsened the depressive like behavior in stress. The effects of the drugs on neural plasticity, measured as long-term potentiation, were also environment-dependent. Overall, we show that the environment affects fluoxetine but not minocycline outcome, indicating that the latter represents a potential alternative to SSRIs to treat depressed patients living in adverse conditions. From a translation perspective, our finding call for considering the drug-by-environment interaction to select the most effective pharmacological treatment.

1. Introduction

Selective serotonin reuptake inhibitors (SSRIs) are the most prescribed drugs for the treatment of Major Depressive Disorder (MDD). Nevertheless, their efficacy is variable and incomplete: 60–70 % of patients do not experience remission and 30–40 % do not show a clinical response [1]. To explain such incomplete efficacy, a novel hypothesis – the undirected susceptibility to change hypothesis – has been recently proposed. It posits that the increase in serotonin levels induced by SSRIs does not affect mood *per se* but, by enhancing neural plasticity, amplifies the influence of the environment on mood. Thus, SSRI outcome depends on the quality of the living environment [2,3]. This hypothesis has been recently demonstrated both at preclinical and clinical levels. In a mouse model of depression, treatment with the SSRI fluoxetine led to an improvement of depressive phenotype in a favorable environment, while having a limited efficacy or even leading to a worsening in a

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stressful environment [4–6]. In patients, the SSRI citalopram has been demonstrated to amplify the influence of the living conditions on mood, improving depressive symptoms according to the socioeconomic status [7,8]. Because of such environment-dependent outcome of SSRIs, other drug classes, having a mechanism of action not directly affecting neural plasticity, might have higher efficacy in adverse living conditions.

Minocycline, a second-generation tetracycline antibiotic able to pass the brain-blood barrier, inhibits the immune system activation [9] and has been found to exert an antidepressant action [10]. Pre-clinical studies have indeed shown that minocycline reduces depression-like behavior and potentiates the effects of different antidepressant drugs [11–13]. Preliminary clinical investigations confirmed these results, demonstrating that minocycline has an antidepressant effect compared to placebo in patients [14,15].

According to the literature, fluoxetine and minocycline appear to have different primary targets and non-overlapping mechanisms of action. The first drug directly inhibits the serotonin transporter [16–18], while the second one has a more complex and diversified action including antioxidant, anti-inflammatory and neuroprotective properties [9]. In addition, fluoxetine has been shown to produce effects that are dependent on the living conditions while, to our knowledge, minocycline effects are not affected by the environment. Therefore, in a drug-by-environment interaction perspective, we hypothesized that the interplay between each one of these drugs and the environment would lead to different outcomes. To test this hypothesis, we exposed C57BL/6 adult female mice to 14 days of chronic unpredictable mild stress to induce a depressive-like phenotype. Afterward, we treated them with vehicle, the SSRI fluoxetine or minocycline for 21 days while the mice were living in either an enriched or a stressful condition. Our prediction was that the effects of fluoxetine are beneficial in the enriched but not in the stressful condition. Conversely, minocycline improves depressive phenotype in both conditions. We performed our experiments on female subjects since MDD is twice as common in women than men [19], expecting that part of such gender difference could be ascribed to physiological features and not only to social and cultural factors.

2. Materials and methods

2.1. Ethical standards

All procedures were carried out in accordance with the European and Italian legislation on animal experimentation (respectively European Directive 2010/63/UE and Decreto Legislativo 26/2014). Animals were examined for signs of discomfort as indicated by the animal care and use guidelines [National Academy of Sciences. Guide for the care and use of laboratory animals, 1998, "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council 2003)]. The Italian Ministry of Health approved the protocol with the permit number D9997.83.

2.2. Animals

Sixtynine C57BL/6 female mice 12–15 weeks old were used (up to 13 animals for experimental conditions). Mice were obtained directly from Charles River Laboratories placed in Calco (Lecco, Italy).

2.3. Housing condition

Mice were kept under reversed 12 light-dark cycle at 22–25 °C. For the entire duration of the experiment, animals were housed in the IntelliCage system (TSE-system, NewBehavior AG, Zürich, Switzerland), which is an apparatus for automatic monitoring of mouse behavior. It consists of a large acrylic cage (20.5 cm high, 58 cm × 40 cm at the top and 55 cm × 37.5 cm at the base, Model 2000 Tecniplast, Buguggiate, VA, Italy) with 4 walls separating each corner from the center so that they form 4 identical triangular conditioning chambers (15 × 15 × 21 cm). Animals have access to each chamber by entering a front hole and only a single mouse can enter a chamber at a time since each one is identified by a transponder. Two drinking bottles were placed in each corner and the access to each solution was prevented by a door, thus, to drink mice had to perform a nosepoke. The system is able to collect data about the number and duration of visits and the number, duration, and side (right or left) of nosepokes and licks.

The floor of the cage is covered with bedding while on the top a food rack is present filled with standard mouse chow (food ad libitum). An additional cage (SocialBox) was used to expand the existing IntelliCage to a multiarea system, thus we increased the number of the subjects tested simultaneously. One week before being moved to the IntelliCage, each animal was injected with a subcutaneous transponder (T—IS 8010 FDX-B Datamars SA, Switzerland). Then, mice have been gradually habituated to the IntelliCage environment for 14 days (habituation period). At the same time, they were habituated also to 0.1 % of saccharin solution. The IntelliCage system allows to phenotype the individual behavioral responses in a group of socially housed mice without any intervention by the experimenter reducing the biases due to animal manipulations and exposure to the testing environment.

2.4. Treatment

After the first 14 days of stressful condition aimed at inducing a depression-like phenotype, mice received for 21 days one of the following treatments: fluoxetine, minocycline, or vehicle while they were exposed either to enriched or stressful environment (Fig. 1).

To avoid the stress due to the manipulation, fluoxetine (Fluoxetine HCl, SantaCruz, USA) and minocycline (Minocycline Hydrocloride crystalline, SigmaAldrich, St Louis, MO, USA) were dissolved in water and saccharin solution avoiding that saccharin preference could affect the amount of drug received. During the treatment period, each experimental group could access only the corner of the IntelliCage administering the treatment to which it was assigned.

The solutions were prepared according to the mouse average weight and daily water consumption to provide respectively an average daily intake of 30 mg/kg of fluoxetine, and 50 mg/kg of minocycline. The average amount of fluoxetine or minocycline administered did not differ among the experimental groups receiving the same compound. Bottles with fluoxetine were wrapped in tin foil to protect the substance from light.

Fluoxetine dose has been selected according to the results previously obtained with the same pharmaceutical form and route of administration in C57BL/6 mice [5,6,20].Such dose allowed to reach an effective fluoxetine serum level of around 150 ng/mL [21]. The dose of minocycline has been also selected based on the literature [22].

2.5. Environmental conditions

Two parallel independent experiments were performed: the first aimed at assessing the effects of treatments in the enriched environment and the second one aimed at assessing the effects of treatments in the stressful environment. In each experiment, the effects of fluoxetine and minocycline were compared with an independent control group receiving the vehicle. In both experiments, mice were first exposed to the stressful condition for 14 days to induce depressive-like behavior and, immediately after, received the treatments for 21 days while exposed to either the enriched or to the stressful condition (Fig.1).

2.5.1. Enriched condition

In the enriched condition, in addition to the different settings (e.g., cage vs operant chambers, [23,24]) provided by the IntelliCage system, mice were given Plexiglas shelters of different colors and shapes (e.g., four red transparent Tecniplast plastic nest boxes) and tissue papers. A new tissue paper was provided every five days, and the plastic shelters were cleaned every week.

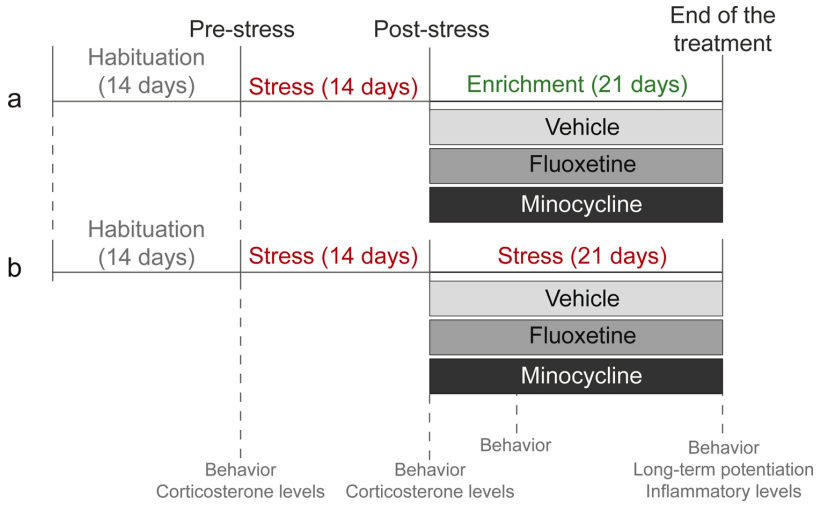

Fig. 1. Experimental design. (a) Treatment in the enriched condition. (b) Treatment in the stressful condition. First, mice have been habituated to the IntelliCage environment during a 14-day period (Habituation). Then, they were exposed to a 14-day period of stress to induce a depression-like phenotype. Afterward, mice received vehicle, fluoxetine or minocycline for 21 days either in the enriched or the stressful condition.

2.5.2. Stressful condition

After the habituation period, mice were exposed to chronic unpredictable mild stress to induce depression-like behavior. In the stressful condition, the IntelliCage system has been used to constantly (h24) expose animals to different stress procedures previously validated through the assessment of behavioral, cellular, and molecular endpoints [5,6,20]. For instance, the stressful conditions exploited in the present study has been shown to increase corticosterone levels, considered a marker of stress response [25]. The stress procedure lasted 14 days and, to prevent the habituation to the stress procedures provided by the IntelliCage, mice were exposed each day to a different, randomly chosen one. The procedures were: short open door: door to access solutions remains open for few seconds; delay: door opens randomly with a delay of 1, 1.5, 2, 2.5 s after the first nosepoke; open door 25 %: door opens only following 25 % of nosepokes; random air puff: randomly 1, 2, 3, or 4 s after the first nosepoke, the animal receives an air puff (2 bar). The duration of each paradigm was randomly 12, 18, or 24 h. During the stressful procedures, red LEDs in the corner were turned on when a mouse entered in and turned off at the end of the visit, the aim is that mice associate this light condition to the probability that one of these stressful events can happen. In addition, during the stressful condition, no shelter or tissue paper was provided.

2.6. Behavioral tests

Behavioral endpoints investigated included liking- and wanting-type anhedonia. The experimental procedures used to phenotype behavior have been selected as they were automatically administered by the IntelliCage avoiding any bias due to the experimenter. We assessed also (i) peripheral corticosterone levels, to verify the effect of the stress exposure and (ii) estrous cycle since the hormonal fluctuation could potentially affect behavioral responses (see Supplementary).

2.6.1. Liking-type anhedonia - saccharin preference

To assess liking-type anhedonia we measured saccharin preference. In each corner of the IntelliCage two bottles were present, one containing tap water and the other containing the saccharin solution; both freely available 24/24 h. The position of water and saccharin in each corner was counterbalanced across the four corners. Saccharin preference was determined as follows: [saccharin solution consumed/(saccharin solution consumed + water consumed)] × 100. Baseline saccharin preference was measured as the mean of the last two days of the habituation period (i.e., Pre-stress). In addition, we measured saccharin preference at the end of the stress period (i.e., Day0), from day 1–5 of the treatment period, and at the end of the treatment period (i.e., Day21; Fig. 1). On the day immediately before the beginning of treatments, all experimental groups showed an overlapping saccharine preference (Fig. 2a, d).

2.6.2. Wanting-type anhedonia - progressive ratio reinforcement schedule

To assess wanting-type anhedonia, i.e., the drive for obtaining a reward, we used the Progressive Ratio reinforcement schedule that utilizes a multiplicative increase in the number of responses (i.e., nosepokes) required to dispense a unit of reinforcement (i.e., saccharin solution). In particular, water was always accessible after one nosepoke while saccharin solution was accessible only after a specific number of nosepokes were performed. Such number increases progressively after each series of 8 visits according to the following sequence: 2, 3, 4, 5, 6, 7, 8, 10, 12, 16, 20, 24. Each test session lasted 48 h or until mice reached the module with 24 nosepokes. The time for performing the nosepokes increased gradually according to the number of nosepokes requested from one to 24 s. The test has been run following the 21 days of treatment, and, to make the mice aware of the testing condition, the green LEDs on the top of each door were kept turned on throughout the session.

2.7. Corticosterone levels

Corticosterone levels were measured in all subjects before and after the chronic unpredictable mild stress procedure. Blood was collected from the tail in the middle of the dark period and the procedure lasted less than 10 min to avoid effects due to the stress of manipulation. The collection of blood consisted of a small superficial cut in the tail. Blood samples were collected in potassium–EDTA coated 10 mL tubes (1.6 mg EDTA/mL blood; Sarstedt, Germany) and kept on ice. Blood samples were then centrifuged at 2500 rpm for 15 min at 4 °C and plasma was transferred to Eppendorf tubes and stored at −80 °C until further analysis. Corticosterone was measured using a commercial ELISA kit (ENZO Life Sciences, ADI-900−097) according to the instruction of the manufacturer. Samples were treated with a 1:100 solution of Steroid

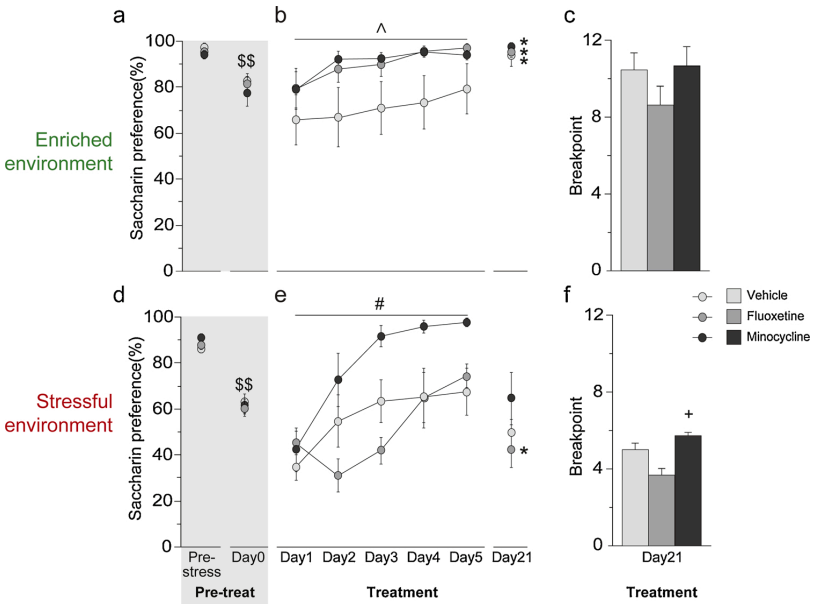

Fig. 2. Effects of treatment with fluoxetine and minocycline on depressive-like behavior. (a,d) In both experiments, the14-day period of chronic unpredictable mild stress induced a depression like phenotype. (b,e) Different effects of fluoxetine and minocycline on liking-type anhedonia in the enriched (b) and stressful (e) conditions during the first five days and after 21 days of treatment. Effects of fluoxetine and minocycline on wanting-type anhedonia in the enriched (c) and the stressful (f) conditions after 21 days of treatment. $$ $p < 0.0001$ vs pre-stress; ^ $p < 0.05$ vs vehicle group, * $p < 0.05$ vs Day0 within each group, # $p < 0.05$ vs fluoxetine and vehicle groups, + $p < 0.05$ vs fluoxetine group. Data are shown as means + s.e.m., n = 7-13.

Displacement Reagent and diluted with ELISA assay buffer to obtain a final dilution of 1:40. 100 µl of sample were pipetted in 96-well plates coated with donkey antibody specific to sheep Ig. Samples were then incubated with 50 µl of alkaline phosphatase-conjugated with corticosterone and 50 µl of sheep polyclonal antibody to corticosterone for 2 h at room temperature on a plate shaker. After 2 h, the plates were washed three times with wash buffer. After the final wash, wells were aspirated and 200 µl of a substrate solution of p-nitrophenyl phosphate in buffer were added to each well. After 1 -h incubation without shaking, 50 µl of stop solution were added to each well, and plates were immediately read at 405 nm, using a microplate reader. Corticosterone concentrations were determined based on the corticosterone standard curve (range 32–20000 pg/ml corticosterone) incubated under a similar condition in each assay. Data were elaborated with GraphPad Prism (GraphPad Software, San Diego, CA, USA). All assays were carried out in duplicate. The sensitivity of the assay was about 26.99 pg/mL. Because of the great variability, the logarithmic transformation was implemented.

2.8. Electrophysiology

To perform electrophysiological experiments, at the end of the treatment period, hippocampal slices were collected. Animals were anesthetized with halothane and decapitated. Whole brains were rapidly removed from the skull and immersed for 10 min in ice-cold artificial cerebrospinal fluid (ACSF), continuously oxygenated with 95 % O2, 5% CO2 to maintain the proper pH (7.4). Transverse 350 µm slices were cut at 4 °C. with a vibratome and the appropriate slices were placed in a chamber containing oxygenated ACSF. After their preparation slices were allowed to recover for 1 h at 30 °C. For field recordings, individual slices were then transferred to the interface slice-recording chamber (BSC1, Scientific System Design Inc) maintained at 30−32 °C and constantly superfused at the rate of 2.5 mL/min. At the beginning of each recording, a concentric bipolar stimulating electrode (SNE-100 × 50 mm long Elektronik–Harvard Apparatus GmbH) was placed in the stratum radiatum for stimulation of Shaffer collateral pathway projection to CA1. Stimuli consisted of 100 µs constant current pulses of variable intensities, applied at 0.05 Hz. A glass micropipette (0.5–1 MΩ) filled with ACSF was placed in the CA1 hippocampal region, at 200–600 µm from the stimulating electrode, in order to measure orthodromically evoked field extracellular postsynaptic potentials (fEPSP). Stimulus intensity was adjusted to evoke fEPSP of amplitude about 50 % of the maximal amplitude with minimal contamination by a population spike. Evoked responses were monitored online, and stable baseline responses were recorded for at least 10 min. Only the slices that showed stable fEPSP amplitudes were included in the experiments. LTP was induced by high-frequency stimulation (HFS, 1 train of stimuli at 100 Hz of 1 s duration), repeated after 30 min. To analyze the time course of the fEPSP slope, the recorded fEPSP was routinely averaged over 1 min (n = 3). The fEPSP slope changes following the LTP induction protocol at 25 and 55 min post tetanus were calculated with respect to those of the baseline (1 min before induction). N/n refers to the number of slices on the total number of mice analyzed. The paired-pulse ratio (PPR) was measured from responses to two synaptic stimuli at 50 ms interstimulus interval. PPR was calculated as the ratio between the fEPSP amplitude evoked by the second stimulus (A2) and that by the first (A1; A2/A1). fEPSP were recorded and filtered (low pass at 1 kHz) with an Axopatch 200A amplifier (Axon Instruments, CA) and digitized at 10k Hz with an A/D converter (Digidata 1322A, Axon Instruments). Data acquisition was stored on a computer using pClamp 9 software (Axon Instruments) and analyzed offline with Clampfit 10 program (Axon Instruments).

2.9. RNA extraction and RT-PCR on the entire hippocampus

Total RNA was obtained from the hippocampi. After RNA reverse transcription, real-time PCR was performed. Sample were normalized to the housekeeping genes Actβ and B2M (see Supplementary).

2.10. Statistical methods

Fluoxetine and minocycline were investigated in two independent experiments aimed at assessing their effects in interaction with either the enriched or the stressful environment. Each experiment included its own control group. All data were analyzed with one-way ANOVA with the statistical software Statview II (Abacus Concepts, CA, USA), to

compare vehicle *versus* fluoxetine and minocycline treatment in each experiment. Time (days) was considered a repeated measure within-subjects, while enriched and stressful conditions were considered independently. *Post-hoc* comparisons were performed using Tukey's test. All mean differences were considered statistically significant when $p < 0.05$.

3. Results and statistical analyses

3.1. Behavior

To investigate depression-like behavior, we assessed liking- and wanting-type anhedonia, previously shown to be affected by chronic unpredictable mild stress and SSRI treatment [4–6]. We investigated the effects of treatment independently in the enriched and the stressful condition and, in each condition, we assessed the effects of the administration of fluoxetine and minocycline.

3.1.1. Treatment in enriched condition, after exposure to stress

The exposure to 14-day period of stress before treatment induced a depression-like phenotype: all experimental subjects showed a significant reduction in saccharin preference (liking-type anhedonia) [$F(1,35) = 28.364$, $p < 0.0001$] (Fig. 2a).

Following the chronic stressful period, exposure to the enriched condition led to a progressive decrease of depression-like responses. Considering the liking-type anhedonia during the first five days of treatment, we observed a significant main effect of the treatments [$F(2,132) = 3.918$, $p = 0.0283$]. *Post-hoc* analysis revealed that fluoxetine and minocycline mice displayed higher saccharin preference compare to the vehicle ($p < 0.05$, Fig. 2b). At the end of the treatment period, we did not observe a significant difference between the experimental groups, neither regarding the liking-and the wanting-type anhedonia [$F(2,34) = 1.215$, $p = 0.3094$] (Fig. 2b-c). These data suggest that 21 days of exposure to the enriched environment allowed all animals to fully recover the effects induced by the exposure to stress, independently from the treatment received.

To investigate the interplay between each treatment and the enriched environment on depression like-behavior in the short term, we analyzed the saccharin preference from day 1–5 within each experimental group. We did not observe a significant improvement of the liking-type anhedonia in the vehicle group, likely because of the short-time interval since the beneficial effects reportedly emerge only after a prolonged exposure to enrichment [26]. We observed a significant improvement of the liking-type anhedonia within the first five days of treatment only in mice receiving minocycline [$F(4,44) = 2.690$, $p = 0.0433$] (Fig. 2b). To assess the long-term effect of the exposure to the enriched environment, we measured the difference in saccharin preference between Day0 and Day21. The results showed that, as expected, each experimental group significantly decreased liking-type anhedonia [vehicle, $F(1,10) = 17.215$, $p = 0.0020$; fluoxetine, $F(1,12) = 6.639$, $p = 0.0243$; minocycline, $F(1,11) = 13.050$, $p = 0.0041$] (Fig. 2a-b).

3.1.2. Treatment in stressful condition, after exposure to stress

The first 14 days of chronic stress were effective in inducing a depression-like profile as indicated by the significant decrease of the saccharin preference dropped from around 90 to 60 percent [$F(1,30) = 114.502$, $p < 0.0001$] (Fig. 2d). During the first five days of treatment, liking-type anhedonia showed statistically significant differences between groups [$F(2,72) = 2.447$, $p = 0.0211$]. *Post-hoc* analysis revealed that mice treated with minocycline showed higher saccharin preference compared to both fluoxetine and vehicle mice ($p < 0.05$, Fig. 2e). After 21 days of treatment in the stressful environment, saccharin preference did not differ between experimental groups (Fig. 2e). However, the minocycline mice showed a significantly reduced wanting-type anhedonia compared to fluoxetine mice, as indicated by a higher breakpoint [$F(2,30) = 4.423$, $p = 0.0207$] (Fig. 2f). Finally, we investigated the effect of the 21-day exposure to the stressful environment in each experimental group. The results showed that fluoxetine mice significantly worsened their liking-type anhedonia over the treatment period [$F(1,9) = 6.059$, $p = 0.0360$] (Fig. 2e).

In both the enriched and stressful conditions, on the first day of the treatment period, all mice showed a reduced preference compared to Day0 likely because, during the treatment period, each experimental group could access only the corner of the IntelliCage administering the treatment to which it was assigned.

To evaluate the potential association between behavior and hormonal fluctuations, we monitored the estrous cycle. No association was found suggesting that the behavioral responses were independent of the hormonal levels (see Supplementary).

3.2. Corticosterone levels

Corticosterone levels measured after the exposure to stress resulted significantly increased [$F(1,29) = 7.641$, $p = 0.0098$] (Fig. 3). Since corticosterone levels start to increase after 5 min of acute stress, we evaluated the correlation between the order of animals during the blood collection and corticosterone levels, but no correlation was found (data not shown).

3.3. Electrophysiology

We explored LTP stimulating Schaffer collaterals with spaced (30 min apart) high-frequency stimulation (HFS) and analyzing LTP amplitudes 25 min after each stimulation. In enriched condition, following the first stimulation, we observed that LTP amplitude was significantly affected by treatment [$F(2, 33) = 3.375$, $p = 0.0464$]. In particular, *post-hoc* analysis revealed that mice treated with minocycline showed higher LTP amplitude (1.1574 ± 0.174) compared to fluoxetine treatment (1.455 ± 0.137). Following the second stimulation, no significant difference between groups arose, although a clear tendency toward an increase in LTP was observed in minocycline treated mice (Fig. 4a). By

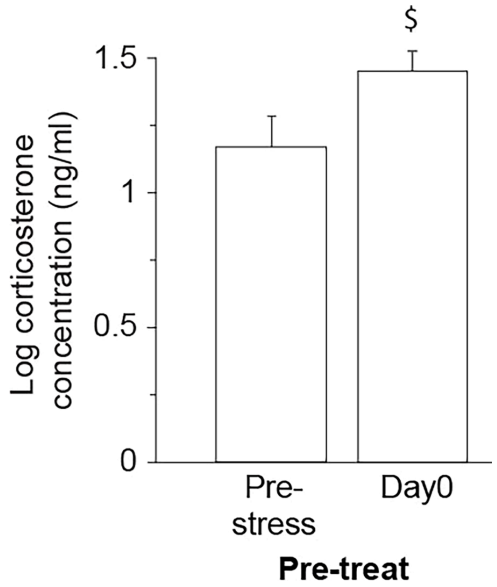

Fig. 3. Peripheral corticosterone levels. Exposure to 14 days of chronic unpredictable mild stress significantly increased the levels of peripheral blood corticosterone. $ $p < 0.01$ vs pre-stress. Data are shown as mean + s.e.m., n = 8-13.

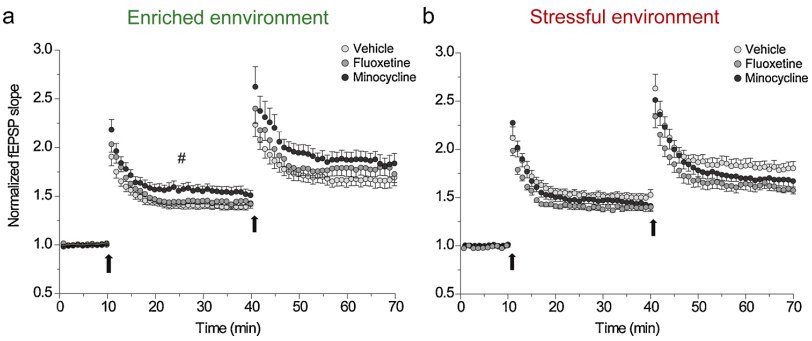

Fig. 4. Hippocampal CA1 LTP in mice exposed either to the enriched or the stressful condition and treated for 21 days as indicated. (a) In the enriched condition, following first tetanus, minocycline induced a significant increase in LTP, while in the stressful environment, (b) no differences arise. Arrows indicate time of application of HFS (100 Hz trains of 1 s duration, 30 min apart); fEPSP, field excitatory postsynaptic potential; # $p < 0.05$ vs fluoxetine and vehicle groups. Data are shown as mean + s.e.m., N/n refers to the number of slices on the total number of mice analyzed, N = 9-15/n = 5-6.

contrast, in the stressful condition, post tetanus LTP amplitude was not significantly affected by treatments (Fig. 4b). Overall, these results indicate that only in enriched condition minocycline significantly potentiates hippocampal plasticity. We thus measured the Paired Pulse Ratio (PPR), a form of short-term plasticity related to neurotransmitter release probability (Zucker, 1989) by stimulating the Schaffer collateral projections to CA1 at 50 ms intervals. In all conditions, PPR was not modified by treatments (data not shown).

3.4. Hippocampal inflammatory levels

To assess the effects of the interaction between the quality of the environment and treatment at molecular level, we measured the hippocampal expression levels of selected inflammatory markers at the end of the treatment period. In both the environmental conditions, fluoxetine and minocycline administration did not affect the cytokines expression levels (see Supplementary).

4. Discussion

Fluoxetine and minocycline had different effects on depressive-like phenotype according to a drug-by-environment interaction. During the first five days of treatment, fluoxetine improved the depressive-like response in the enriched but not in the stressful environment, while minocycline led to an improvement in both environmental conditions. After 21 days, in enrichment, fluoxetine, minocycline and vehicle groups showed an overlapping significant improvement, likely because of the beneficial effect of the enrichment. By contrast, in stress, fluoxetine worsened the depressive like behavior (Fig. 2). Though no clear relationship between behavioral changes and neural plasticity modifications emerged, the effect of treatments on LTP was found to be dependent on the environment as well (Fig. 4).

It is worth noting that, despite the prevalence of MDD is almost twice in women than men [27], most of the preclinical studies focused on males . In the present study, we investigated for the first time the interplay between the environment and the SSRI treatment in female mice. This is important because the physiological and behavioral features of depressive disorder profoundly differ between the two sexes [28,29].

4.1. Fluoxetine treatment outcome is dependent on the quality of the environment

The exposure to chronic stress was effective to induce the depressive-like phenotype, as indicated by the significantly increased liking-type anhedonia (Fig. 2a, d) and serum corticosterone levels (Fig. 3), both widely considered endophenotypes of depression [30–32]. Chronic stress reportedly activates the hypothalamic-pituitary-adrenal axis and increased glucocorticoid levels have been found in both depressed patients and animal models of depression [33–37]. During the first five days of treatment in the enriched condition, fluoxetine administration significantly improved the anhedonic profile, increasing saccharin preference over water. By contrast, in the stressful environment, fluoxetine effect did not differ from vehicle, both groups showing almost no preference for the sweet solution. These results confirm previous studies demonstrating that SSRI administration in a favorable environment leads to a reduction of depressive symptoms while, in a stressful environment, the treatment has limited beneficial effects [5,6,20]. The role of the environment in determining the SSRI outcome could explain the apparently discordant evidence on the effects of this class of drugs, some studies reporting an improvement of depression endophenotypes [38–45], while others describing opposite results [46–54]. The environment-dependence of the outcome of treatments affecting serotonin levels is increasingly exploited to explain the results in the literature [55–58] and several studies suggest to exploit the interaction between increased neurobehavioral plasticity and the environment to improve the clinical outcome in major depression [3,6,59,60].

Following 21 days of enriched condition, both fluoxetine and vehicle mice showed similarly high saccharin preference, which overlapped the pre-stress levels (Fig. 2b). Such lack of difference is likely due to the beneficial effect of the prolonged exposure to the enrichment. By contrast, in the stressful condition, 21 days of fluoxetine treatment worsened the anhedonic profile compared to the levels immediately before the treatment period (Fig. 2e), in line with our hypothesis of the potential detrimental effect of fluoxetine when administered in an adverse environment [2,20].

4.2. Minocycline antidepressant action is not dependent on the quality of the environment

Contrary to fluoxetine, during the first five days of treatment, minocycline exerted a beneficial effect independently from the quality of the environment: treated subjects displayed a higher saccharin preference compared to vehicle both in the enriched and stressful conditions (Fig. 2b, e). The different effects of the two compounds are in line with their different mechanisms of action: while fluoxetine directly modulates serotonin levels and consequently neural plasticity [61–63], minocycline targets a variety of biological processes though which it exerts an anti-inflammatory and anti-oxidant action and is able to block microglial activity [64–66]. In addition, minocycline does not directly affect the serotoninergic system [11,65]. However, after 21 days, also minocycline treatment did not produce any effect compared to vehicle, indicating that the beneficial action of this compound is not detectable in the long-term. This suggests that minocycline has a fast but short-time efficacy. Such rapid action might be due to its ability to reduce microglial activity when microglial proliferation and activation is maximal, i.e. during the beginning of the exposure to chronic stress [64,67,68]. For wanting-type anhedonia, no difference between treated groups and

vehicle was found after 21 days of treatment. However, minocycline significantly reduced anhedonia compared to fluoxetine, further suggesting that minocycline is more effective in adverse conditions (Fig. 2f).

4.3. Effects of treatments on neural plasticity

To investigate possible neural correlates of the behavioral effects of treatments, we focused on neural plasticity measured as hippocampal LTP since the latter has been involved in antidepressant action and is reportedly affected by fluoxetine and minocycline treatments [69–73]. We assessed LTP in two consecutive stimulations to measure the effect of cumulative recruitment of potentiated synapses and possible LTP saturation. LTP amplitude was not reduced by chronic stress, as previously reported in males [20]. This might be due to the reported different response to stress in males and females [74], the latter being more resistant to the neurobiological effects of chronic stress [75,76].

