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Effects of acute and sub-chronic glucocorticoid treatments on hippocampal neurons of wild type and dystrophin-deficient *DMD^{mdx} mice:* an *in vitro* and *in vivo* study

Dottorando:
Francesca Cosmi

Docente guida
Prof.ssa Maria Egle De Stefano
Coordinatore
Prof.ssa Giulia De Lorenzo

“I do not know what I may appear to the world, but to myself I seem to have been only like a boy playing on the seashore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.”
Sir Isaac Newton

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Glossary

Ach: acetylcholine
ACTH: adrenocorticotrophic hormone
ADHD: attention-deficit/hyperactivity disorder
BDNF: brain-derived neurotrophic factor
BLA: basolateral amygdala
BSA: bovine serum albumin
cAMP: cyclic adenosine monophosphate
Cav1: caveolin 1
Cav2: caveolin 2
CNS: central nervous system
CORT: corticosterone
CRF: corticotrophin-releasing factor
CRH: corticotrophin releasing hormone
DBD: DNA-binding domain
DEX: dexamethasone
DG: dentate gyrus
DGC: dystrophin glycoprotein complex
DMD: Duchenne Muscular Dystrophy
DMSO: dimethyl sulfoxide
E18: embryonic day 18
ECM: extracellular matrix
EdU: 5-ethynyl-2'-deoxyuridine
GC: glucocorticoids
GILZ: glucocorticoid-induced leucine zipper
GJIC: gap junction intercellular communication
GPCRs: G-protein coupled receptors
GR: glucocorticoid receptor
GRE: glucocorticoid response element
HDAC: histone deacetylase
HPA: hypothalamus-pituitary-adrenal axis
HSP: heat shock proteins
IL-2: interleukin 2

IQ: intelligence quotient
LBD: ligand-binding domain
LTP: long-term potentiation
MAPK: mitogen-activated protein kinase
mGR: membrane-associated GR
MR: mineralcorticoid receptor
MT1X: metallothionein 1X
nAChRs: nicotinic acetylcholine receptor
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NGF: nerve growth factor
nGRE: negative glucocorticoid response element
NLS: nuclear localization signals
NOS: nitric oxide synthase
NPSC: neural pluripotent stem cell
NTD: N-terminal domain
OD: optical density
PI3K: phosphoinositide 3-kinase
PKA: protein kinase A
PNS: peripheral nervous system
PS: population spike
PVN: paraventricular nucleus
SCG: superior cervical ganglion
ANS: autonomous nervous system
CNS: central nervous system
SGZ: subgranular zone
STD: short-term depression
STP: short-term potentiation
14 DIV: 14 days in vitro

Abstract

Duchenne muscular dystrophy (DMD) is a lethal X-linked disease characterized by progressive muscular wasting due to lack of full-length dystrophin (Dp427), a cytoskeletal protein expressed in muscle and selected brain regions (i.e. hippocampus). Dp427 binds to a large multi-proteic complex (Dystrophin Glycoprotein Complex, DGC), endowed with structural and functional properties, as the modulation of several intracellular signaling pathways. The presence of the dystrophin-DGC in areas involved in cognitive functions suggests that lack of Dp427 may be responsible for the neurological disturbances described in DMD patients. These could be further aggravated by the glucocorticoid (GC) therapeutic treatments of the muscular inflammation in DMD patients. As the hippocampus is one major GC target, in this study I analyzed whether *in vitro* (acute) and *in vivo* (acute and sub-chronic) treatments with either corticosterone (CORT) or dexamethasone (DEX) affected the already compromised hippocampal neuron physiology. Under any conditions we analyzed several parameters of the neuronal response to GCs: a) protein levels of the glucocorticoid receptor (GR) and of its phosphorylated (active) form *p*GR; b) mRNA levels of GR and GILZ; c) changes in the intensity of GR and *p*GR immunohistochemistry; d) protein levels of GR intracellular signaling effectors (i.e. caveolin 1, ERK 1/2); e) proliferation of hippocampal neural progenitor cells (NPC) (*in vivo* sub-chronic treatment only). In both *in vitro* and *in vivo* studies, *mdx* mouse hippocampal neurons respond differently than wild type to GC treatments. The general picture emerging is that they could be more sensitive to GCs and, therefore, more predisposed to be damaged. In fact, even acute GC administrations elicit a response similar to the more damaging

chronic administration: i.e. reduction in GR levels, increase in the ration *p*GR/GR, possible reduction in GR gene expression, all aspects that are connotative of a chronic stress response. During high level of stress, which correspond to high and prolonged levels of secreted GCs, several physiological responses are altered, including those typical of hippocampal activity: i.e. synaptic plasticity, cognitive functions. These are accompanied by a reversal of the GC effects on hippocampal neurons: from the promotion of neuronal activity, and hence of its inhibitory control over the HPA axis, to its reduction, with consequent depression of HPA axis activity and increase in GC secretion. These are the basis for psychopathologies, as post-traumatic disorders. Therefore, the already compromised activity of the hippocampus in dystrophic subjects could be further damaged even by mild doses of GC, amplifying the risks for serious neural hilliness. Another crushing data is that sub-chronic treatments with DEX induce an increase in the proliferation of NPC in adult hippocampus, in contrast to what occurs in the wild type. This de-regulation of precursor cell cycle, responsible for of glia and neuronal self-renewal in adult brains, could further compromised hippocampal physiology.
In conclusion, in the hope that new therapies could extend the life span of the young DMD patients, it is important to go deeper in the comprehension of how hippocampus and other brain areas affected by DMD, respond to anti-inflammatory (GCs) treatments.

Introduction

1. The Duchenne Muscular Dystrophy and the dystrophin protein

The Duchenne Muscular Dystrophy (DMD) is the most common form of muscular dystrophy and the second most common genetically inherited disease, affecting approximately 1 in 3500 live male births (1). The disease is characterized by a progressive and devastating muscular degeneration, which primarily affects skeletal muscles, and subsequently cardiac and respiration muscles, causing premature death of DMD patients within their 20s (2). The disease is caused by the lack of a large cytoskeletal protein of 427 KDa called dystrophin (Dp427) (3).

The dystrophin gene, localized on the short arm of the X chromosome, is one of the largest human genes so far described, comprising almost 0.1% of the genome, (4) and consisting of 79 exons (5) encoding a primary transcript of 2400 kilo-bases. Due to its large size, this gene has a high mutation probability, so that nearly one third of DMD cases are non-familial (6). The dystrophin gene is quite complex, hosting at least eight independent and tissue-specific promoters. The full-length dystrophin isoform (Dp427), for instance, is transcribed by three independently regulated promoters, labelled as B (brain), M (striated muscle), or P (Purkinje cell), the respective letters reflecting the major sites of expression (7). Apart from the Dp427, five additional isoforms exist, produced by activation of different promoters along the gene or by alternative mRNA splicing. These shorter isoforms are named according to their molecular weights: Dp260 (predominantly in the retina), Dp140 (central nervous system and kidneys), Dp116 (peripheral nervous system), Dp71 (most tissues, but not muscles), and Dp40 (brain) (8, 9, 10).

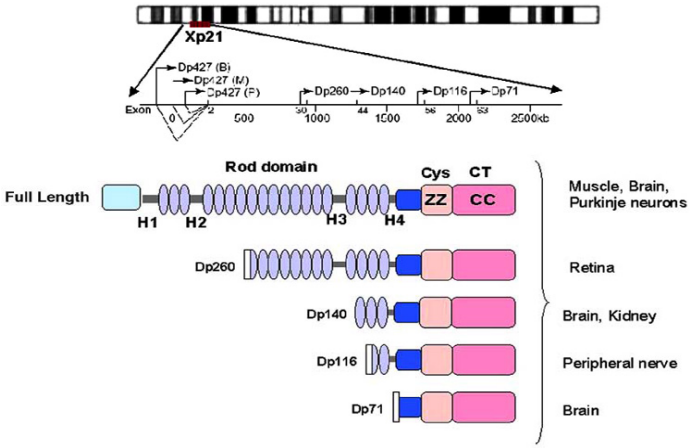

Fig 1: Schematic diagram showing the position of all promoters within the dystrophin gene and the molecular structure of the dystrophin isoforms. Tissue distribution of dystrophin and its isoforms are also indicated (11)

Muscle dystrophin is localized at the cytoplasmic face of the sarcolemma membrane and consists of an N-terminal actin-binding domain, a central large rod-like domain composed of spectrin-like repeats, and a cysteine rich C-terminus (Fig. 1) that connects to a multiproteic complex called the dystrophin-associated glycoprotein complex (DGC) (Fig. 2).

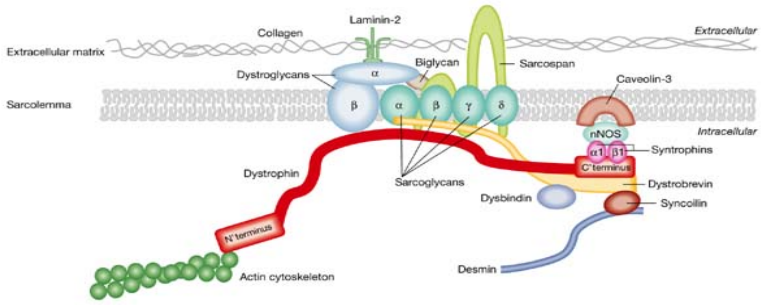

Fig 2: The dystrophin-associated glicoprotein complex in skeletal muscles (12)

Central protein of the DGC is dystroglycan (DG), composed by the transmembrane β-DG and the extracellular α-DG, sarcoglycans (α, β, γ and δ), sarcospan, syntrophins (α1, β1, β2, γ1, γ2), and dystrobrevin (13). In this complex, while the β-DG binds to dystrophin, which in turn links to the cortical actin filaments, the α-DG binds to extracellular matrix (ECM) proteins, as laminin. Therefore, the DGC provides a link between cytoskeleton and ECM (14), which is thought to protect the muscle plasma membrane (sarcolemma) from mechanical damages. Sarcoglycan is the second transmembrane component of the DGC, connected to β-DG and dystrobrevin. On the cytoplasmic side, instead, dystrophin directly interacts with syntrophins and dystrobrevin, which recruit other scaffolding proteins onto which signalling proteins (i.e. nitric oxide synthase) and ion channels are anchored to the plasma membrane (15). In this way, the DGC provides both a physical and functional connection between the internal and external environment of muscle cells.

In DMD, the absence of Dp427 results in the destabilization of the DGC, which not only hinders muscle integrity, but also induce secondary changes reflecting an impairment in intracellular signalling, as could be the reduction in nitric oxide synthesis and, hence, protein nitrosylation (16).

2. Dystrophin expression and localization in the nervous system

Selected neuronal populations (within hippocampus, cortex, cerebellum, autonomic ganglia) and glial cells (i.e. oligodendrocytes, Schwann cells) of both central and peripheral nervous systems also express Dp427, some of its isoforms and proteins composing the DGC (17). In brain, the Dp427 is primarily located in the hippocampus, prefrontal cortex, amygdala and cerebellum (18, 19). Here, similarly to skeletal muscles, it associates to the DGC, although several brain DGC variants exist because of the various dystrophin isoforms, alternatively spliced variants, and the presence of other DGC components, such as β-dystrobrevin, ε-sarcoglycan, and the γ-syntrophins (Fig. 3), which are not expressed in muscle. Moreover, the Dp427-DGC localizes not only in domains along the plasma membrane, but also at post-synaptic specializations, in which the complex does not bind to laminin but to the pre-synaptic protein neurexin (Fig. 3) (15).

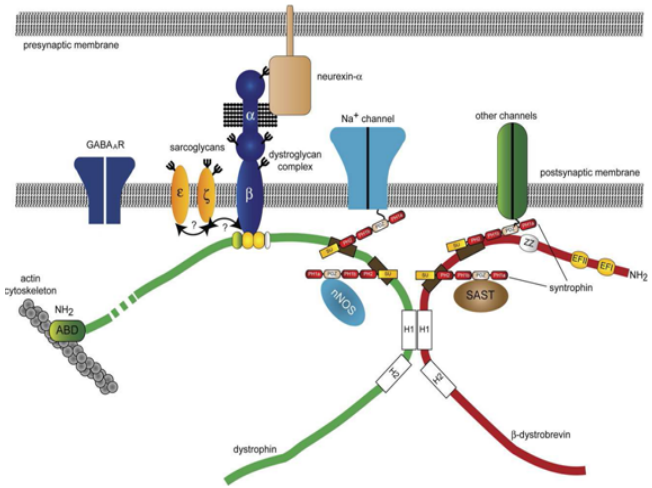

Fig 3: Neuronal DGC localized within post-synaptic specializations. When Dp427-DGC are localized in the post-synaptic domains, dystrophin binds to β-dystrobrevin and the dimer β-DG/α-DG, which in turn associates with the pre-synaptic protein neurexin-α. This contributes to synaptic stabilization. In addition, part of the DGC is also α1- and γ1-syntrophins, absent in muscles. These proteins, by binding Dp427 and dystrobrevin, are involved in the stabilization of ion channels within the membranes, among which voltage-gated Na^+ channels. In addition, syntrophin also bind and stabilize at the DGC the neuronal nitric oxide synthase. The all complex is also implicated in the stabilization of post-synaptic receptors, as $GABA_A$ receptors. (Abbreviations: DGC dystrophin-associated glycoprotein complex; nNOS neuronal nitric oxide synthase; SAST syntrophin-associated serine/threonine kinase; ABD actin-binding domain; EFI/EFII EF hand domains; ZZ zinc finger domain; H1/H2 helical domains) (15)

Due to these differences, the DGC in the nervous system is often referred to as a DGC-like (15, 20). This complex associates not only with the Dp427, but also with all its shorter informs. Localization of full-length and short dystrophin

isoforms is cell-specific: for example, Dp427 is mainly neuronal, although has also been described in oligodendrocytes; Dp260 and Dp140 are highly represented in the retina, especially in neurons of the inner nuclear layers (21); Dp140 has also been found in perivascular astrocytes, along the Dp71, the most ubiquitous dystrophin isoform (22, 16, 8, 23); Dp116 is mainly expressed by Schwann cells of peripheral nerves.

Full-length dystrophin is mainly localized in specific neuronal populations, as hippocampal neurons (all regions), cortical pyramidal cells, and cerebellar Purkinje cells (24). Major localization in adult individuals (both human and animal models) is at the postsynaptic sites, which has suggested a role in the maintenance of synaptic structure and function (16). The Dp427 has been found to co-localize with the γ-aminobutyric acid type A ($GABA_A$) receptors possibly through their linkage to syntrophins and gephyrin. This would prevent receptor diffusion (25), playing a critical role in their clustering, stabilization and synaptic signal transmission (26, 27, 1, 28, 29, 30).

In both autopsy brains of DMD patients and *mdx* mice (elective animal model of DMD), absence of Dp427 does not induce gross anatomical alterations, but a number of diversified cellular and sub-cellular abnormalities, among which: 50% decrease in cortical neuron number and neural shrinkage (31), abnormal dendritic development (32), altered post-synaptic density organization and pre-synaptic ultrastructure in hippocampus, mainly in the CA1 region (33, 34, 35, 17). In addition, in *mdx* mice, loss of Dp427 has been associated with a reduction in 40–70% of $GABA_A$ receptor clusters in CA1 and CA3 hippocampal neurons (containing the alpha-2 subunit), cerebellar Purkinje cells (containing the alpha-1 subunit), amygdala and cerebral cortex (containing both alpha-1 and

alpha-2 subunits) (36, 26, 37). $GABA_A$ alpha-1 and/or alpha-2 receptor subunit gene expression was also decreased in these brain regions. In brain, dystrophin deficiency has also been associated to a reduction in the response to nicotine in a passive avoidance memory task (engaging hippocampal activity) (38). This suggested a role of Dp427 in the stabilization of nicotinic acetylcholine receptor (nAChR) subtypes, similarly to $GABA_A$ and glutamatergic receptors. Whether all these alterations are the morpho-functional basis of cognitive dysfunctions has been a matter of discussion for long time, others failed to demonstrate this correlation (39, 17).

Because brain dystrophin is more abundant in the hippocampus compared to other sub-cortical areas (19), selective behavioural deficits involving hippocampal function were predicted to occur in the *mdx*. Indeed, several studies showed that dystrophin deficiency in *mdx* mice is associated with impaired memory retention at long delays, in certain procedural learning and spatial alternation tasks (40, 41), suggesting a role for Dp427 in the consolidation of certain forms of long-term memories. Absence of Dp427 also causes unbalanced calcium homeostasis (42), with consequent alterations in hippocampal long-term potentiation (LTP) (43), a form of plasticity widely believed to be critical for memory formation. Intriguingly, dystrophin deficiency in CA1 hippocampal neurons appeared to facilitate induction of short-term potentiation (STP) and depression (STD) of the glutamatergic transmission (41, 29), with no apparent alteration in the maintenance of the LTP phase (41). Recently, brain dystrophin was found in association with a sub-population of $GABA_A$ receptors at inhibitory synapses (36, 44) and the abnormal enhancement of hippocampal STP and STD was shown to be occluded by a $GABA_A$-receptor antagonist (29), suggesting that decreased inhibitory tone may be one possible

mechanism of the altered plasticity in dystrophin-deficient neurons. At the functional level, this induces alterations in other mechanisms of plasticity, such as the LTP of the population spike (PS), with important consequences on the output of the neuronal circuitry.

Duchenne De Boulogne was the first describing cognitive limitations in boys with DMD (45). General intelligence among boys with DMD is one standard deviation below the normal population mean IQ and mental retardation has been reported in approximately one third (34.8%) of patients (46). Moreover, boys with DMD can be affected by attention-deficit/hyperactivity disorders (ADHD) (1.7 %), autism spectrum disorders (3.1 %) and reading problems (20 % moderate, 20 % severe) (47). Mutations in the dystrophin gene can even cause intellectual disability in the absence of muscular dystrophy (48). However, since these cognitive deficits do not seem to depend on the location of the gene mutations, a clear genotype-phenotype correlation for cognitive impairment in DMD has yet been established (17, 20). As the gene encodes several dystrophin isoforms, the number of affected gene products and/or cell-type specific isoforms may correlate with the occurrence and severity of cognitive impairment (49, 20).

DMD also associates to peripheral nervous system (PNS) alterations. Autonomic dysfunctions have been reported in DMD patients (50), and a number of morphological and functional alterations have been reported by studies conducted in our laboratory on the sympathetic neurons of the superior cervical ganglion (SCG) of *mdx* mice. Specifically, loss of Dp427 induces an early (since post-natal day 0, P0) and persistent reduction in the SCG noradrenergic innervation of iris and heart, compared to wild type mice, which associates

with a significant loss in muscle-innervating ganglionic neurons between P5 and P10 (51). Moreover, all *mdx* mouse ganglionic neurons, regardless of the type of target they innervate, showed reduced defasciculation and terminal branching (51). De-regulated protein levels for components of the nerve growth factor (NGF) signalling system (i.e., NGF receptors TrkA and p75 NTR) (52), unbalanced proNGF/mature NGF ratio (52), and reduction in the NGF-mediated intracellular signalling cascade were also described (53). Moreover, a reduction in intra-ganglionic $\alpha 3, \beta 2/\beta 4$ nAChR stabilization (54) and activity (55), as well a different modulation in the expression of genes encoding proteins involved in neuron survival and differentiation (56) were also reported.

3. *Therapeutic approaches for DMD treatment*

Although DMD was discovered more than twenty years ago, there is currently no cure to resolve the disease. In fact, the most widespread pharmacological therapy focuses on the use of a glucocorticoid-based treatment, aimed to reducing muscle inflammation and the activity of the immune system, to alleviate the dystrophic phenotype and increase motor activity in DMD affected patients (57). The action of these drugs can improve muscle strength, ambulation and cardiac activity. However, this therapy is associated with multiple side effects, such as weight gain, CNS disorders, gastrointestinal and metabolic disorders (58). To date, new therapies based on genetic techniques, able to restore the products of the *dmd* gene, are being tested. In fact, by acting on the dystrophin pre-mRNA, it can induce the exclusion of exon containing the mutation and generate a truncated and partially functional protein. However, the presence of a semi-functional dystrophin improves the patient's motor skills, reducing the severity of

symptoms and lengthening life expectancy, but there were no significant improvements in cognitive function following treatment with drugs, mainly because the administration is intramuscular and the drug has a local action (59).
One of the main objectives in this area is to find a non-invasive treatment, effective both on the muscular and the nervous phenotype.

4. ***The glucocorticoid-mediated stress signalling***

4.1 Glucocorticoids and their receptors

Glucocorticoids (GC) are steroid hormones and the end product of following the activation of the hypothalamus-pituitary-adrenal (HPA) axis, which regulates stress responses. GC, like all steroid hormones, are synthesized from cholesterol, which undergoes multiple transformations in a multi-enzymatic process called steroidogenesis. GCs are neurosteroids, a large group of steroid hormones, which includes allopregnanolone and allotetrahydrodeoxycorticosterone (THDOC), testosterone-derived androgens (androstanediol) and estradiol. Neurosteroidogenesis occurs in the brain regions such as cortex, hippocampus, and amygdala. The type of steroid hormone synthesized in a particular endocrine gland is determined by the combination of the enzymes it expresses. The main enzymes for the synthesis of glucocorticoids are: 17-hydroxylase, 3β-hydroxysteroid dehydrogenase, 21-hydroxylase and 11β-hydroxylase (60).