Fluoxetine did not affect LTP in the enriched and stressful conditions. The lack of effect in enrichment is in line with previous studies [20] and could be due to the overlapping effects of treatment and enrichment on neural plasticity, both inducing an LTP increase [20,71,77]. By contrast, the lack of fluoxetine effect in the stressful condition could be ascribed to the five weeks of stressful procedure that flattened potential effects of treatment. In addition, long-term fluoxetine treatment might produce different outcomes in females and males [20].

Minocycline treatment enhanced LTP during the first stimulation in the enriched condition. This effect appears to be due to microglial inhibition that is reportedly associated to LTP enhancement [78,79]. By contrast, minocycline did not affect LTP in the stress condition, suggesting that its effect on hippocampal plasticity might be temporarily restricted to the first five days of treatment, paralleling behavioral results. Accordingly, in stress conditions, microglia status has been shown to go through dynamic alterations which are a function of the duration of the stress exposure and the anti-depressive action of minocycline has been found to be maximal at short term [68]. This could also account for the differences between depressive-like phenotype and neural plasticity modifications observed. In addition, besides the CA1 hippocampal plasticity, other cellular mechanisms and brain area could contribute to the behavioral phenotype.

The assessment of the expression levels of inflammatory markers (i.e., IL-1β, IL-6, and TNF-α) following 21 days of fluoxetine or minocycline treatment revealed no effect of stress or treatment (Fig. S1). Though these results appear controversial, previous studies have already reported that chronic stress does not affect, or even decrease, inflammatory levels [80]. The duration of the procedure may account for these results since cytokine expression has been reported to be up-regulated after five but not after 10 days of stress [81]. Moreover, stress produces a less pronounced increase of the hippocampal cytokine levels in C57BL6 mouse females than in males [81]. Regarding the unexpected lack of effect of minocycline on inflammatory markers, our results are in line with a recent clinical study reporting that minocycline does not affect inflammation in patients, suggesting that the therapeutic action of this drug might not rely on a direct modulation of the immune system activity [15].

This study presents limitations. We did not perform a pharmacokinetic analysis to assess the potential interaction between fluoxetine and minocycline. However, previous studies indicate that these compounds do not interact, and minocycline does not directly affect the serotonergic system [11]. The assessment of different doses of fluoxetine and minocycline would have provided a better characterization of their effects on depression-like phenotype. The assessment of the adult hippocampal neurogenesis would have better illustrated the effects of the stress exposure and treatment administration since chronic stress and serotoninergic antidepressants reportedly modify neurogenesis in the dentate gyrus [20,82]. The analysis of brain areas other than the hippocampus, such as the prefrontal cortex, would have provided a more exhaustive characterization of fluoxetine and minocycline effects [83,84]. Finally, the analysis of the wanting-type anhedonia at the end of the first five days of treatment would have provided a more comprehensive behavioral phenotyping of the drug-by-environment effects. Thus, further studies aimed at a more comprehensive characterization of the neurobehavioral effects of fluoxetine and minocycline at different time-points are warranted.

5. Conclusions

Our findings highlight the relevance of the drug-by-environment interaction showing that at short-term minocycline is effective in both the enriched and stressful conditions, while fluoxetine has a beneficial effect only in enrichment. At long-term, in the stressful condition, minocycline has limited efficacy, but fluoxetine has even detrimental effects. Thus, minocycline appears overall more effective when subjects live in an unfavorable condition. In a translation perspective, the beneficial effects of minocycline, which are independent from the quality of living conditions, are particularly relevant because the onset and progression of MDD are associated with stress [85–88] and, often is not possible to change the patient's environmental conditions because of personal history and complex life circumstances.

Finally, here we confirm and further show the key role of the living environment in psychiatric treatment, in particular for those compounds leveraging on the enhancement of neural and behavioral plasticity such as SSRIs [2,6,20,59]. We thus underpin the relevance of the patient's living conditions in the selection of the most effective pharmacological treatment and advocate for considering the key role of the environemnt in the precision medicine paradigm in the psychiatric field.

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Author statement

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Branchi Igor: Conceptualization, Investigation, Writing - Original Draft, Writing - Review & Editing, Supervision, Funding acquisition.

Author contributions

Poggini Silvia and **Matte Bon Gloria**: participation in the design of the study, performing most of the experimental procedures, analyzing data, writing of the manuscript, **Golia Maria Teresa**: performing experimental procedures and analyzing data, **Viglione Aurelia** and **Naomi Ciano Albanese**, performing part of the experimental procedures; **Poleggi Anna**: performing experimental procedures and discussing data; **Limatola Cristina**: reviewing the data and the manuscript, **Maggi Laura**: discussing data, writing and reviewing the manuscript, **Branchi Igor**: conceptualization of the study, result interpretation, writing of the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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References

[1] M.H. Trivedi, A.J. Rush, S.R. Wisniewski, A.A. Nierenberg, D. Warden, L. Ritz, G. Norquist, R.H. Howland, B. Lebowitz, P.J. McGrath, K. Shores-Wilson, M. M. Biggs, G.K. Balasubramani, M. Fava, S.D.S. Team, Evaluation of outcomes with citalopram for depression using measurement-based care in STAR*D: implications for clinical practice, Am. J. Psychiatry 163 (1) (2006) 28–40.

[2] I. Branchi, The double edged sword of neural plasticity: increasing serotonin levels leads to both greater vulnerability to depression and improved capacity to recover, Psychoneuroendocrinology 36 (3) (2011) 339–351.

[3] I. Branchi, A. Giuliani, Shaping therapeutic trajectories in mental health: instructive vs. Permissive causality, Eur. Neuropsychopharmacol. (2020).

[4] S. Alboni, S. Poggini, S. Garofalo, G. Milior, H. El Hajj, C. Lecours, I. Girard, S. Gagnon, S. Boisjoly-Villeneuve, N. Brunello, D.P. Wolfer, C. Limatola, M. E. Tremblay, L. Maggi, I. Branchi, Fluoxetine treatment affects the inflammatory response and microglial function according to the quality of the living environment, Brain Behav. Immun. (2016).

[5] I. Branchi, S. Santarelli, S. Capoccia, S. Poggini, I. D'Andrea, F. Cirulli, E. Alleva, Antidepressant treatment outcome depends on the quality of the living environment: a pre-clinical investigation in mice, PLoS One 8 (4) (2013), e62226.

[6] S. Poggini, M.T. Golia, S. Alboni, G. Milior, L.P. Sciarria, A. Viglione, G. Matte Bon, N. Brunello, S. Puglisi-Allegra, C. Limatola, L. Maggi, I. Branchi, Combined fluoxetine and metformin treatment potentiates antidepressant efficacy increasing IGF2 expression in the dorsal Hippocampus, Neural Plast. 2019 (2019), 4651031.

[7] F. Chiarotti, A. Viglione, A. Giuliani, I. Branchi, Citalopram amplifies the influence of living conditions on mood in depressed patients enrolled in the STAR*D study, Transl. Psychiatry 7 (3) (2017), e1066.

[8] A. Viglione, F. Chiarotti, S. Poggini, A. Giuliani, I. Branchi, Predicting antidepressant treatment outcome based on socioeconomic status and citalopram dose, Pharmacogenomics J. (2019).

[9] N. Garrido-Mesa, A. Zarzuelo, J. Galvez, Minocycline: far beyond an antibiotic, Br. J. Pharmacol. 169 (2) (2013) 337–352.

[10] R. Yirmiya, N. Rimmerman, R. Reshef, Depression as a microglial disease, Trends Neurosci. 38 (10) (2015) 637–658.

[11] M. Molina-Hernandez, N.P. Tellez-Alcantara, J. Perez-Garcia, J.I. Olivera-Lopez, M.T. Jaramillo-Jaimes, Desipramine or glutamate antagonists synergized the antidepressant-like actions of intra-nucleus accumbens infusions of minocycline in male Wistar rats, Prog. Neuropsychopharmacol. Biol. Psychiatry 32 (7) (2008) 1660–1666.

[12] S. Arakawa, Y. Shirayama, Y. Fujita, T. Ishima, M. Horio, K. Muneoka, M. Iyo, K. Hashimoto, Minocycline produced antidepressant-like effects on the learned helplessness rats with alterations in levels of monoamine in the amygdala and no changes in BDNF levels in the hippocampus at baseline, Pharmacol. Biochem. Behav. 100 (3) (2012) 601–606.

[13] N.N. Burke, D.M. Kerr, O. Moriarty, D.P. Finn, M. Roche, Minocycline modulates neuropathic pain behaviour and cortical M1-M2 microglial gene expression in a rat model of depression, Brain Behav. Immun. 42 (2014) 147–156.

[14] J.D. Rosenblat, R.S. McIntyre, Efficacy and tolerability of minocycline for depression: a systematic review and meta-analysis of clinical trials, J. Affect. Disord. 227 (2018) 219–225.

[15] M.A. Nettis, G. Lombardo, C. Hastings, Z. Zajkowska, N. Mariani, N. Nikkheslat, C. Worrell, D. Enache, A. McLaughlin, M. Kose, L. Sforzini, A. Bogdanova, A. Cleare, A.H. Young, C.M. Pariante, V. Mondelli, Augmentation therapy with minocycline in treatment-resistant depression patients with low-grade peripheral inflammation: results from a double-blind randomised clinical trial, Neuropsychopharmacology (2021).

[16] S.H. Preskorn, Clinically relevant pharmacology of selective serotonin reuptake inhibitors. An overview with emphasis on pharmacokinetics and effects on oxidative drug metabolism, Clin. Pharmacokinet. 32 (Suppl 1) (1997) 1–21.

[17] W. Xue, P. Wang, B. Li, Y. Li, X. Xu, F. Yang, X. Yao, Y.Z. Chen, F. Xu, F. Zhu, Identification of the inhibitory mechanism of FDA approved selective serotonin reuptake inhibitors: an insight from molecular dynamics simulation study, Phys. Chem. Chem. Phys. 18 (4) (2016) 3260–3271.

[18] J.P. Feighner, Mechanism of action of antidepressant medications, J. Clin. Psychiatry 60 (Suppl 4) (1999) 4–11, discussion 12-13.

[19] C. Kuehner, Gender differences in unipolar depression: an update of epidemiological findings and possible explanations, Acta Psychiatr. Scand. 108 (3) (2003) 163–174.

[20] S. Alboni, R.M. van Dijk, S. Poggini, G. Milior, M. Perrotta, T. Drenth, N. Brunello, D.P. Wolfer, C. Limatola, I. Amrein, F. Cirulli, L. Maggi, I. Branchi, Fluoxetine effects on molecular, cellular and behavioral endophenotypes of depression are driven by the living environment, Mol. Psychiatry 22 (4) (2017) 552–561.

[21] S.C. Dulawa, K.A. Holick, B. Gundersen, R. Hen, Effects of chronic fluoxetine in animal models of anxiety and depression, Neuropsychopharmacology 29 (7) (2004) 1321–1330.

[22] M.A. Di Castro, F. Trettel, G. Milior, L. Maggi, D. Ragozzino, C. Limatola, The chemokine CXCL16 modulates neurotransmitter release in hippocampal CA1 area, Sci. Rep. 6 (2016) 34633.

[23] A. Codita, A.H. Mohammed, A. Willuweit, A. Reichelt, E. Alleva, I. Branchi, F. Cirulli, G. Colacicco, V. Voikar, D.P. Wolfer, F.J. Buschmann, H.P. Lipp, E. Vannoni, S. Krackow, Effects of spatial and cognitive enrichment on activity pattern and learning performance in three strains of mice in the IntelliMaze, Behav. Genet. 42 (3) (2012) 449–460.

[24] S. Muthuraju, S. Taha, S. Pati, M. Rafique, H. Jaafar, J.M. Abdullah, Normabaric hyperoxia treatment improved locomotor activity of C57BL/6J mice through enhancing dopamine genes following fluid-percussion injury in striatum, Int. J. Biomed. Sci. 9 (4) (2013) 194–204.

[25] G. Milior, C. Lecours, L. Samson, K. Bisht, S. Poggini, F. Pagani, C. Deflorio, C. Lauro, S. Alboni, C. Limatola, I. Branchi, M.E. Tremblay, L. Maggi, Fractalkine receptor deficiency impairs microglial and neuronal responsiveness to chronic stress, Brain Behav. Immun. 55 (2016) 114–125.

[26] M. Leger, E. Paizanis, K. Dzahini, A. Quiedeville, V. Bouet, J.C. Cassel, T. Freret, P. Schumann-Bard, M. Boulouard, Environmental enrichment duration differentially affects behavior and neuroplasticity in adult mice, Cereb. Cortex 25 (11) (2015) 4048–4061.

[27] A.J. Ferrari, F.J. Charlson, R.E. Norman, S.B. Patten, G. Freedman, C.J. Murray, T. Vos, H.A. Whiteford, Burden of depressive disorders by country, sex, age, and year: findings from the global burden of disease study 2010, PLoS Med. 10 (11) (2013), e1001547.

[28] M.G. Oyola, R.J. Handa, Hypothalamic-pituitary-adrenal and hypothalamic-pituitary-gonadal axes: sex differences in regulation of stress responsivity, Stress 20 (5) (2017) 476–494.

[29] H.F. Figueiredo, C.M. Dolgas, J.P. Herman, Stress activation of cortex and hippocampus is modulated by sex and stage of estrus, Endocrinology 143 (7) (2002) 2534–2540.

[30] T. Strekalova, R. Spanagel, D. Bartsch, F.A. Henn, P. Gass, Stress-induced anhedonia in mice is associated with deficits in forced swimming and exploration, Neuropsychopharmacology 29 (11) (2004) 2007–2017.

[31] B. Planchez, A. Surget, C. Belzung, Animal models of major depression: drawbacks and challenges, J. Neural Transm. Vienna (Vienna) 126 (11) (2019) 1383–1408.

[32] H. Bonilla-Jaime, S. Retana-Marquez, G. Vazquez-Palacios, J. Velazquez-Moctezuma, Corticosterone and testosterone levels after chronic stress in an animal model of depression, Neuropsychobiology 48 (2) (2003) 55–58.

[33] S. Alboni, F. Tascedda, D. Corsini, C. Benatti, F. Caggia, G. Capone, N. Barden, J. M. Blom, N. Brunello, Stress induces altered CRE/CREB pathway activity and BDNF expression in the hippocampus of glucocorticoid receptor-impaired mice, Neuropharmacology 60 (7-8) (2011) 1337–1346.

[34] S. Chourbaji, C. Brandwein, M.A. Vogt, C. Dormann, P. Gass, Evaluation of effects of previous exposure to an acute stressor before testing for depression-like behaviours in mice, Stress 11 (2) (2008) 170–175.

[35] F. Holsboer, The corticosteroid receptor hypothesis of depression, Neuropsychopharmacology 23 (5) (2000) 477–501.

[36] M. Ising, S. Horstmann, S. Kloiber, S. Lucae, E.B. Binder, N. Kern, H.E. Kunzel, A. Pfennig, M. Uhr, F. Holsboer, Combined dexamethasone/corticotropin releasing hormone test predicts treatment response in major depression - a potential biomarker? Biol. Psychiatry 62 (1) (2007) 47–54.

[37] C.B. Nemeroff, E. Widerlov, G. Bissette, H. Walleus, I. Karlsson, K. Eklund, C. D. Kilts, P.T. Loosen, W. Vale, Elevated concentrations of CSF corticotropin-releasing factor-like immunoreactivity in depressed patients, Science 226 (4680) (1984) 1342–1344.

[38] S. Alboni, C. Benatti, G. Capone, D. Corsini, F. Caggia, F. Tascedda, J. Mendlewicz, N. Brunello, Time-dependent effects of escitalopram on brain derived neurotrophic factor (BDNF) and neuroplasticity related targets in the central nervous system of rats, Eur. J. Pharmacol. 643 (2–3) (2010) 180–187.

[39] J.C. Brenes, J. Fornaguera, The effect of chronic fluoxetine on social isolation-induced changes on sucrose consumption, immobility behavior, and on serotonin and dopamine function in hippocampus and ventral striatum, Behav. Brain Res. 198 (1) (2009) 199–205.

[40] D.J. David, B.A. Samuels, Q. Rainer, J.W. Wang, D. Marsteller, I. Mendez, M. Drew, D.A. Craig, B.P. Guiard, J.P. Guilloux, R.P. Artymyshyn, A.M. Gardier, C. Gerald, I. A. Antonijevic, E.D. Leonardo, R. Hen, Neurogenesis-dependent and -independent effects of fluoxetine in an animal model of anxiety/depression, Neuron 62 (4) (2009) 479–493.

[41] M.E. Kozisek, D. Middlemas, D.B. Bylund, Brain-derived neurotrophic factor and its receptor tropomyosin-related kinase B in the mechanism of action of antidepressant therapies, Pharmacol. Ther. 117 (1) (2008) 30–51.

[42] F. Possamai, J. dos Santos, T. Walber, J.C. Marcon, T.S. dos Santos, C. Lino de Oliveira, Influence of enrichment on behavioral and neurogenic effects of antidepressants in Wistar rats submitted to repeated forced swim test, Prog. Neuropsychopharmacol. Biol. Psychiatry 58 (2015) 15–21.

[43] S. Sammut, I. Bethus, G. Goodall, R. Muscat, Antidepressant reversal of interferon-alpha-induced anhedonia, Physiol. Behav. 75 (5) (2002) 765–772.

[44] M. Tonissaar, T. Mallo, M. Eller, R. Haidkind, K. Koiv, J. Harro, Rat behavior after chronic variable stress and partial lesioning of 5-HT-ergic neurotransmission: effects of citalopram, Prog. Neuropsychopharmacol. Biol. Psychiatry 32 (1) (2008) 164–177.

[45] C.C. Weber, G.P. Eckert, W.E. Muller, Effects of antidepressants on the brain/plasma distribution of corticosterone, Neuropsychopharmacology 31 (11) (2006) 2443–2448.

[46] M. Goekint, B. Roelands, E. Heyman, R. Njemini, R. Meeusen, Influence of citalopram and environmental temperature on exercise-induced changes in BDNF, Neurosci. Lett. 494 (2) (2011) 150–154.

[47] J.P. Jacobsen, A. Mork, The effect of escitalopram, desipramine, electroconvulsive seizures and lithium on brain-derived neurotrophic factor mRNA and protein

expression in the rat brain and the correlation to 5-HT and 5-HIAA levels, Brain Res. 1024 (1–2) (2004) 183–192.
[48] A. Klomp, L. Vaclavu, G.F. Meerhoff, L. Reneman, P.J. Lucassen, Effects of chronic fluoxetine treatment on neurogenesis and tryptophan hydroxylase expression in adolescent and adult rats, PLoS One 9 (5) (2014), e97603.
[49] M.W. Marlatt, M.C. Potter, T.A. Bayer, H. van Praag, P.J. Lucassen, Prolonged running, not fluoxetine treatment, increases neurogenesis, but does not alter neuropathology, in the 3xTg mouse model of Alzheimer's disease, Curr. Top. Behav. Neurosci. 15 (2013) 313–340.
[50] X. Miro, S. Perez-Torres, F. Artigas, P. Puigdomenech, J.M. Palacios, G. Mengod, Regulation of cAMP phosphodiesterase mRNAs expression in rat brain by acute and chronic fluoxetine treatment. An in situ hybridization study, Neuropharmacology 43 (7) (2002) 1148–1157.
[51] M.A. Prendergast, D.P. Yells, S.E. Balogh, S.R. Paige, S.E. Hendricks, Fluoxetine differentially suppresses sucrose solution consumption in free-fed and food-deprived rats–reversal by amantadine, Med. Sci. Monit. 8 (10) (2002) BR385–BR390.
[52] Q. Shen, R. Lal, B.A. Luellen, J.C. Earnheart, A.M. Andrews, B. Luscher, Gamma-Aminobutyric acid-type A receptor deficits cause hypothalamic-pituitary-adrenal axis hyperactivity and antidepressant drug sensitivity reminiscent of melancholic forms of depression, Biol. Psychiatry 68 (6) (2010) 512–520.
[53] M.V. Wu, J.L. Shamy, G. Bedi, C.W. Choi, M.M. Wall, V. Arango, M. Boldrini, R. W. Foltin, R. Hen, Impact of social status and antidepressant treatment on neurogenesis in the baboon hippocampus, Neuropsychopharmacology 39 (8) (2014) 1861–1871.
[54] T.S. Zetterstrom, Q. Pei, T.R. Madhav, A.L. Coppell, L. Lewis, D.G. Grahame-Smith, Manipulations of brain 5-HT levels affect gene expression for BDNF in rat brain, Neuropharmacology 38 (7) (1999) 1063–1073.
[55] J. Lima, T. Sharp, D.M. Bannerman, S.B. McHugh, Enhanced discriminative aversive learning and amygdala responsivity in 5-HT transporter mutant mice, Transl. Psychiatry 9 (1) (2019) 139.
[56] S. Matias, E. Lottem, G.P. Dugue, Z.F. Mainen, Activity patterns of serotonin neurons underlying cognitive flexibility, Elife 6 (2017).
[57] S.B. Yoo, B.T. Kim, J.Y. Kim, V. Ryu, D.W. Kang, J.H. Lee, J.W. Jahng, Adolescence fluoxetine increases serotonergic activity in the raphe-hippocampus axis and improves depression-like behaviors in female rats that experienced neonatal maternal separation, Psychoneuroendocrinology 38 (6) (2013) 777–788.
[58] D. Kovacs, X. Gonda, P. Petschner, A. Edes, N. Eszlari, G. Bagdy, G. Juhasz, Antidepressant treatment response is modulated by genetic and environmental factors and their interactions, Ann. Gen. Psychiatry 13 (2014) 17.
[59] S.T. Wilkinson, P.E. Holtzheimer, S. Gao, D.S. Kirwin, R.B. Price, Leveraging neuroplasticity to enhance adaptive learning: the potential for synergistic somatic-behavioral treatment combinations to improve clinical outcomes in depression, Biol. Psychiatry 85 (6) (2019) 454–465.
[60] J.K. Forsyth, P. Bachman, D.H. Mathalon, B.J. Roach, R.F. Asarnow, Augmenting NMDA receptor signaling boosts experience-dependent neuroplasticity in the adult human brain, Proc. Natl. Acad. Sci. U. S. A. 112 (50) (2015) 15331–15336.
[61] J.F. Maya Vetencourt, A. Sale, A. Viegi, L. Baroncelli, R. De Pasquale, O.F. O'Leary, E. Castren, L. Maffei, The antidepressant fluoxetine restores plasticity in the adult visual cortex, Science 320 (5874) (2008) 385–388.
[62] E. Castren, Is mood chemistry? Nat. Rev. Neurosci. 6 (3) (2005) 241–246.
[63] C. Kraus, E. Castren, S. Kasper, R. Lanzenberger, Serotonin and neuroplasticity - Links between molecular, functional and structural pathophysiology in depression, Neurosci. Biobehav. Rev. 77 (2017) 317–326.
[64] M. Hinwood, R.J. Tynan, J.L. Charnley, S.B. Beynon, T.A. Day, F.R. Walker, Chronic stress induced remodeling of the prefrontal cortex: structural re-organization of microglia and the inhibitory effect of minocycline, Cereb. Cortex 23 (8) (2013) 1784–1797.
[65] C.U. Pae, D.M. Marks, C. Han, A.A. Patkar, Does minocycline have antidepressant effect? Biomed. Pharmacother. 62 (5) (2008) 308–311.
[66] J.K. Soczynska, R.B. Mansur, E. Brietzke, W. Swardfager, S.H. Kennedy, H. O. Woldeyohannes, A.M. Powell, M.S. Manierka, R.S. McIntyre, Novel therapeutic targets in depression: minocycline as a candidate treatment, Behav. Brain Res. 235 (2) (2012) 302–317.
[67] M. Hinwood, J. Morandini, T.A. Day, F.R. Walker, Evidence that microglia mediate the neurobiological effects of chronic psychological stress on the medial prefrontal cortex, Cereb. Cortex 22 (6) (2012) 1442–1454.
[68] T. Kreisel, M.G. Frank, T. Licht, R. Reshef, O. Ben-Menachem-Zidon, M.V. Baratta, S.F. Maier, R. Yirmiya, Dynamic microglial alterations underlie stress-induced depressive-like behavior and suppressed neurogenesis, Mol. Psychiatry 19 (6) (2014) 699–709.
[69] F.J. Rubio, E. Ampuero, R. Sandoval, J. Toledo, F. Pancetti, U. Wyneken, Long-term fluoxetine treatment induces input-specific LTP and LTD impairment and structural plasticity in the CA1 hippocampal subfield, Front. Cell. Neurosci. 7 (2013) 66.
[70] H.C. Dringenberg, L.R. Branfield Day, D.H. Choi, Chronic fluoxetine treatment suppresses plasticity (long-term potentiation) in the mature rodent primary auditory cortex in vivo, Neural Plast. 2014 (2014), 571285.
[71] J.W. Wang, D.J. David, J.E. Monckton, F. Battaglia, R. Hen, Chronic fluoxetine stimulates maturation and synaptic plasticity of adult-born hippocampal granule cells, J. Neurosci. 28 (6) (2008) 1374–1384.
[72] R. Griffin, R. Nally, Y. Nolan, Y. McCartney, J. Linden, M.A. Lynch, The age-related attenuation in long-term potentiation is associated with microglial activation, J. Neurochem. 99 (4) (2006) 1263–1272.
[73] M.C. Liu, X.Q. Liu, W. Wang, X.F. Shen, H.L. Che, Y.Y. Guo, M.G. Zhao, J.Y. Chen, W.J. Luo, Involvement of microglia activation in the lead induced long-term potentiation impairment, PLoS One 7 (8) (2012), e43924.
[74] R. Verma, Y.P. Balhara, C.S. Gupta, Gender differences in stress response: role of developmental and biological determinants, Ind. Psychiatry J. 20 (1) (2011) 4–10.
[75] Y. Lin, G.J. Ter Horst, R. Wichmann, P. Bakker, A. Liu, X. Li, C. Westenbroek, Sex differences in the effects of acute and chronic stress and recovery after long-term stress on stress-related brain regions of rats, Cereb. Cortex 19 (9) (2009) 1978–1989.
[76] V. Luine, J. Gomez, K. Beck, R. Bowman, Sex differences in chronic stress effects on cognition in rodents, Pharmacol. Biochem. Behav. 152 (2017) 13–19.
[77] S.M. Ohline, W.C. Abraham, Environmental enrichment effects on synaptic and cellular physiology of hippocampal neurons, Neuropharmacology 145 (Pt A) (2019) 3–12.
[78] S. Innes, C.M. Pariante, A. Borsini, Microglial-driven changes in synaptic plasticity: a possible role in major depressive disorder, Psychoneuroendocrinology 102 (2019) 236–247.
[79] M. Liu, J. Li, P. Dai, F. Zhao, G. Zheng, J. Jing, J. Wang, W. Luo, J. Chen, Microglia activation regulates GluR1 phosphorylation in chronic unpredictable stress-induced cognitive dysfunction, Stress 18 (1) (2015) 96–106.
[80] A. Bartolomucci, P. Palanza, S. Parmigiani, T. Pederzani, E. Merlot, P.J. Neveu, R. Dantzer, Chronic psychosocial stress down-regulates central cytokines mRNA, Brain Res. Bull. 62 (3) (2003) 173–178.
[81] J. McWhirt, M. Sathyanesan, D. Sampath, S.S. Newton, Effects of restraint stress on the regulation of hippocampal glutamate receptor and inflammation genes in female C57BL/6 and BALB/c mice, Neurobiol. Stress 10 (2019), 100169.
[82] S.A. Small, S.A. Schobel, R.B. Buxton, M.P. Witter, C.A. Barnes, A pathophysiological framework of hippocampal dysfunction in ageing and disease, Nature reviews, Neuroscience 12 (10) (2011) 585–601.
[83] B.D. Hare, R.S. Duman, Prefrontal cortex circuits in depression and anxiety: contribution of discrete neuronal populations and target regions, Mol. Psychiatry 25 (11) (2020) 2742–2758.
[84] W. Liu, T. Ge, Y. Leng, Z. Pan, J. Fan, W. Yang, R. Cui, The role of neural plasticity in depression: from Hippocampus to prefrontal cortex, Neural Plast. 2017 (2017), 6871089.
[85] K.S. Kendler, J.M. Hettema, F. Butera, C.O. Gardner, C.A. Prescott, Life event dimensions of loss, humiliation, entrapment, and danger in the prediction of onsets of major depression and generalized anxiety, Arch. Gen. Psychiatry 60 (8) (2003) 789–796.
[86] K.S. Kendler, L.M. Karkowski, C.A. Prescott, Causal relationship between stressful life events and the onset of major depression, Am. J. Psychiatry 156 (6) (1999) 837–841.
[87] G.M. Slavich, M.R. Irwin, From stress to inflammation and major depressive disorder: a social signal transduction theory of depression, Psychol. Bull. 140 (3) (2014) 774–815.
[88] L. Yang, Y. Zhao, Y. Wang, L. Liu, X. Zhang, B. Li, R. Cui, The effects of psychological stress on depression, Curr. Neuropharmacol. 13 (4) (2015) 494–504.

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Microglial-glucocorticoid receptor depletion alters the response of hippocampal microglia and neurons in a chronic unpredictable mild stress paradigm in female mice

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ABSTRACT

Chronic psychological stress is one of the most important triggers and environmental risk factors for neuropsychiatric disorders. Chronic stress can influence all organs via the secretion of stress hormones, including glucocorticoids by the adrenal glands, which coordinate the stress response across the body. In the brain, glucocorticoid receptors (GR) are expressed by various cell types including microglia, which are its resident immune cells regulating stress-induced inflammatory processes. To study the roles of microglial GR under normal homeostatic conditions and following chronic stress, we generated a mouse model in which the GR gene is depleted in microglia specifically at adulthood to prevent developmental confounds. We first confirmed that microglia were depleted in GR in our model in males and females among the cingulate cortex and the hippocampus, both stress-sensitive brain regions. Then, cohorts of microglial-GR depleted and wild-type (WT) adult female mice were housed for 3 weeks in a standard or stressful condition, using a chronic unpredictable mild stress (CUMS) paradigm. CUMS induced stress-related behavior in both microglial-GR depleted and WT animals as demonstrated by a decrease of both saccharine preference and progressive ratio breakpoint. Nevertheless, the hippocampal microglial and neural mechanisms underlying the adaptation to stress occurred differently between the two genotypes. Upon CUMS exposure, microglial morphology was altered in the WT controls, without any apparent effect in microglial-GR depleted mice. Furthermore, in the standard environment condition, GR depleted-microglia showed increased expression of pro-inflammatory genes, and genes involved in microglial homeostatic functions (such as *Trem2*, *Cx3cr1* and *Mertk*). On the contrary, in CUMS condition, GR depleted-microglia showed reduced expression levels of pro-inflammatory genes and increased neuroprotective as well as anti-inflammatory genes compared to WT-microglia. Moreover, in microglial-GR depleted mice, but not in WT mice, CUMS led to a significant reduction of CA1 long-term potentiation and paired-pulse ratio. Lastly, differences in adult hippocampal neurogenesis were observed between the genotypes during normal homeostatic conditions, with microglial-GR deficiency increasing the formation of newborn neurons in the dentate gyrus

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subgranular zone independently from stress exposure. Together, these findings indicate that, although the deletion of microglial GR did not prevent the animal's ability to respond to stress, it contributed to modulating hippocampal functions in both standard and stressful conditions, notably by shaping the microglial response to chronic stress.

1. Introduction

Stress is defined as the perturbation of the physiological homeostasis or psychological well-being by a real or interpreted threat. While the stress response is a necessary survival mechanism required for the adaptation to environmental challenges, it can have many adverse effects when it persists or is repeated over time (McEwen, 2008). Chronic stress is associated with an increased risk of developing adverse health conditions that include cardiovascular diseases (Steptoe and Kivimäki, 2012), neuropsychiatric disorders (Davis et al., 2017), and neurodegenerative diseases (Esch et al., 2002). In our modern society thriving on performance and efficiency, chronic stress is an increasingly important public health concern.

Stress activates the hypothalamic–pituitaryadrenal axis (HPA axis), which controls the production of glucocorticoids (GCs) by the adrenal cortex (Herman et al., 2016). GCs are a class of steroid hormones that regulate the physiological (e.g., metabolic, cardiovascular, and immune) and behavioral (e.g., emotional, cognitive, and motor) response to stress (Smith and Vale, 2006). The main GC in rodents is corticosterone (CORT), which acts by binding to mineralocorticoid receptors (MR) and GC receptors (GR). MR and GR cooperatively regulate the stress response (de Kloet et al., 2008; Orchinik et al., 1991). Nevertheless, MR is mainly recruited under basal conditions and GR upon stress, since MR has a 10-fold higher affinity for CORT (Reul and Kloet, 1985). CORT has an anti-inflammatory effect that depends on its concentration and duration of exposure. Chronically elevated CORT levels, as measured upon chronic stress, repress mRNA levels of the neuronal chemokine fractalkine and its microglial CX3C chemokine receptor 1 (CX3CR1) –a signaling pathway that restrains neuroinflammation and modulates the stress response– while increasing mRNA levels of the pro-inflammatory cytokine interleukin (IL)-1β in adult rat hippocampus (Sorrells et al., 2014). Long-term increase of CORT levels is also well-known to cause maladaptive neuronal plasticity, notably in the hippocampus. It has been shown that chronically elevated circulating CORT levels induce hippocampal dendritic retractions in ovariectomized females rats (McLaughlin et al., 2005) as well as neuronal loss and dendritic atrophy, leading to an overall reduction of hippocampal volume in male rodents (Lee et al., 2009; Sapolsky et al., 1985; Woolley et al., 1990). In female and male mice, chronic stress was also associated with a reduction of adult neurogenesis in the dentate gyrus (DG), the main region where new neurons are generated throughout life (Goshen et al., 2008; Murray et al., 2008).

Microglia, the resident macrophages of the brain, play an essential role in the maintenance and remodeling of neuronal networks. These cells modulate hippocampal neurogenesis by producing pro-neurogenic factors and phagocytosing the excess of newborn cells undergoing apoptosis (Sierra et al., 2014). Their processes are highly dynamic and make frequent contacts with neuronal cells at synapses (Tremblay et al., 2010). Microglia-synapse interactions were shown to be important for the formation, maintenance and elimination of synapses, contributing to processes such as synaptic plasticity, learning, and adaptation to the environment (Tay et al., 2017). Microglia are extremely sensitive to homeostatic changes in their microenvironment such as those resulting from chronic stress exposure. Several studies have revealed that microglia undergo important morphological and functional changes in response to chronic psychological stress (reviewed in (Picard et al., 2021)). In mice, different models of chronic stress were shown to exacerbate microglial phagocytosis, notably of synaptic structures (Lehmann et al., 2016; Milior et al., 2016; Wohleb et al., 2018). Stressed microglia also contribute to neuroinflammation via their secretion of various mediators. Indeed, in a paradigm of repeated social defeat, acutely isolated microglia from male mice had increased mRNA levels of IL-1β (Wohleb et al., 2011). These modifications in response to chronic stress suggest microglial involvement in stress-induced neuronal deficits and behavioral maladaptation. However, while microglia abundantly express GR as demonstrated in rodent models (Sierra et al., 2008; Tanaka et al., 1997), the role of microglial GR under homeostatic conditions and in the stress response remains largely undetermined.

To explore the role of microglial GR in the response to stress, we generated a mouse model in which microglia specifically lack GR. We hypothesized that disrupting microglial GR signaling will modify their homeostatic functions, with consequences on the neurobehavioral response to stress. We crossed GRflox mice with Cx3cr1CreERT2 mice, resulting in a tamoxifen-inducible conditional depletion of GR from CX3CR1-expressing cells. Mice were treated with tamoxifen at adulthood to prevent developmental confounds, and the experiments were initiated one month after recombination, to target microglia selectively considering the higher turnover rate of peripheral myeloid cells that can also express CX3CR1 and transit to the brain upon stress (Lawson et al., 1992; Wohleb et al., 2013). Females were specifically examined considering their greater stress vulnerability and HPA axis dysregulation, as well as higher predisposition to stress-induced anxiety and depression compared with males (Heck and Handa, 2019). Female mice exposed to chronic unpredictable stress also presented increased glucocorticoid resistance compared to males, which might contribute to their vulnerability to stress-related disorders (Palumbo et al., 2020). The microglial-GR (mGR) depleted female mice and their wild-type (WT) controls were exposed either to standard housing conditions or chronic unpredictable mild stress (CUMS) using the Intellicage system, a high throughput apparatus designed for the automated behavioral phenotyping of mice (Kiryk et al., 2020). Our analyses focused on the hippocampus considering its high expression levels of GR and its key involvement in the stress response (de Kloet et al., 2005). We investigated microglial density, distribution and morphology, as well as gene expression. We also assessed hippocampal neurogenesis, short- and long-term neuronal plasticity as well as basal synaptic transmission at Schaffer collateral-Cornu Ammonis 1 (CA1) synapses and in pyramidal neurons to provide insights into neural plasticity mechanisms underlying the response to CUMS (Tay et al., 2017).

2. Methods

2.1. Animals

All experiments were approved and performed under the guidelines of Université Laval's animal ethics committees, the Canadian Council on Animal Care, the European Directive (2010/63/UE), and the Italian legislation on animal experimentation (Decreto Legislativo 26/2014). Animals were examined for signs of discomfort as indicated by the animal care and use guidelines [National Academy of Sciences. Guide for the care and use of laboratory animals, 1998, "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council 2003)]. They were housed at 22–25 °C under a 12-h light–dark cycle with *ad libitum* access to food and water. The Cx3cr1CreERT2 mice were obtained from the European Mouse Mutant Archive with authorization from Prof. Steffen Jung (Weizmann Institute of Science, Rehovot, Israël) and crossed with Nr3c1 fl/fl (GRflox) mice purchased from The Jackson Laboratory (strain No. 021021). To induce

recombination in Cx3cr1CreERT2+/−:Nr3c1fl/fl mice, at adulthood (6.5 to 8.5 weeks of age), the animals were treated orally with 10 mg of tamoxifen (Sigma-Aldrich) (dissolved in 1:10 ethanol/corn oil) (microglial-GR depletion tamoxifen group) twice with a 2-day interval. As controls, Cx3cr1CreERT2+/−:Nr3c1fl/fl mice were treated with oil (microglial-GR depletion oil group), Cx3cr1CreERT2−/−:Nr3c1fl/fl mice with tamoxifen (WT tamoxifen group), and Cx3cr1CreERT2−/−:Nr3c1fl/fl mice with oil (WT oil group). The animals were then allowed a resting period of 7–9 weeks, housed 3–5 per cage. This resting period allowed to selectively target microglia due to their longer lifespan compared with peripheral myeloid cells that can also express CX3CR1 (Füger et al., 2017). As described below, the animals were then either sacrificed to characterize microglial-GR depletion or transferred to the Intellicage system to study the effects of CUMS *versus* standard housing conditions, followed by behavioral, electrophysiological, cellular, or molecular analyses.

The physiological and behavioral features of the stress response profoundly differ between sexes (Figueiredo et al., 2002; Oyola and Handa, 2017). Numerous recent findings show that gonadal hormones might play a role in determining microglial response to stress, which could shape neurobiological and behavioral outcomes (Picard et al., 2021). Females have been reported to be more vulnerable to chronic stress and display an increased predisposition to stress-induced anxiety and depression (Heck and Handa, 2019). Therefore, considering both the higher vulnerability and the limited knowledge available from the literature, we decided in the current study to investigate the effects of microglial-GR deficiency in female subjects.

2.2. Characterization of microglial GR depletion

We first characterize microglial GR depletion in both males and females to confirm our model using immunofluorescence (Fourgeaud et al., 2016; Zhou et al., 2020). Naïve male and female mice were anesthetized with a mix of ketamine [80 mg/kg]/xylazine [10 mg/kg] and blood was transcardially flushed with ice-cold phosphate-buffered saline [50 mM, pH 7.40] (PBS). Brains were post-fixed in 4% paraformaldehyde (PFA; Electron Microscopy Sciences) overnight at 4 °C. After cryoprotection in 10%, 20% and 30% sucrose solutions (24 h in each, at 4 °C), the brains were cut into 30 μm-thick coronal sections using a freezing microtome (Leica SM 2000R). Sections were stored in cryoprotectant solution (30% (v/v) glycerol and 30% (v/v) ethylene glycol in PBS) at −20 °C until use.

Sections containing the cingulate cortex (Bregma 1.41 mm to 1.93 mm) and the ventral hippocampus (Bregma −2.91 mm to −3.39 mm) were selected based on the stereotaxic atlas of Paxinos and Franklin (4th edition). We chose these regions, both part of the limbic system, considering their involvement in the regulation of emotional behavior and response to stress (Fanselow and Dong, 2010; McEwen and Gianaros, 2010). For immunofluorescence staining, the free-floating sections were first incubated in citrate buffer [0.1 M] at 70 °C for 40 min to retrieve antigens. After cooling down, the sections were permeabilized with 0.3% (v/v) H_2O_2 in PBS for 5 min and then washed with PBS. They were then placed in 0.1% $NaBH_4$ solution for 30 min to block non-specific staining and washed. Afterward, the sections were incubated in a blocking solution of 10% (v/v) fetal calf serum with 3% (w/v) bovine serum albumin (BSA) and 0.03% (v/v) Triton X-100 in PBS for 1 hr at room temperature (RT). All primary and secondary antibodies were diluted in blocking buffer. Sections were incubated with anti-IBA1 antibody (ionized calcium-binding adapter molecule 1, 1:150, catalog: MABN92, EMD Millipore) and anti-GR antibody (1:800, catalog: sc-1004, Santa Cruz Biotechnology) at 4 °C overnight. Sections were then rinsed in Tris-buffered saline [50 mM] (TBS) containing 0.01% (v/v) Triton X-100 (TBS-T), and incubated with a donkey anti-mouse Alexa Fluor® 488-conjugated (1:300, catalog: A21202, Life Technologies) and a goat anti-rabbit Alexa Fluor® 568-conjugated (1:300, catalog: A11011, Life Technologies) secondary antibodies for 90 min at RT. Sections were washed in TBS, counter-stained with DAPI (1:20000, Thermo-scientific), mounted on slides and cover-slipped in anti-fading medium (Fluoromount-G, catalog: 0100-01, SouthernBiotech).

Imaging was performed in the cingulate cortex and ventral hippocampal DG polymorphic layer and CA1 *stratum radiatum* (*str rad*) with a Zeiss Axio Imager M2 epifluorescence microscope. The pictures were acquired with an AxioCamMR3 camera (Zeiss, Oberkochen, Germany) using a 20x objective. The analysis of IBA1/GR colocalization was performed blind to the experimental conditions using Photoshop CC 2018 on 2–3 sections/region/animal (n = 3–5 animals/experimental group). Every IBA1-positive (+) cell in the pictures in the region of interest was analyzed to assess colocalization with GR in the cell body. For figure presentation, microglial cells were imaged using a Zeiss LSM-880 confocal microscope. Z-stacks were acquired with a 63× objective and the Airyscan module was used for the IBA1 channel. Each stack contained ~95 slices and focus stacking was performed using Zen 3.1 software (Blue edition, Zeiss).

2.3. Chronic unpredictable mild stress (CUMS) paradigm

Following the confirmation of our model, we exposed WT (Cx3cr1CreERT2−/−: Nr3c1fl/fl) and mGR depleted (Cx3cr1CreERT2+/−: Nr3c1fl/fl) female mice treated with tamoxifen to CUMS (n = 9–13 animals/group). For the entire duration of the experiment, female mice were housed in the Intellicage system (TSE-system, NewBehavior AG, Zürich, Switzerland), which is an apparatus designed for the automatic monitoring of mouse behavior. It consists of a large acrylic cage (20.5 cm high, 58 cm × 40 cm at the top and 55 cm × 37.5 cm at the base, Model 2000 Tecniplast) with 4 walls separating each corner from the center so that they form 4 identical triangular conditioning chambers (15 × 15 × 21 cm). In this system, animals have access to each chamber by entering a front hole and only a single mouse can enter each chamber at a time since each one is identified by a transponder. The system can collect data about the number and duration of visits and number, duration, and side (right or left) of nose pokes and licks.

The floor of the cage is covered with bedding and contains four sleeping shelters in the center while a food rack on the top is filled with standard mouse chow (food *ad libitum*). One week before being moved to the Intellicage, each animal was injected with a subcutaneous transponder (T-IS 8010 FDX-B; Datamars SA). Mice have been gradually habituated to the Intellicage environment for 14 days. At the same time, they were habituated to drinking a 0.1% saccharin solution for a later assay of taste preferences and motivation.

2.3.1. Standard condition

The standard condition consisted of housing mice in the Intellicage in the absence of stressful procedures. In particular, mice were socially housed in the Intellicage and exposed to Plexiglas shelters of different colours and shapes (four red transparent Tecniplast plastic nest boxes), and to tissue paper. New tissue paper was provided every 5 days and the plastic shelters were cleaned every week.

2.3.2. Stressful condition

After the habituation period, mice were exposed to CUMS procedures to induce depression-like behavior. CUMS lasted three weeks and was expected to induce an approximate 30% decrease of saccharin preference. During CUMS, mice were exposed each day to a different randomly chosen stressor to prevent habituation. The stressors were administered by the Intellicage system, without any physical intervention by the experimenter, and comprised either: Short open door: door to access water or saccharin solution remains open for only 1.5 s; Delay: door opens only with a delay of 1, 1.5, 2, 2.5 s after the first nosepoke; Open door 25%: door opens only following 25% of nosepokes; Air puff: when the mouse performs a visit, it has a 20% chance to receive an air puff (2 bar) that lasts 1 s or until the animal leaves the corner and the doors

remained closed. During the stressful phase, to drink more, the animals have to leave the corner and start a new visit. The duration of each stressor was randomly 12, 18 or 24 hrs. During the stress-inducing procedures, red LEDs in the corner were turned on when a mouse entered in and turned off at the end of the visit, in order for mice to associate this light condition to the probability of a random stressful event happening. Additionally, during the stressful paradigm, no shelter or tissue paper was provided.

This stressful paradigm, consisting in the constant (24 hrs) exposure of the animals to various stressors, has been previously validated through the assessment of behavioral, cellular, and molecular endpoints (Alboni et al., 2017; Branchi et al., 2013; Poggini et al., 2019). Indeed, the stressful conditions exploited in the present study have been shown to increase basal CORT levels, a marker of stress response in males (Milior et al., 2016).

2.4. Behavioral tests and physiological assessments

The experimental procedures used to phenotype behavior were automatically administered by the Intellicage system, thus avoiding any bias or additional stress due to the presence of experimenter. These included saccharine preference and progressive ratio schedule. In addition, immediately before and at the end of their housing in the Intellicage, the animals were measured for body weight changes. Following the exposure to standard or stressful conditions, plasmatic CORT levels were measured to assess the HPA axis activity and identify potential dysfunctions. Finally, to evaluate the potential association between behavior and hormonal fluctuations, we monitored the estrous cycle of all mice (see Supplementary Methods).

2.4.1. Bodyweight

At the end of the habituation period and following the exposure either to the standard or stressful conditions, the bodyweight of the animals was measured at the beginning of the dark phase.

2.4.2. Saccharin preference

To saccharin preference was determined. In each corner of the Intellicage two bottles were present, one containing tap water and the other containing 0.1% saccharin solution; both freely available at all time. Water and saccharin solutions were swapped daily. The position of water and saccharin in each corner was counterbalanced across the four corners. Saccharin preference was determined as follows: (saccharin solution consumed/ saccharin solution consumed + water consumed) × 100. We measured the baseline saccharin preference on the last two days of the habituation period and at the end of the exposure either to the standard or stressful conditions.

2.4.3. Progressive Ratio schedule

To assess the effort to obtaining a reward, we used the progressive ratio reinforcement schedule that utilizes a multiplicative increase in the number of responses (i.e., nosepokes) required to dispense a unit of reinforcement (i.e., saccharin). In particular, water was always accessible after one nosepoke while saccharin solution was accessible only after a specific number of nosepokes performed in a given amount of time. Such number increased progressively after each series of 8 visits according to the following scheme: 2, 3, 4, 5, 6, 7, 8, 10, 12, 16, 20, 24. The time to perform the nosepokes was progressively increased according to the required number of nosepokes from two to 24 s. Mice were exposed to this task immediately before and after the exposure to the environmental condition (i.e., standard *versus* CUMS paradigm). To allow mice to recognize the session period, the three LEDs on the top of each door were switched on. Each test session lasted two days or until mice reached the module with 24 nosepokes. The animals have been trained on the progressive ratio reinforcement schedule during the habituation phase and before the testing in order to assess their learning performance and control differences in cognitive abilities. The parameter considered was the 'breakpoint', defined as the highest number of consecutive nose pokes performed in a single visit to achieve the access to the bottle.

2.4.4. Locomotor activity – Number of corner visits

To assess the homecage locomotor activity, we measured the number of corner visits per day, both during the habituation period and CUMS.

2.4.5. CORT levels

Plasma CORT levels, which follow a marked circadian rhythm, have been assessed in all animals at the end of the dark phase when the hormonal levels are lowest and do not differ between males and females (Lightman and Conway-Campbell, 2010), at the end of behavioral assessment. In unanesthetized animals, blood was collected from the tail 3 hrs before lights on. The bleeding procedure consisted of a small and superficial cut on the tail. Blood samples were collected individually in potassium–ethylenediaminetetraacetic acid (EDTA) coated 10 ml tubes (1.6 mg EDTA/ml blood: Sarstedt). All samples were kept on ice and later centrifuged at 3000 rpm for 15 min at 4 °C. Blood plasma was transferred to Eppendorf tubes for CORT determination and stored at −80 °C until analysis. Plasma CORT levels were quantified by enzyme immunoassay according to the manufacturer's recommendations (Corticosterone Elisa kit, Enzo Life Sciences). Samples were diluted 1:40 before analysis, and data were analyzed by sigmoidal 4-parameter logistic curve fit using Prism 6 (Graphpad). The sensitivity of the assay was 27.0 pg/ml and the intra-assay percent coefficients of variations were between 3.35% and 3.82%.

2.5. Immunohistochemistry staining

2.5.1. Hippocampal adult neurogenesis

24 hrs after the end of the Intellicage paradigm, a cohort of animals was anesthetized with ketamine [80 mg/kg]/xylazine [10 mg/kg] and perfused transcardially with 4% PFA to assess hippocampal neurogenesis. Brains were post-fixed in 4% PFA overnight at 4 °C and cryoprotected in 10%, 20% and 30% sucrose solution (24 hrs each concentration). The brains were divided into the hemispheres, and right hemispheres were cryoprotected with 30% sucrose, frozen and cut into 40 μm-thick horizontal sections using a manual microtome (HM 430 Sliding Microtome); series of every 8th section were collected and stored in cryoprotection solution (30 % (v/v) glycerol and 30% (v/v) ethylene glycol in PBS) at −20 °C until further processing. Sequential horizontal sections containing the hippocampus across the mouse brain (every ~ 320 μm, ~ 8–9 sections per mice) were collected, mounted in the correct anatomical order and Giemsa-stained (Giemsa stock solution 1.09204.0500, Merck, Darmstadt, Germany). The stained sections were differentiated 10 s in 1% acetic acid, dehydrated and coverslipped. For the neurogenesis assessment, free-floating sections were heat-treated for 40 min in citrate buffer (Target Retrieval Solution, DAKO; 1:10, pH 6.0) for epitope retrieval. After pre-incubation in 2% normal serum with 0.25% Triton in TBS for 1 hr at RT, the sections were incubated overnight at 4 °C with primary antibodies against Ki-67 (1:300, polyclonal Mouse-anti-Ki-67, BD Pharming) or doublecortin (DCX) (1:2000, Rabbit polyclonal DCX, Abcam ab18723). Incubation in secondary antibody (1:300 for Ki-67 and 1:1000 for DCX) was followed by a 1hr incubation with avidin–biotin complex (ABC) solution (Vectastain). Finally, sections were stained with 3,3′-diaminobenzidine (DAB) and mounted. DCX stained sections were counterstained with hematoxylin solution and all sections were dehydrated and coversliped.

The total number of DCX + cells was estimated in the polymorphic layer of the DG with the optical fractionator using the Stereoinvestigator software (MicroBrightField Inc. Williston, USA) with a 63x oil-immersion lens (ZEISS Plan-Apochromat 63x/1.40 Oil DIC). Cells were counted in a frame of 35 μm × 35 μm with x- and y- step sizes of 75 μm. Total cell numbers (N) were calculated using the formula: $N = \sum Q \cdot 1\ asf\ 1\ ssf$ where $\sum Q$ is the total number of cells counted, *asf* is the area

sampling fraction and *ssf* the section sampling fraction. All Ki-67 + cells were counted manually, stained cells in the top focal plane were not considered to prevent over-estimation. Total cell number estimates were calculated by multiplying the cell counts by the section sampling fraction.

2.5.2. Microglial density, distribution and morphology

For the analysis of microglial density, spacing and morphology, ventral hippocampal sections (Bregma −2.91 mm to −3.39 mm) were incubated in citrate buffer at 70 °C for 40 min for antigen retrieval. After the sections had cooled down, the free-floating sections were placed in 0.1% $NaBH_4$ solution for 30 min and washed. They were incubated in a blocking solution of 10% donkey serum with 0.5% gelatin and 0.1% Triton X-100 in PBS for 1 hr at RT. Sections were then incubated with anti-IBA1 (1:150, catalog: MABN92, EMD Millipore) and anti-TMEM119 (Transmembrane Protein 119) (1:300, catalog: ab209064, Abcam) antibodies at 4 °C overnight, rinsed in PBS(T) 0.01%, and then incubated with an Alexa Fluor® 555-conjugated (1:300, catalog: A31570, Invitrogen) and an Alexa Fluor® 647-conjugated (1:300, catalog: A31573, Invitrogen) secondary antibodies for 90 min at RT. All primary and secondary antibodies were diluted in the same blocking buffer. Sections were washed in PBS, counter-stained with DAPI (1:20000, Thermoscientific), mounted on slides, then coverslipped with anti-fading medium (Fluoromount-G, SouthernBiotech).

6 sections/mice in 6 mice per experimental group were used for imaging. For density, distribution and infiltration analyses, sections containing the hippocampal DG polymorphic layer and CA1 *str rad* were imaged in a single plane at 20x magnification using an Axio Imager M2 epifluorescence microscope equipped with an AxioCamMR3 camera (Zeiss, Oberkochen, Germany) and mosaics were created with Zen 3.1 software (Blue edition, Zeiss). For the morphological analysis, the hippocampal DG polymorphic layer and CA1 *str rad* were imaged using a Quorum WaveFX Spinning disc confocal microscope. Z-stacks were acquired with a 20x objective and an ORCA-R2 camera (Hamamatsu, 512 × 512 pixels). Each stack contained ~ 30 slices (1 µm each) and focus stacking was performed using Volocity software (Version 5.4, PerkinElmer).

Microglial density, spacing, and morphology were analyzed in all the captured images from the 6 sections/mice in 6 mice per experimental group. The analysis was performed blind to the experimental conditions with ImageJ software (National Institutes of Health) as previously described (Tremblay et al., 2012). Density and spacing were calculated, respectively using the "analyze particles" and "nearest neighbour distance" plugins of ImageJ. Cellular density was defined as the total number of cells divided by the total area (cells/mm^2) for each region of interest in each animal. Spacing index was calculated as the square of the average nearest neighbour distance (NND) multiplied by microglial density per region of interest and animal. For the infiltration analysis, total counts of IBA1+/TMEM119+ (microglia) and IBA1+/TMEM119 negative (−) (peripheral/perivascular macrophages) cells were compiled per region of interest and animal, using the analyze particles plugin (Ibanez González et al., 2019). Morphology was analyzed for 15–20 microglial cells per animal, selected based on their complete visualization. Soma and arborization areas were determined using the "drawing" and "measure" tools in ImageJ and converted in μm^2. A morphological index was then determined using the formula: soma area/arborization area was also compiled (Ibanez González et al., 2019; Tremblay et al., 2012). The larger the value, the greater the soma size was in relation to the arbor size. Circularity and solidity were next determined using the shape descriptors plugins in ImageJ as described in Bordeleau et al, 2020 (Bordeleau et al., 2020). Briefly, circularity was calculated by: $4\pi\times(\text{Area}/\text{Perimeter}^2)$ for which a value of 1.0 represent a perfect circle. Solidity was calculated by dividing the cell area by the convex cell area for which a value closer to 1.0 indicates a more convex shape.

2.6. Isolation of $CD11b^+$ cells from hippocampus and real-time polymerase chain reaction

Hippocampus of mice previously perfused with saline to eliminate circulating peripheral immune cells were cut into small pieces and single-cell suspension was achieved in Hank's balanced salt solution (HBSS). The tissue was further mechanically dissociated using a glass wide-tipped pipette and processed for cluster of differentiation (CD)11b + cells extraction with a magnetic-activated cell sorting (MACS) system (Garofalo et al., 2017). Briefly, CD11b + cells were magnetically labelled with CD11b MicroBeads and the cell suspension was loaded onto a MACS Column placed in the magnetic field of a MACS Separator. CD11b + cells were eluted as the positive fraction. Live CD11b + cells were assessed by flow cytometry and the purity was ~ 99% (Garofalo et al., 2017).

RNA was isolated with the RNeasy Mini Kit and processed for real-time polymerase chain reaction (rt-PCR) (Qiagen) in duplicate, and the intra-assay coefficient of variation was below 30%. The quality and yield of RNAs were verified using the NANODROP One system (Thermo Scientific). Reverse transcription reaction of CD11b + cells collected by MACS was performed in a thermocycler (MJ Mini Personal Thermal Cycler; Biorad) using IScript TM Reverse Transcription Supermix (Biorad) according to the manufacturer's protocol, under the following conditions: incubation at 25 °C for 5 min, reverse transcription at 42 °C for 30 min, inactivation at 85 °C for 5 min. rt-PCR was carried out in a I-Cycler IQ Multicolor rt-PCR Detection System (Biorad) using SsoFast EvaGreen Supermix (Biorad) according to the manufacturer's instructions. The PCR protocol consisted of 40 cycles of denaturation at 95 °C for 30 s and annealing/extension at 60 °C for 30 s. For quantification analysis, the comparative cycle threshold (Ct) method was used. The ΔCt was calculated by the difference between Ct values of each gene of interest and the Ct value of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), then normalized with the non-stressed WT group (reference group) to obtain the ΔΔCt. Relative quantification was performed using the $2^{\Delta\Delta C}$t method and expressed as fold change in arbitrary values. The primers used are listed in Table 1.

2.7. Electrophysiology

2.7.1. Extracellular field recordings

Animals were anesthetized by inhalation of halothane (Sigma-Aldrich) and then decapitated. The whole brain was rapidly removed from the skull and immersed in ice-cold artificial cerebrospinal fluid (ACSF) solution containing (in mM): NaCl 125, KCl 4.4, $CaCl_2$ 2.5, $MgSO_4$ 1.5, NaH2PO_4 1, $NaHCO_3$ 26 and glucose 10. ACSF was continuously oxygenated with 95% O_2 + 5% CO_2 to maintain a pH close to 7.4. Following removal, the brain tissues were blocked on the stage of a vibrating microtome (Thermo Scientific) and 350 µm thick slices were cut in ice-cold ACSF. The hippocampal slices were then transferred to an incubation chamber containing oxygenated ACSF, where they recovered for 1 hr at 30 °C prior to field recording. Individual slices were then transferred to an interface slice-recording chamber (BSC1, Scientific

Table 1
Primers sequence for real-time polymerase chain reaction experiments.

Gene	Primer Forward	Primer Reverse
Arg1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
Cd68	AGAACTTACGGAAGCACCCA	GGCAGATATGCAGTCCCATT
Gapdh	TCGTCCCGTAGACAAAATGG	TTGAGGTCAATGAAGGGGTC
Il1b	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Tnfa	GTGGAACTGGCAGAAGAG	CCATAGAACTGATGAGAGG
Fizz-1	CAGGTCTGGCAATTCTTCTGAA	GTCTTGCTCATGTGTGTAAGTGA
Bdnf	CGGC GCCCATGAAAGAAGTA	AGACCTCTCGAA CCTGCCCT
Trem2	ATGGGACCTCTCCACCAGTT	TCACGTACCTCCGGGTCCA
Mertk	GATTCTGGCCAGCACAACAGA	GAGATATCCGGTAGCCCACCA
Cx3cr1	TGACTGGCACTTCCTGCAGA	AGGGCGTAGAAGACGGACAG

System Design Inc) with a total fluid dead space of about 3 ml, to perform experiments within 1–6 hrs after slice preparation. They were maintained at 30–32 °C and constantly superfused with ACSF at the rate of 2 ml/min using a peristaltic pump. A concentric bipolar stimulating electrode (SNE-100 × 50 mm long, Elektronik–Harvard Apparatus GmbH) was placed in the CA1 *str rad* to stimulate Schaffer collateral fibers. Stimuli consisted of 100 μs constant current pulses of variable intensity, applied at 0.05 Hz. An ACSF-filled glass micropipette (0.5–1 MΩ) was placed in the CA1 region, at 200–600 μm from the stimulating electrode, in order to measure orthodromically-evoked field extracellular postsynaptic potentials (fEPSP). Stimulus intensity was adjusted to evoke fEPSPs of amplitude about 50% of maximal amplitude with minimal contamination by a population spike. Evoked responses were monitored online, and stable baseline responses were recorded for at least 10 min. Only the slices that showed stable fEPSP amplitudes were included in the experiments. LTP was induced by high-frequency stimulation (HFS, 1 train of stimuli at 100 Hz, of 1 s duration). To analyze the time course of fEPSP amplitude, the recorded fEPSP was routinely averaged over 1 min (n = 3). fEPSP amplitude changes following the LTP induction protocol were calculated with respect to the baseline.

fEPSPs were recorded and filtered (low pass at 1 kHz) with an Axopatch 200 A amplifier (Axon Instruments, CA) and digitized at 10 kHz with an A/D converter (Digidata 1322 A, Axon Instruments). Data acquisition was stored on a computer using pClamp 9 software (Axon Instruments) and analyzed off-line with Clampfit 10 program (Axon Instruments).

2.7.2. Patch-clamp recordings

Patch clamp recordings were performed as described in Basilico et al., 2019 (Basilico et al., 2019). Briefly, animals were decapitated under halothane anesthesia. Whole brains were removed and immersed for 10 min in ice-cold ACSF solution containing (in mM): KCl 2.5, $CaCl_2$ 2.4, $MgCl_2$ 1.2, $NaHPO_4$ 1.2, glucose 11, $NaHCO_3$ 26, glycerol 250. The ACSF was continuously oxygenated with 95% O_2 and 5% CO_2 to maintain the physiological pH. Horizontal 250 μm thick hippocampal slices were cut at 4 °C, using a Vibratome (DSK, Dosaka EM), and placed in a chamber filled with oxygenated ACSF containing (in mM): NaCl 125, KCl 2.5, $CaCl_2$ 2, $MgCl_2$ 1, $NaHPO_4$ 1.2, $NaHCO_3$ 26 and glucose 10. Before use, slices were allowed to recover for at least 1 hr at RT. All recordings were performed at RT on slices submerged in ACSF and perfused with the same solution in the recording chamber.

Evoked excitatory postsynaptic currents (EPSC) were recorded from CA1 pyramidal neurons using the patch-clamp technique in whole-cell configuration at −70 mV. Patch pipettes (3–5 MΩ) were filled with intracellular solution containing (in mM): Cs-methanesulfonate 135, HEPES 10, MgATP 2, NaGTP 0.3, $CaCl_2$ 0.4, $MgCl_2$ 2, QX-314 2, BAPTA 5 (pH adjusted to 7.3 with CsOH). Bicuculline methochloride (10 μM) was added to extracellular solution to block $GABA_A$ receptors. EPSC were evoked by electrical stimulation with theta glass tubes filled with external solution. Stimulating electrodes were connected to the stimulus isolation unit (Iso-stim A320, WPI) and placed in the *str rad*, 100–150 μm from the patched CA1 neuron, to activate the Schaffer collaterals projecting to CA1. Synaptic responses were evoked by stimulating for 100 μs at 0.1 Hz, the stimulus intensity was adjusted accordingly to the experiment. AMPA-mediated EPSC were evoked by paired-pulse stimulations (interval 50 ms) to determine the paired-pulse ratio (PPR), calculated as the ratio between the amplitude evoked by the second stimulus (A2) over the first (A1; A2/A1). The input/output (I/O) of the AMPA component was determined at −70 mV, stimulating Schaffer collaterals at increasing intensities (0.1–10 mA). Each pulse of a given intensity was repeated 6 times to obtain an average response. To determine the AMPA/NMDA ratio, stimulus strength was adjusted to obtain at −70 mV stable AMPA-mediated EPSC with an amplitude corresponding to the 50% of the maximum response. The NMDA current was recorded at + 40 mV from the same neuron, using the same stimulus strength. During the analysis, NMDA peak amplitude was measured with a delay of 25 ms from the AMPA peak. Recordings were made with a patch-clamp amplifier (Axopatch 200A, Molecular Devices), filtered at 2 kHz, digitized (10 kHz) and acquired using pClamp 10.0 software (Molecular Devices); the analysis was performed off-line using Clampfit 10 (Molecular Devices).