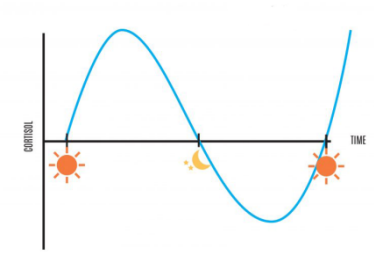

Fig. 4: Schematic ultradian fluctuation of GCs in healthy individuals

Two types of receptors mediate the GC effects: the mineralcorticoid receptor (MR) and the glucocorticoid receptor (GR), both expressed in several brain regions. Among these areas there is the hippocampus, in which the great abundance of GC receptors makes it an important relay for both stress appraisal and adaptative processes. Through MR, GCs influence the brain's appraisal of novel information and memory retrieval, and thereby influence behavioural coping responses. As GC concentrations increase in response to stressors, GR are activated to promote stress adaptation, reallocation of energy resources in preparation for future events and recovery of the system (61). One important target of GCs is the BDNF-signalling, which crucially contributes to the modulation of axonal guidance, synaptic plasticity and neurite outgrowth (62). MR, GR and the BDNF receptor, TrkB, are co-expressed in hippocampal neurons, supporting this region as the primary site of immediate interactions between the GC- and BDNF-signalling pathways (63). Ultradian fluctuations of GCs (Fig. 4) drive GR activation and reactivation, whereas MR occupancy is more constant and promotes excitability (64). This balance has implications for the genomic and non-genomic activity of adrenal steroids within target cells.

The GR belongs to the nuclear receptor superfamily, which includes receptors for steroid hormones (e.g. glucocorticoids, estrogens, androgens and mineralocorticoids) as well as receptors for other hydrophobic molecules, such as

prostaglandins, fatty acids and thyroid hormones. Nuclear receptors share a similar structural organization and mode of action, *via* transcriptional activation (transactivation) or repression of transcription (trans-repression). The GR consists of three main domains; the N-terminal domain (NTD), the DNA-binding domain (DBD) and the ligand-binding domain (LBD), with a hinge region between the LBD and DBD. This modular structure is broadly conserved across the nuclear receptor superfamily. The DBD is the most conserved region across the nuclear hormone receptor superfamily. It contains two "zinc finger" structural motifs, the N-terminal zinc finger binds specifically to the DNA response element (glucocorticoid response element, GRE), and the second is thought to be involved in protein-protein interactions, such as when the receptor forms a dimer. Once a dimer of GR binds GRE, it can recruit co-regulators and chromatin-remodelling complexes that modulate gene transcription rates by affecting the activity of RNA polymerase II (65). The LBD is localized at the C-terminal end of the protein. The structure of the binding pocket consists of 12 α-helices and four β-sheets that form a hydrophobic pocket to which the steroid hormone preferentially binds. This conformational structure is dependent on the co-association of the chaperone protein hsp90, which maintains the receptor in the open position in order to accept ligand binding (66). Once a GC binds the LBD, a conformational change leads to the dissociation of chaperones and exposure of the nuclear localization signals. Post-translational modifications of the NTD occur *via* phosphorylation and sumoylation. Human GR phosphorylation occurs at serine residues 203 and 211 in response to hormone binding, and is thought to influence receptor localization within the cell, with phosphorylation of Ser203 causing the receptor to be cytoplasmic, and phosphorylation of Ser211 promoting

translocation into the nucleus (67). GR phosphorylation changes its interaction with cofactors and influence target gene expression (68).

4.2 The glucocorticoid receptor genomic response

Steroid hormones have a typical arrangement of four cycloalkane rings, and are derived from enzymatic processing of cholesterol. Their lipophilic nature allows them to enter the cell by diffusion through the plasma membrane, in order to bind the cytoplasmic GR, which is held in the open conformation by heterocomplex of chaperones, such as immunophilins and heat shock proteins (hsp). As said, this binding induces a receptor conformational change, dissociation of chaperone proteins, receptor phosphorylation and exposure of nuclear localization signals (NLS). This signal is recognized by importins, which are proteins for the import into the nucleus, and translocated into this compartment (69). Here, GRs dimerize and bind, *via* the zinc finger motifs to the GRE, which are conserved signals upstream to target genes. This binding can either enhance or repress gene transcription, depending on the GRE present and the availability of other transcription factors. If GRE is a promoter of transcription, bound GRs recruit co-activators and other transcription factors, leading to increased chromatin remodelling, recruitment of further co-factor, and eventual recruitment of RNA polymerase 2 (RNA Pol II). In case of a "negative" GRE (nGRE), GR binding causes repression of target gene transcription, possibly by blocking the promoter site, or other transcription factor binding sites. Rogatsky and collaborators (70) identified some genes directly regulated by GC binding to GRE. These include glucocorticoid-induced leucine zipper (GILZ), IGFBP1, and metallothionein 1X (MT1X). In particular, GILZ is involved in cell proliferation, epithelial sodium channel activity (71), and

plays key roles in GC signal-modulation, control of protein trafficking and signalling, modulation of T-lymphocyte and of other immune cell activation, IL-2 production and apoptosis (72, 73). GILZ interacts, inhibiting, with NF-κB, AP-1, Raf-1 and Ras, which are negative regulators of GC signalling, as they normally inhibit GC target gene expression, as trans-repression of pro-inflammatory genes (i.e., cytokines and their receptors, nitric oxide synthase, COX-2). Treatments with dexamethasone (DEX), a potent GR synthetic agonist, up-regulate GILZ expression, which mimics some of the GC effects, adding further immunosuppressive effects (74).

4.3 The glucocorticoid receptor non-genomic signalling

GC genomic response is necessarily a rather long process. Gene transcription and expression, along with post-translational processing of proteins, may take several hours, with the minimum time for an effect to be measurable of at least 15 minutes. However, GC also elicit a number of fast responses (within a few seconds to minutes), which are not sensitive to inhibition of both transcription and protein synthesis, and that can be observed also in cells that don't have a nucleus, such as platelets. These non-genomic signalling can be investigated by using GR inhibitors, such as RU-486, which inhibit the classical ligand-binding activation of cytosolic GR, or by stimulation with DEX conjugated to BSA (DEX:BSA). BSA complexing gives rise to a compound unable to cross the plasma membrane and approach intracellular receptors, hence triggering a signalling cascade, which must be *via* a membrane-associated receptor (75, 76, 77) (Fig. 5).

Several mechanisms have been proposed for the non-genomic effects of GC signalling. These include effects mediated by a membrane-associated GR (mGR), *via* GC ligand-binding to a different receptor to the GR, such as glutamate receptors, or

acting *via* G-protein coupled receptors, or having a direct effect on the membrane (75, 78). Effects of GC signalling to the cytosolic GR have also been proposed to elicit non-genomic effects via proteins released when the receptor complex is disrupted upon ligand binding. The effects observed have included activation of second-messenger systems, changes in ion flow, and activation of kinase pathways (79). Xiao and collaborators (80) demonstrated that GC rapidly and non-genomically activate ERK1/2, JNK, and p38 mitogen-activated protein kinases (MAPK) in GR-deficient neurons, but had no detectable effects on their neuronal viability. CORT rapidly activates both the cAMP-PKA pathways and the ERK1/2 pathway in neurons, important in the hippocampal response to stress (81, 82). Evidence for the involvement of the PI3K pathway has not yet been studied in the brain.

Over the last decade, highly ordered plasma membrane micro-domains with particular lipid and protein composition have been identified. There is evidence that these domains, termed "lipid rafts," orchestrate some control over intracellular signalling pathways and mediate cross talk between membrane-associated receptors (83). Current evidence suggests that the estrogen receptor is associated with a particular sub-set of lipid rafts termed "caveolae" (84). Caveolin-1, the major protein component of caveolae, has been implicated as a structural scaffold for the oligomerization and organization of cytoplasmic signal complexes (85). Interaction with, and modulation by, caveolin-1 has been shown in many signal transduction pathways, including those regulated by mGR. Caveolae can act as signalling organizers, and several works established a role for Cav1 in a rapid GC signalling pathway that triggers MAPK activation in embryonic mouse neural progenitor cells (NPC) cultures. In this case, one of the consequences of Cav1-dependent activation of MAPK by GCs

is an inhibition of intercellular communication between NPSCs coupled by gap junction (86). Studies with other steroid hormone receptors (i.e., androgen and estrogen receptor) have revealed mechanisms of cross talk between the genomic and the rapid GR responses (87).

MAPKs are a superfamily of serine-threonine kinases composed by the well-characterized extracellular signal-regulated kinases (ERK1 and ERK2 MAPK isoforms of 44- and 42-kDa, respectively), the c-Jun-NH2-terminal protein kinase/stress-activated protein kinase (JNK/SAPK) and the p38 MAPK (88). They are activated by diverse stimuli ranging from cytokines, growth factors, neurotransmitters, hormones, as well as under conditions of cellular stress and cell adherence. They play a key role in the regulation of cell growth, differentiation, cytoskeletal function, and gene expression (89, 90). As demonstrated in PC12 cells, GCs might act *via* its membrane receptor and activate ERK1/2 MAPK. ERK1/2 MAPK sustained activation results in its nuclear translocation (91, 92), which is then capable of regulating gene transcription itself (93, 94).

Current evidence suggests the estrogen receptor interact with caveolin-1 (84, 95). In addition, association of the GR with membrane lipid rafts has also been proposed (96), as confirmed by immunoprecipitation studies showing co-precipitation of GR and caveolin-1 (97). Moreover, as a control, loss of caveolin expression prevents GC inhibition of cell proliferation, an effect mediated by blocking the cells in G1/S transition (97). Recent data support integration of different signalling cascades to determine integrated cell responses (98).

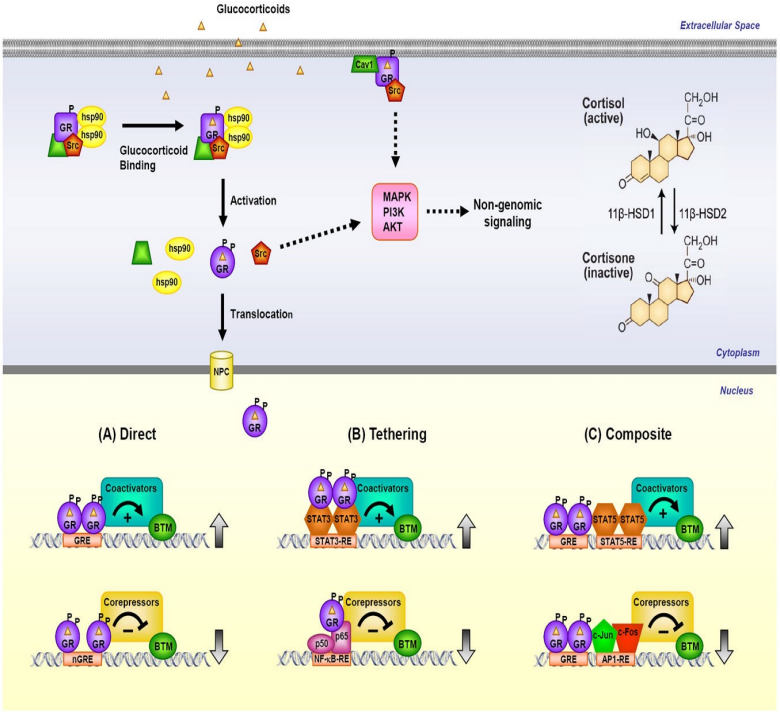

Fig 5: GR signalling pathways. Glucocorticoid-activated GR regulates gene expression by three main routes: (A) binding directly to DNA, (B) tethering itself to other DNA-bound transcription factors, (C) binding directly to DNA and interacting with neighbouring DNA-bound transcription factors. GR can also signal using a non-genomic pathway, by using membrane-bound GR and activating various intracellular signalling kinases. (77)

5. The stress response

Stress represents a condition that implies a modification of homeostasis, which may occur through a number of different events regulating emotion, behaviour, cognition as well as physical health. Indeed, stress represents the main environmental components for the susceptibility to mental illness, although it is known that the response to stress is

modulated by the genetic signature, as well as by a number of other factors, including earlier exposure to adverse life events that may have 'primed' the brain toward enhanced susceptibility. The majority of studies have reported that exposures to stress or elevated levels of corticosteroids impair performance on memory tasks dependent on the hippocampus (99, 100). Memory impairments have also been reported in transgenic mice with elevated corticosterone (CORT) production, due to central overexpression of corticotrophin-releasing factor (CRF). Recent findings of stress altering the firing properties of place cells in the hippocampus (101), which are thought to support spatial navigation and memory, are consistent with the stress effects on spatial memory tasks. The discovery of a relationship between stress and hippocampal LTP is significant, because it offers both a testable synaptic mechanism that may explain stress effects on memory and a "neurophysiological marker" to compare behavioural results from studies that use different stress paradigms. For example, a chronic restraint stress causes reduction in hippocampal volume from the pre-stress size (102), which determines reduction in the number of dendritic spines and branches of pyramidal neurons in the CA3 (103) and suppression of the production of new granule neurons in the dentate gyrus (DG) (104). The abundant presence of the GR in rodent and human hippocampus makes this brain structure, together with the hypothalamic paraventricular nucleus (PVN), very sensitive to the action of GCs and key to the regulation of the stress response (61, 105).

GRs are central in the regulation of stress response and other situation where GC levels are elevated (106, 61, 107), since basal levels of CORT will not occupy all the receptors, leaving some vacant for signalling the response to stress. Prolonged exposures to CORT have also been shown to cause

morphological and molecular changes, reduced neurogenesis and impaired synaptic plasticity in the hippocampus, physiological outcomes which are thought to precipitate hippocampus-dependent memory impairments and anxiety- and depression-like behaviours (108) (Fig. 6). These long-term effects of chronic CORT elevation on the hippocampus have been hypothesized to occur via epigenetic mechanisms (i.e., DNA methylation and histone modification) in the HPA axis (109).

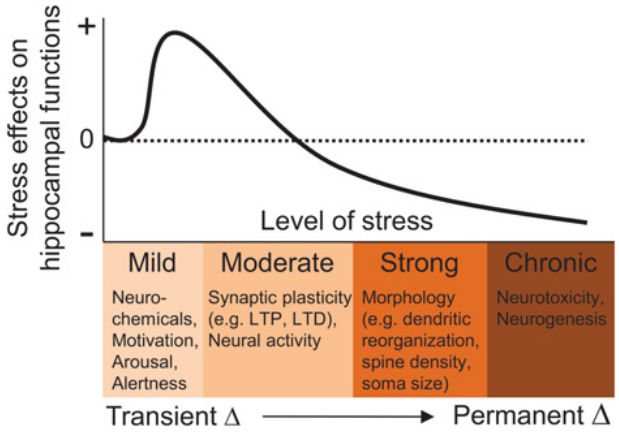

Fig. 6: Biological effects of stress on the hippocampus. As the severity (intensity, duration) of stress increases, alterations in neurochemicals, synaptic plasticity, neural activity, cytoarchitecture, and neurogenesis occur in the hippocampus that can influence subsequent cognitive functions, such as learning and memory, and contribute to psychopathologies. + and – represent an increase and decrease in hippocampal functioning, respectively. (110)

In response to stressful or threatening situations, corticosteroids are released by the HPA axis (Fig 7). Neuronal signalling to the PVN of the hypothalamus in response to

perceived danger causes the release of corticotrophin releasing hormone (CRH) and vasopressin, which stimulates the release of adrenocorticotrophic hormone (ACTH) from the pituitary gland into the blood stream. This is carried to the adrenal glands, above the kidneys, where it stimulates production of corticosteroids, such as mineralocorticoids and GC, from cholesterol by the adrenal cortex (61).

HPA axis activity is organized in a circadian rhythm with high levels in the morning, enabling individuals to cope with energy demands ahead of them. It is important to note that rapid HPA axis activation by acute stress and the subsequent turn-off of the HPA axis by the negative feedback response of CORT is healthy, as it helps an individual to cope with the stressor (111) (Fig. 7).

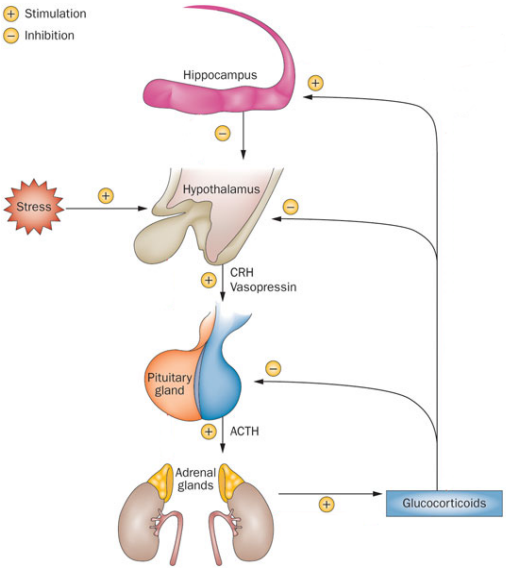

Fig 7: Under normal conditions, the presence of a stressor stimulates the

paraventricular nucleus of the hypothalamus to release CRH and vasopressin, which induce the release of ACTH from the anterior pituitary gland. ACTH stimulates the synthesis and release of glucocorticoids from the adrenal cortex. As a result of the deleterious effects of long-term exposure to glucocorticoids, a strict glucocorticoid-feedback mechanism, acting at the pituitary, hypothalamic and hippocampal levels, is fundamental to modulate the activity of the HPA axis. Particularly, the activation of hippocampal glucocorticoid-receptor-expressing neurons exerts a potent inhibition of the HPA axis. *Modified from Vitale et al., 2013*

In order to respond quickly to dangerous stimuli, the body must produce an adaptive behavioural response such as increased locomotion and risk assessment. A physiological response is also produced by the sympathetic action of the autonomic nervous system to produce the so-called "fight or flight" response *via* acetylcholine (Ach), epinephrine and norepinephrine signalling.

Corticosteroid signalling in the brain modulates the stress response, with mineralocorticoids affecting appraisal of the situation, and GC acting on consolidation of information (112). The hippocampal formation is a powerful upstream modulator of the HPA axis stress response and consequent emotional states (114). Imbalances in circulating levels of GCs can lead to defects in hippocampal functioning, and *vice versa* (106). The production of GC has an inhibitory effect on the HPA axis, inhibiting the release of CRH and ACTH, to decrease the release of further corticosteroids, this occurs in a delayed genomic manner, but effects have been found to occur within 5-15 minutes. The neurons of the PVN of the hypothalamus have a high number of GRs, which would account for the genomic inhibitory effect on the HPA axis. The production of ACTH at the pituitary is also inhibited in a fast, as well as delayed manner, and although the mechanism is still unclear (113), GR-dependent and GR-independent signalling may be involved (114).

6. ***Glucocorticoid effects on adult hippocampal neurogenesis***

The hippocampal formation is a powerful upstream modulator of the HPA axis stress response and consequent emotional states (115). Imbalances in circulating levels of GCs and stress context can lead to defects in hippocampal functioning and *vice versa* (106); among these is adult neurogenesis, which occurs at the DG level (116, 117). On the other hand, correct hippocampal neurogenesis is important in the hormonal regulation of stress response, as it gives rise to a small subset of neurons that are critical for a powerful negative control of the hippocampus over the HPA axis. Defects in hippocampus structure and function may be manifested by HPA-axis deregulation (118), which is associated with many forms of affective disorders (119). Conversely, enhancing neurogenesis can improve mood-related behaviour and restore central control over stress response systems (120).

The hippocampus is divided into three main sub-regions, named CA1, CA3, and DG. Neuronal cells derived from these three sub-regions are connected by the so-called tri-synaptic pathways. It is generally assumed, but not proven, that information processing by this pathway is crucially involved in learning and memory formation. Neurogenesis occurs only in the DG and there is no evidence that other hippocampal regions generate new neurons (121). Adult hippocampal neurogenesis refers to neural stem/progenitor cells (NSPCs) present in the sub-granular zone (SGZ) of the hippocampal DG, where they continue to produce new neurons during adulthood in many species, including human (122, 123). New neurons have been implicated in various hippocampus-related functions and disorders, such as spatial learning and memory, pattern separation, epilepsy, anxiety, depression and dementia (124, 125, 126, 127, 128). Recent reports further indicate that new

neurons also play an important role in HPA axis feedback regulation after stress: mice lacking new hippocampal neurons have a slower recovery of CORT levels to baseline after stress. Other studies also indicated that hippocampal new born neurons play an important role in stress regulation, as increased adult hippocampal neurogenesis exerts antidepressant effects, improves behaviour and regulation of stress response (118, 120, 129, 130, 131).