2.8. Statistical analyses

For GR microglial deficiency characterization and microglial analyses (density, distribution, morphology), as well as peripheral/perivascular myeloid cells, statistical analyses were conducted using 2-way ANOVA with the software Prism (GraphPad, Version 8). *Post-hoc* comparisons were performed using Bonferroni's correction for GR microglial deficiency characterization to compare the other groups to the WT oil group and Tukey's correction for the microglial analyses. Sample size (n) refers to the number of animals in each experiment, except for the microglial morphology analysis where it refers to the cell number, as previously conducted by our group (Hui et al., 2018; Lecours et al., 2020; Milior et al., 2016). Intellicage, neurogenesis and electrophysiological experiments were analyzed with 2-way ANOVAs with the software Statview II (Abacus Concepts) or R and Sigmaplot software, considering genotype (WT and microglial-GR depletion) and environmental condition (standard, stress) as the between-subject variables. *Post-hoc* comparisons were performed using Tukey's correction (Milior et al., 2016). Microglial gene expression data were tested for equal variances and then analyzed with 2-way ANOVAs with Sigmaplot software and *post-hoc* comparisons were performed using Holm-Sidak's correction. All mean differences were considered statistically significant when $p < 0.05$. All reported values are mean ± standard error of the mean (SEM).

3. Results

We investigated the effects of microglial-GR depletion on microglia and neuronal circuits, at steady-state and in response to CUMS using female mice, considering that they are more vulnerable to chronic stress and have a higher predisposition to stress-induced anxiety and depression compared to males (Heck and Handa, 2019). The animals were habituated to the Intellicage system for two weeks, and then exposed to a stressful (CUMS) or standard (without stress) housing environment for three additional weeks (Fig. 1). After sacrifice of the animals, cellular, molecular and electrophysiological analyses were performed in the hippocampal CA1 *str rad* and the DG polymorphic layer, two key regions

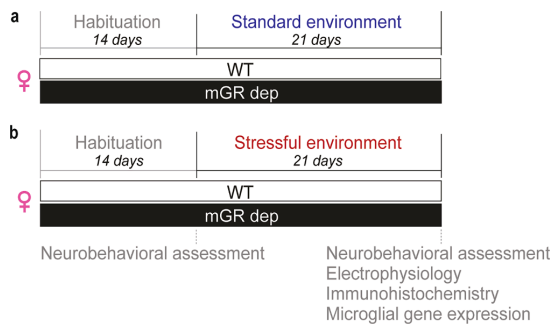

Fig. 1. Schematic representation of the experimental design. Following 14-days period of habituation to the Intellicage system, the mice were exposed either to (a) standard or (b) stressful housing environment for 21 days. Immediately after the habituation phase and the environmental condition exposure, physiological and behavioral assessments were performed. At the end of the experiment, we performed a series of electrophysiological, immunohistological, and gene expression measures. mGR dep: microglial-glucocorticoid receptor depletion, WT: wild-type.

involved in the adaptation to stress (McEwen, 2001).

3.1. The microglial-GR depletion mouse model almost completely suppresses microglial GR expression

To characterize our mouse model, we first verified that microglia were depleted for GR in both male and female mice using a double immunofluorescent staining against the microglial marker IBA1 and GR. The observations were made in the cingulate cortex, CA1 *str rad* and DG polymorphic layer of WT *versus* mGR depleted animals that received either tamoxifen to induce microglial-GR deletion or oil as a control. In mGR depleted female mice, we observed a significant decrease in the proportion of microglia expressing GR following tamoxifen administration in the three regions compared to the WT oil group (Fig. 2), the cingulate cortex (78.05 ± 2.19 % (WT oil) *vs* 6.17 ± 1.64% (mGR depletion Tam) [$F_{(1,9)} = 553.9$, $p < 0.0001$]) (Supplementary Fig. 1a), the CA1 *str rad* (78.13 ± 1.09% (WT oil) *vs* 4.54 ± 2.28 % (mGR depletion Tam) [$F_{(1,9)} = 482.0$, $p < 0.0001$]) and DG polymorphic layer (97.78 ± 0.48 % (WT oil) *vs* 4.44 ± 4.44 % (mGR depletion Tam) [$F_{(1,9)} = 126.7$, $p < 0.0001$]) (Fig. 2). The same decrease was observed in males in the cingulate cortex (80.50 ± 2.16 % (WT oil) *vs* 5.91 ± 0.10 % (mGR depletion Tam) [$F_{(1,10)} = 204.3$, $p < 0.0001$]) and the CA1 *str rad* (78.17 ± 0.77 % (WT oil) *vs* 4.17 ± 0.21 % (mGR depletion Tam) [$F_{(1,11)} = 328.4$, $p < 0.0001$]) (Supplementary Fig. 1b-c). The tamoxifen treatment did not induce a complete depletion, which is consistent with what was previously observed in other models of tamoxifen-induced depletion in microglia (Goldmann et al., 2013). Tamoxifen *versus* oil also induced a moderate increase in microglial GR expression in the female WT animals, among the CA1 *str rad* (78.13 ± 1.09 % (WT oil) *vs* 91.10 ± 2.34 % (WT Tam) [$F_{(1,9)} = 482.0$, $p < 0.0001$]) and the cingulate cortex (78.05 ± 2.19 % (WT oil) *vs* 92.05 ± 1.73 % (WT Tam) [$F_{(1,9)} = 482.0$, $p < 0.0001$]).

3.2. CUMS affects the neurobehavioral profile independently from the genetic background

At the end of the habituation period, we measured the bodyweight of the animals and we did not observe a significant difference between the genotypes [$F_{(1,41)} = 0.224$, $p = 0.6386$] (Fig. 3a). At the end of the experiment, we calculated the difference of body weight between before and after the exposure to the different conditions. We observed a significant effect of stress exposure, with both mGR depleted and WT mice exposed to CUMS showing a significant reduction of body weight compared to the animals in standard condition [$F_{(1,38)} = 100.7$, $p < 0.0001$] (Fig. 3b). The increase in basal peripheral CORT levels after CUMS failed to reach statistical significance [$F_{(1,32)} = 2.139$, $p = 0.153$] (Fig. 3c).

When we assessed the saccharine preference on the last two days of habituation, immediately before the animals were exposed to the standard or stressful paradigms, both mGR depleted and WT mice showed a strong preference for the saccharin solution [$F_{(1,41)} = 3.990$, $p = 0.0524$] (Fig. 3d). Following the exposure to the standard condition, both genotypes maintained a clear preference for the saccharin solution. By contrast, the exposure to the CUMS induced a significant decrease of saccharine preference, with no difference between mGR depleted and WT animals [$F_{(1,38)} = 15.00$, $p < 0.0001$] (Fig. 3e).

During the habituation phase, WT and mGR depleted mice were trained to progressive ratio. During training, they did not differ in the breakpoint reached [$F_{(1,41)} = 1.843$, p = 0.1821], suggesting that the depletion of the microglial GR does not impair associative learning. During the first progressive ratio aimed at assessing the motivation response, immediately before stress, no difference in the breakpoint reached between mGR depleted and WT mice was found [$F_{(1,39)} = 3.383$, $p = 0.0735$] (Fig. 3f). As expected, CUMS significantly affected the anhedonic response, stressed mice showing a significantly lower breakpoint compared to animals exposed to standard condition [$F_{(1,37)} = 7.612$, $p = 0.0090$]. No interaction effect between genotype and condition was found [$F_{(1,37)} = 6.456$, $p = 0.9936$] (Fig. 3g).

Finally, we investigated the locomotor activity of the animals as measured by the number of corner visits per day. We did not observe an effect of genotype on activity neither during the habituation period nor the stress exposure (data not shown).

All the neurobehavioral parameters assessed were not affected by the estrous cycle (data not shown).

3.3. Microglial-GR depletion affects microglial morphology in a region-specific manner

To provide insights into the consequences of microglial GR depletion on brain immunity, we next evaluated microglial density and distribution, as well as perivascular and infiltrating peripheral myeloid cells, in the CA1 *str rad* and the DG polymorphic layer using a double immunofluorescent staining against IBA1 (labels all myeloid cells including microglia) and TMEM119 (specific to microglia). We found that microglial density, NND and spacing index (compiling the average nearest neighbour and microglial density) (Fig. 4), as well as perivascular and infiltrating peripheral myeloid cells (proportion of cells only IBA1 +) (Table 2) were not modified by CUMS or microglial GR

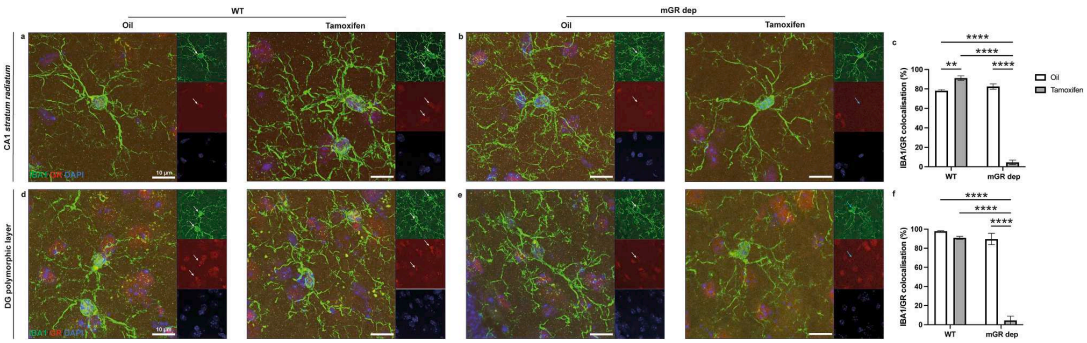

Fig. 2. Characterization of microglial-GR depleted induced by tamoxifen. 4 weeks after the administration of tamoxifen or the vehicle, we used a double immunofluorescence with IBA1 and GR to confirm microglial depletion of GR in the CA1 *str rad* (a, b, c) and DG polymorphic layer (d, e, f). The pictures were acquired using a confocal microscope (Zeiss LSM-880 with Airyscan) at a 63x magnification. Scale bar is equivalent to 10 µm. Data are shown as mean ± standard error of the mean (n = 3–4 mice/group). White arrow: IBA1+/GR + cell, blue arrow: IBA1+/GR- cell, DG: dentate gyrus, GR: glucocorticoid receptor, mGR dep: microglial-glucocorticoid receptor depletion, WT: wild-type. $^{**}p < 0.01$, $^{****}p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

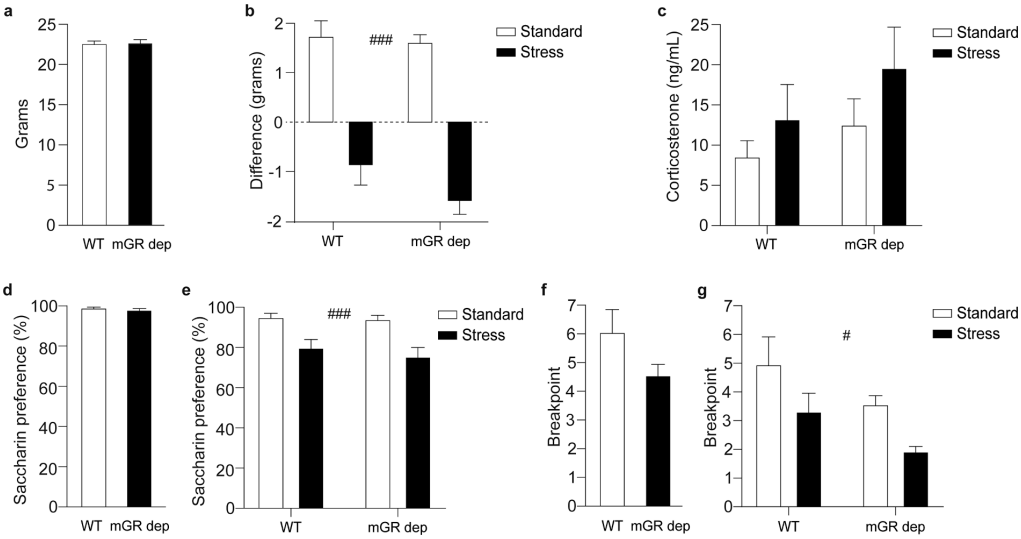

Fig. 3. Physiological and behavioral responses of WT and microglial-GR depleted mice exposed to CUMS. (a) At the end of the habituation period, we did not observe difference of body weight between the groups. (b) Following the environment exposure, mGR dep and WT mice exposed to CUMS showed a significant reduction of body weight compared to the animals in standard condition. (c) The exposure to CUMS did not induced an increase in corticosterone levels. (d) During the habituation phase, both groups showed strong preference for the saccharin solution. (e) The exposure to the CUMS induced a significant decrease of saccharine preference, with no difference between genotypes. (f) Before the exposure to the different environmental conditions, mGR depleted mice displayed a lower number of break point compared to the WT, but no significant difference was found. (g) At the end of the environmental condition, main effect of both genotype and condition was found. Data are shown as mean ± standard error of the mean (n = 9–13 mice per group). CUMS: chronic unpredictable mild stress, mGR dep: microglial-glucocorticoid receptor depletion, WT: wild-type #$p < 0.05$ and ### $p < 0.0001$ standard *vs* stress.

deficiency in both regions.

In the CA1 *str rad*, microglial arborizations area increased with CUMS in WT animals (2278 ± 76.83 μm^2 *vs* 2705 ± 72.77 μm^2, $p = 0.0013$; Fig. 5c). However, this increase was not observed with CUMS in mGR depleted animals (2714 ± 95.31 μm^2 *vs* 2616 ± 79.96 μm^2). In agreement with this result, microglial morphological index (soma area/arborizations area) was significantly decreased following stress in WT animals (0.0314 ± 0.0013 a.u *vs* 0.0263 ± 0.0009 a.u., $p = 0.0056$; Fig. 5d), but not in mGR depleted animals (0.0284 ± 0.0010 a.u. *vs* 0.0289 ± 0.0012 a.u.). Microglial arborizations were also larger in mGR depleted animals compared to WT animals in standard housing condition (2713.918 ± 95.305 μm^2 *vs* 2277.628 ± 76.828 μm^2, $p = 0.0011$; Fig. 5c). Microglial morphology did not differ significantly between genotypes and environmental conditions in the DG polymorphic layer (Fig. 5 g, h and Table 2).

3.4. Microglial-GR depletion affects microglial inflammatory gene expression upon CUMS

To determine if GR deficiency modulated microglial inflammatory phenotype at steady-state and upon CUMS, we sorted microglial (CD11b +) cells from hippocampus of mGR depleted or WT mice and analyzed the expression of different pro- or anti-inflammatory genes and of genes involved in microglial homeostatic functions (Triggering receptor expressed on myeloid cells 2 (*Trem2*), myeloid-epithelial-reproductive tyrosine kinase (*Mertk*) and *Cx3cr1*). In standard condition, mGR depleted-microglia expressed higher levels of pro-inflammatory genes (*Il1b* and tumor necrosis factor α (*Tnfa*)) (Fig. 6b, c), and increased the expression of genes involved in phagocytic activity and in the modeling of synaptic transmission (cluster of differentiation *Cd68, Trem2*, *Mertk* and *Cx3cr1*) (Fig. 6a, g, h, i). Furthermore, CUMS increased the expression of anti-inflammatory genes (*Arg-1* and *Fizz-1*) (Fig. 6d, e) in mGR depleted *vs* WT microglia, and reduced the expression of pro-inflammatory (*Tnfa*), phagocytic (*Cd68)* as well as other functional (*Trem2*, *Mertk* and *Cx3cr1*) genes (Fig. 6). There was no change in *Bdnf* (Brain-derived neurotrophic factor).

3.5. Microglial GR deficiency alters adult hippocampal neurogenesis

Microglia are known to influence adult hippocampal neurogenesis and this interaction can be affected by chronic stress (Sierra et al., 2014). To evaluate whether the microglial GR deficiency had an impact on this neurogenesis, we used immunochemistry against Ki-67 (a marker of proliferating cells) and DCX (a marker of newborn neurons). The analysis of Ki-67 + cells density revealed that the exposure to either standard or stressful conditions does not affect proliferation [$F_{(1,16)} = 0.637$, $p = 0.4363$] (Fig. 7a). By contrast, DCX + cells density was affected by the genotype [$F_{(1,15)} = 5.888$, $p = 0.0283$], but no interaction was found [$F_{(1,15)} = 1.725$p = 0.20875]. *Post-hoc* analysis revealed that, in the standard condition, DCX + cells number is significantly increased in mGR depleted mice compared to WT (Fig. 7b), suggesting an increased number of immature neurons ($p < 0.05$). Exposure to CUMS led to the normalization of adult neurogenesis: mGR depleted mice exposed to stress showed a similar number of DCX + cells as in WT mice exposed to standard conditions.

3.6. Microglial GR deficiency affects CA1 hippocampal plasticity, increasing sensitivity to stress

To investigate the effect of microglial GR gene depletion on hippocampal plasticity processes, we explored CA1 plasticity inducing long-term potentiation (LTP) by a single Schaffer collateral stimulation (1 train of stimuli at 100 Hz) in WT and mGR depleted mice exposed to standard or stressful conditions. Notably, in WT mice, exposure to stress did not produce any effect, whereas it reduced the amplitude of LTP in mGR depleted mice. An interaction between genotype and

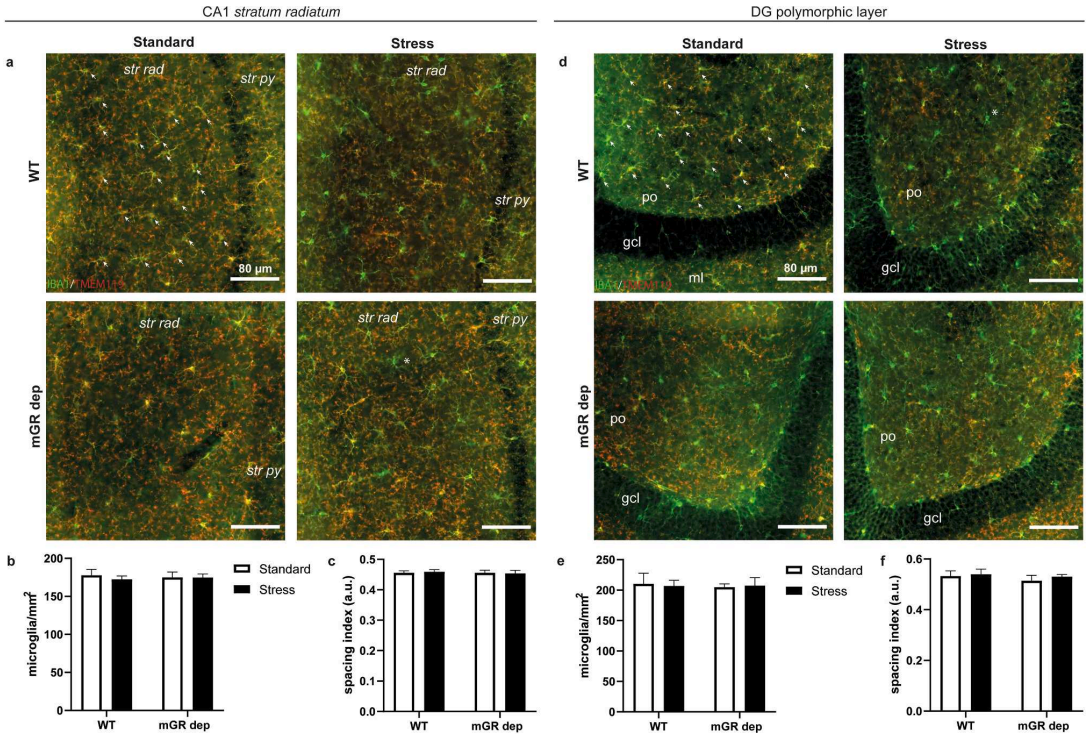

Fig. 4. Density and distribution of microglia, perivascular, and infiltrating myeloid cells in WT *versus* microglial-GR depleted mice exposed to CUMS. Representative epifluorescence pictures at a 20x magnification showing IBA1- (green) and TMEM119- (red) stained microglia in CA1 *str rad* (a) and polymorphic layer of the dentate gyrus (d) in the four groups. Scale bar is equivalent to 80 µm. White arrows: IBA1+/TMEM119- cells. No difference was observed for the microglial density and the spacing index in CA1 *str rad* (b,c) and in the polymorphic layer of the DG (e,f). (n = 6 mice per group). Data are shown as mean ± standard error of the mean. a.u. = arbitrary units, CUMS: chronic unpredictable mild stress, DG: dentate gyrus, mGR dep: microglial-glucocorticoid receptor depletion, po = polymorphic layer, gcl = granule cell layer, *str rad* = *stratum radiatum*, *str py* = *stratum pyramidale*, WT: wild-type. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

environmental condition additionally emerged [$F_{(1,53)} = 4.958$, $p = 0.0300$]. In particular, *post-hoc* analysis revealed that under standard condition, the experimental groups did not differ in their LTP amplitude (WT, 1.499 ± 0.044; mGR depletion, 1.547 ± 0.0390) (Fig. 8a) but in stressful condition, mGR depleted mice showed a significant decrease in LTP amplitude (1.413 ± 0.038) compared to both mGR depleted and WT mice in standard condition ($p = 0.018$) (Fig. 8a) and to stressed WT mice (1.539 ± 0.036, $p = 0.0396$).

By means of patch-clamp recordings, we measured the PPR, a form of short-term plasticity related to neurotransmitter release probability (Zucker, 1989). We determined the ratio between the amplitude evoked by a second stimulus over the first, by stimulating the Schaffer collaterals projections to CA1 at 50 ms intervals. As shown in Fig. 8b, in WT mice, stress did not modify PPR value, by contrast, it significantly reduced the value in the mGR depleted mice. We found an effect of genotype in stress condition ($p = 0.0260$) and *post-hoc* analysis showed that in mGR depleted mice, exposure to stress reduced PPR value (1.027 ± 0.115) compared to the standard housing condition (1.552 ± 0.151, $p = 0.0080$).

3.7. Microglial GR deficiency affects hippocampal synaptic connectivity independently of stress

We stimulated the CA1 Schaffer collaterals using a graded stimulation and recorded the resulting AMPA responses. As shown in Fig. 8c, we compared I/O curves between genotypes exposed to different environmental stimuli. We observed that overall WT mice at different stimulus intensity displayed a significantly weaker peak currents compared to mGR depleted mice [$F_{(1,253)} = 7.533$, $p = 0.0070$] in both environmental conditions, indicating that in the absence of GR, hippocampal synapses showed higher synaptic connectivity compared to WT mice.

We then measured the amplitude of AMPA receptor (AMPAR) and NMDA receptor (NMDAR)-mediated components of postsynaptic currents at −70 mV and + 40 mV of holding potential, respectively, to determine AMPA/NMDA ratio (Hoshiko et al., 2012). This ratio evaluated in WT and mGR depleted mice did not differ between experimental conditions (Fig. 8d).

4. Discussion

In the present study, we developed a depletion model of microglial GR inducible by tamoxifen during adulthood. Using that model, we show that microglial GCs-GR signaling plays an important role in modulating microglial phenotype and hippocampal plasticity in both standard and stressful conditions. Indeed, mGR depleted mice had an increased microglial arborizations and higher number of newborn cells, as well as an increased expression of microglial gene-related to synaptic plasticity and inflammation. Moreover, contrary to WT mice, mGR depleted mice exposed to CUMS had a decreased LTP and PPR and their microglia displayed a shift towards an anti-inflammatory gene profile. Behavioral characterization further revealed that CUMS induced a

Table 2
Microglial distribution, perivascular and infiltrating myeloid cells and microglial arborizations properties in WT *versus* mGR depleted mice exposed to CUMS. % Infiltration: Average percentage of IBA1+/TMEM119- cells on total myeloid cells count, a.u.: arbitrary unit, CUMS: chronic unpredictable mild stress, DG: dentate gyrus, mGR dep: microglial-glucocorticoid receptor depletion, NND: nearest neighbor distance, *St rad*: *stratum radiatum*, WT: wild-type.

Parameters		Mean ± standard error of the mean				*F*	*p*
		Standard		Stress			
		WT	mGR dep	WT	mGR dep		
CA1 *St rad*	NND (a.u.)	50.846±0.898	51.339±0.899	51.896±0.858	51.246 ± 0.523	Condition*Genotype: 0.4972 Condition: 0.3482 Genotype: 0.0093	Condition*Genotype: 0.4889 Condition: 0.5617 Gentoype: 0.9241
	% Infiltration	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.407 ± 0.996	Condition*Genotype: 0.1667 Condition: 0.1667 Genotype: 0.1667	Condition*Genotype: 0.6874 Condition: 0.6874 Genotype: 0.6874
	Arborizations circularity (a.u.)	0.482 ± 0.012	0.488 ± 0.013	0.494 ± 0.010	0.491 ± 0.011	Condition*Genotype: 0.1448 Condition: 0.3911 Genotype: 0.0101	Condition*Genotype: 0.7038 Condition: 0.9199 Genotype: 0.5321
	Arborizations solidity (a.u.)	0.811 ± 0.006	0.820 ± 0.007	0.822 ± 0.005	0.820 ± 0.006	Condition*Genotype: 0.8006 Condition: 0.7718 Genotype: 0.2075	Condition*Genotype: 0.3715 Condition: .0.3802 Genotype: 0.6490
DG polymorphic layer	NND (a.u.)	51.015 ± 1.764	50.488 ± 1.120	51.516 ± 0.896	51.535 ± 1.691	Condition*Genotype: 0.0371 Condition: 0.2982 Genotype: 0.0320	Condition*Genotype: 0.8491 Condition: 0.5911 Genotype: 0.8598
	% Infiltration	0.000 ± 0.000	0.000 ± 0.000	0.407 ± 0.996	0.340 ± 0.833	Condition*Genotype: 0.0026 Condition: 0.3307 Genotype: 0.0026	Condition*Genotype: 0.9597 Condition: 0.5716 Genotype: 0.9597
	Arborizations circularity (a.u.)	0.544 ± 0.010	0.560 ± 0.011	0.518 ± 0.011	0.524 ± 0.011	Condition*Genotype: 0.1908 Condition: 8.288 Genotype: 1.097	Condition*Genotype: 0.3325 Condition: **0.0042** Genotype: 0.2956
	Arborizations solidity (a.u.)	0.846 ± 0.005	0.855 ± 0.005	0.833 ± 0.006	0.830 ± 0.005	Condition*Genotype: 1.329 Condition: 13.460 Genotype: 0.3546	Condition*Genotype: 0.2498 Condition: **0.0003** Genotype: 0.5518

decrease of both saccharine preference and progressive ratio breakpoint, suggesting stress-related behaviors that are not prevented by the mGR depletion. However, it is possible that GR depletion affected other behaviors involving the corticolimbic regions that we did not test, such as the anxiety (Wohleb, 2016).

Tamoxifen induced a moderate increase in microglial GR expression in the female WT animals compared to vehicle controls. This increase might relate to tamoxifen's modulation of estrogen receptors (Singh et al., 2008), which are known to be expressed by microglia (Sierra et al., 2008) and to interact with GR (Vahrenkamp et al., 2018). Moreover, estradiol is known to possess a protective effect against some of the deleterious effects of stress (e.g., synaptic transmission impairments, dendritic atrophy) (Garrett and Wellman, 2009; Wei et al., 2014), a phenomenon that might be prevented by tamoxifen considering its anti-estrogenic properties (Singh et al., 2008). To consider these possible effects of tamoxifen, all of our animals exposed to standard or CUMS environments did receive tamoxifen, including WT groups. While tamoxifen may have altered baseline responses of the outcomes measured, and may interact with CUMS, we observed differences between the WT and mGR depleted groups, pointing to a role of microglial GR in modulating these reactions to CUMS. Nevertheless, replicating an equivalent microglia-specific depleted model using a different approach would shed more light onto the possible effects of tamoxifen.

4.1. Microglial-GR signaling deficiency alters microglial and neuronal properties at steady-state

Our results demonstrate that the microglial GR plays an important role in modulating microglial functions under normal homeostatic conditions. Indeed, microglia from mGR depleted mice differed from those of WT mice by showing an increased arborizations in the CA1 *str rad* while no difference was observed in the DG polymorphic layer. The morphology of GR-deficient microglia was previously characterized by Carrillo-de Sauvage *et al* using $GR^{LysMCre}$ mutant mice in which both microglia and peripheral macrophages were conditionally deficient in GR. They did not observe differences in the microglial surface area within the cerebral cortex under normal physiological conditions (Carrillo-de Sauvage et al., 2013). Thus, the observed increase of microglial arborizations that we measured could be specific to the CA1 *str rad*. This is consistent with the known heterogeneity of microglia between brain regions (Tan et al., 2020). The increased microglial arborizations area was associated with an increase in microglial gene expression of *Mertk*, which can regulate microglial process surveillance and phagocytosis (Fourgeaud et al., 2016), and an increase of *Cd68* that is associated with phagolysosomal activity (Lecours et al., 2020). This could indicate a higher activity of microglia notably in extracellular debris clearance and synaptic remodeling (Lecours et al., 2020). Moreover, GR depleted microglia had increased gene expression of *Cx3cr1* and *Trem2*, both involved in microglial migration, phagocytosis and remodeling of synaptic transmission (Castro-Sánchez et al., 2019; Filipello et al., 2018; Maggi et al., 2011; Mazaheri et al., 2017). These results suggest that GR depleted microglia might be more involved in synaptic plasticity, and that microglial GR has a role in maintaining microglia in a surveillant state. However, the physiological and functional changes associated with microglial hyper-ramification are still not well understood and warrant further investigation. Consistent with their homeostatic microglial functional changes, mGR depleted mice presented an alteration in the hippocampal synaptic connectivity at steady state. Higher

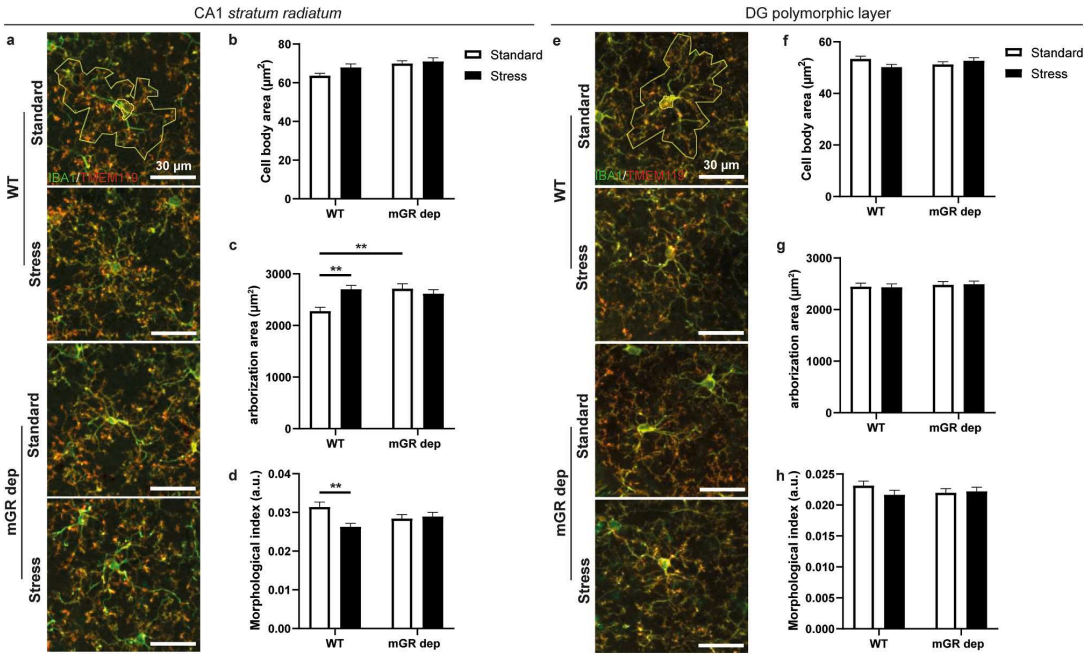

Fig. 5. Microglial cell body and arborizations area, as well as morphological index in WT *versus* microglial-GR depleted mice exposed to CUMS. Representative confocal pictures showing the morphology of IBA1- (green) and TMEM119- (red) stained microglia in the four groups, in the *CA1 str rad* (a) and the DG polymorphic layer (e) at a 20x magnification. Scale bar is equivalent to 30 µm. Immunofluorescence of IBA1 (red) and TMEM119 (green) allowed the analysis of microglial cell body area (b,f), arborizations area (c,g), and morphological index (d,h) in the four groups, in both regions. Data are shown as mean ± standard error of the mean. (n = 90–105 microglial cells in 6 mice per group). a.u. = arbitrary units, CUMS: chronic unpredictable mild stress, DG: dentate gyrus, mGR dep: microglial-glucocorticoid receptor depletion, *str rad* = *stratum radiatum*, WT: wild-type. $^{**}p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

AMPA I/O curves in the mGR depleted compared to the WT mice were measured after stimulation of the Schaffer collaterals, and could be explain by a higher number of functional synapses. This could be linked with the increased microglial *Tnfa* we observed in the mGR depleted mice. Indeed, it was shown that glial *Tnfa* promotes the expression of AMPA glutamate receptors in neurons (Stellwagen et al., 2005; Stellwagen and Malenka, 2006). Another potential cause behind the neuronal connectivity change could be the increased number of DCX + cells in mGR depleted mice compared to the WT mice. Microglia play a key role in the regulation of neurogenesis by phagocytosing the excess of newborn cells (Sierra et al., 2010) and releasing neurotrophic factors such as insulin-like growth factor 1 (IGF-1) that promote neurogenesis (Gemma and Bachstetter, 2013). Considering the increase of microglial *Mertk* and *Cd68*, the observed increase in newly generated cells did not likely result from a deficit in microglial phagocytic engulfment of newborn cells (Fourgeaud et al., 2016). Instead, it could arise from a decreased microglial release of trophic factors (e.g., IL-7, IL-11) that can promote their differentiation (Sierra et al., 2014). These effects could also be mediated by the increase in *Cx3cr1*, which is known to be involved in hippocampal neurogenesis. Indeed, male mice deficient in CX3CR1 presented a decrease in hippocampal neurogenesis (Bachstetter et al., 2011), as well as in the functional connectivity (Basilico et al., 2019), which suggests an implication of the receptor in this process. It has been widely reported that elevated levels of GCs reduce adult hippocampal neurogenesis, inhibiting cell proliferation, differentiation, and survival (Bessa et al., 2009; Egeland et al., 2015; Gould et al., 1998; Morais et al., 2014). Notwithstanding, knowledge into the role of microglial GRs in these neurogenic processes, including cellular proliferation and differentiation, remains limited. Nevertheless, the high expression of GR on microglia suggests that GCs can affect neurogenesis via both neuronal and microglial GRs.