Both acute and chronic stress can suppress one or more phases of adult hippocampal neurogenesis (132, 133, 117). For example, predator stress rapidly raises GC levels, which cause significant reductions in hippocampal NSPC proliferation, (134, 135). While many, if not all, of these effects are generally attributed to increased GC levels, a simple interpretation of the effects of corticosteroids on the regulation of adult hippocampal neurogenesis is difficult to provide and it is important to realize that many other variables can influence both hippocampal neurogenesis and the way GCs regulate it (136, 117). Therefore, the reactions of the endocrine system to stress, and the results of that activation on neuronal function, are modulated by other events and may vary in complex ways. The sum of this pattern determines how a given stressor alters adult hippocampal neurogenesis in a given individual at any one time. Multiple stages of the neurogenic process are affected, including proliferation, as subsequent neuronal differentiation, connections to output pathways (e.g. CA3) and dendritic growth. Stress not only reduces NSPC proliferation and adult hippocampal neurogenesis, it may also control subsequent NSPC fate specification and differentiation through the action of the GR, which has important consequences for hippocampal network connectivity, function and behaviour (137). Direct effects of GCs on NSPCs have been demonstrated in the absence of known stressors, showing that the GR plays a

central role in mediating the direct effect of CORT on hippocampal NSPCs, as selective reduction in GR expression in new born cells results in altered neurogenesis (137). Clearly, the effects of stress and stress hormones on adult neurogenesis are complex. Firstly, neuronal stem cells in the SGZ of the DG are located in a specialized microenvironment, the so-called neurogenic niche, consisting of numerous different cell types, including astrocytes, ependymal cells, blood vessels, interneurons, oligodendrocytes, and myeloid cells, i.e., microglia cells and dendritic cells. All this cell types may modulate adult neurogenesis. For example, depending on the type of microglia and on the challenge, activated microglia cells release cytokines that may have detrimental or beneficial effects on adult neurogenesis (138). Also, these cell types express GRs indicating that stress-induced GC elevation targets these cells, which may modulate the rate of neurogenesis. Secondly, the nature of the stressor is an important factor.

NSPC express both GR and cell-specific mechanisms regulating its activity at the level of intracellular trafficking, suggesting an important biological function for the GR in these cells (139, 140). Consistent with a role in NSPC differentiation, knocking down GR expression in the new born cells results in increased NSPC differentiation (137), demonstrating that direct effects of CORT on these cells via the GR exist as well.

An important question that emerges is what type of environmental factors regulating adult neurogenesis also affect GR levels in neuronal progenitor cells. Interestingly, early life events, such as maternal separation and parental care, are known to reduce GR levels at adult age by epigenetic programming of the GR promoter (141, 142, 143, 144) and associate with impaired adult neurogenesis (145, 146). Aging is associated with lower rates of hippocampal neurogenesis (147), impaired negative feedback of CORT on the HPA axis, and

reduced levels of the GR (148, 149). Chronic stress is another factor negatively affecting both GR levels and adult neurogenesis attenuating multiple excitatory and inhibitory signalling cascades through the GR (150, 151, 152).
GCs inhibit cell differentiation and synaptic development in the various brain regions, resulting in alterations in neural fine structure and functions (153, 154, 155). Prenatal stress diminishes neurogenesis in the DG of juvenile rhesus monkeys (156) and, throughout postnatal life, GCs exert suppressive effects on cell proliferation in the DG. Moreover, persistently high CORT levels decrease cell proliferation in the adult rat DG (157). DEX has been shown to produce cell loss in the pyramidal layers and DG (158) and elicit various effects on neural cells. It has also been reported to cause an arrest of HT-22 cells (mouse hippocampal neuronal cell line) in the G1 phase of the cell cycle (159) and inhibit platelet-derived growth factor-induced differentiation of HiB5 cells (hippocampal stem cell line) (160). The sensitivity of DG to glucocorticoids is of particular concern because DG is also an important brain center involved in the synaptic plasticity. The two aspects of neurogenesis, which includes proliferation and differentiation, could be independently affected by GC activation (161).

7. Glucocorticoids and DMD

CNS alterations associated to DMD are reported in patients and animal models. However, differently from muscles, nervous system defects are mainly established prenatally and difficult to identify, as functional alterations become apparent during growth. Different anatomical alterations induce diversified neurological disorders with different degree of severity. Among these, children and adolescent with DMD are at great risk of developing depression and anxiety disorders (162).

To date, there is no proximity to cure for DMD

neurological aspects, but new pharmacological treatments are succeeding in extending patients life span. Despite different innovative therapeutic approaches are in the stage of clinical trial, the treatment based on corticosteroid administration, which reduces skeletal muscle recurrent inflammation, continues to be the main adopted therapy (163). In healthy individuals, increase in GC synthesis and release follows the coordinated activation of the HPA and autonomic system. The ubiquitous expression of GR and the multitude of functions of GCs confer this system an essential role in the response to stress and restoration of homeostasis. Precise regulation of the HPA axis activity is very important for the organism; indeed, chronic exposure to GCs end up in various adverse side effects, such as osteoporosis, diabetes, hypertension, neurodegeneration (164) and inhibition of neurogenesis (111, 165, 161). On the other hand, a deficient HPA axis is observed in a wide range of autoimmune and inflammatory disease. GCs reach every organ by way of the circulation and cross the blood brain barrier, which allows the coordination of brain and body functions. Several experimental evidences underline the relevance of corticosteroids as structural modulators in the limbic areas. Both acute (single) and chronic (repeated) stress stimuli induce prominent changes in neuronal activity and synaptic functions, in hippocampus and medial prefrontal cortex, which rely on neuronal remodelling (i.e. dendrite shortening and pruning) (166). A great number of genes are also inhibited directly by GCs, through the formation of a repressor complex containing histone deacetylases (HDACs). Alteration, at any level, in GC mode of action is observed also in aging and in neurodegenerative diseases (167).

Aim and Scope

Goal of this research project is to explore the effects that increased levels of GCs exert in brain regions particularly susceptible to stressful stimuli, such as hippocampus, in DMD. The animal model used has been the *mdx* mouse, a genetic model of the disease in which a spontaneous point mutation in the exon 23 induces an anticipated stop codon in the dystrophin gene, resulting in absence of full-length dystrophin synthesis. Numerous experimental evidences are shedding a light on the role that Dp427 plays in different brain regions. Among these are hippocampus and cerebellum, which are field of demonstrated brain physiological failures and neurological disorders in both DMD patients and animal models. These areas are in part overlapping with those recognized as major targets of GCs, which are released at high levels in response to both emotional and physical stressors. This makes imperative to better investigate whether the mode of action of GCs in the brain of wild type and dystrophic mice is similar, or whether important differences related to dysfunction of the DGC complex, determined by lack of Dp427, can be revealed. Since DMD patients are subjected to repeated and prolonged application of corticosteroids, directed at lowering recurrent muscular inflammatory events, it is of some importance identifying factors, which could aggravate the already compromised neurological conditions of young DMD patients.

The first part of the project, conducted on wild type and *mdx* mouse hippocampal neurons *in vitro*, has been centered to uncover the response of these neurons, isolated from their physiological context and from other influence, to acute administration of different concentrations of CORT.

The second part of the project was performed by using an *in vivo* approach, and investigates the effect of acute and sub-chronic treatment with GCs on adult *mdx* and wild type

mice. CORT and DEX were used, respectively. In all experimental conditions, GR phosphorylation, expression, synthesis and cellular localization has been used as a marker of the response to GCs by hippocampus. This choice was based on the characteristic physiological regulation of GR levels by circulating GCs: GR modulation not only represents one of the first responses to GC, but it is also highly modulated by levels of circulating hormones and time of exposure.

The final part of the research compares the effects of a sub-chronic exposure to DEX on the proliferation rate of NPCS localized in the sub-granular zone of adult wild type and *mdx* mice hippocampus. This quantitative study was performed by labelling dividing cells with green fluorescent EdU, injected intra-peritoneum alone or in combination with DEX. The dramatic impact on hippocampal physiology by elevated and/or persistent levels of GC, and the negative regulation they have on both NPCS proliferation and subsequent neurogenesis in healthy brains has been well cleared by several studies.

The results obtained by this research highlights important aspects of the response of dystrophic mouse hippocampus, which could suggest future lines of research on the impact that GC-based therapeutic treatments have on the already compromised brain areas of DMD patients.

Materials and Methods

Animals

Wild type and genetically dystrophic *mdx/mdx* C57/Bl/10 mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were used. All procedures were conducted in accordance with guidelines established by National Istitute of Health and in accordance with the Code of Ethics of the EU directive 2010/63/EU. The experimental protocols were approved by The Ethical Committee of Animal Research of the Italian Ministry of Public Health.

Mice were housed in cages (max five per cage) and were maintained on a 12 h light–dark cycle with free access to food and water.

Primary hippocampal neuron cell cultures

Hippocampal neurons were cultured using reagents from Sigma-Aldrich (Milan, Italy), unless otherwise indicated. Briefly, hippocampi were dissected from embryonic day 18 (E18) mice embryos in Ca^{2+}- and Mg^{2+}-free HBSS, containing 3 mM HEPES (GIBCO, Milan, Italy) and antibiotics (100 unit/ml penicillin and 0.1 mg/ml streptomycin). Hippocampi were washed 5 times in HBSS and then incubated, for 15 min at 37 °C, with 0.5 % trypsin and 100 μg/ml DNAse in Ca^{2+}- and Mg^{2+}-free HBSS, transferred in fresh HBSS and washed 5 times. Dissociated cells were then obtained by mechanical dissociation, counted and plated on 35 mm Petri dishes (for Western blot, 1 x 10^6 cells per Petri dish) or on 12 mm glass coverslip (for immunofluorescence, 1 x 10^5 per coverslip), previously coated with 100 μg/mL poly-L-lisine (4 h at 37 °C) and 3 μg/mL laminin (overnight at 37 °C). Cells were grown in Neurobasal medium (GIBCO, Milan, Italy) containing antibiotics, B27 serum-free supplement (Invitrogen, Milan,

Italy), 0.5 mM glutamine and 23 μM glutamate, for 14 days (14 DIV), in humidified atmosphere, at 37 °C with 5 % CO_2

In vitro glucocorticoid administration

After 14 DIV, both wild type and *mdx* mouse hippocampal neurons were incubated for 1 h at 37 °C with either 1 μM or 10 μM corticosterone (CORT) (Sigma-Aldrich), diluted in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and glutamate free medium. Control cultures wee incubated with vehicle alone. Some of the cultures were, instead, incubated, for 1 h at 37 °C, with 10 nM dexamethasone (DEX) a more powerful GR activator, and used for immunocytochemical experiments (see the *"Immunofluorescence in vitro"* section)

In vivo glucocorticoid administration

Acute treatment. Six-seven week-old male wild type and *mdx* mice were randomly subdivided in three experimental groups: the CORT group (n=9/genotype), which received a single intra-peritoneal injection of 40 mg/kg of CORT, diluted in saline and containing 15 % of hydroxypropyl-h-cyclodextrin (HBC; Sigma Chemical), 0.1 % dimethyl sulfoxide (DMSO) and 0.1 % Tween-20; the vehicle group (VC) group (n=9/genotype), which was injected with vehicle alone; the control (CTRL) group (n=9/genotype), which was left untreated. Injections were delivered between 9:30 and 11:30 h. Mice were killed by decapitation after either 90 min or 6 h from injection, following Isoflurane (Merial, Milan, Italy) anaesthesia. In the case of mRNA and protein level evaluation, the hippocampi were rapidly dissected out and stored at –80 °C until use. Three mice/genotype of the CTRL and 90 min CORT groups were used for immunohistochemistry. In this case, mice were perfused as described in the appropriate section (see the "*Immunohistochemistry"* section).