Microglia deficient in GR additionally presented at steady-state an increased expression of *Il1b* and *Tnfa,* which are considered to be pro-inflammatory cytokines (Liu and Quan, 2018). At steady-state, GR is known to promote anti-inflammatory actions through its inhibition of the transcription of pro-inflammatory genes (Escoter-Torres et al., 2019). Thus, its absence could induce the transcription of inflammatory genes that have normally been inhibited, with possible consequences on the mechanisms underlying the response of the brain to environmental challenges such as CUMS.

4.2. Role of microglial GCs-GR signaling in response to CUMS

The main finding of our study is that the impairment of microglial GCs-GR signaling leads to an alteration of microglial morphology and gene profile, as well as synaptic plasticity (LTP and PPR), but does not affect the behavioral response to CUMS exposure. The CUMS paradigm did not induce a significant increase in CORT, which could be due to sex differences. Studies of HPA axis dysregulation in rodent depression models reports that CORT is not always increased in females as it is in males. In a chronic isolation/restrain model, the stress resulted in increased adrenocorticotropic hormone levels, without changes in CORT levels in female mice (Dong et al., 2020). Another study using foot-shock stress revealed a higher CORT increase in socially-isolated male compared to female rats (Pisu et al., 2016).

In terms of microglial changes, CUMS did not modify density in the CA1 *str rad* or the DG polymorphic layer in both genotypes. There was very marginal myeloid cell infiltration, only observed in the stress

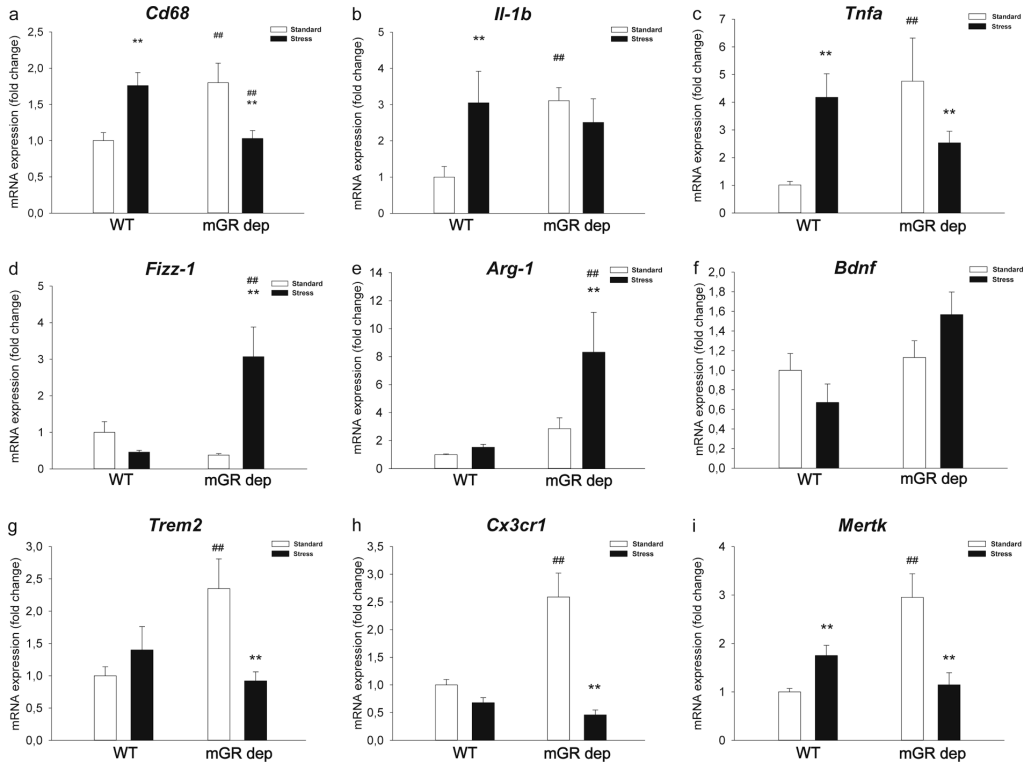

Fig. 6. Gene expression changes in WT *versus* microglial-GR depleted mice exposed to CUMS. RT-PCR in CD11b^{+} cells sorted from hippocampus of WT or microglial-GR depleted mice of (a) *cd68*, (b) *il-1b*, (c) *tnfa*, (d) *fizz-1*, (e) *arg-1*, (f) *bdnf*, (g) *trem2*, (h) *cx3cr1* and (i) *mertk*. Data are shown as mean ± standard error of the mean (n = 5 mice per group). Two-way ANOVA with Holm-sidak *post-hoc* test was used for the analysis. * Standard *vs* stress, # WT standard *vs* mGR depleted standard or WT stress *vs* mGR depleted stress. CUMS: chronic unpredictable mild stress, mGR dep: microglial-glucocorticoid receptor depletion, Rt-PCR: Real-time polymerase chain reaction, WT: wild-type. *# $p < 0.05$ **## $p < 0.01$.

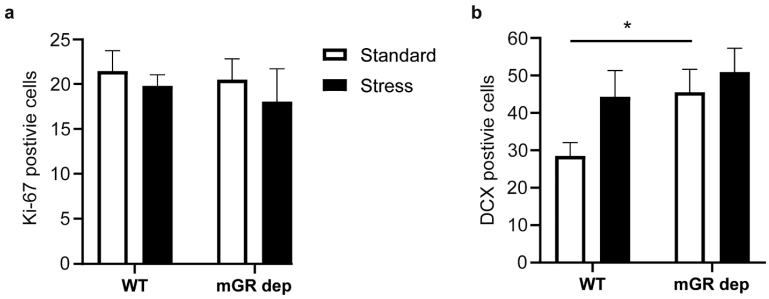

Fig. 7. Adult neurogenesis levels in WT *versus* microglial-GR depleted mice exposed to CUMS. (a) Ki-67 and (b) DCX positive cells in the dentate gyrus. In the standard condition, DCX positive cells number is significantly lower in mGR depleted mice compared to WT. Data are shown as mean ± standard error of the mean (n = 6 mice per group). CUMS: chronic unpredictable mild stress, DCX: doublecortin, mGR dep: microglial-glucocorticoid receptor depletion, WT: wild-type. *$p < 0.05$.

condition in both genotypes. This is consistent with studies showing chronic stress can promote the recruitment of peripheral cells in males and female mice, although this was observed in a repeated social defeat paradigm (Weber et al., 2019; Yin et al., 2019), which is a stronger, more physical stressor (Golden et al., 2011). We also found that WT animals had an increase in the microglial arborizations area in the CA1 *str rad*, but not in the DG polymorphic layer. Microglial hyper-ramification following chronic stress was previously observed in the DG in mice (Hellwig et al., 2016), as well as in the medial prefrontal cortex (mPFC) (Hinwood et al., 2013; Hinwood et al., 2012) and CA3 of rats (Franklin et al., 2018). However, some studies reported a decrease in microglial arborizations in CA1 *str rad* of male mice following CUMS (Milior et al., 2016) or de-ramification in the mPFC (Horchar and Wohleb, 2019), medial amygdala, paraventricular nucleus, DG and hippocampus of male mice following repeated social defeat (Wohleb et al., 2014a; Wohleb et al., 2014b; Wohleb et al., 2011). These different results could

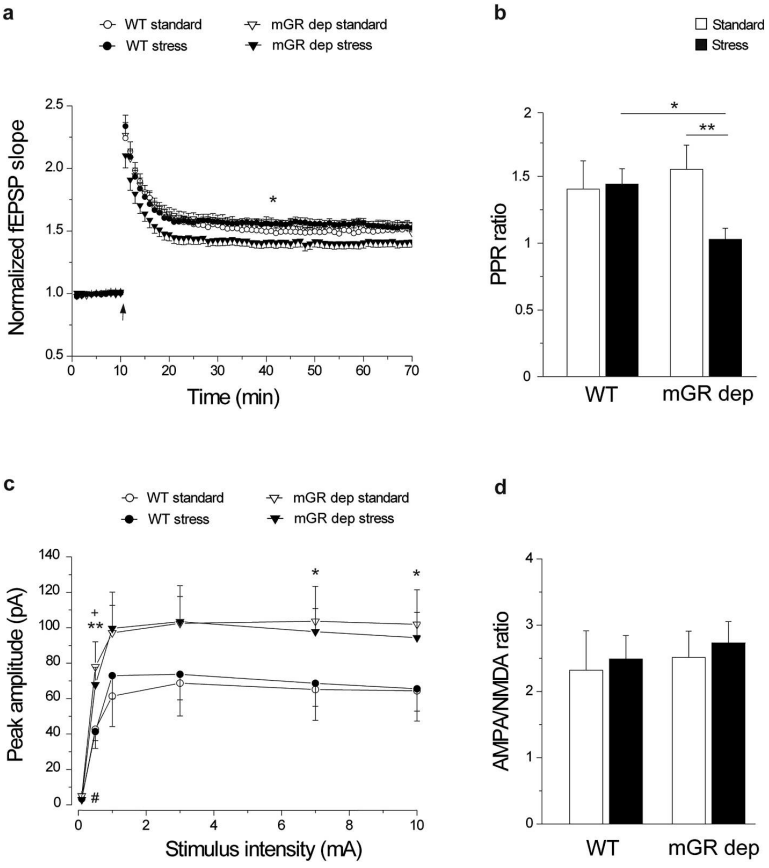

Fig. 8. CA1 basal responses and synaptic plasticity in WT *versus* microglial-GR depleted mice exposed to CUMS. (a) LTP of fEPSP slope from extracellular records made from WT and mGR depleted mice exposed to standard or stress condition (WT standard n = 16 slices/7 mice, white circle; WT stress n = 14 slices/7 mice, black circles; mGR depleted standard n = 14 slices/6 mice, white triangle; mGR depleted stress n = 15 slices/6 mice, black triangle). Time course of slope values from responses evoked at 0.05 Hz and normalized as detailed in the Methods. Arrows indicate LTP induction (HFS, 1 train of stimuli at 100 Hz, of 1 s duration). Data are means ± standard error of the mean. * $p < 0.05$ mGR depleted stress *vs* mGR depleted standard or WT stress. (b) Histogram representing EPSC recorded in CA1 pyramidal cells in WT and mGR dep mice exposed to standard (white, n = 13 slices/7 mice and n = 14 slices/4 mice, respectively) and stressful (black, n = 16 slices/5 mice and n = 24 slices/6 mice, respectively) environment following paired-pulse stimulation of Schaffer collaterals (interstimulus interval 50 ms; average of 6 traces). Data are mean ± standard error of the mean. * $p < 0.05$, ** $p < 0.01$. (c) Relationship between stimulus intensity and evoked peak amplitudes of EPSC recorded at − 70 mV from WT and mGR depleted mice exposed to standard and stress condition (WT standard n = 13 slices/6 mice, white circle; WT stress n = 16 slices/5 mice, black circles; mGR dep standard n = 14 slices/4 mice, white triangle; mGR depleted stress n = 23 slices/6 mice, black triangle). * $p < 0.05$ ** p 0.01 wt *vs* mGR depleted, + $p < 0.05$ wt standard *vs* mGR depleted standard, # $p < 0.05$ mGR depleted standard *vs* mGR depleted stress. (d) Bar chart illustrating the ratio between AMPA current (evoked at − 70 mV) and NMDA (evoked at + 40 mV) in CA1 synapses, determined measuring EPSC amplitude in WT and mGR depleted following standard or stressful condition stimulation (WT standard n = 8 slices/5 mice, WT stress n = 13 slices/5 mice, mGR dep standard n = 12 slices/4 mice and mGR depleted stress n = 16 slices/6 mice). CUMS: chronic unpredictable mild stress, fEPSC: field excitatory postsynaptic currents, HFS: high-frequency stimulation, LTP: long-term potentiation, mA: milliampere, mGR dep: microglial-glucocorticoid receptor depletion, pA: peak amplitude, PPR: paired-pulse ratio, s: seconds, WT: wild-type.

be due to a myriad of factors including the stress paradigms and their duration, the sex, and the brain regions studied. In fact, it is widely documented that varied stress models have profoundly different effects on neuroimmune function as well as behavior (Deak et al., 2015; Patchev and Patchev, 2006; Picard et al., 2021), which further highlights the complexity and context-dependent nature of the stress response.

Interestingly, the lack of microglial GCs-GR signaling impaired the microglial morphological response to CUMS in the CA1 *str rad*. Hippocampal microglial hyper-ramification was proposed to be induced by an increase of GCs signaling. Van Olst *et al* showed that enhancing GCs levels using slow-release GC pellets in adult male mice directly increased CD11b immunolabeling and microglial ramifications in the molecular layer of the DG (van Olst et al., 2018). This work showed a relationship wherein administration of GCs alters microglial morphology, albeit directly test whether this was through microglial GR; wherein, conversely, our works shows that an absence of microglial GR impaired microglial morphological response to CUMS. Our results are also in line with a recent study by Horchar and Wohleb, which demonstrated that administration of an antagonist of GR (RU486) limited the microglial morphological alterations in the PFC of male mice undergoing CUMS (Horchar and Wohleb, 2019). Together, this implies both a necessity and sufficiency for GR-GCs interactions to alter microglia morphology in response to chronic stress or repeated CORT exposure. Contrary to our findings, GR blockade prevented chronic stress-induced behavioral despair (Horchar and Wohleb, 2019). This might be due to the fact that RU486 is also an antagonist of progesterone receptors (Cadepond et al., 1997) and can inhibit GR in other cell types of the CNS, including neurons (Sousa et al., 1989), oligodendrocytes, and astrocytes (Vielkind et al., 1990). It is also possible that the microglial modifications we observe do not translate into behavioral changes because they are compensated by other CNS cells. GR in astrocytes was recently shown in male mice to be important for mediating the brain response to GCs, mainly for stress-induced memory formation (Tertil et al., 2018).

Moreover, CUMS induced an increase in microglial *Cd68*, *Il1b* and *Tnfa* gene expression, which is in agreement with previous studies showing an increase in microglial pro-inflammatory cytokines upon exposure to various chronic stress paradigm, such as CUMS (Intellicage) (Alboni et al., 2016) and repeated social defeat (Weber et al., 2019). In contrast, mGR depleted mice exposed to CUMS showed an increased

microglial gene expression of *Fizz1* and *Arg1*, both known to have anti-inflammatory functions (Cherry et al., 2014), and a decrease of *Cd68* and *Tnfa*. While microglia are partially insensitive to CORT in mGR depleted mice, they could be responding to the inflamed microenvironment (Munhoz et al., 2008) caused by chronic stress through other receptors and respond to it by increasing their anti-inflammatory properties.

We did not observe any change in neurogenesis in the DG polymorphic layer following exposure to CUMS for both genotypes, which is in disagreement with previous studies showing a decrease of neurogenesis following CUMS (Eid et al., 2020) and repeated water-immersion and restraint stress (Shimizu et al., 2019) in female mice. However, unlike our study, the mice were ovariectomized in both studies, which supports a putative protective role of estrogen. Moreover, genes involved in the positive regulation of neurogenesis were shown to be upregulated in female mice upon CUMS (Lotan et al., 2018). This gene regulation could protect females from the decrease in neurogenesis often observed in male mice after chronic stress (Ferragud et al., 2010; Mitra et al., 2006; Schloesser et al., 2010).

At the neuronal circuit level, WT controls showed no modification with CUMS exposure, which is in contradiction with the literature showing that hippocampal LTP is impaired after chronic stress (Joëls and Krugers, 2007; Kim and Diamond, 2002). The effects of stress on the neuronal excitability of the female brain remain largely unknown. Only a few studies have been conducted in females and the mechanisms underlying sex differences have not yet been clarified in the context of stress response. It has been reported that chronic stress impairs behavioral responses in the males but not female rats (Lin et al., 2008; Zuena et al., 2008), as well as induces sexual dimorphism in synaptic innervation of hippocampal subiculum (Carvalho-Netto et al., 2011; Andrade et al., 2000). Contrary to males, female rats also did not exhibit the same severity of apical dendritic atrophy seen after stress exposure (Galea et al., 1997). Interestingly, CUMS results in a significant decrease in pCREB expression, involved in long-term synaptic plasticity and memory, specifically in male DG (Lin et al., 2008). These results suggest that females are less susceptible to the aversive effects of chronic stress. Nevertheless, more studies are needed to unravel the mechanisms underlying these important sex differences. Hippocampal LTP is known to be sexually dimorphic under basal conditions, with females having a lower amplitude than males in rats (Monfort et al., 2015). In this regard, our's and other's groups have previously demonstrated that following CUMS in male rodents, hippocampal short and long-term plasticity are impaired (Joëls and Krugers, 2007; Kim and Diamond, 2002; Milior et al., 2016). Here we show for the first time that, following the same environmental paradigm (CUMS), that females do not have reduced cellular hippocampal neuronal plasticity. This observation is in line with the idea of male–female differences in the susceptibility to chronic stress. Following CUMS exposure, mGR depleted mice exhibited a higher sensitivity to stress, displayed at the neuronal circuit level by a decrease in hippocampal PPR and LTP. The mechanism underlying the absence of LTP modulation by Intellicage induced CUMS in females and the sensitization to stress of mGR depleted mice, however, requires further investigation. One possible explanation behind the CUMS-induced LTP and PPR alterations observed in mGR depleted mice could result from differences observed in microglial morphology and homeostatic functions, in genes related to inflammation, as well as in neuronal connectivity. Further studies examining a possible compensatory mechanism in the neurons or other cells, i.e., an increased expression of the receptor that would sensitize them to stress, are also warranted. Moreover, while we found minimal physiological and neurobiological effects of CUMS in the WT mice hippocampus, we still observed a behavioral response in both genotypes with a decrease in both saccharine preference and motivated behavior, which suggest the CUMS paradigm might induce a neurobiological response recruiting other brain regions such as the nucleus accumbens, which is involved in the sucrose preference (Scheggi et al., 2018). Overall, the mechanisms underlying modifications in short- and long-term plasticity of mGR depleted mice need further investigations.

5. Conclusion

Overall, our results demonstrate that microglial GR signaling contributes to neurogenesis and hippocampal synaptic connectivity while maintaining microglia in a surveillant state under steady-state condition. Our results also suggest that the lack of microglial GCs-GR does not alter the behavioral response to CUMS exposure, yet the underlying plasticity mechanisms and microglial gene profile are modified. These neural changes may compensate the deletion of microglial GR, allowing for an overall behavioral response to stress that is similar to the controls. Thus, mGR depleted mice still have the behavioral capacity to adapt to environmental challenges. These results support other findings (Frank et al., 2019; Hellwig et al., 2016; Kreisel et al., 2014; Milior et al., 2016) indicating that microglia are key players regulating the central nervous system response to chronic stress. Further studies are warranted to better understand the mechanisms underlying the effects of microglial GCs-GR signaling deficiency and to determine whether similar or different results would be seen in males and in other brain regions involved in the stress response (McEwen et al., 2015). Long-term consequences of chronic stress would also need to be investigated in our animal model. This study therefore suggests a mechanistic basis that could be used to modulate microglial activity and could help alleviate symptoms or prevent more severe neuropsychiatric disorders caused by chronic stress in females.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2021.07.022.

References

Alboni, S., Poggini, S., Garofalo, S., Milior, G., El Hajj, H., Lecours, C., Girard, I., Gagnon, S., Boisjoly-Villeneuve, S., Brunello, N., Wolfer, D.P., Limatola, C., Tremblay, M.-È., Maggi, L., Branchi, I., 2016. Fluoxetine treatment affects the inflammatory response and microglial function according to the quality of the living environment. Brain Behav. Immun. 58, 261–271. https://doi.org/10.1016/j.bbi.2016.07.155.

Alboni, S., van Dijk, R.M., Poggini, S., Milior, G., Perrotta, M., Drenth, T., Brunello, N., Wolfer, D.P., Limatola, C., Amrein, I., Cirulli, F., Maggi, L., Branchi, I., 2017. Fluoxetine effects on molecular, cellular and behavioral endophenotypes of depression are driven by the living environment. Mol. Psychiatry 22 (4), 552–561. https://doi.org/10.1038/mp.2015.142.

Andrade, José.P., Madeira, M.D., Paula-Barbosa, M.M., 2000. Sexual dimorphism in the subiculum of the rat hippocampal formation. Brain Res. 875 (1-2), 125–137. https://doi.org/10.1016/S0006-8993(00)02605-6.

Bachstetter, A.D., Morganti, J.M., Jernberg, J., Schlunk, A., Mitchell, S.H., Brewster, K. W., Hudson, C.E., Cole, M.J., Harrison, J.K., Bickford, P.C., Gemma, C., 2011. Fractalkine and CX3CR1 regulate hippocampal neurogenesis in adult and aged rats. Neurobiol. Aging 32 (11), 2030–2044. https://doi.org/10.1016/j.neurobiolaging.2009.11.022.

Basilico, B., Pagani, F., Grimaldi, A., Cortese, B., Di Angelantonio, S., Weinhard, L., Gross, C., Limatola, C., Maggi, L., Ragozzino, D., 2019. Microglia shape presynaptic properties at developing glutamatergic synapses. Glia 67 (1), 53–67. https://doi.org/10.1002/glia.23508.

Bessa, J.M., Ferreira, D., Melo, I., Marques, F., Cerqueira, J.J., Palha, J.A., Almeida, O.F. X., Sousa, N., 2009. The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. Mol. Psychiatry 14 (8), 764–773. https://doi.org/10.1038/mp.2008.119.

Bordeleau, M., Lacabanne, C., Fernández de Cossío, L., Vernoux, N., Savage, J.C., González-Ibáñez, F., Tremblay, M.-È., 2020. Microglial and peripheral immune priming is partially sexually dimorphic in adolescent mouse offspring exposed to maternal high-fat diet. J. Neuroinflamm. 17, 264. https://doi.org/10.1186/s12974-020-01914-1.

Branchi, I., Santarelli, S., Capoccia, S., Poggini, S., D'Andrea, I., Cirulli, F., Alleva, E., Hashimoto, K., 2013. Antidepressant treatment outcome depends on the quality of the living environment: a pre-clinical investigation in mice. PLoS ONE 8 (4), e62226. https://doi.org/10.1371/journal.pone.0062226.

Cadepond, F., Ulmann, A., Baulieu, E.-E., 1997. RU486 (MIFEPRISTONE): Mechanisms of Action and Clinical Uses. Annual Review of Medicine 48, 129–156. https://doi.org/10.1146/annurev.med.48.1.129.

Carrillo-de Sauvage, M.Á., Maatouk, L., Arnoux, I., Pasco, M., Sanz Diez, A., Delahaye, M., Herrero, M.T., Newman, T.A., Calvo, C.F., Audinat, E., Tronche, F., Vyas, S., 2013. Potent and multiple regulatory actions of microglial glucocorticoid receptors during CNS inflammation. Cell Death Differ. 20 (11), 1546–1557. https://doi.org/10.1038/cdd.2013.108.

Carvalho-Netto, E.F., Myers, B., Jones, K., Solomon, M.B., Herman, J.P., 2011. Sex differences in synaptic plasticity in stress-responsive brain regions following chronic variable stress. Physiology & Behavior, 2010 Neurobiology of Stress Workshop 104, 242–247. https://doi.org/10.1016/j.physbeh.2011.01.024.

Castro-Sánchez, S., García-Yagüe, Ángel.J., Kügler, S., Lastres-Becker, I., 2019. CX3CR1-deficient microglia shows impaired signalling of the transcription factor NRF2: implications in tauopathies. Redox Biol. 22, 101118. https://doi.org/10.1016/j.redox.2019.101118.

Cherry, J.D., Olschowka, J.A., O'Banion, M., 2014. Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. J. Neuroinflamm. 11 (1), 98. https://doi.org/10.1186/1742-2094-11-98.

Davis, M.T., Holmes, S.E., Pietrzak, R.H., Esterlis, I., 2017. Neurobiology of Chronic Stress-Related Psychiatric Disorders: Evidence from Molecular Imaging Studies. Chronic Stress (Thousand Oaks) 1. https://doi.org/10.1177/2470547017710916.

de Kloet, E.R., Joëls, M., Holsboer, F., 2005. Stress and the brain: from adaptation to disease. Nat. Rev. Neurosci. 6 (6), 463–475. https://doi.org/10.1038/nrn1683.

de Kloet, E.R., Karst, H., Joëls, M., 2008. Corticosteroid hormones in the central stress response: quick-and-slow. Front. Neuroendocrinol. 29 (2), 268–272. https://doi.org/10.1016/j.yfrne.2007.10.002.

Deak, T., Quinn, M., Cidlowski, J.A., Victoria, N.C., Murphy, A.Z., Sheridan, J.F., 2015. Neuroimmune mechanisms of stress: sex differences, developmental plasticity, and implications for pharmacotherapy of stress-related disease. Stress 18 (4), 367–380. https://doi.org/10.3109/10253890.2015.1053451.

Dong, Y., Wang, X., Zhou, Y., Zheng, Q., Chen, Z., Zhang, H., Sun, Z., Xu, G., Hu, G., 2020. Hypothalamus-pituitary-adrenal axis imbalance and inflammation contribute to sex differences in separation- and restraint-induced depression. Horm Behav 122, 104741. https://doi.org/10.1016/j.yhbeh.2020.104741.

Egeland, M., Zunszain, P.A., Pariante, C.M., 2015. Molecular mechanisms in the regulation of adult neurogenesis during stress. Nat. Rev. Neurosci. 16 (4), 189–200. https://doi.org/10.1038/nrn3855.

Eid, R.S., Lieblich, S.E., Duarte-Guterman, P., Chaiton, J.A., Mah, A.G., Wong, S.J., Wen, Y., Galea, L.A.M., 2020. Selective activation of estrogen receptors α and β: Implications for depressive-like phenotypes in female mice exposed to chronic unpredictable stress. Horm. Behav. 119, 104651. https://doi.org/10.1016/j.yhbeh.2019.104651.

Esch, T., Stefano, G.B., Fricchione, G.L., Benson, H., 2002. The role of stress in neurodegenerative diseases and mental disorders. Neuro Endocrinol. Lett. 23, 199–208.

Escoter-Torres, L., Caratti, G., Mechtidou, A., Tuckermann, J., Uhlenhaut, N.H., Vettorazzi, S., 2019. Fighting the fire: mechanisms of inflammatory gene regulation by the glucocorticoid receptor. Front Immunol 10, 1859. https://doi.org/10.3389/fimmu.2019.01859.

Fanselow, M.S., Dong, H.-W., 2010. Are the dorsal and ventral Hippocampus functionally distinct structures? Neuron 65 (1), 7–19. https://doi.org/10.1016/j.neuron.2009.11.031.

Ferragud, A., Haro, A., Sylvain, A., Velázquez-Sánchez, C., Hernández-Rabaza, V., Canales, J.J., 2010. Enhanced habit-based learning and decreased neurogenesis in the adult hippocampus in a murine model of chronic social stress. Behav. Brain Res. 210 (1), 134–139. https://doi.org/10.1016/j.bbr.2010.02.013.

Figueiredo, H.F., Dolgas, C.M., Herman, J.P., 2002. Stress activation of cortex and hippocampus is modulated by sex and stage of estrus. Endocrinology 143, 2534–2540. https://doi.org/10.1210/endo.143.7.8888.

Filipello, F., Morini, R., Corradini, I., Zerbi, V., Canzi, A., Michalski, B., Erreni, M., Markicevic, M., Starvaggi-Cucuzza, C., Otero, K., Piccio, L., Cignarella, F., Perrucci, F., Tamborini, M., Genua, M., Rajendran, L., Menna, E., Vetrano, S., Fahnestock, M., Paolicelli, R.C., Matteoli, M., 2018. The microglial innate immune receptor TREM2 is required for synapse elimination and normal brain connectivity. Immunity 48 (5), 979–991.e8. https://doi.org/10.1016/j.immuni.2018.04.016.

Fourgeaud, L., Través, P.G., Tufail, Y., Leal-Bailey, H., Lew, E.D., Burrola, P.G., Callaway, P., Zagórska, A., Rothlin, C.V., Nimmerjahn, A., Lemke, G., 2016. TAM receptors regulate multiple features of microglial physiology. Nature 532 (7598), 240–244. https://doi.org/10.1038/nature17630.

Frank, M.G., Fonken, L.K., Watkins, L.R., Maier, S.F., 2019. Microglia: Neuroimmune-sensors of stress. Semin. Cell Devel. Biol. SI: Calcium Signall. 94, 176–185. https://doi.org/10.1016/j.semcdb.2019.01.001.

Franklin, T.C., Wohleb, E.S., Zhang, Y., Fogaça, M., Hare, B., Duman, R.S., 2018. Persistent increase in microglial RAGE contributes to chronic stress-induced priming of depressive-like behavior. Biol. Psychiatry 83 (1), 50–60. https://doi.org/10.1016/j.biopsych.2017.06.034.

Füger, P., Hefendehl, J.K., Veeraraghavalu, K., Wendeln, A.-C., Schlosser, C., Obermüller, U., Wegenast-Braun, B.M., Neher, J.J., Martus, P., Kohsaka, S., Thunemann, M., Feil, R., Sisodia, S.S., Skodras, A., Jucker, M., 2017. Microglia turnover with aging and in an Alzheimer's model via long-term in vivo single-cell imaging. Nat. Neurosci. 20 (10), 1371–1376. https://doi.org/10.1038/nn.4631.

Galea, L.A.M., McEwen, B.S., Tanapat, P., Deak, T., Spencer, R.L., Dhabhar, F.S., 1997. Sex differences in dendritic atrophy of CA3 pyramidal neurons in response to chronic restraint stress. Neuroscience 81 (3), 689–697. https://doi.org/10.1016/S0306-4522(97)00233-9.

Garofalo, S., Porzia, A., Mainiero, F., Di Angelantonio, S., Cortese, B., Basilico, B., Pagani, F., Cignitti, G., Chece, G., Maggio, R., Tremblay, M.-E., Savage, J., Bisht, K., Esposito, V., Bernardini, G., Seyfried, T., Mieczkowski, J., Stepniak, K., Kaminska, B., Santoni, A., Limatola, C., 2017. Environmental stimuli shape microglial plasticity in glioma. Elife 6. https://doi.org/10.7554/eLife.33415.

Garrett, J.E., Wellman, C.L., 2009. Chronic stress effects on dendritic morphology in medial prefrontal cortex: sex differences and estrogen dependence. Neuroscience 162 (1), 195–207. https://doi.org/10.1016/j.neuroscience.2009.04.057.

Gemma, C., Bachstetter, A.D., 2013. The role of microglia in adult hippocampal neurogenesis. Front. Cell Neurosci. 7, 229. https://doi.org/10.3389/fncel.2013.00229.