Sub-chronic treatment: Six-seven weeks-old male wild type and *mdx* mice were randomly subdivided in three experimental groups: the DEX group (n=8 /genotype), which received a daily intra-peritoneal injection of 100 μg/kg dexamethasone (DEX) dissolved in saline as described by Kim *et al*. (161), for 9 consecutive days; the VC group (n=8 /genotype), which was injected with vehicle alone; the CTRL group (n=4 /genotype), which was left untreated. Injections were always delivered between 9:30 and 11:30 h. Twenty four hours after the last DEX injection, mice were sacrificed by decapitation, as described for the acute treatment, hippocampi were rapidly dissected out and stored at -80 °C until use.

Plasma corticosterone measurement

Animals were euthanized by rapid decapitation in a benchtop fume hood. Trunk blood was collected into tubes, and samples were centrifuged at 3000 rpm for 20 min at 4 °C; sera were collected and stored at -80 °C until assayed. Plasma corticosterone was measured by specific radioimmunoassay. A single experimenter was responsible in acquiring all blood samples. CORT was measured in 5 μl plasma sample using a commercial radioimmunoassay (RIA) kit (ImmunChemTM 125I Corticosterone RIA, MP Biomedicals, Orangeburg, NY) with 200 μl trace and 200 μl antibody as described by De Filippis *et al*. (168). The sensitivity of the assay was 7.7 ng/ml. All samples to be statistically compared were run in the same assay to avoid inter-assay variability.

Real-Time PCR

RNA extraction and retro-transcription

Total RNA was extracted from both cultured neurons (n=10) and brain tissue (n=6/9) by using Directzol RNA MiniPrep Kit

(Zymo Research, Irvine, USA), following manufacturer's instructions. Briefly, cell and tissue (a piece) samples were homogenated in 300 µl of TRIzol buffer, monophasic solution of phenol and isothiocyanate guanidine, and then was added the same volume of ethanol 100%. Each sample was transferred in a Zymo-Spin IIC spin column, within a 2ml collection tube, centrifuged at 10000 -14000 rpm for 1 min and the flow-through was discarded. After rinse, was added at the column the Dnasi I, for 15 min at RT. Dnasi I was diluted in DNA digestion buffer 1:16. After digestion and rinse with Direct-zol RNA PreWash and RNA Wash Buffer, the column was transferred in a sterile tube and the RNA was eluted in 30 µl di Dnase/Rnase-Free Water. RNA was quantified by using the Thermo Scientific NanoDrop 2000 spectrophotometer.
Retrotrascription was performed using miScript II RT Kit (QIAGEN, Hilden, Germania). Mix solution was prepared into a sterile, nuclease-free tube (10% di miScript Nucleics Mix, 20% di miScript HiFlex Buffer, 5% di miScript Reverse Transcriptase Mix), to which was added 150 ng of RNA sample template. Samples were gently mixed and incubated for 60 min at 35 °C. The reaction was blocked by heating samples at 70 °C for 5 min.

Quantitative RT-PCR
For each sample, were amplified 10 ng di cDNA (QuantiTech fast SYBR Green PCR Kit, Quiagen) using mix solutions composed by 2X QuantiTech SYBR Green PCR Master Mix and 10X QuantiTech Primer Assay in ultrapure water. Three technical repetitions have been made for each primer. cDNA was amplified at 95 °C for 15 min, 94 °C for 15'', 55 °C for 30'', 72 °C for 30'', 95 °C for 15'', and finally a *melting* phase at 65-95°C for 15'' in a 7500Fast real-time PCR (Applied

Biosystem). Total number of cycles was 40. Primers pairs sequences are shown below:
Eif1α: Mm_Eif1_1_SG QuantiTect (QT02280348 Qiagen, Germany) GR: Mm_Nr3c1_1_SG QuantiTect (QT00160349 Qiagen, Germany) Dsip: Rn_Tsc22d3_1_SG QuantiTect (QT00404075 Qiagen, Germany) ATP50: CAACCGCCCTGTACTGTGCT (fw) and GGATTCAGAACAGCCAGAGACAC (rw)**;**

Western Immunoblot

Primary antibodies

Antibodies used for Western Immunoblot were: rabbit anti-GR (D6H2L), rabbit anti-*p*GR-Ser 211, rabbit anti-p44/42 MAPK (Erk1/2), rabbit anti-phospho-p44/42 MAPK (*p*Erk1/2 Thr202/Tyr204) and rabbit anti-caveolin1 (Cav1), were from Cell Signaling Technology (Leiden, Netherlands) and diluted 1:1000 in 5 % BSA (Bovine Serum Albumin), 0.1 % Tween 20 in TBS (Tris Buffered Saline) 1X. Mouse anti-caveolin2 (Cav2) (1:500; BD Transduction Laboratories, Milan, Italy), rabbit anti-β-actin (1:1000; AbCam, Cambridge, UK), and mouse anti-GAPDH (Glyceraldehyde Phosphate Dehydrogenase) (1:5000; AbCam, Cambridge, UK), were diluted in 3 % BSA, 0.1 % Tween20, 0.05% NaN_3 in TBS 1X.

Preparation of cell lysates and tissue extracts

Cell lysates were obtained from hippocampal neuron cultures (10^6/Petri dishes) (n=3/genotype/treatment). After removing the culture medium, neurons were washed with PBS and Petri dishes placed on ice. Cells were lysed with 50 µl RIPA buffer (50 mM Tris/HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 % SDS, 1 % Triton X-100, 10 µl/ml cocktail of protease inhibitors, 1 mM PMSF, 0.2 mM Na_3VO_4 and 1 mM NaF),

applied for ~5 min before scraping cells and transferring them in a 1.5 ml tube.
Tissue extracts were obtained from six-seven weeks-old male wild type and *mdx* mouse hippocampi (n=6-9/genotype/treatment) previously stored at -80 °C. A piece of tissue was placed in 400 µl of RIPA buffer and sonicated for 30 sec at a frequency of 30 kHz in an UP100H Ultrasonic Processor (Dr. Hielsher GmbH, Teltow, Germany).

Electrophoresis
Both cell lysates and tissue extracts were centrifuged at 14000 rpm for 15 min at 4 °C and a measured aliquot of the supernatant was used to determine protein concentration by using Micro BCA Kit (Pierce, Rockford, IL, USA). Loading buffer (4X: 200 mM Tris/HCl pH 6.8, 4% SDS, 30% glycerol, 4% β-mercaptoethanol, 4% blue bromophenol) was added to the homogenates to a final 1X concentration. Twenty µg of protein of hippocampal neuron lysates and twenty five µg of hippocampal tissue homogenates were electrophoresed at 4 °C on a 10% SDS–PAGE gel (25 mA for each gel) in running buffer (Tris 25 mM, Glycine 192 mM, SDS 0,1% in distilled water) along with a pre-stained molecular weight markers (AbCam, Cambridge, UK) (245 KDa, 180 KDa, 135 KDa, 100 KDa, 75 KDa, 63 KDa, 48 KDa, 35 KDa, 25 KDa, 20KDa, 17 KDa, 11 KDa) and then transferred onto a nitrocellulose membrane (2 h, RT at 70 V) by using a transfer buffer made of Tris 50 mM, Glycine 380 mM, SDS 0.1%, Methanol 20 % in distilled water.

Protein revelation
Membranes were first incubated, for 2h at RT, in 5% dry milk (DM) in 1X TTBS (20 mM Tris HCl, pH 7.4; 500 mM NaCl;

0.05 % Tween20), to block non-specific binding sites, and then with either of the primary antibodies, overnight at 4 °C. After thorough rinse in buffer, membranes were incubated for 1h at RT in either anti-rabbit IgG or anti-mouse IgG secondary antibody, conjugated with horseradish peroxidase (HRP), diluted 1:5000 in 2.5 % DM in 1X TTBS. Antibody binding sites were detected by chemiluminescence (ECL) (Immunological Science) using luminol/peroxidase detection reagents. The intensity of the bands was evaluated by densitometric analysis using the ImageJ Software. The optical density (OD) of each protein band was normalized against the OD of actin or GAPDH bands, used as internal reference standards.

Immunofluorescence in vitro

Control CORT- and DEX-treated hippocampal cell cultures (n= 3 per genotype and treatment) were immunolabelled for GR, *p*GR and cleaved Caspase-3. Control and glucocorticoid-treated cultures (CORT and DEX) were washed in PBS and then fixed in 4% paraformaldehyde and 4 % sucrose in PBS, for 10 min at RT. After a rinse (3 x 10 min) in PBS, cells were incubated in the blocking solution [1 % BSA, 10 % NGS (Jackson Immuno Research, Suffolk, England) and 0.5 % Triton X-100 in PBS] for 1h at RT. Primary antibodies, rabbit anti-GR (1:1000), rabbit anti-*p*GR (1:1000), anti-caspase 3-cleaved (1:300) (Cell Signaling Technology, Leiden, Netherlands) and mouse anti-NeuN (1:100) (Millipore, Darmstadt, Germany) were diluted in 1 % BSA, 1 % NGS, 0.2 %Triton X-100 in PBS and incubated overnight at 4 °C. After a rinse (3 x 10 min) in PBS, cultures were incubated with the appropriate secondary antibody, either goat anti mouse IgG

Alexa Fluor 488 (Molecular Probes, Invitrogen), or goat anti rabbit IgG Cy3 (Jackson Immuno Research) diluted 1:1000 and 1:800, respectively, for 1h at RT. After a rinse in PBS (3 x 10 min), cell cultures were incubated in the nuclear stating Hoechst 33342 (Invitrogen, Carlsbad, CA), 1:10000 in PBS rinsed again (3 x 10 min) and mounted on glass slides with the ProLong Gold Antifade Reagent (Invitrogen). Cultures were viewed at an optical microscope (AxioScop2, Carl Zeiss, Jena, Germany) photographed with a video camera (AxioCam MRc5 Carl Zeiss, Jena, Germany), by using the AxioVision 4.8.2 SP3 software. Images were adjusted for brightness and contrast with the Photoshop software for aesthetic purposes only. GR and pGR immunofluorescence was analysed for intensity and localization, while caspase-3 immunopositive neurons were photographed at a 63X oil objective (Carl Zeiss, Jena, Germany) and counted. The percentages of apoptotic neurons after CORT treatment in both wild type and *mdx* mouse neuron culture were statistically analysed by the χ^2 – test.

EdU incorporation and detection

Six-seven weeks-old male wild type and *mdx* mice, sub-chronically injected with either DEX or saline, received from day 6 to day 9 (in combination with DEX or saline) (Fig 8) a daily intraperitoneal injection of 50 mg/kg b.w. of 5-ethynyl-2'-deoxyuridine (EdU, The Click-iT® EdU Assay, Invitrogen, Carlsbad, CA).