Golden, S.A., Covington, H.E., Berton, O., Russo, S.J., 2011. A standardized protocol for repeated social defeat stress in mice. Nat. Protoc. 6 (8), 1183–1191. https://doi.org/10.1038/nprot.2011.361.

Goldmann, T., Wieghofer, P., Müller, P.F., Wolf, Y., Varol, D., Yona, S., Brendecke, S.M., Kierdorf, K., Staszewski, O., Datta, M., Luedde, T., Heikenwalder, M., Jung, S., Prinz, M., 2013. A new type of microglia gene targeting shows TAK1 to be pivotal in CNS autoimmune inflammation. Nat. Neurosci. 16 (11), 1618–1626. https://doi.org/10.1038/nn.3531.

Goshen, I., Kreisel, T., Ben-Menachem-Zidon, O., Licht, T., Weidenfeld, J., Ben-Hur, T., Yirmiya, R., 2008. Brain interleukin-1 mediates chronic stress-induced depression in mice via adrenocortical activation and hippocampal neurogenesis suppression. Mol. Psychiatry 13 (7), 717–728. https://doi.org/10.1038/sj.mp.4002055.

Gould, E., Tanapat, P., McEwen, B., Flügge, G., Fuchs, E., 1998. Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. Proceedings of the National Academy of Sciences of the United States of America. https://doi.org/10.1073/PNAS.95.6.3168.

Heck, A.L., Handa, R.J., 2019. Sex differences in the hypothalamic–pituitary–adrenal axis' response to stress: an important role for gonadal hormones. Neuropsychopharmacology 44 (1), 45–58. https://doi.org/10.1038/s41386-018-0167-9.

Hellwig, S., Brioschi, S., Dieni, S., Frings, L., Masuch, A., Blank, T., Biber, K., 2016. Altered microglia morphology and higher resilience to stress-induced depression-like behavior in CX3CR1-deficient mice. Brain Behav. Immun. 55, 126–137. https://doi.org/10.1016/j.bbi.2015.11.008.

Herman, J.P., McKlveen, J.M., Ghosal, S., Kopp, B., Wulsin, A., Makinson, R., Scheimann, J., Myers, B., 2016. Regulation of the hypothalamic-pituitary-adrenocortical stress response. Compr Physiol. 6, 603–621. https://doi.org/10.1002/cphy.c150015.

Hinwood, M., Morandini, J., Day, T.A., Walker, F.R., 2012. Evidence that microglia mediate the neurobiological effects of chronic psychological stress on the medial prefrontal cortex. Cereb. Cortex 22 (6), 1442–1454. https://doi.org/10.1093/cercor/bhr229.

Hinwood, M., Tynan, R.J., Charnley, J.L., Beynon, S.B., Day, T.A., Walker, F.R., 2013. Chronic stress induced remodeling of the prefrontal cortex: structural re-organization of microglia and the inhibitory effect of minocycline. Cereb. Cortex 23 (8), 1784–1797. https://doi.org/10.1093/cercor/bhs151.

Horchar, M.J., Wohleb, E.S., 2019. Glucocorticoid receptor antagonism prevents microglia-mediated neuronal remodeling and behavioral despair following chronic unpredictable stress. Brain Behav. Immun. 81, 329–340. https://doi.org/10.1016/j.bbi.2019.06.030.

Hoshiko, M., Arnoux, I., Avignone, E., Yamamoto, N., Audinat, E., 2012. Deficiency of the microglial receptor CX3CR1 impairs postnatal functional development of thalamocortical synapses in the barrel cortex. J. Neurosci. 32 (43), 15106–15111. https://doi.org/10.1523/JNEUROSCI.1167-12.2012.

Hui, C.W., St-Pierre, M.-K., Detuncq, J., Aumailley, L., Dubois, M.-J., Couture, V., Skuk, D., Marette, A., Tremblay, J.P., Lebel, M., Tremblay, M.-È., 2018. Nonfunctional mutant Wrn protein leads to neurological deficits, neuronal stress, microglial alteration, and immune imbalance in a mouse model of Werner syndrome. Brain Behav. Immun. 73, 450–469. https://doi.org/10.1016/j.bbi.2018.06.007.

Ibanez González, F., Picard, K., Bordelau, M., Sharma, K., Bisht, K., Tremblay, M.-È., 2019. Immunofluorescence staining using IBA1 and TMEM119 for microglial density, morphology and peripheral myeloid cell infiltration analysis in mouse brain. JoVE (J. Visual. Exp.), e60510. https://doi.org/10.3791/60510.

Joëls, M., Krugers, H.J., 2007. LTP after stress: up or down? Neural Plast. 2007, 1–6. https://doi.org/10.1155/2007/93202.

Kim, J.J., Diamond, D.M., 2002. The stressed hippocampus, synaptic plasticity and lost memories. Nat. Rev. Neurosci. 3 (6), 453–462. https://doi.org/10.1038/nrn849.

Kiryk, A., Janusz, A., Zglinicki, B., Turkes, E., Knapska, E., Konopka, W., Lipp, H.-P., Kaczmarek, L., 2020. IntelliCage as a tool for measuring mouse behavior – 20 years perspective. Behav. Brain Res. 388, 112620. https://doi.org/10.1016/j.bbr.2020.112620.

Kreisel, T., Frank, M.G., Licht, T., Reshef, R., Ben-Menachem-Zidon, O., Baratta, M.V., Maier, S.F., Yirmiya, R., 2014. Dynamic microglial alterations underlie stress-induced depressive-like behavior and suppressed neurogenesis. Mol. Psychiatry 19 (6), 699–709. https://doi.org/10.1038/mp.2013.155.

Lawson, L.J., Perry, V.H., Gordon, S., 1992. Turnover of resident microglia in the normal adult mouse brain. Neuroscience 48 (2), 405–415. https://doi.org/10.1016/0306-4522(92)90500-2.

Lecours, C., St-Pierre, M.-K., Picard, K., Bordeleau, M., Bourque, M., Awogbindin, I.O., Benadjal, A., Ibanez, F.G., Gagnon, D., Cantin, L., Parent, M., Di Paolo, T., Tremblay, M.-E., 2020. Levodopa partially rescues microglial numerical, morphological, and phagolysosomal alterations in a monkey model of Parkinson's disease. Brain Behav. Immun. 90, 81–96. https://doi.org/10.1016/j.bbi.2020.07.044.

Lee, T., Jarome, T., Li, S.-J., Kim, J.J., Helmstetter, F.J., 2009. Chronic stress selectively reduces hippocampal volume in rats: a longitudinal MRI study. NeuroReport 20, 1554–1558. https://doi.org/10.1097/WNR.0b013e328332bb09.

Lehmann, M.L., Cooper, H.A., Maric, D., Herkenham, M., 2016. Social defeat induces depressive-like states and microglial activation without involvement of peripheral macrophages. J. Neuroinflamm. 13 (1) https://doi.org/10.1186/s12974-016-0672-x.

Lightman, S.L., Conway-Campbell, B.L., 2010. The crucial role of pulsatile activity of the HPA axis for continuous dynamic equilibration. Nat. Rev. Neurosci. 11 (10), 710–718. https://doi.org/10.1038/nrn2914.

Lin, Y., Westenbroek, C., Bakker, P., Termeer, J., Liu, A., Li, X., Ter Horst, G.J., 2008. Effects of long-term stress and recovery on the prefrontal cortex and dentate gyrus in male and female rats. Cereb. Cortex 18 (12), 2762–2774. https://doi.org/10.1093/cercor/bhn035.

Liu, X., Quan, N., 2018. Microglia and CNS interleukin-1: beyond immunological concepts. Front. Neurol. 9, 8. https://doi.org/10.3389/fneur.2018.00008.

Lotan, A., Lifschytz, T., Wolf, G., Keller, S., Ben-Ari, H., Tatarsky, P., Pillar, N., Oved, K., Sharabany, J., Merzel, T.K., Matsumoto, T., Yamawaki, Y., Mernick, B., Avidan, E., Yamawaki, S., Weller, A., Shomron, N., Lerer, B., 2018. Differential effects of chronic stress in young-adult and old female mice: cognitive-behavioral manifestations and neurobiological correlates. Mol. Psychiatry 23 (6), 1432–1445. https://doi.org/10.1038/mp.2017.237.

Maggi, L., Scianni, M., Branchi, I., D'Andrea, I., Lauro, C., Limatola, C., 2011. CX(3)CR1 deficiency alters hippocampal-dependent plasticity phenomena blunting the effects of enriched environment. Front. Cell Neurosci. 5, 22. https://doi.org/10.3389/fncel.2011.00022.

Mazaheri, F., Snaidero, N., Kleinberger, G., Madore, C., Daria, A., Werner, G., Krasemann, S., Capell, A., Trümbach, D., Wurst, W., Brunner, B., Bultmann, S., Tahirovic, S., Kerschensteiner, M., Misgeld, T., Butovsky, O., Haass, C., 2017. TREM2 deficiency impairs chemotaxis and microglial responses to neuronal injury. EMBO Rep 18, 1186–1198. https://doi.org/10.15252/embr.201743922.

McEwen, B.S., 2001. Plasticity of the hippocampus: adaptation to chronic stress and allostatic load. Ann. N. Y. Acad. Sci. 933, 265–277. https://doi.org/10.1111/j.1749-6632.2001.tb05830.x.

McEwen, B.S., 2008. Central effects of stress hormones in health and disease: understanding the protective and damaging effects of stress and stress mediators. Eur. J. Pharmacol. 583 (2-3), 174–185. https://doi.org/10.1016/j.ejphar.2007.11.071.

McEwen, B.S., Bowles, N.P., Gray, J.D., Hill, M.N., Hunter, R.G., Karatsoreos, I.N., Nasca, C., 2015. Mechanisms of stress in the brain. Nat. Neurosci. 18 (10), 1353–1363. https://doi.org/10.1038/nn.4086.

McEwen, B.S., Gianaros, P.J., 2010. Central role of the brain in stress and adaptation: links to socioeconomic status, health, and disease. Ann. N. Y. Acad. Sci. 1186, 190–222. https://doi.org/10.1111/j.1749-6632.2009.05331.x.

McLaughlin, K.J., Baran, S.E., Wright, R.L., Conrad, C.D., 2005. Chronic stress enhances spatial memory in ovariectomized female rats despite CA3 dendritic retraction: Possible involvement of CA1 neurons. Neuroscience 135 (4), 1045–1054. https://doi.org/10.1016/j.neuroscience:2005.06.083.

Milior, G., Lecours, C., Samson, L., Bisht, K., Poggini, S., Pagani, F., Deflorio, C., Lauro, C., Alboni, S., Limatola, C., Branchi, I., Tremblay, M.-E., Maggi, L., 2016. Fractalkine receptor deficiency impairs microglial and neuronal responsiveness to chronic stress. Brain Behav. Immun. Microglia Physiol. Behav. 55, 114–125. https://doi.org/10.1016/j.bbi.2015.07.024.

Mitra, R., Sundlass, K., Parker, K.J., Schatzberg, A.F., Lyons, D.M., 2006. Social stress-related behavior affects hippocampal cell proliferation in mice. Physiol. Behav. 89 (2), 123–127. https://doi.org/10.1016/j.physbeh.2006.05.047.

Monfort, P., Gomez-Gimenez, B., Llansola, M., Felipo, V., 2015. Gender differences in spatial learning, synaptic activity, and long-term potentiation in the hippocampus in rats: molecular mechanisms. ACS Chem. Neurosci. 6 (8), 1420–1427. https://doi.org/10.1021/acschemneuro.5b00096.

Morais, Mónica, Santos, P.AR., Mateus-Pinheiro, A., Patrício, P., Pinto, L., Sousa, N., Pedroso, P., Almeida, S., Filipe, A., Bessa, João.M., 2014. The effects of chronic stress on hippocampal adult neurogenesis and dendritic plasticity are reversed by selective MAO-A inhibition. J. Psychopharmacol. 28 (12), 1178–1183. https://doi.org/10.1177/0269881114553646.

Munhoz, C.D., García-Bueno, B., Madrigal, J.L.M., Lepsch, L.B., Scavone, C., Leza, J.C., 2008. Stress-induced neuroinflammation: mechanisms and new pharmacological targets. Braz. J. Med. Biol. Res. 41 (12), 1037–1046. https://doi.org/10.1590/S0100-879X2008001200001.

Murray, F., Smith, D.W., Hutson, P.H., 2008. Chronic low dose corticosterone exposure decreased hippocampal cell proliferation, volume and induced anxiety and depression like behaviours in mice. Eur. J. Pharmacol. 583 (1), 115–127. https://doi.org/10.1016/j.ejphar.2008.01.014.

Orchinik, M., Murray, T., Moore, F., 1991. A corticosteroid receptor in neuronal membranes. Science 252 (5014), 1848–1851. https://doi.org/10.1126/science:2063198.

Oyola, M.G., Handa, R.J., 2017. Hypothalamic-pituitary-adrenal and hypothalamic-pituitary-gonadal axes: sex differences in regulation of stress responsivity. Stress 20 (5), 476–494. https://doi.org/10.1080/10253890.2017.1369523.

Palumbo, M.C., Dominguez, S., Dong, H., 2020. Sex differences in hypothalamic–pituitary–adrenal axis regulation after chronic unpredictable stress. Brain Behav. 10 (4) https://doi.org/10.1002/brb3.v10.410.1002/brb3.1586.

Patchev, V.K., Patchev, A.V., 2006. Experimental models of stress. Dialogues Clin. Neurosci. 8, 417–432.

Picard, K., St-Pierre, M.-K., Vecchiarelli, H.A., Bordeleau, M., Tremblay, M.-Ève., 2021. Neuroendocrine, neuroinflammatory and pathological outcomes of chronic stress: A story of microglial remodeling. Neurochem. Int. 145, 104987. https://doi.org/10.1016/j.neuint.2021.104987.

Pisu, M.G., Garau, A., Boero, G., Biggio, F., Pibiri, V., Dore, R., Locci, V., Paci, E., Porcu, P., Serra, M., 2016. Sex differences in the outcome of juvenile social isolation on HPA axis function in rats. Neuroscience 320, 172–182. https://doi.org/10.1016/j.neuroscience.2016.02.009.

Poggini, S., Golia, M.T., Alboni, S., Milior, G., Sciarria, L.Pepè., Viglione, A., Matte Bon, G., Brunello, N., Puglisi-Allegra, S., Limatola, C., Maggi, L., Branchi, I., 2019. Combined fluoxetine and metformin treatment potentiates antidepressant efficacy increasing IGF2 expression in the dorsal hippocampus [WWW Document]. Neural Plasticity 2019, 1–12. https://doi.org/10.1155/2019/4651031.

Reul, J.M.H.M., Kloet, E.R.DE., 1985. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117 (6), 2505–2511. https://doi.org/10.1210/endo-117-6-2505.

Sapolsky, R.M., Krey, L.C., McEwen, B.S., 1985. Prolonged glucocorticoid exposure reduces hippocampal neuron number: implications for aging. J. Neurosci. 5 (5), 1222–1227. https://doi.org/10.1523/JNEUROSCI.05-05-01222.1985.

Scheggi, S., De Montis, M.G., Gambarana, C., 2018. Making sense of rodent models of anhedonia. Int. J. Neuropsychopharmacol. 21, 1049–1065. https://doi.org/10.1093/ijnp/pyy083.

Schloesser, R.J., Lehmann, M., Martinowich, K., Manji, H.K., Herkenham, M., 2010. Environmental enrichment requires adult neurogenesis to facilitate the recovery from psychosocial stress. Mol. Psychiatry 15 (12), 1152–1163. https://doi.org/10.1038/mp.2010.34.

Shimizu, S., Ishino, Y., Takeda, T., Tohyama, M., Miyata, S., 2019. Antidepressive effects of kamishoyosan through 5-HT1AReceptor and PKA-CREB-BDNF signaling in the hippocampus in postmenopausal depression-model mice. Evid. Based Complement. Alternat. Med. 2019, 1–11. https://doi.org/10.1155/2019/9475384.

Sierra, A., Gottfried-Blackmore, A., Milner, T.A., McEwen, B.S., Bulloch, K., 2008. Steroid hormone receptor expression and function in microglia. Glia 56 (6), 659–674. https://doi.org/10.1002/glia.20644.

Sierra, A., Encinas, J.M., Deudero, J.J.P., Chancey, J.H., Enikolopov, G., Overstreet-Wadiche, L.S., Tsirka, S.E., Maletic-Savatic, M., 2010. Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. Cell Stem Cell 7 (4), 483–495. https://doi.org/10.1016/j.stem.2010.08.014.

Sierra, A., Beccari, S., Diaz-Aparicio, I., Encinas, J.M., Comeau, S., Tremblay, M.-Ève., 2014. Surveillance, phagocytosis, and inflammation: how never-resting microglia influence adult hippocampal neurogenesis. Neural Plast 2014, 1–15. https://doi.org/10.1155/2014/610343.

Singh, M.N., Martin-Hirsch, P.L., Martin, F.L., 2008. The multiple applications of tamoxifen: an example pointing to SERM modulation being the aspirin of the 21st century. Med. Sci. Monit. 14, RA144-148.

Smith, S.M., Vale, W.W., 2006. The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. Dial. Clin. Neurosci. 8, 383.

Sorrells, S.F., Munhoz, C.D., Manley, N.C., Yen, S., Sapolsky, R.M., 2014. Glucocorticoids increase excitotoxic injury and inflammation in the hippocampus of adult male rats. Neuroendocrinology 100 (2-3), 129–140. https://doi.org/10.1159/000367849.

Sousa, R.J., Tannery, N.H., Lafer, E.M., 1989. In situ hybridization mapping of glucocorticoid receptor messenger ribonucleic acid in rat brain. Mol. Endocrinol. 3 (3), 481–494. https://doi.org/10.1210/mend-3-3-481.

Stellwagen, D., Beattie, E.C., Seo, J.Y., Malenka, R.C., 2005. Differential regulation of AMPA receptor and GABA receptor trafficking by tumor necrosis factor-α. J. Neurosci. 25, 3219–3228. https://doi.org/10.1523/JNEUROSCI.4486-04.2005.

Stellwagen, D., Malenka, R.C., 2006. Synaptic scaling mediated by glial TNF-α. Nature 440 (7087), 1054–1059. https://doi.org/10.1038/nature04671.

Steptoe, A., Kivimäki, M., 2012. Stress and cardiovascular disease. Nat. Rev. Cardiol. 9 (6), 360–370. https://doi.org/10.1038/nrcardio.2012.45.

Tan, Y.-L., Yuan, Y., Tian, L., 2020. Microglial regional heterogeneity and its role in the brain. Mol. Psychiatry 25 (2), 351–367. https://doi.org/10.1038/s41380-019-0609-8.

Tanaka, J., Fujita, H., Matsuda, S., Toku, K., Sakanaka, M., Maeda, N., 1997. Glucocorticoid- and mineralocorticoid receptors in microglial cells: the two receptors mediate differential effects of corticosteroids. Glia 20 (1), 23–37.

Tay, T.L., Savage, J.C., Hui, C.W., Bisht, K., Tremblay, M.-Ève., 2017. Microglia across the lifespan: from origin to function in brain development, plasticity and cognition. J. Physiol. (Lond.) 595 (6), 1929–1945. https://doi.org/10.1113/JP272134.

Tertil, M., Skupio, U., Barut, J., Dubovyk, V., Wawrzczak-Bargiela, A., Soltys, Z., Golda, S., Kudla, L., Wiktorowska, L., Szklarczyk, K., Korostynski, M., Przewlocki, R., Slezak, M., 2018. Glucocorticoid receptor signaling in astrocytes is required for aversive memory formation. Transl. Psychiatry 8, 1–11. https://doi.org/10.1038/s41398-018-0300-x.

Tremblay, M.-È., Lowery, R.L., Majewska, A.K., 2010. Microglial Interactions with Synapses Are Modulated by Visual Experience. PLoS Biology 8, e1000527. https://doi.org/10.1371/journal.pbio.1000527.

Tremblay, M.-Ève., Zettel, M.L., Ison, J.R., Allen, P.D., Majewska, A.K., 2012. Effects of aging and sensory loss on glial cells in mouse visual and auditory cortices. Glia 60 (4), 541–558. https://doi.org/10.1002/glia.22287.

Vahrenkamp, J.M., Yang, C.-H., Rodriguez, A.C., Almomen, A., Berrett, K.C., Trujillo, A. N., Guillen, K.P., Welm, B.E., Jarboe, E.A., Janat-Amsbury, M.M., Gertz, J., 2018. Clinical and genomic crosstalk between glucocorticoid receptor and estrogen receptor α in endometrial cancer. Cell Rep. 22 (11), 2995–3005. https://doi.org/10.1016/j.celrep.2018.02.076.

van Olst, L., Bielefeld, P., Fitzsimons, C.P., de Vries, H.E., Schouten, M., 2018. Glucocorticoid-mediated modulation of morphological changes associated with aging in microglia. Aging Cell 17 (4), e12790. https://doi.org/10.1111/acel.2018.17.issue-410.1111/acel.12790.

Vielkind, U., Walencewicz, A., Levine, J.M., Bohn, M.C., 1990. Type II glucocorticoid receptors are expressed in oligodendrocytes and astrocytes. J. Neurosci. Res. 27 (3), 360–373. https://doi.org/10.1002/jnr.490270315.

Weber, M.D., McKim, D.B., Niraula, A., Witcher, K.G., Yin, W., Sobol, C.G., Wang, Y., Sawicki, C.M., Sheridan, J.F., Godbout, J.P., 2019. The influence of microglial elimination and repopulation on stress-sensitization induced by repeated social defeat. Biol. Psychiatry 85, 667–678. https://doi.org/10.1016/j.biopsych.2018.10.009.

Wei, J., Yuen, E.Y., Liu, W., Li, X., Zhong, P., Karatsoreos, I.N., McEwen, B.S., Yan, Z., 2014. Estrogen protects against the detrimental effects of repeated stress on glutamatergic transmission and cognition. Mol. Psychiatry 19 (5), 588–598. https://doi.org/10.1038/mp.2013.83.

Wohleb, E.S., 2016. Neuron-microglia interactions in mental health disorders: "for better, and for worse". Front. Immunol. 7 https://doi.org/10.3389/fimmu.2016.00544.

Wohleb, E.S., Hanke, M.L., Corona, A.W., Powell, N.D., Stiner, L.M., Bailey, M.T., Nelson, R.J., Godbout, J.P., Sheridan, J.F., 2011. β-adrenergic receptor antagonism prevents anxiety-like behavior and microglial reactivity induced by repeated social defeat. J. Neurosci. 31 (17), 6277–6288. https://doi.org/10.1523/JNEUROSCI.0450-11.2011.

Wohleb, E.S., McKim, D.B., Shea, D.T., Powell, N.D., Tarr, A.J., Sheridan, J.F., Godbout, J.P., 2014a. Re-establishment of anxiety in stress-sensitized mice is caused by monocyte trafficking from the spleen to the Brain. Biol. Psychiatry 75 (12), 970–981. https://doi.org/10.1016/j.biopsych.2013.11.029.

Wohleb, E.S., Powell, N.D., Godbout, J.P., Sheridan, J.F., 2013. Stress-induced recruitment of bone marrow-derived monocytes to the brain promotes anxiety-like behavior. J. Neurosci. 33 (34), 13820–13833. https://doi.org/10.1523/JNEUROSCI.1671-13.2013.

Wohleb, E.S., Patterson, J.M., Sharma, V., Quan, N., Godbout, J.P., Sheridan, J.F., 2014b. Knockdown of interleukin-1 receptor type-1 on endothelial cells attenuated stress-induced neuroinflammation and prevented anxiety-like behavior. J. Neurosci. 34 (7), 2583–2591. https://doi.org/10.1523/JNEUROSCI.3723-13.2014.

Wohleb, E.S., Terwilliger, R., Duman, C.H., Duman, R.S., 2018. Stress-induced neuronal colony stimulating factor 1 provokes microglia-mediated neuronal remodeling and depressive-like behavior. Biol. Psychiatry 83 (1), 38–49. https://doi.org/10.1016/j.biopsych.2017.05.026.

Woolley, C.S., Gould, E., McEwen, B.S., 1990. Exposure to excess glucocorticoids alters dendritic morphology of adult hippocampal pyramidal neurons. Brain Res. 531 (1-2), 225–231. https://doi.org/10.1016/0006-8993(90)90778-A.

Yin, W., Gallagher, N.R., Sawicki, C.M., McKim, D.B., Godbout, J.P., Sheridan, J.F., 2019. Repeated social defeat in female mice induces anxiety-like behavior associated with enhanced myelopoiesis and increased monocyte accumulation in the brain. Brain Behav. Immun. 78, 131–142. https://doi.org/10.1016/j.bbi.2019.01.015.

Zhou, X., Wahane, S., Friedl, M.-S., Kluge, M., Friedel, C.C., Avrampou, K., Zachariou, V., Guo, L., Zhang, B., He, X., Friedel, R.H., Zou, H., 2020. Microglia and macrophages promote corralling, wound compaction and recovery after spinal cord injury via Plexin-B2. Nat. Neurosci. 23 (3), 337–350. https://doi.org/10.1038/s41593-020-0597-7.

Zucker, R.S., 1989. Short-term synaptic plasticity. Annu. Rev. Neurosci. 12 (1), 13–31. https://doi.org/10.1146/annurev.ne.12.030189.000305.

Zuena, A.R., Mairesse, J., Casolini, P., Cinque, C., Alemà, G.S., Morley-Fletcher, S., Chiodi, V., Spagnoli, L.G., Gradini, R., Catalani, A., Nicoletti, F., Maccari, S., Cookson, M.R., 2008. Prenatal restraint stress generates two distinct behavioral and neurochemical profiles in male and female rats. PLoS ONE 3 (5), e2170. https://doi.org/10.1371/journal.pone.0002170.

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Minocycline treatment improves cognitive and functional plasticity in a preclinical mouse model of major depressive disorder

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ABSTRACT

Major depressive disorder (MDD) is a chronic, recurring, and potentially life-threatening illness, which affects over 300 million people worldwide. MDD affects not only the emotional and social domains but also cognition. However, the currently available treatments targeting cognitive deficits in MDD are limited. Minocycline, an antibiotic with anti-inflammatory properties recently identified as a potential antidepressant, has been shown to attenuate learning and memory deficits in animal models of cognitive impairment. Here, we explored whether minocycline recovers the deficits in cognition in a mouse model of depression. C57BL6/J adult male mice were exposed to two weeks of chronic unpredictable mild stress to induce a depressive-like phenotype. Immediately afterward, mice received either vehicle or minocycline for three weeks in standard housing conditions. We measured anhedonia as a depressive-like response, and place learning to assess cognitive abilities. We also recorded long-term potentiation (LTP) as an index of hippocampal functional plasticity and ran immunohistochemical assays to assess microglial proportion and morphology. After one week of treatment, cognitive performance in the place learning test was significantly improved by minocycline, as treated mice displayed a higher number of correct responses when learning novel spatial configurations. Accordingly, minocycline-treated mice displayed higher LTP compared to controls. However, after three weeks of treatment, no difference between treated and control animals was found for behavior, neural plasticity, and microglial properties, suggesting that minocycline has a fast but short effect on cognition, without lasting effects on microglia. These findings together support the usefulness of minocycline as a potential treatment for cognitive impairment associated with MDD.

1. Introduction

Major depressive disorder (MDD) is one of the leading causes of global disability worldwide [1] affecting over 300 million people [2]. Although MDD primarily involves mood disturbance, up to 70% of the patients suffer from cognitive deficits [3–7], including impairment in learning and memory, executive functioning, processing speed, attention, and concentration. These symptoms are highly heterogeneous in severity, duration, onset, and treatment response [8] and characterize not only the acute phase of the illness, but may persist in remission and

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worsen over time with repeated episodes [9–16].

Currently, few pharmacological treatments yielding direct pro-cognitive effects in depressed patients are available [8]. Vortioxetine is the first antidepressant approved by the Food and Drug Administration specifically targeting cognition [17] and able to improve multiple cognitive domains [18–22]. The therapeutic effect of vortioxetine depends on its modulation of a wide range of targets, including serotoninergic, dopaminergic, and noradrenergic neurotransmitters [19,23]. However, alternatives to this drug are still very limited. Different compounds, from serotonin and norepinephrine reuptake inhibitors to ketamine and psychostimulants, have been explored for their capability to improve cognitive function. However, their efficacy and implementation in clinics are still debated [8].

Compelling evidence indicates that cognitive dysfunctions in mood disorders are characterized by high levels of pro-inflammatory markers and activation of the immune system response [24–26]. Therefore, there is a rising interest in anti-inflammatory drugs as an add-on or stand-alone treatment for cognitive deficits associated with MDD [27,28]. Minocycline is an antibiotic able to pass the brain-blood barrier that exerts both anti-inflammatory and antidepressant actions [29,30]. The beneficial effects of this treatment have been reported both in preclinical and clinical studies. Recent evidence shows that minocycline is able to attenuate deficits in learning and memory in animal models of neurodegeneration [31] and cognitive impairment [32]. In addition, its administration as an add-on has been associated with improvement in both negative symptoms and executive functioning in patients with schizophrenia [33,34]. Thus, we hypothesized that this drug represents a promising treatment for cognitive impairment associated with MDD. To test this hypothesis, we exposed C57BL/6J adult male mice to two weeks of chronic unpredictable mild stress to induce a depressive-like phenotype. Afterward, the experimental subjects received either vehicle or minocycline for three weeks while living in standard housing conditions. Our prediction was that minocycline treatment favors the restoring of cognitive functions. We measured long-term potentiation and changes in microglia as these cells are reportedly involved in minocycline central action [35]. We performed behavioral phenotyping by exploiting the IntelliCage system, an apparatus that allows for an automated, high-throughput, and continuous phenotyping of animal behavior in home-cage [36,37].

2. Materials and methods

2.1. Ethical standards

All procedures were carried out in accordance with the European and Italian legislation on animal experimentation (respectively European Directive 2010/63/UE and *Decreto Legislativo* 26/2014). Animals were examined for signs of discomfort as indicated by the animal care and use guidelines ("Guide for the care and use of laboratory animals", National Research Council 2003). The Italian Ministry of Health approved the protocol with permit number D9997.83.

2.2. Animals

Fifty-two C57BL/6J male mice 12–15 weeks old were used (animals for experimental conditions). Mice were obtained directly from Charles River Laboratories located in Calco (Lecco, Italy).

2.3. Housing conditions

Mice were kept under a reversed 12 light-dark cycle at 22–25 °C. For the entire duration of the experiment, animals were housed in the IntelliCage system (TSE-system, NewBehavior AG, Zürich, Switzerland), which is an apparatus for automatic monitoring and measuring of mouse behavior. Intellicages are large acrylic cages (20.5 cm high, 58 cm × 40 cm at the top and 55 cm × 37.5 cm at the base, Model 2000 Tecniplast, Buguggiate, VA, Italy) with 4 walls separating each corner from the center so that they form 4 identical triangular conditioning chambers (15 × 15 × 21 cm). Animals have access to each chamber by entering a front hole and only a single mouse can enter a chamber at a time since each one is identified by a transponder. Two drinking bottles were placed in each corner and access to each solution was prevented by a door, thus, to drink mice had to perform a nosepoke. The system is able to collect data about the number and duration of visits as well as the number, duration, and side (right or left) of the nosepokes and licks.

The floor of the cage was covered with bedding while on the top a food rack was filled with standard mouse chow (food *ad libitum*). An additional cage (SocialBox) was used to expand the existing IntelliCage to a multi-area system, allowing to increase the number of subjects tested simultaneously. One week before being moved to the IntelliCage, each animal was injected with a subcutaneous transponder (T-IS 8010 FDX-B Datamars SA, Switzerland). Then, mice were gradually habituated to the IntelliCage environment for 14 days (habituation period) and to 0.1% of saccharin solution. The IntelliCage system allows to phenotype individual behavioral responses in a group of socially housed mice without any intervention by the experimenter reducing biases due to animal manipulation and exposure to the testing environment.

2.4. Treatment

After the first 14 days of stressful conditions aimed to induce a depression-like phenotype, mice received minocycline or vehicle for 21 days while they were exposed to the standard environment (Fig. 1). On the day immediately before the beginning of treatment, we split the animals in order to create two experimental groups with an overlapping behavioral profile.

To avoid stress due to the manipulation, minocycline (Minocycline Hydrocloride crystalline, SigmaAldrich, St Louis, MO, USA) was dissolved both in water and saccharin solution. During the treatment period, mice from each experimental group could access only the corner of the IntelliCage administering the treatment to which it was assigned. The solutions were prepared according to the mouse average weight and daily water consumption to provide an average daily intake of 50 mg/kg of minocycline. The dose was selected based on the literature since it has been reported to suppress the activation of microglia in different mouse models of disease [38–40]. All the solutions were prepared fresh every 2 days because of the instability of minocycline in aqueous solution [41,42].

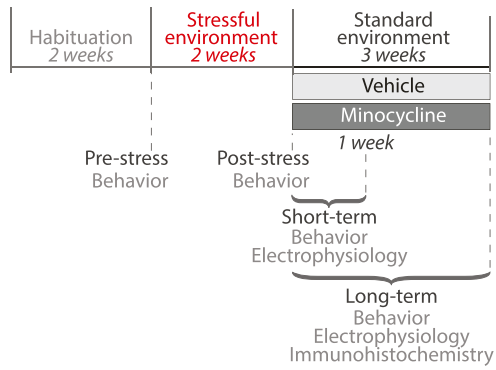

Fig. 1. Experimental design. Following two weeks of habituation to the Intellicage, animals were exposed to two weeks of chronic unpredictable mild stress, to induce the depression-like behavior. Then mice were treated with vehicle or minocycline while they were exposed to the standard condition for three weeks.

2.5. Environmental conditions

Mice were first exposed to the stressful condition for 14 days to induce the depressive-like behavior and, immediately after, received the treatment for 21 days while kept under the standard condition (Fig. 1).

2.5.1. Standard condition

In the standard condition, in addition to the different settings (e.g., cage *vs* operant chambers), provided by the IntelliCage system, mice were given Plexiglas shelters of different colors and shapes (e.g., four red transparent Tecniplast plastic nest boxes) and tissue papers [43,44]. A new tissue paper was provided every five days, and the plastic shelters were cleaned every week.

2.5.2. Stressful condition

After the habituation period, mice were exposed to chronic unpredictable mild stress to induce depressive-like behavior. In stressful conditions, the IntelliCage system was used to constantly (24 hrs) expose animals to different stress procedures previously validated through the assessment of behavioral, cellular, and molecular endpoints [45–47]. The stressful conditions exploited in the present study have been shown to increase corticosterone levels, considered a marker of the stress response [48]. The stresses were delivered for 14 days and, to prevent habituation to the stress procedures, mice were exposed each day to a different stressful stimulus, randomly chosen. The procedures were: short open door: door to access solutions remains open for few seconds; delay: door opens randomly with a delay of 1, 1.5, 2, 2.5 s after the first nosepoke; open door 25%: door opens only following 25% of nosepokes; random air puff: randomly 1, 2, 3, or 4 s after the first nosepoke, the animal receives an air puff (2 bar). The duration of each paradigm was randomly 12, 18, or 24 hrs.

Finally, during the stressful procedures, no shelter or tissue paper

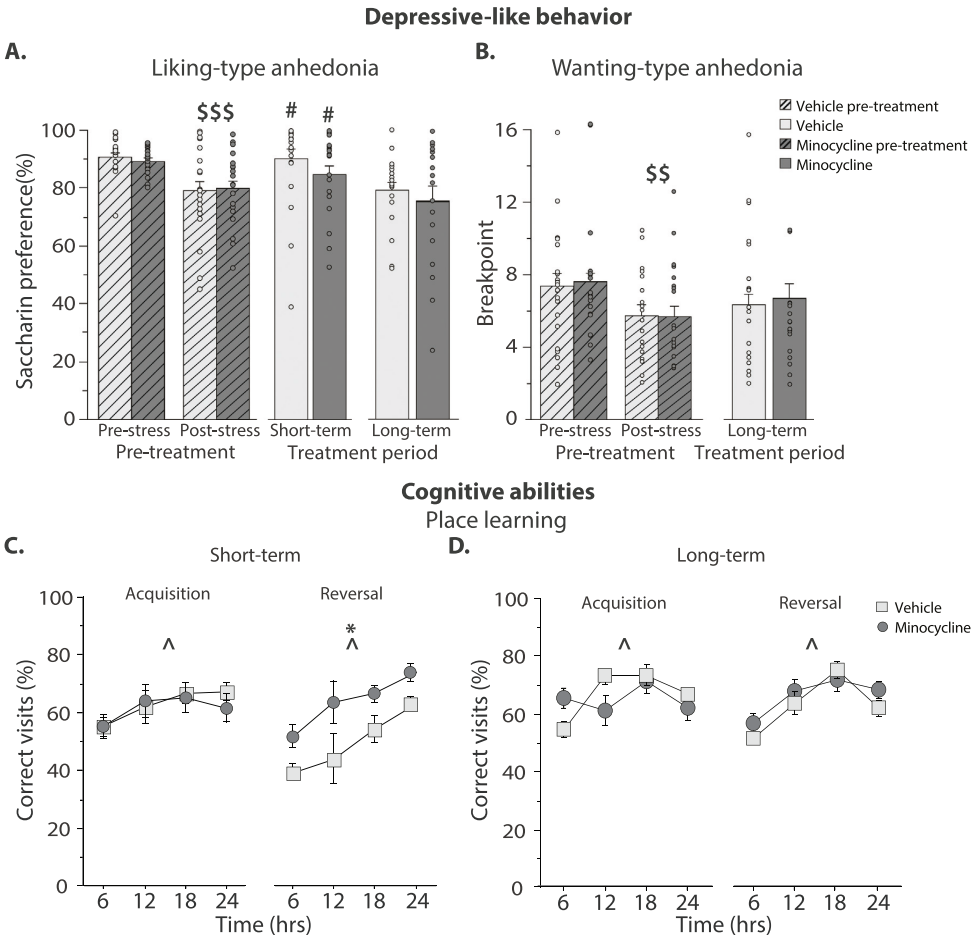

Fig. 2. Depressive like-behavior. **A.** The exposure to two weeks of chronic mild stress induced a depressive-like profile as indicated by the significant decrease of saccharin preference displayed by the animals housed in the Intellicage system. When the animals switched to the standard condition, an increase in their saccharin preference was detected, although such improvement did not last long,. n = 18–19 per group. **B.** The exposure to chronic mild stress led to a significant increase in wanting-type anhedonia; following three weeks of standard condition, the motivation of the animals increased, n = 17–18 per group. **C.** During the acquisition phase of the first place learning test, both vehicle- and minocycline-treated mice progressively increased their visits in the target corner. **D.** By contrast, in the reversal phase, minocycline-treated mice displayed a higher number of correct visits. When we run the second place learning test at the end of the treatment period, we observed that all the mice learned and re-learned the correct spatial task with no difference between the two experimental groups. $$ $p < 0.05$ and $$$ $p < 0.0001$ vs pre-stress; # $p < 0.05$ vs post-stress within the same group; ^ $p < 0.05$ main effect of the time, * $p < 0.05$ main effect of the treatment, data shown as mean +s.e.m., n = 21 per group.

was provided. Thus, we can consider that the animals were under a continuous stress-like condition.

2.6. Behavioral tests

All experimental subjects underwent all testing procedures at all time points. These included liking- and wanting-type anhedonia, and place learning. The experimental procedures used to phenotype the selected behaviors were automatically administered by the IntelliCage avoiding any bias due to the experimenter. It is worth noting that the protocols and the tests we ran through the Intellicage system have been largely validated by us and other research groups [49–53].

2.6.1. Liking-type anhedonia - saccharin preference

To assess liking-type anhedonia we measured saccharin preference. In each corner of the IntelliCage two bottles were present, one containing tap water and the other containing the saccharin solution; both freely available 24/24 hrs. The position of the water and saccharin bottles in each corner was counterbalanced across the four corners. Saccharin preference was determined as follows: [saccharin solution consumed/ (saccharin solution consumed + water consumed)] × 100. Baseline saccharin preference was measured as the mean of the last two days of the habituation period (i.e., Pre-stress), at the end of the stress exposure, (i.e., Post-stress), and following one (Short-term) and three weeks of treatment (Long-term; Fig. 2).

2.6.2. Wanting-type anhedonia - progressive ratio reinforcement schedule

To assess wanting-type anhedonia, i.e., the drive for obtaining a reward, we used the progressive ratio reinforcement schedule that utilizes a multiplicative increase in the number of responses (i.e., nosepokes) required to dispense a unit of reinforcement (i.e., saccharin solution). In particular, water was always accessible after one nosepoke while saccharin solution was accessible only after a specific number of nosepokes occurred. This number increases progressively after each series of eight visits according to the following sequence: 2, 3, 4, 5, 6, 7, 8, 10, 12, 16, 20, and 24. Each test session lasted 48 hrs or until mice reached the module with 24 nosepokes. The time for performing the nosepokes increased gradually according to the number of nosepokes requested from one to 24 s. The test has been run immediately before and after the stress exposure, and following 3 weeks of treatment (Long-term). To make the mice aware of the testing condition, the green LEDs on the top of each door were kept turned on throughout the session.

2.6.3. Cognitive domain – place learning test

Place learning consisted of a simple spatial task in which mice were allowed to drink in one corner, instead of all four. During the first phase (acquisition), the mice learned which corner was rewarded and which was not, leading to a visiting preference for the rewarded corner (% correct visits). In particular, the doors were closed in all corners but one. Following 24 hrs, the corner in which the bottles were accessible was switched to the respective opposite corner for 24 hrs (reversal). The animals were tested both after 3 days (Short-term) and 3 weeks (Long-term) of treatment.

2.7. Electrophysiology

To perform electrophysiological experiments, at the end of the treatment period, hippocampal slices were collected. Animals were anesthetized with halothane and decapitated. Whole brains were rapidly removed from the skull and immersed for 10 min in ice-cold artificial cerebrospinal fluid (ACSF), continuously oxygenated with 95% O_2 and 5% CO_2 to maintain the proper pH (7.4). Transverse 350 μm slices were cut at 4 °C with a vibratome and the appropriate slices were placed in a chamber containing oxygenated ACSF. After their preparation slices were allowed to recover for 1 hr at 30 °C. For field recordings, individual slices were then transferred to the interface slice-recording chamber (BSC1, Scientific System Design Inc) maintained at 30–32 °C and constantly superfused at a rate of 2.5 mL/min. At the beginning of each recording, a concentric bipolar stimulating electrode (SNE-100 ×50 mm long Elektronik–Harvard Apparatus GmbH) was placed in the *stratum radiatum* for stimulation of Shaffer collateral pathway projection to CA1. Stimuli consisted of 100 μs constant current pulses of variable intensities, applied at 0.05 Hz. A glass micropipette (0.5–1 MΩ) filled with ACSF was placed in the CA1 hippocampal region, at 200–600 μm from the stimulating electrode, in order to measure orthodromically evoked field extracellular postsynaptic potentials (fEPSP). Stimulus intensity was adjusted to evoke fEPSP of amplitude about 50% of the maximal amplitude with minimal contamination by a population spike. Evoked responses were monitored online, and stable baseline responses were recorded for at least 10 min. Only the slices that showed stable fEPSP amplitudes were included in the experiments. LTP was induced by high-frequency stimulation (HFS, 1 train of stimuli at 100 Hz of 1 s duration), repeated after 30 min. To analyze the time course of the fEPSP slope, the recorded fEPSP was routinely averaged over 1 min (n = 3). The fEPSP slope changes following the LTP induction protocol at 25 and 55 min post-tetanus were calculated with respect to those of the baseline (1 min before induction). N/n refers to the number of slices on the total number of mice analyzed. The paired-pulse ratio (PPR) was measured from responses to two synaptic stimuli at 50 ms interstimulus interval. PPR was calculated as the ratio between the fEPSP amplitude evoked by the second stimulus (A2) and that by the first (A1; A2/A1). fEPSP were recorded and filtered (low pass at 1 kHz) with an Axopatch 200 A amplifier (Axon Instruments, CA) and digitized at 10k Hz with an A/D converter (Digidata 1322 A, Axon Instruments). Data acquisition was stored on a computer using pClamp 9 software (Axon Instruments) and analyzed offline with Clampfit 10 program (Axon Instruments). For each recording, we routinely normalize LTP value to the baseline, measuring the increment of the fEPSP slope 25 min post-tetanus compared to the baseline (1 min before induction). This normalization procedure allows comparing experiments with different basal values of fEPSP slope. Once normalized, we use statistical analysis (one-way ANOVA) to compare the amplitude of LTP at 25 min post-tetanus in all the slices of vehicle-treated versus the values of LTP measured in slices obtained by minocycline-treated animals, for both the first and the second stimulation.

2.8. Tissue collection, section preparation, and immunofluorescence

Mice were placed in a box containing ~ 3% isoflurane (Iso-Vet) and injected with ketamine (160 mg/kg, Biowet Pulawy) and xylazine (Sedazin, Biowet, 20 mg/kg). Under deep anesthesia, they were perfused transcardially using a perfusion pump (BQ80S Microflow Variable-Speed Peristaltic Pump, Golander) which infused phosphate-buffered saline (PBS) through the left ventricle, until the fluid coming out of the heart was clear. For immunohistochemistry, mice were perfused with cold 4% (w/v) paraformaldehyde (PFA) in PBS (20–30 mL) followed by PBS. Mice were decapitated, then brains were rapidly removed and stored in 4 °C PFA for 48 hrs. Subsequently, brains were transferred into 30% sucrose (w/v) in PBS for 48 hrs and frozen in a Tissue Freezing Medium (Leica) at − 80 °C.

Brains were cut into 12 μm slices taken rostrocaudally using a cryostat (Microm HM525, Thermo Scientific) at − 18ºC and 4–5 sections were placed on polysine™ slides (ThermoScientific). From the prefrontal lobe, sections were prepared from a Bregma 2.34–1.94 mm, and from dorsal hippocampi and habenula slices were collected within a Bregma 1.22–2.30 (Paxinos & Franklin, 2001). Sections were stored at − 80ºC until further processing.

For immunofluorescence, sections were thawed for 2 hrs, washed 3 times with PBS for 5 min with gentle rocking, then incubated with a blocking serum (D9663, Sigma, 10% in Tris-Buffered Saline, TBS) for 2 hrs. Sections were incubated with the primary antibodies recognizing TMEM119 (1:1000, ab209064 Abcam), Iba1 (1:200, E291118 Novus),

Ki-67 (1:1000, ab66155 Abcam), and Doublecortin (DCX; 1:1000, ab18723 Abcam), diluted in 3% block serum in TBS overnight at 4°C. After washing three times with PBS, secondary antibodies: donkey anti-rabbit AlexaFluor 488 or donkey anti-goat AlexaFluor 555 (1:1000, Thermofisher Scientific) were applied for 2 hrs at room temperature. After washing 3 times with PBS, cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole, 1:1000 in PBS, #D-9542, Sigma), washed and cover-slipped (Medlab, Marienfield) in fluorescence mounting medium (#S3023, Dako).

2.9. Cell quantification and confocal analysis

Fluorescent pictures were acquired using a fluorescent microscope (Leica DM4000B, Leica CTR6500, ebq100) and the Leica App Suite 2.8.1 software. We used 40x objective to obtain 2088 × 1560 pixels counting frames and collected 5 images per area per animal, in particular in three subareas of the medial prefrontal cortex (mPFC), cingulate cortex (CG), prelimbic cortex (PL), and infralimbic cortex(IL), and in three subareas of the dorsal hippocampus, CA1, CA3 and dentate gyrus (DG). We focused on these specific brain regions since it has been reported that the dorsal hippocampus is involved in the response to different antidepressant treatments and that following stress exposure morphological changes occur in this area [47,54].

Each image was collected in a given channel (blue, red, and green) in order to combine them during the image analysis.

Images were analyzed using the Fiji software (Image J 1.52p Wayne Rasband NIH, USA, 64 bits). A composite image was created with separate channels: green, red and blue for double-stained microglia (TMEM119, Iba1, and DAPI), or green and blue for DCX -positive cells (DCX and DAPI) or Ki-67. Percentages of positive cells were calculated based on the number of DAPI-stained nuclei per image, each area was averaged per animal.

Z-stacks (step size = 0.21 μm) of 20 cells (double-positive for TMEM119 and Iba1) per area/mice (around 2400 pictures) were acquired using the 63x immersion objective of a confocal microscope and numerical aperture 1.4 (LSM800 Zeiss Airyscan) with Zeiss ZEN software. The pictures were analyzed using the Fiji software (Image J 1.52p Wayne Rasband NIH, USA, 64-bits) as described (González Ibáñez et al., 2019). Measurements of the body size and branching area per cell were calculated.

To determine whether the treatment affects the percentage and morphology of microglia we selected two established microglial markers: Ionized calcium-binding adaptor molecule 1 (Iba1) and TMEM119, and we analyzed their immunoreactivity (IR). Iba1 is expressed by microglia and macrophages in health and disease, showing increased expression upon environmental challenges such as chronic stress [55]. TMEM119 is considered a microglia-specific transmembrane protein of unknown function [56]. This marker was shown to be relatively stable across various conditions with some exceptions [57]. We analyzed 20 cells/area/animal using confocal microscopy.

2.10. Statistical methods

The statistical analyses have been performed using the software Statview II (Abacus Concepts, CA, USA) and the R program (version 2022.07.1). All data were analyzed with one-way ANOVA to compare vehicle versus minocycline treatment. Stress exposure (Pre- and Post-), time (hrs and min), and sub-regions of each brain area were considered repeated measures within subjects. Concerning the Place learning test, the two phases (acquisition and reversal) have been considered independent measures. *Post-hoc* comparisons were performed using Tukey's test. All mean differences were considered statistically significant at $p < 0.05$. The final version of the graphs has been obtained with Adobe Illustrator software (version CC 2017).

3. Results

3.1. Minocycline improved cognitive abilities in the short-term

3.1.1. Depressive-like behavior

To evaluate the depressive-like phenotype, we assessed liking- and wanting-type anhedonia, previously shown to be affected by exposure to chronic unpredictable mild stress [46,47,58]. As expected, we found that following two weeks of chronic stress, all the experimental subjects displayed (i) a significant reduction in saccharin preference [$F(1,40) = 39.691$, $p < 0.0001$] and (ii) a significant decrease in motivation [$F(1, 40) = 15.338$, $p = 0.0003$, Fig. 2A-B].

The switch to the standard condition led to an improvement of the depressive-like symptoms, both vehicle- and minocycline-treated mice increased their saccharin preference [respectively $F(1,20) = 11.639$, $p = 0.0028$ and $F(1,20) = 4.639$, $p = 0.0436$], with no difference between the two treatment groups [$F(1,40) = 0.643$, $p = 0.4273$]. The prolonged exposure to the standard condition led to an increase in liking-type anhedonia, and the long-term preference for the saccharin solution of both vehicle- and minocycline-treated mice was around 75% with no difference between the two experimental groups [$F(1,35) = 0.388$, $p = 0.5376$; Fig. 2 A]. Accordingly, the wanting-type anhedonia was improved by the exposure to the standard condition for three weeks, although we did not observe a significant difference between vehicle- and minocycline-treated mice [$F(1,33) = 0.094$, $p = 0.7608$; Fig. 2B].

3.1.2. Cognitive abilities

Within the first week of treatment, we found the main effect of time in the acquisition phase, both vehicle- and minocycline-treated mice progressively increased their percentage of correct visits, [$F(1120) = 6.244$, $p = 0.0006$], with no difference between the experimental groups [$F(1,40) = 0.048$, $p = 0.8285$; Fig. 2 C]. A main effect of treatment emerged in the reversal phase, minocycline-treated mice displayed a significantly higher number of correct visits compared with vehicle-treated mice [$F(1,40) = 9.827$, $p = 0.0032$], suggesting that treatment enhanced cognitive performance. In addition, we found a main effect of time as well, with both groups significantly increasing the number of correct visits [$F(1120) = 9.951$, $p < 0.0001$; Fig. 2 C]. In both the acquisition and reversal phases of the place learning test at the end of the treatment period, the animals showed a significant increase in their number of correct visits over time, indicating intact learning abilities and the capability of the animals to learn new spatial tasks [respectively $F(1,90) = 7.257$, $p = 0.0002$ and $F(1,96) = 18.719$, $p < 0.0001$; Fig. 2D].

3.2. Functional plasticity has been improved by minocycline treatment in the short-term

We explored LTP stimulating Schaffer collaterals with spaced (30 min apart) high-frequency stimulation (HFS) and analyzing LTP amplitudes 25 min after each stimulation. Following one week of treatment in the standard condition, during the second stimulation, we observed that LTP amplitude was significantly affected by treatment [$F(1,11) = 4.572$, $p = 0.05$]. In particular, minocycline-treated mice showed higher LTP amplitude (1.877 ± 0.0479, $n = 7$ slice/3 mice) compared to vehicle-treated mice (1.654 ± 0.0984, $n = 8$ slice/3 mice; Fig. 3 A). By contrast, at the end of the treatment period, post tetanus LTP amplitude was not significantly affected by treatments (Fig. 3B, vehicle: $n = 18$ slice/8 mice; minocycline: $n = 16$ slice/7 mice).

3.3. Minocycline treatment does not affect microglial proportion and morphology in the long-term

When we analyzed microglial proportion and morphology following three weeks of treatment, we found a co-localization of TMEM119 IR with Iba1 IR. Quantification of the percentage of double-positive $Iba1^{+}Tmem119^{+}$ cells over all DAPI-positive cells revealed no

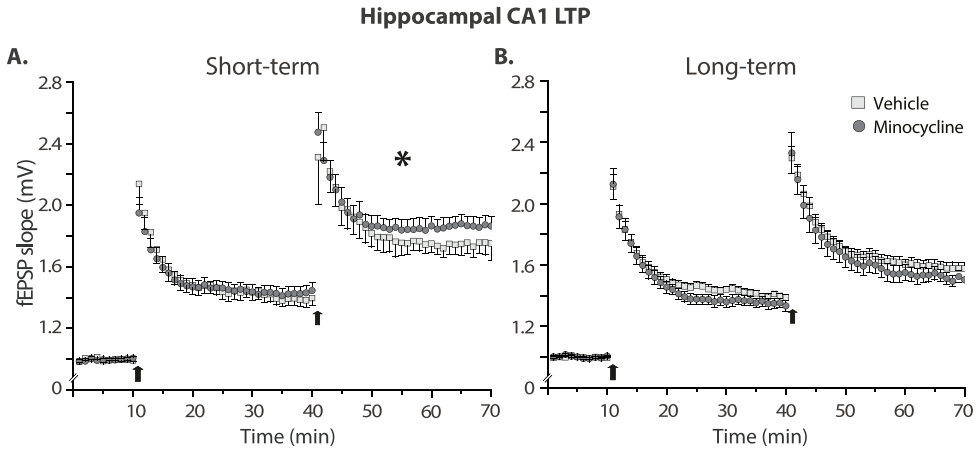

Fig. 3. Hippocampal CA1 LTP. LTP of fEPSP slope from extracellular records made from vehicle- (white square; 8 slices /3 mice) and minocycline- (dark circle) treated mice (7 slices/3 mice) following one week (Short-term, A) or three weeks (Long-term, B) of treatment. Time course of slope values from responses evoked at 0.05 Hz and normalized as detailed in the Methods. Arrows indicate LTP induction (HFS, 1 train of stimuli at 100 Hz, of 1 s duration). **A.** After one week of treatment, the electrophysiological assessment showed that, following two trains of stimulation, minocycline-treated mice displayed higher LTP compared to vehicle, indicating that minocycline increased neuronal plasticity. **B.** After 3 weeks of treatment, we did not observe any differences between minocycline and vehicle-treated animals, following both first and second stimulation fEPSP, field excitatory post-synaptic potential; *p = 0.05 vs vehicle, data shown as mean +s.e.m., n = 7–8 mice.

statistically significant difference between vehicle- and minocycline-treated mice [$F(1,20) = 0.001$, $p = 0.9991$ and $F(1,20) = 2.390$, $p = 0.1173$] in the three selected subareas of the medial prefrontal cortex, nor in the three selected subareas of the dorsal hippocampus (Fig. 4A).

Double-positive Iba1^{+}TMEM119^{+} cells were then selected for the quantification of the microglial cell body and branching area. We did not find statistically significant differences in the microglia soma size in the mPFC [$F(1,16) = 0.667$, $p = 0.5268$] or in the dorsal hippocampus [$F(1,14) = 0.415$, $p = 0.6680$; Fig. 5A].

When the branching of microglial processes was analyzed in the mPFC, there was no difference between vehicle- and minocycline-treated mice [$F(1,16) = 1.685$, $p = 0.2167$]. Similarly, there were no significant differences in the branching area in the hippocampus [$F(1,16) = 2.195$, $p = 0.1437$] between the two experimental groups (Fig. 5B).

These results suggest that minocycline treatment might not interfere with microglial physiological and immune functions, notably pertaining to their surveillance of the parenchyma, in the long run.

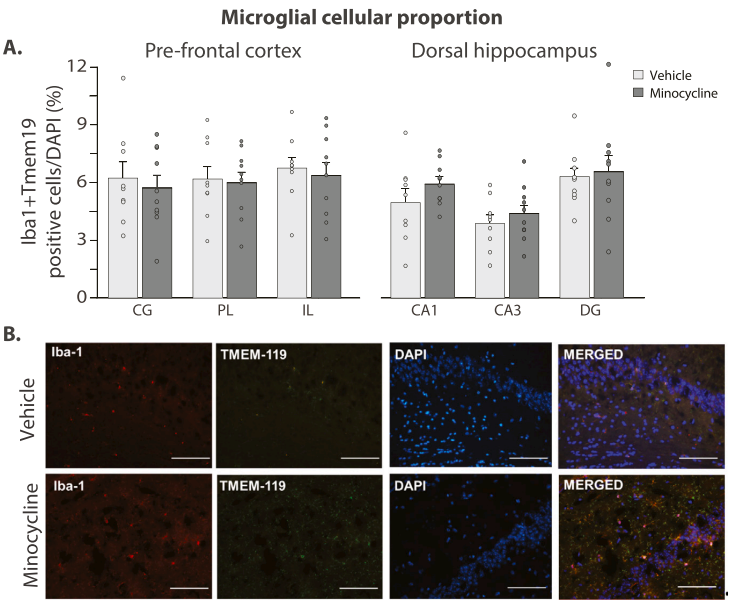

Fig. 4. Microglial proportion. **A.** Percentages of Iba1 and TMEM119 positive cells (over all DAPI-positive cells) in different areas of the prefrontal cortex and dorsal hippocampus. No significant differences in the number of double-positive cells between vehicle- and minocycline-treated mice were detected at the end of the treatment period in the standard condition. Data shown as mean +s.e.m., n = 5 per group. **B.** Representative images for assessment of a number of double-positive microglia. Photomicrographs from the hippocampus of a vehicle- (on the top) and a minocycline- (on the bottom) treated subjects: separate staining for Iba1, TMEM119, DAPI (to visualize nuclei) and merged. scale bar= 100 μm.

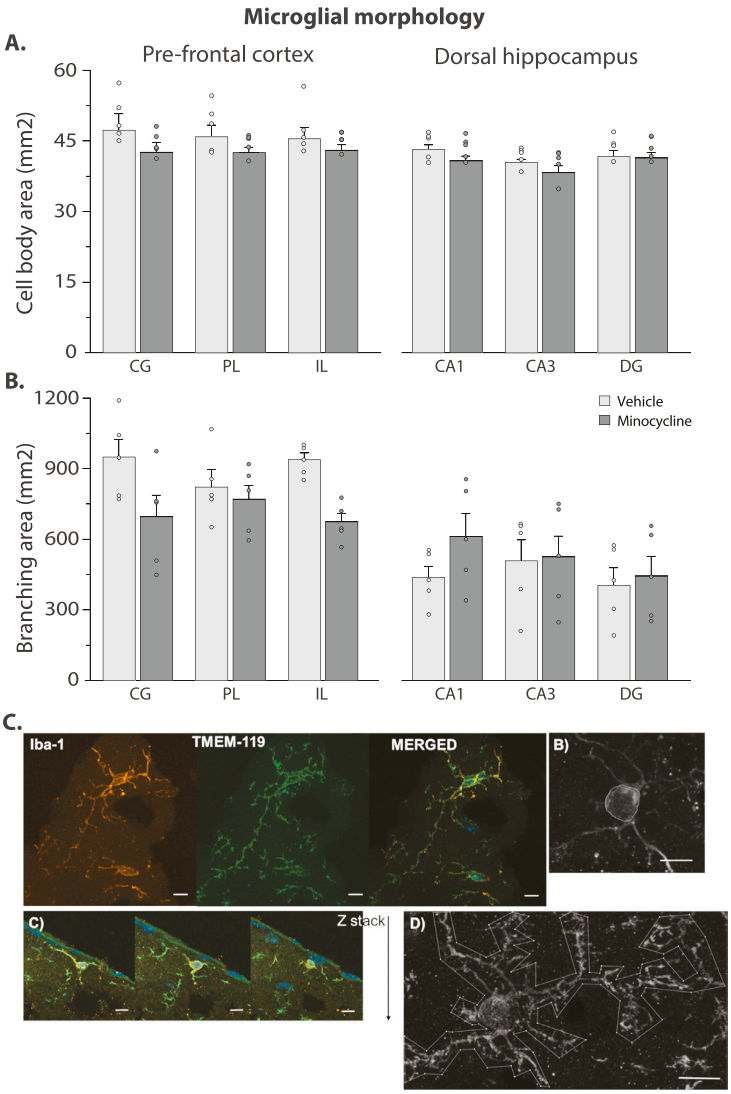

Fig. 5. Microglial morphology. Three weeks after the end of the treatment period, the morphology of microglial cells (TMEM119 $^{+}$Iba1 $^{+}$ cells) was assessed by measuring the size of their cell body area (**A**) and branching area (**B**) in the medial prefrontal cortex (mPFC) and in the hippocampus using confocal microscopy. The analyses did not reveal the statistical difference between the two experimental groups, although we noticed that in the prefrontal cortex minocycline treated mice showed smaller microglial branching areas compared to vehicle animals. Data shown as mean +s.e.m. n = 4–5 per group. **C.** Representative images of microglia for morphology assessment. Photomicrographs of two microglial cells with a large and a small tree size; staining for Iba1, TMEM119, and merged. An example of the morphology assessment showed a drawing encompassing the soma size area to calculate the area covered. View of microglia at the z-axis in the mPFC cortex. An example of a morphology analysis showed drawing the tree size area to calculate the area covered. Scale bar 10 μm. CG, cingulate cortex; PL, prelimbic cortex; IL, infralimbic cortex; DG, dentate gyrus.

4. Discussion

Minocycline significantly enhanced cognitive abilities and neural plasticity in the short-term compared to vehicle. By contrast, both experimental groups equally improved their depressive-like profile, likely because during treatment they were no more exposed to a stressful environment.

As previously reported [46,47,58], the exposure to chronic stress was effective at inducing a depressive-like phenotype, as indicated by the significant increase in liking- and wanting-type anhedonia. In the short-term from the end of the stressful condition, all the tested animals showed increased saccharin preference. However, such an increase was no more evident in the long-term (Fig. 2A-B). This observation is in line with previous studies showing that the termination of the stressful condition improves the depressive-like phenotype, but a standard environment is not supportive enough to produce a long-lasting reduction of the anhedonic response [46,59–61]. In this study, we did not find a significant difference in saccharin preference between minocycline- and vehicle-treated mice. Although a recent systematic review reported that this drug reduces the anhedonic-like behavior in rodents, the studies reporting an increased preference for the sweet solution following minocycline administration did not consider naïve animals, but individuals with an experimentally enhanced inflammatory state, such as those exposed to chronic stress, olfactory bulbectomized, or affected by type-1 diabetes [62]. These preclinical findings have been confirmed in a recent randomized clinical trial demonstrating that add-on treatment with minocycline is effective only in depressed patients with high baseline levels of inflammation [63].

Minocycline treatment improved learning and memory in the place learning test in the short term as the treatment significantly increased the ability to learn a new spatial task. These results are concordant with those by Naderi and colleagues, who reported that minocycline administration for only seven days helps experimental subjects recover the memory deficits induced by cerebral ischemia/reperfusion [32]. A

similar effect has been described in a model of intracerebral hemorrhage: the treatment with minocycline for one week led to an improvement of the neurobehavioral performances and reduced cellular apoptosis and glial cell reactivity [64]. In Alzheimer's disease rodent models, minocycline administration attenuated the memory impairments shown during the Morris Water Maze test and counteracted neuronal cell death [31]. Finally, the treatment has been proven to counteract the age-associated deterioration of memory and to ameliorate cognitive performance (e.g., spatial learning) in both adult and old mice [65,66]. Although minocycline mechanism of action remains unclear, it has been hypothesized that the observed beneficial effects were associated with anti-inflammatory and antioxidant properties [31,32,64,65]. Indeed, the drug can downregulate microglial-driven inflammation and reduce neuronal death triggered by exposure to stress. Three weeks after stress, no difference between the minocycline and vehicle mice was found, all subjects displayed an increase in the number of correct responses during both the acquisition and reversal phases (Fig. 2 C), suggesting that both groups recover learning and memory abilities in long-term. The rapid but short effect of minocycline on cognition after exposure to chronic stress could be explained by its ability to reduce microglial proliferation and reactivity when these phenomena are at a high level, which happens during and immediately after stress, when pathological conditions such as oxidative stress, cell damage, and death are in place [67–69]. Accordingly, a number of studies reported both neuronal and behavioral improvements following minocycline administration mostly in preclinical models of diseases associated with persistently increased inflammation [70–75]. These results are in line with the clinical evidence as a study showed that minocycline enhances the learning functions during navigation only in participants with a high body mass index, associated with higher inflammatory levels, while inducing cognitive impairments in participants with low or normal body mass index associated with lower immune activation [76].