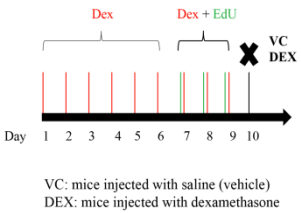

Fig. 8: Schematic representation of Dex and EdU treatment plan

EdU was dissolved in 0.9 % NaCl and 0.1 % DMSO and filtered through a 0.2 μm

syringe filter, in sterile conditions. Mice (n=4 /genotype) were sacrificed 24 h after the last DEX and EdU injections. Mice were deeply anesthetized with a mixture of Zoletil-100 (20 mg/Kg b.w.; Virbac, France) and Rompun® (8 mg/Kg b.w.; Bayer, Canada), diluted in saline, and perfused via the ascending aorta with a Ringer solution 1X (10X: 85g NaCl, 2.5g KCl, 2g $NaHCO_3$ per 1 l), followed by ~ 100 ml of fixative composed of freshly depolymerized 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were rapidly removed, cryoprotected for 2-3 d in a 30 %sucrose/saline solution, frozen on dry ice and stored at 80 °C until use. Coronal serial sections (30 µm/section) were cut at a freezing microtome (Leica), through the entire antero-posterior extension of the hippocampus, and collected free floating. Part of the series were immediately mounted on glass slides, while unused series were placed in an anti-freezing solution (NaCl 150 mM, PVP-40 250mM, Ethylene glycol 30%, Sucrose 50mM in PB 0,1 M) and stored at -80 °C until use. Sections were permealised with 0.5 % Triton X-100 in PBS for 20 min, rinsed twice in 3 % BSA in PBS, and then incubated for 30 min at RT and in the dark, with the Click-iT™ reaction cocktail containing: Click-iT™ reaction buffer, CuSO4, Alexa Fluor® 488 Azide, and reaction buffer additive. Successively, sections were rinsed in 3 % BSA in PBS and incubated with 5 µg/mL Hoechst 33342 in PBS, for 30 min, to allow nuclear staining. Slides were finally washed twice in PBS and coverslipped with the Prolong Gold Antifade Reagent mounting media.

Cell counting and statistical analysis

Hippocampi were viewed and photographed with a video camera at a Zeiss (AxioScop2, Carl Zeiss, Jena, Germany) fluorescent microscope, with a 20X objective, which allows including one entire hemi-hippocampus. EdU-positive cell

(EdU$^+$) were counted at the dentate gyrus (DG) level by using the Image J software: briefly, the DG area, evidenced by the Hoechst staining (as reported in Fig. 9), was manually outlined in one out of 5 brain sections (150 μm apart), throughout the entire hippocampus. EdU$^+$ cells were counted in the focal plane in which they appeared at their largest size, within the traced area, and final values were expressed as the number of EdU$^+$ cells/DG volume. DG reference volume was determined by summing the traced DG areas of each section and multiplying this number by the distance between the chosen sections.

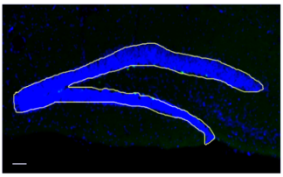

Fig 9: representative image of DG area, manually outlined by using Image J software. Scale bar: 100 μm

Peroxidase anti-peroxidase immunohistochemistry

Primary antibodies

Primary antibodies used for immunohistochemistry were: rabbit anti-GR (1:800) rabbit anti-*p*GR-Ser 211 (1:500)

Immunostaining

Control and 90 min CORT-injected wild type and *mdx* (n=3 /genotype/treatment) were perfused, as described above, with 4% freshly depolymerized paraformaldehyde in 0.1 M PB. Brains were dissected out, cryoprotected in 30 % sucrose in saline, for 2-3 days at 4 °C, and then cut at a freezing microtome in serial sagittal section (30μm), collected free floating in 0.1 M PB. Endogenous peroxidases were blocked by incubating sections, for 10 min at RT, with 10 % methanol, 3 % H_2O_2 in Tris 0.5 M pH 7.6. After 3 x 5 min rinses in buffer, sections were first incubated in 5 % DM, 0.5 % Triton X-100 in 0.1 M PB for 1h at RT, to block unspecific labelling, and

then with one of the primary antibodies, appropriately diluted in 1 % DM, 0.2 % Triton X-100 in 0.1 M PB, for two overnights 4°C. After a rinse (3 x 10 min) in 0.1 M PB, sections were incubated, for 1 ha at RT, with a secondary antibody raised in goat and directed against the IgG of the animal species in which the first antibody was raised: Goat anti-Rabbit IgG (1:100) or goat anti-mouse IgG (1:100) (Covance, Princeton, NJ, USA). After further rinses in 0.1 M PB (3 x 10 min), sections were incubated with the tertiary antibody raised in the species of the primary antibody and directed against the enzyme peroxidase. These formed complexes, called peroxidase-anti-peroxidase (PAP), which amplified the signal: rabbit PAP (1:100) and mouse PAP (1:100). After 3 x 10 min rinses in buffer, antibody binding sites were revealed by a 10 min incubation, in the dark at RT, in 0.05 % 3-3'-diaminobenzidine (DAB; Sigma) and 0.01 % H_2O_2 in 0.1 M PB. After a thorough final rinse, sections were placed on glass slides and mounted with Eukitt balsam. Sections were viewed at a Zeiss AxioScop2 light microscope and images acquired by a video camera, using the AxioVision 4.8.2 SP3 software.

Statistical Analysis

Unless differently indicated, all data were analysed by the two tails Student's *t*-test. Differences were considered statistically significant for $p \leq 0.05$. Data are expressed as a mean ± standard error of the mean (SEM).

Results

In vitro treatments

To evaluate the response of wild type and *mdx* mouse hippocampal neurons to acute GC administration, devoid of other systemic influences, primary neuron cultures were obtained from both genotype hippocampi and grown for 14 days. Either 1 μM CORT, 10 μM CORT or vehicle alone (DMSO) were added for 1 h to culture media, and cells incubated for 1 h in incubator (37°C, 5% CO2). Modulation by GCs on GR activation (protein levels of phosphorylated GR), synthesis (protein levels of total GR) and expression (mRNA levels) were evaluated.

GR protein levels in the hippocampus of E18 mdx mice are significantly lower compared to wild type

GCs are present in both maternal and foetal circulation and, in most species, increase in concentration towards term (169). In general, GC concentrations are higher in the mother than the foetus, and maternal GCs enter the placenta blood stream and reach the foetus. In mice, for example, 70–80 % of the circulating GCs in foetuses is of maternal origin, as foetal adrenal cortex is relatively inactive, or incapable of steroidogenesis (170). Environmental stressors, which raise maternal GC levels, also increase foeto-placental GC exposure (169). GCs can, therefore, cross the placenta in both directions, depending on the concentration gradient, and their bioavailability is modulated by the tissue 11β-hydroxysteroid dehydrogenase (11β-HSD) activity (171). In foetuses, GCs have even a broader range of functions, during both normal and adverse conditions, than adults (172). Towards term, they act as the primary maturational signal in the developmental sequence that prepares the foetus for the new challenges of extra uterine

life. However, by changing foetal tissue development, early exposure to excess glucocorticoids modifies the phenotype, with life-long physiological consequences (173). Based on these assumptions, we evaluated the baseline levels of GR and *p*GR in E18 mice hippocampi, the pre-natal date at which hippocampal cell cultures were prepared. This at the aim to ascertain whether there was already an ongoing modulation of the two protein levels consequent to recurrent mild inflammatory events occurring in DMD mice. As shown in Fig 10A, A', total GR protein levels in *mdx* mice were significantly lower compared to wild type, while no differences were present in the *p*GR protein levels. However, by comparing the ratio of *p*GR/total GR in each genotype, a higher proportion of GR is phosphorylated in *mdx* mouse neurons compared to wild type.

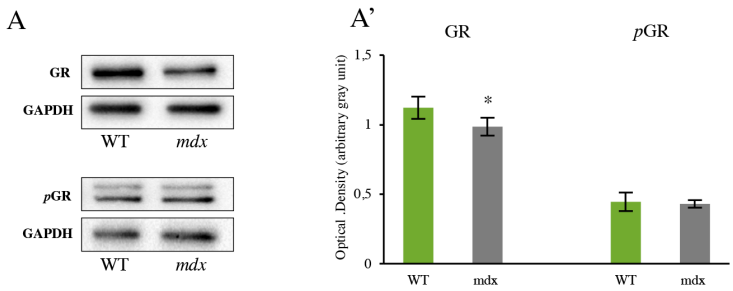

Fig 10. GR and *p*GR protein levels in E18 wild type and *mdx* mouse hippocampus. **A**: representative Western immunoblots of GR and *p*GR proteins in E18 wild type and *mdx* mice. **A'**: Corresponding densitometric analyses. Data are represented as the mean ± SEM of the optical densities (O.D.) of the GR and *p*GR immunopositive bands. Data are analysed by the two tails Student's *t*-test. n = 4 independent experiments. *p ≤ 0.05 WT vs *mdx*.

This difference may reflect a classical behavioural response of

the hippocampus to elevated, or long-lasting, exposure to GCs, which induce down-regulation of receptor expression/synthesis. To explore the hypothesis that this difference was due to elevated CORT levels in *mdx* pregnant mothers compared to wild type, one preliminary CORT radioimmunoassay (RIA) experiment, using trunk blood samples, has been performed. As expected from literature data (174) this first experiment showed high CORT levels in the wild type female mice (1275,248 ng/ml), due by enhanced activity of HPA axis after mid-gestation in healthy condition. CORT levels in the *mdx* pregnant female were higher (1669,903 ng/ml) than wild type, possibly due mild muscular degeneration also occurring in *mdx* female mice. This data, however, need to be confirmed and statistically analysed by further experiments.

GR and pGR protein levels after acute corticosterone treatment
GCs act on target cells by binding to both cytoplasmic and membrane receptors, and this binding determines receptor phosphorylation, which indicates its activation (77). Phosphorylation, which occurs mainly at Ser211, correlates with the GR transcriptional activity (68). As reported in Fig 11A', a significant increase in *p*GR levels was observed solely in wild type mouse neurons after 1 μM CORT treatment, compared to vehicle treated neurons. At this concentration, *mdx* mouse hippocampal cells showed just a tendency to increase *p*GR levels compared to vehicle, but this difference never reached significance. No differences were observed, for both genotypes, when neurons were treated with 10 μM CORT. Representative Western immunoblot are shown in Fig. 11A

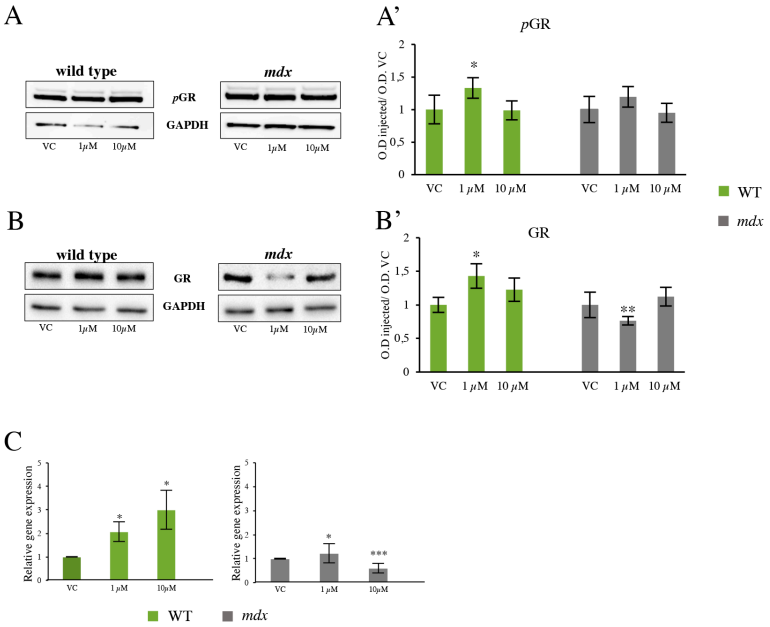

Fig 11: *p*GR and GR protein levels, and modulation of GR gene expression after 1 h CORT treatment.