To investigate neural modifications underlying the minocycline effects on cognition, we assessed hippocampal neural plasticity measured as LTP, which reflects some mechanisms involved in learning and memory [77–79], and in particular, in place learning, considered a hippocampal-dependent task [80]. We found that minocycline enhanced LTP in the short-, but not in the long-term (Fig. 3). It is worth noting that this temporal profile overlaps with the behavioral effects of minocycline, suggesting that the enhanced performance during the learning and memory task is associated with enhanced plasticity and LTP.

The immunohistochemical results showed a lack of minocycline effect on microglial morphology and proportion in the hippocampus and pre-frontal cortex following three weeks of treatment (Fig. 4 A). This is in line with our behavioral and electrophysiological results indicating a recovery within the first week after stress. Accordingly, previous studies found that minocycline action depends on the inflammatory status and that microglial reactivity is mainly affected in the short-term after stress, when an immune system response occurs [68,81–83]. However, here, the relation between inflammatory response and behavioral profile is still speculative because further data on microglial functional states at different time points are still warranted.

The present study has some limitations. First, the lack of a non-stressed group did not allow to assess the effect of chronic stress by comparing stressed and non-stressed animals. However, we could measure the stress effect in a within-subjects analysis by comparing the behavioral profile of the animals immediately before and after the stress exposure. Second, we did not measure microglial proportion and morphology following the first week of treatment, when we observed the minocycline effects at behavioral and electrophysiological levels, which hinders potential microglial modifications underlying its efficacy in the short-term. In addition, we did not measure learning and memory immediately after the end of chronic stress to assess the behavioral response before treatment. This choice was motivated by the need to avoid excessive training due to the repetition of the same task.

The rising attention on cognitive symptoms associated with MDD is pointing out the need to develop novel antidepressant treatments able to target this domain in addition to the emotional one. In the present study, we showed that minocycline treatment can enhance neural plasticity and improve learning and memory abilities with a fast but short efficacy suggesting, from a translational perspective, that this drug is a promising candidate to treat cognitive impairments in depressed patients.

These results are of even greater impact when considering that treating cognitive residual symptoms has been shown to be pivotal to preventing new depressive episodes and the reportedly associated increased risk of neurodegenerative disorders [84].

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CRediT authorship contribution statement

Silvia Poggini: participation in the design of the study, performing most of the experimental procedures, analyzing data, writing of the manuscript; Maria Banqueri Lopez: performing experimental procedures, analyzing data, writing of the manuscript; Naomi Ciano Albanese: performing experimental procedures and analyzing data; Maria Teresa Golia and Fernando González Ibáñez: performing part of the experimental procedures; Marie-Eve Tremblay, Maciej Lalowski and Martin Furhmann, Cristina Limatola: reviewing the manuscript; Laura Maggi: discussing data, writing and reviewing the manuscript; Bozena Kaminska and Igor Branchi: conceptualization of the study, results interpretation, writing of the manuscript.

Competing interests

Declarations of interest: none.

Data availability

Data will be made available on request.

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References

[1] H.A. Whiteford, L. Degenhardt, J. Rehm, A.J. Baxter, A.J. Ferrari, H.E. Erskine, et al., Global burden of disease attributable to mental and substance use disorders: findings from the Global Burden of Disease Study 2010, Lancet 382 (2013) 1575–1586.
[2] World Health Organization. Depression and Other Common Mental Disorders: Global Health Estimates. 2017.
[3] T. Chakrabarty, G. Hadjipavlou, R.W. Lam, Cognitive dysfunction in major depressive disorder: assessment, impact, and management, Focus 14 (2016) 194–206.
[4] G. Perini, M. Cotta Ramusino, E. Sinforiani, S. Bernini, R. Petrachi, A. Costa, Cognitive impairment in depression: recent advances and novel treatments, Neuropsychiatr. Dis. Treat. 15 (2019) 1249–1258.
[5] B. Ravnkilde, P. Videbech, K. Clemmensen, A. Egander, N.A. Rasmussen, R. Rosenberg, Cognitive deficits in major depression, Scand. J. Psychol. 43 (2002) 239–251.
[6] T. Tran, M. Milanovic, K. Holshausen, C.R. Bowie, What is normal cognition in depression? Prevalence and functional correlates of normative versus idiographic cognitive impairment, Neuropsychology 35 (2021) 33–41.

[7] S. Wagner, B. Doering, I. Helmreich, K. Lieb, A. Tadic, A meta-analysis of executive dysfunctions in unipolar major depressive disorder without psychotic symptoms and their changes during antidepressant treatment, Acta Psychiatr. Scand. 125 (2012) 281–292.

[8] Z. Pan, C. Park, E. Brietzke, H. Zuckerman, C. Rong, R.B. Mansur, et al., Cognitive impairment in major depressive disorder, CNS Spectr. 24 (2019) 22–29.

[9] V.M. Dotson, S.M. McClintock, P. Verhaeghen, J.U. Kim, A.A. Draheim, S. M. Syzmkowicz, et al., Depression and Cognitive Control across the Lifespan: a Systematic Review and Meta-Analysis, Neuropsychol. Rev. 30 (2020) 461–476.

[10] M. Semkovska, L. Quinlivan, T. O'Grady, R. Johnson, A. Collins, J. O'Connor, et al., Cognitive function following a major depressive episode: a systematic review and meta-analysis, Lancet Psychiatry 6 (2019) 851–861.

[11] Å. Hammar, E.H. Ronold, G.Å. Rekkedal, Cognitive impairment and neurocognitive profiles in major depression—a clinical perspective, Front Psychiatry (2022) 13.

[12] H.J. Conradi, J. Ormel, P. de Jonge, Presence of individual (residual) symptoms during depressive episodes and periods of remission: a 3-year prospective study, Psychol. Med. 41 (2011) 1165–1174.

[13] B.T. Baune, R. Miller, J. McAfoose, M. Johnson, F. Quirk, D. Mitchell, The role of cognitive impairment in general functioning in major depression, Psychiatry Res. 176 (2010) 183–189.

[14] M. Halvorsen, R.S. Hoifodt, I.N. Myrbakk, C.E. Wang, K. Sundet, M. Eisemann, et al., Cognitive function in unipolar major depression: a comparison of currently depressed, previously depressed, and never depressed individuals, J. Clin. Exp. Neuropsychol. 34 (2012) 782–790.

[15] H.R. Snyder, Major depressive disorder is associated with broad impairments on neuropsychological measures of executive function: a meta-analysis and review, Psychol. Bull. 139 (2013) 81–132.

[16] B.J. Hasselbalch, U. Knorr, L.V. Kessing, Cognitive impairment in the remitted state of unipolar depressive disorder: a systematic review, J. Affect. Disord. 134 (2011) 20–31.

[17] D. Bennabi, E. Haffen, V. Van Waes, Vortioxetine for cognitive enhancement in major depression: from animal models to clinical research, Front. Psychiatry 10 (2019) 771.

[18] B.T. Baune, L.B. Sluth, C.K. Olsen, The effects of vortioxetine on cognitive performance in working patients with major depressive disorder: a short-term, randomized, double-blind, exploratory study, J. Affect. Disord. 229 (2018) 421–428.

[19] M.J. Blumberg, S.R. Vaccarino, S.J. McInerney, Procognitive effects of antidepressants and other therapeutic agents in major depressive disorder: a systematic review, J. Clin. Psychiatry (2020) 81.

[20] A.R. Mahableshwarkar, J. Zajecka, W. Jacobson, Y. Chen, R.S. Keefe, A randomized, placebo-controlled, active-reference, double-blind, flexible-dose study of the efficacy of vortioxetine on cognitive function in major depressive disorder, Neuropsychopharmacol.: Off. Publ. Am. Coll. Neuropsychopharmacol. 40 (2015) 2025–2037.

[21] R.S. McIntyre, S. Lophaven, C.K. Olsen, A randomized, double-blind, placebo-controlled study of vortioxetine on cognitive function in depressed adults, Int. J. Neuropsychopharmacol. 17 (2014) 1557–1567.

[22] E. Vieta, L.B. Sluth, C.K. Olsen, The effects of vortioxetine on cognitive dysfunction in patients with inadequate response to current antidepressants in major depressive disorder: a short-term, randomized, double-blind, exploratory study versus escitalopram, J. Affect. Disord. 227 (2018) 803–809.

[23] M. Sowa-Kucma, P. Panczyszyn-Trzewik, P. Misztak, R.R. Jaeschke, K. Sendek, K. Styczen, et al., Vortioxetine: a review of the pharmacology and clinical profile of the novel antidepressant, Pharmacol. Rep.: PR 69 (2017) 595–601.

[24] C.L. Raison, L. Capuron, A.H. Miller, Cytokines sing the blues: inflammation and the pathogenesis of depression, Trends Immunol. 27 (2006) 24–31.

[25] J.D. Rosenblat, E. Brietzke, R.B. Mansur, N.A. Maruschak, Y. Lee, R.S. McIntyre, Inflammation as a neurobiological substrate of cognitive impairment in bipolar disorder: Evidence, pathophysiology and treatment implications, J. Affect. Disord. 188 (2015) 149–159.

[26] M.J. Stuart, B.T. Baune, Chemokines and chemokine receptors in mood disorders, schizophrenia, and cognitive impairment: a systematic review of biomarker studies, Neurosci. Biobehav. Rev. 42 (2014) 93–115.

[27] J. Krogh, M.E. Benros, M.B. Jorgensen, L. Vesterager, B. Elfving, M. Nordentoft, The association between depressive symptoms, cognitive function, and inflammation in major depression, Brain Behav., Immun. 35 (2014) 70–76.

[28] J.D. Rosenblat, D.S. Cha, R.B. Mansur, R.S. McIntyre, Inflamed moods: a review of the interactions between inflammation and mood disorders, Prog. Neur Psychopharmacol. Biol. Psychiatry 53 (2014) 23–34.

[29] N. Garrido-Mesa, A. Zarzuelo, J. Galvez, Minocycline: far beyond an antibiotic, Br. J. Pharm. 169 (2013) 337–352.

[30] R. Yirmiya, N. Rimmerman, R. Reshef, Depression as a microglial disease, Trends Neurosci. 38 (2015) 637–658.

[31] Y. Choi, H.S. Kim, K.Y. Shin, E.M. Kim, M. Kim, H.S. Kim, et al., Minocycline Attenuates Neuronal Cell Death and Improves Cognitive Impairment in Alzheimer's Disease Models, Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol. 32 (2007) 2393–2404.

[32] Y. Naderi, M. Sabetkasaei, S. Parvardeh, T.M. Zanjani, Neuroprotective effect of minocycline on cognitive impairments induced by transient cerebral ischemia/reperfusion through its anti-inflammatory and anti-oxidant properties in male rat, Brain Res. Bull. 131 (2017) 207–213.

[33] L. Zhang, H. Zheng, R. Wu, T.R. Kosten, X.Y. Zhang, J. Zhao, The effect of minocycline on amelioration of cognitive deficits and pro-inflammatory cytokines levels in patients with schizophrenia, Schizophr. Res. 212 (2019) 92–98.

[34] Y. Levkovitz, S. Mendlovich, S. Riwkes, Y. Braw, H. Levkovitch-Verbin, G. Gal, et al., A double-blind, randomized study of minocycline for the treatment of negative and cognitive symptoms in early-phase schizophrenia, J. Clin. Psychiatry 71 (2010) 138–149.

[35] T.L. Tay, J.C. Savage, C.W. Hui, K. Bisht, M.E. Tremblay, Microglia across the lifespan: from origin to function in brain development, plasticity and cognition, J. Physiol. 595 (2017) 1929–1945.

[36] G. Cisbani, S. Poggini, N. Laflamme, V. Pons, M.E. Tremblay, I. Branchi, et al., The Intellicage system provides a reproducible and standardized method to assess behavioral changes in cuprizone-induced demyelination mouse model, Behav. Brain Res. 400 (2021), 113039.

[37] S. Krackow, E. Vannoni, A. Codita, A.H. Mohammed, F. Cirulli, I. Branchi, et al., Consistent behavioral phenotype differences between inbred mouse strains in the IntelliCage, Genes Brain Behav. 9 (2010) 722–731.

[38] M.A. Di Castro, F. Trettel, G. Milior, L. Maggi, D. Ragozzino, C. Limatola, The chemokine CXCL16 modulates neurotransmitter release in hippocampal CA1 area, Sci. Rep. 6 (2016) 34633.

[39] Y. Hou, G. Xie, X. Liu, G. Li, C. Jia, J. Xu, et al., Minocycline protects against lipopolysaccharide-induced cognitive impairment in mice, Psychopharmacology 233 (2016) 905–916.

[40] E. Ozaki, C. Delaney, M. Campbell, S.L. Doyle, Minocycline suppresses disease-associated microglia (DAM) in a model of photoreceptor cell degeneration, Exp. Eye Res. 217 (2022), 108953.

[41] S. Hersch, K. Fink, J.P. Vonsattel, R.M. Friedlander, Minocycline is protective in a mouse model of Huntington's disease, Ann. Neurol. 54 (841) (2003) 2–3 (author reply).

[42] V.W. Yong, J. Wells, F. Giuliani, S. Casha, C. Power, L.M. Metz, The promise of minocycline in neurology, Lancet Neurol. 3 (2004) 744–751.

[43] A. Codita, A.H. Mohammed, A. Willuweit, A. Reichelt, E. Alleva, I. Branchi, et al., Effects of spatial and cognitive enrichment on activity pattern and learning performance in three strains of mice in the IntelliMaze, Behav. Genet. 42 (2012) 449–460.

[44] S. Muthuraju, S. Taha, S. Pati, M. Rafique, H. Jaafar, J.M. Abdullah, Normabaric hyperoxia treatment improved locomotor activity of C57BL/6J mice through enhancing dopamine genes following fluid-percussion injury in striatum, Int. J. Biomed. Sci.: IJBS 9 (2013) 194–204.

[45] S. Alboni, R.M. van Dijk, S. Poggini, G. Milior, M. Perrotta, T. Drenth, et al., Fluoxetine effects on molecular, cellular and behavioral endophenotypes of depression are driven by the living environment, Mol. Psychiatry 22 (2017) 552–561.

[46] I. Branchi, S. Santarelli, S. Capoccia, S. Poggini, I. D'Andrea, F. Cirulli, et al., Antidepressant treatment outcome depends on the quality of the living environment: a pre-clinical investigation in mice, PloS One 8 (2013), e62226.

[47] S. Poggini, M.T. Golia, S. Alboni, G. Milior, L.P. Sciarria, A. Viglione, et al., Combined fluoxetine and metformin treatment potentiates antidepressant efficacy increasing IGF2 expression in the dorsal hippocampus, Neural Plast. 2019 (2019) 4651031.

[48] G. Milior, C. Lecours, L. Samson, K. Bisht, S. Poggini, F. Pagani, et al., Fractalkine receptor deficiency impairs microglial and neuronal responsiveness to chronic stress, Brain, Behav., Immun. 55 (2016) 114–125.

[49] B. Albuquerque, A. Haussler, E. Vannoni, D.P. Wolfer, I. Tegeder, Learning and memory with neuropathic pain: impact of old age and progranulin deficiency, Front. Behav. Neurosci. 7 (2013) 174.

[50] K. Jastrzebska, M. Walczak, P.E. Cieslak, L. Szumiec, M. Turbasa, D. Engblom, et al., Loss of NMDA receptors in dopamine neurons leads to the development of affective disorder-like symptoms in mice, Sci. Rep. 6 (2016) 37171.

[51] A. Kiryk, A. Janusz, B. Zglinicki, E. Turkes, E. Knapska, W. Konopka, et al., IntelliCage as a tool for measuring mouse behavior - 20 years perspective, Behav. Brain Res. 388 (2020), 112620.

[52] N. Kulesskaya, N.N. Karpova, L. Ma, L. Tian, V. Voikar, Mixed housing with DBA/2 mice induces stress in C57BL/6 mice: implications for interventions based on social enrichment, Front. Behav. Neurosci. 8 (2014) 257.

[53] A. Puscian, S. Leski, T. Gorkiewicz, K. Meyza, H.P. Lipp, E. Knapska, A novel automated behavioral test battery assessing cognitive rigidity in two genetic mouse models of autism, Front. Behav. Neurosci. 8 (2014) 140.

[54] R. Fujikawa, S. Jinno, Identification of hyper-ramified microglia in the CA1 region of the mouse hippocampus potentially associated with stress resilience, Eur. J. Neurosci. (2022).

[55] M.A. Calcia, D.R. Bonsall, P.S. Bloomfield, S. Selvaraj, T. Barichello, O.D. Howes, Stress and neuroinflammation: a systematic review of the effects of stress on microglia and the implications for mental illness, Psychopharmacology 233 (2016) 1637–1650.

[56] M.L. Bennett, F.C. Bennett, S.A. Liddelow, B. Ajami, J.L. Zamanian, N.B. Fernhoff, et al., New tools for studying microglia in the mouse and human CNS, Proc. Natl. Acad. Sci. USA 113 (2016) E1738–E1746.

[57] E. Vankriekelsvenne, U. Chrzanowski, K. Manzhula, T. Greiner, A. Wree, A. Hawlitschka, et al., Transmembrane protein 119 is neither a specific nor a reliable marker for microglia, Glia 70 (2022) 1170–1190.

[58] S. Alboni, S. Poggini, S. Garofalo, G. Milior, H. El Hajj, C. Lecours, et al., Fluoxetine treatment affects the inflammatory response and microglial function according to the quality of the living environment, Brain Behav., Immun. (2016).

[59] S. Poggini, G. Matte Bon, M.T. Golia, N. Ciano Albanese, A. Viglione, A. Poleggi, et al., Selecting antidepressants according to a drug-by-environment interaction: a comparison of fluoxetine and minocycline effects in mice living either in enriched or stressful conditions, Behav. brain Res. 408 (2021), 113256.

[60] H. Huang, Q. Wang, X. Guan, X. Zhang, Y. Zhang, J. Cao, et al., Effects of enriched environment on depression and anxiety-like behavior induced by early life stress: a comparison between different periods, Behav. Brain Res. 411 (2021), 113389.
[61] H. Wurbel, Ideal homes? Housing effects on rodent brain and behaviour, Trends Neurosci. 24 (2001) 207–211.
[62] D.J. Reis, E.J. Casteen, S.S. Ilardi, The antidepressant impact of minocycline in rodents: a systematic review and meta-analysis, Sci. Rep. 9 (2019) 261.
[63] M.A. Nettis, G. Lombardo, C. Hastings, Z. Zajkowska, N. Mariani, N. Nikkheslat, et al., Augmentation therapy with minocycline in treatment-resistant depression patients with low-grade peripheral inflammation: results from a double-blind randomised clinical trial, Neuropsychopharmacol.: Off. Publ. Am. Coll. Neuropsychopharmacol. (2021).
[64] C. Power, S. Henry, M.R. Del Bigio, P.H. Larsen, D. Corbett, Y. Imai, et al., Intracerebral hemorrhage induces macrophage activation and matrix metalloproteinases, Ann. Neurol. 53 (2003) 731–742.
[65] R.A. Kohman, T.K. Bhattacharya, C. Kilby, P. Bucko, J.S. Rhodes, Effects of minocycline on spatial learning, hippocampal neurogenesis and microglia in aged and adult mice, Behav. Brain Res. 242 (2013) 17–24.
[66] Y. Jiang, Y. Liu, C. Zhu, X. Ma, L. Ma, L. Zhou, et al., Minocycline enhances hippocampal memory, neuroplasticity and synapse-associated proteins in aged C57 BL/6 mice, Neurobiol. Learn. Mem. 121 (2015) 20–29.
[67] M. Hinwood, J. Morandini, T.A. Day, F.R. Walker, Evidence that microglia mediate the neurobiological effects of chronic psychological stress on the medial prefrontal cortex, Cereb. Cortex 22 (2012) 1442–1454.
[68] M. Hinwood, R.J. Tynan, J.L. Charnley, S.B. Beynon, T.A. Day, F.R. Walker, Chronic stress induced remodeling of the prefrontal cortex: structural re-organization of microglia and the inhibitory effect of minocycline, Cereb. Cortex 23 (2013) 1784–1797.
[69] T. Kreisel, M.G. Frank, T. Licht, R. Reshef, O. Ben-Menachem-Zidon, M.V. Baratta, et al., Dynamic microglial alterations underlie stress-induced depressive-like behavior and suppressed neurogenesis, Mol. Psychiatry 19 (2014) 699–709.
[70] V. Brundula, N.B. Rewcastle, L.M. Metz, C.C. Bernard, V.W. Yong, Targeting leukocyte MMPs and transmigration: minocycline as a potential therapy for multiple sclerosis, Brain J. Neurol. 125 (2002) 1297–1308.
[71] M. Chen, V.O. Ona, M. Li, R.J. Ferrante, K.B. Fink, S. Zhu, et al., Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease, Nat. Med. 6 (2000) 797–801.
[72] Y. Du, Z. Ma, S. Lin, R.C. Dodel, F. Gao, K.R. Bales, et al., Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease, Proc. Natl. Acad. Sci. USA 98 (2001) 14669–14674.
[73] D.L. Smith, B. Woodman, A. Mahal, K. Sathasivam, S. Ghazi-Noori, P.A. Lowden, et al., Minocycline and doxycycline are not beneficial in a model of Huntington's disease, Ann. Neurol. 54 (2003) 186–196.
[74] M. Tomas-Camardiel, I. Rite, A.J. Herrera, R.M. de Pablos, J. Cano, A. Machado, et al., Minocycline reduces the lipopolysaccharide-induced inflammatory reaction, peroxynitrite-mediated nitration of proteins, disruption of the blood-brain barrier, and damage in the nigral dopaminergic system, Neurobiol. Dis. 16 (2004) 190–201.
[75] D.C. Wu, V. Jackson-Lewis, M. Vila, K. Tieu, P. Teismann, C. Vadseth, et al., Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease, J. Neurosci. Off. J. Soc. Neurosci. 22 (2002) 1763–1771.
[76] S.C. Berens, C.M. Bird, N.A. Harrison, Minocycline differentially modulates human spatial memory systems, Neuropsychopharmacol.: Off. Publ. Am. Coll. Neuropsychopharmacol. 45 (2020) 2162–2169.
[77] V. Bouet, T. Freret, P. Dutar, J.M. Billard, M. Boulouard, Continuous enriched environment improves learning and memory in adult NMRI mice through theta burst-related-LTP independent mechanisms but is not efficient in advanced aged animals, Mech. Ageing Dev. 132 (2011) 240–248.
[78] A. Matynia, S.A. Kushner, A.J. Silva, Genetic approaches to molecular and cellular cognition: a focus on LTP and learning and memory, Annu. Rev. Genet. 36 (2002) 687–720.
[79] D. Pereda, I. Al-Osta, A.E. Okorocha, A. Easton, N.A. Hartell, Changes in presynaptic calcium signalling accompany age-related deficits in hippocampal LTP and cognitive impairment, Aging Cell 18 (2019), e13008.
[80] M.J. Galsworthy, I. Amrein, P.A. Kuptsov, I.I. Poletaeva, P. Zinn, A. Rau, et al., A comparison of wild-caught wood mice and bank voles in the intellicage: assessing exploration, daily activity patterns and place learning paradigms, Behav. Brain Res. 157 (2005) 211–217.
[81] Y. Han, L. Zhang, Q. Wang, D. Zhang, Q. Zhao, J. Zhang, et al., Minocycline inhibits microglial activation and alleviates depressive-like behaviors in male adolescent mice subjected to maternal separation, Psychoneuroendocrinology 107 (2019) 37–45.
[82] R. Scholz, M. Sobotka, A. Caramoy, T. Stempfl, C. Moehle, T. Langmann, Minocycline counter-regulates pro-inflammatory microglia responses in the retina and protects from degeneration, J. Neuroinflamm. 12 (2015) 209.
[83] J. Abraham, P.D. Fox, C. Condello, A. Bartolini, S. Koh, Minocycline attenuates microglia activation and blocks the long-term epileptogenic effects of early-life seizures, Neurobiol. Dis. 46 (2012) 425–430.
[84] E.S. Paykel, Partial remission, residual symptoms, and relapse in depression, Dialog. Clin. Neurosci. 10 (2008) 431–437.

RESPONSE TO REVIEWERS

Reviewer Dr. Francesca Cirulli, Center for Behavioral Sciences and Mental Health, Istituto Superiore di Sanità, Rome Italy

Report

This is a very good doctoral thesis exploring the role of the environment in determining resilience/risk for mental disorders. The thesis mainly focuses on adolescence, a critical life stage when psychopathology often manifests. The experimental work included preclinical studies that investigated: i) the neurobiological bases of depression-like behavior in adolescence with the main focus on the role of the immune system; ii) a drug x environment interaction strategy to unravel the potential role of environmental conditions on antidepressant efficacy and iii) attest whether previous social experiences can redirect SSRI treatment outcome. The work is original and well-described. There is a very clear logic and a good wealth of techniques. I would only suggest the candidate to better introduce (in a few paragraphs) in the Introductory section the role of the immune system in the regulations object of the thesis.

I thank Doctor Cirulli for the suggestion, in order to clarify the role of the immune system in the onset and progression of depression I added a new section (page 14) in the General introduction and discussed the issue as follows:

"*Brain-immune crosstalk in the onset of adolescent depression*

The relative contribution of biological and cultural factors to adolescent-onset depression and to the sex-dependent vulnerability to depression is not yet clear (Conley and Rudolph, 2009). An increasing number of preclinical and clinical findings implicate the brain-immune crosstalk as a key determinant in the onset and treatment of psychiatric disorders – from depression to schizophrenia (Branchi, 2011; Branchi et al., 2021; Bullmore, 2018; Milaneschi et al., 2020; Pariante, 2016; Thibaut, 2018; Viglione et al., 2019). In particular, the expression of inflammatory cytokines in the brain has been reported to be involved in the pathogenesis of and susceptibility to depression, indicating adolescence as a unique stage of immune development (Granata et al., 2022; Mariani et al., 2021). The link between disturbances in the immuno-inflammatory system and the

etiology and pathophysiology of several psychiatric disorders has been specifically explored, making the field of immunopsychiatry rise (Dantzer et al., 2008; Leboyer et al., 2016a; Leboyer et al., 2016b; Pariante, 2017). Some authors have hypothesized that an increase in inflammatory markers occurs before the onset of depressive symptoms, suggesting that immune system activation precedes psychopathology (Gimeno et al., 2009; Liu et al., 2019; Pasco et al., 2010). Others proposed an opposite temporal relationship, suggesting that psychiatric disorders cause an impairment in immunocompetence, leading to an increased vulnerability to infectious diseases (Copeland et al., 2012; Dantzer, 2012) or reported a lack of correlation (Copeland et al., 2012; Dantzer, 2012; Levine et al., 1999). In addition, only 30% of depressed patients show high inflammatory levels (Raison et al., 2006) and, in turn, depression does not always follow an immune activation (Raison and Miller, 2011). Since stress increases both immune response and the risk of psychiatric disorders, exposure to stressful conditions, including childhood adversity, has been hypothesized to represent the common factor triggering the increase in both inflammatory markers and the likelihood of depression (Benros et al., 2013; Garcia-Bueno et al., 2008; Muller et al., 2019). Adverse childhood events occurring in late childhood and adolescence, but not earlier in life, were recently reported to be associated with persistent immune abnormalities (Iob et al., 2022), suggesting immune signaling may be particularly influential during the adolescent period and that it may play a crucial role in the onset of psychopathologies."

Reviewer Prof. Judith Homberg, Radboud University Medical Center Donders Institute for Brain, Cognition, and Behaviour Nijmegen The Netherlands

Report

The thesis focusses on depression during adolescence, it's treatment by SSRIs as function of the environment, and finally the subjective experience of the environment that determines how individuals respond to SSRI treatment. The work complements the existing theory that SSRI treatment does not unequivocally exerts antidepressant effects, but that this depends on the quality of the environment, with anti-depressive outcomes if the environment is positive and depressive outcomes if the environment is (perceived) negative. In this thesis this work is extended to depression in adolescence and the notion that it is about how the environment is being perceived. Especially social buffering alters the perception of the environment and contributes to resilience or a beneficial response to SSRI treatment. It is clear that this work has high societal relevance, given that depression is one of the most common disorders we have. The COVID-19 pandemic has further illustrated this. Also, the discussion about SSRI treatment during adolescence (potential increased suicides) is well addressed. The three chapters nicely complement each other. The experiments are well-designed and illustrated with figures. The data presentation is transparent, with the individual data points shown in the graphs. It is also great that male and female mice have been tested, as there is a bias to studying only males in neuroscience research. If find the effects found in chapter 3, with the standard housing versus communal nesting effects on the response to fluoxetine particularly compelling. The final discussion is also nice. Potentially, it could be worthful to add also a discussion on study limitations and an outlook for future research. For instance, how can the findings from this study be translated to humans? How do we define/create positive environments for humans? Overall, the thesis demonstrates that the candidate is able to independently conduct scientiftic research. Some small comments Page 86: "13 male and 36 C57BL/6 mice" > Is female missing before 36? Page 86: "On PND-1 litters were culled to 3 male and 3 female pups and a cross-fostering procedure was performed to

minimize the number of subjects" > how does cross-fostering contribute to minimize the number of subjects.

I thank Professor Homberg for the suggestions, the limitations of this study are now discussed in a new paragraph (page 151) of the General discussion and conclusions section as follows:

"Limits

The limitations of the present work include, first, the lack of data concerning the estrous cycle in female subjects. However, vaginal cell sampling imposes stress on the experimental subjects, and previous studies have reported that the effect of the estrous cycle in several behavioral tests, such as open field or fear conditioning, is limited: behavioral parameters were not affected across all four estrous phases (Hiroi and Neumaier, 2006; Lovick and Zangrossi, 2021; Meziane et al., 2007).

A further limitation concerning the study exploring the immune system activation after social isolation, is the assessment of inflammatory markers only in the central nervous system, though in clinical settings peripheral levels are routinely measured. Therefore, future studies assessing both central and peripheral markers will have a high translational value and will help illustrate the link between central and peripheral inflammation.

Finally, in the study aimed at assessing the role of subjective appraisal in determining the antidepressant outcome, we focused mainly on hippocampal BDNF, though other brain regions and molecular markers could have also been considered. However, BDNF regulation in the hippocampus is considered the gold standard reference to assess the action and effects of serotoninergic antidepressants (Castren and Monteggia, 2021; Duman et al., 2021)."

The outlook for future research, focused on the translational perspective of the study, is now integrated into the paragraph "General conclusions and future perspectives" (page 153) of the General discussion and conclusions section and discussed as follows:

"*General conclusions and future perspectives*

Our findings confirm and further describe the key role of context in adolescent neurodevelopment in both sexes and in driving the outcome of psychoactive compounds, especially those leveraging on the enhancement of neural and behavioral plasticity such as SSRIs (Alboni et al., 2017; Branchi, 2011; Poggini et al., 2019; Wilkinson et al., 2019).

In a translational perspective, modifications in the living conditions/lifestyle and/or of the subjective appraisal should be considered to improve therapeutic intervention and predict treatment outcomes in both adolescence and adulthood, as previously suggested by the study of Viglione and collaborators (Viglione et al., 2019). We thus underpin the relevance of the patient's living conditions in the selection of the most effective pharmacological treatment and advocate for considering the key role of context in the precision medicine paradigm in the psychiatric field.

*The availability of several longitudinal studies such as The Resilience, Ethnicity and AdolesCent Mental Health (REACH) Study, The National Community Mental Health Care Database (NCMHCD), Adolescent Brain Cognitive Development (ABCD) Study, the Covid-Mind Network, The Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study and the Combining medications to enhance depression outcomes (CO-MED) study, will allow to further explore the interaction between environmental conditions, subjective appraisal, and their interaction at a clinical level to setup novel and effective therapeutic strategies.*"

As suggested by Professor Homberg, I added the missed word "female" on page 88 and clarified the rationale of the cross-fostering procedure as follows (page 88): "On PND-1 litters were culled to 3 male and 3 female pups and a cross-fostering procedure was performed to limit the number of mates needed".

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