A and **B**: representative Western immunoblots of *p*GR and GR protein levels in lysates from wild type and *mdx* E18 hippocampal neurons, cultured for 14 days and treated either with vehicle (VC), 1 µM CORT or 10 µM CORT. **A'** and **B'**: Corresponding densitometric analyses. Data are represented as the mean ± SEM of the optical densities (O.D.) of the bands immunopositive for *p*GR and total GR. **C**: relative gene expression of the GR gene 1 h after vehicle and CORT treatments. Data are represented as the mean ± SEM of the relative gene expression. All data were analysed by the two tail Student's *t*-test. n = 10 independent experiments. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001 vs VC.

GR activation is associated with the modulation of its own expression, and this event is thought to be involved in the negative feedback mechanism that the hippocampus plays on the HPA axis and hence, on GCs synthesis and release (175). Total GR protein levels were, then, analysed. As shown in the representative Western immunoblot (Fig. 11B) and corresponding densitometric analyses (Fig. 11B'), acute administration of 1 μM of CORT to wild type neurons induced a significant increase in the total GR levels, compared its respective control vehicle. In contrast, *mdx* mouse neurons underwent a significant reduction in GR levels. Once again, high CORT concentration (10 μM) did not induce changes in GR levels, for both genotypes.

GR mRNA levels are differently modulated in wild type and mdx mouse hippocampal neurons after acute treatment with high CORT concentrations

As expected following a genomic response to acute GR activation (176), GR mRNA levels in the wild type mouse neurons, evaluated by real time RT-PCR, increased in a CORT dose-dependent manner compared to vehicle (Fig. 11 C), although with a high variability at the 10 μM concentration. Differently, in *mdx* mouse neuron culture, a slight, but significant, increase was observed only after 1 μM incubation, whereas a significant reduction was observed when 10 μM were used (Fig. 11C).

These results suggest that, despite GR phosphorylation, dystrophic mouse neurons do not respond as a to a classical acute receptor activation, as wild type mouse neurons do. Instead, type of response resembles more what could be expected following a chronic administration, which progressively inverts the outcome of the genomic response,

inhibiting GR activity in hippocampal neurons more than promoting it.

Acute administration of high corticosterone concentrations promotes neuronal death

From these first results, appeared clear that, even in wild type mouse neuron cultures, the response to elevated CORT concentrations did not induce an increase in the levels of both *p*GR and GR, suggesting the 10 μM concentration could be lethal for cultured neurons, because not physiological (177). Therefore, to investigate this aspect, we conducted a quantitative study on the number of apoptotic neurons present in the three experimental conditions, by immunolabelling hippocampal neuron cultures for caspase 3-cleaved (Casp 3), a common apoptotic marker (Fig. 12A).

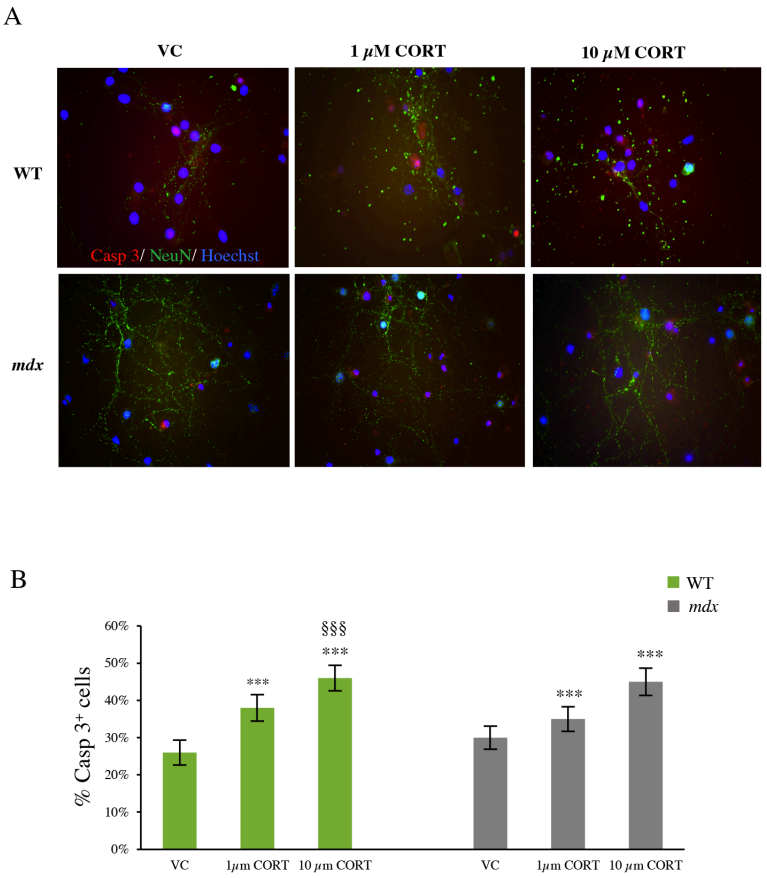

Fig 12. Quantitative analysis of apoptotic neurons in VC and CORT treated hippocampal neuron cultures. A: representative images of 14 DIV hippocampal wild type and *mdx* neurons from E18 mouse embryos, incubated for 1 h with either vehicle (VC), 1 μM CORT or 10 μM CORT. Neurons were co-immunolabelled for caspase 3-cleaved (Casp 3, red), an apoptotic marker, and NeuN (green), a neuronal marker. Nuclei were stained with the Hoechst nuclear dye (blue). **B**: quantitative analysis of caspase^{+} cells in the three conditions. Data are represented as the mean

percentage of apoptotic neurons ± SEM, and analysed by the χ-squared test. N = 10 independent experiments. ***p ≤ 0.001 vs CTRL; §§§p ≤ 0.001, 1 μM vs 10 μM within the same genotype.

The percentage of apoptotic neurons after both 1 μM and 10 μM CORT incubation significantly higher compared to the respective control, in both genotypes [wild type: 26 % (VC) *vs* 38 % (1) *vs* 45 % (10); *mdx*: 30 % (VC) *vs* 35 % (1) *vs* 46 % (10)], with a significant increase intra-genotype between the 1 μM and 10 μM conditions (Fig. 12B). These results confirmed the hypothesis that acute CORT treatment increases the physiological apoptotic activity of hippocampal neurons consequent to culturing procedures, an increase that at the 10 μM concentration doubles the number observed in control conditions.

Immunofluorescence analysis confirms glucocorticoid effects on hippocampal neurons disclosed by biochemical analyses

As described in the previous section, wild type and *mdx* mouse hippocampal neurons respond differently to acute administration of CORT. To further verify these results, we co-immunolabelled vehicle, 1 μM CORT and 10 nM DEX-treated hippocampal neurons for tubulin βIII (Tuj1; a neuronal marker) with either *p*GR or GR. DEX is a synthetic, specific and potent analogue of GCs, which we used as a positive control of hippocampal response to CORT treatment. Immunolabelling revealed a prevalent localization of GR within the nucleus, in all experimental conditions (Fig. 13). In wild type hippocampal mouse neurons, both intensity of immunolabelling and number of labelled nuclei increased slightly after both treatments, being somewhat more intense after 1 h DEX incubation. Differently, labelling in *mdx* mouse neuron culture remained apparently unchanged after GC treatment. Immunolabelling for *p*GR (Fig.

14), instead, was both nuclear and cytoplasmic, labelling cell somata and neurites, and often associated to the plasma membrane (enlargement in Fig. 14). In wild type mouse hippocampal neurons, the intensity of *p*GR immunolabelling increased after both treatments, being more intense after DEX incubation. Differently, in *mdx* mouse hippocampal neurons, the increase in *p*GR intensity of immunolabelling after both incubations was less prominent compared to wild type.

This data confirms the previously obtained biochemical results.

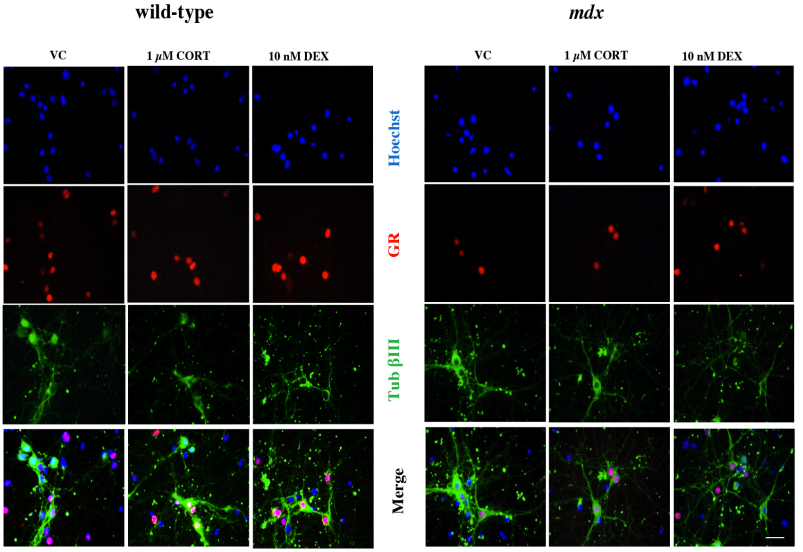

Fig 13. Qualitative analysis of GR intensity of immunolabelling in vehicle, CORT and DEX-treated hippocampal neuron cultures. 14DIV wild type and *mdx* mouse hippocampal neurons from E18 embryos are co-immunolabelled for βIII tubulin (Tuj1, green) and GR (red). GR immunofluorescence is prevalently localized within cell nuclei, in all experimental conditions: vehicle (VC), 1 μM CORT and 10 nM DEX. Both GC treatments induce a slight increase in the intensity of immunolabelling only in the wild type mouse neuron cultures. Nuclei are stained the Hoechst

dye (blue). Scale bar: 30 µm.

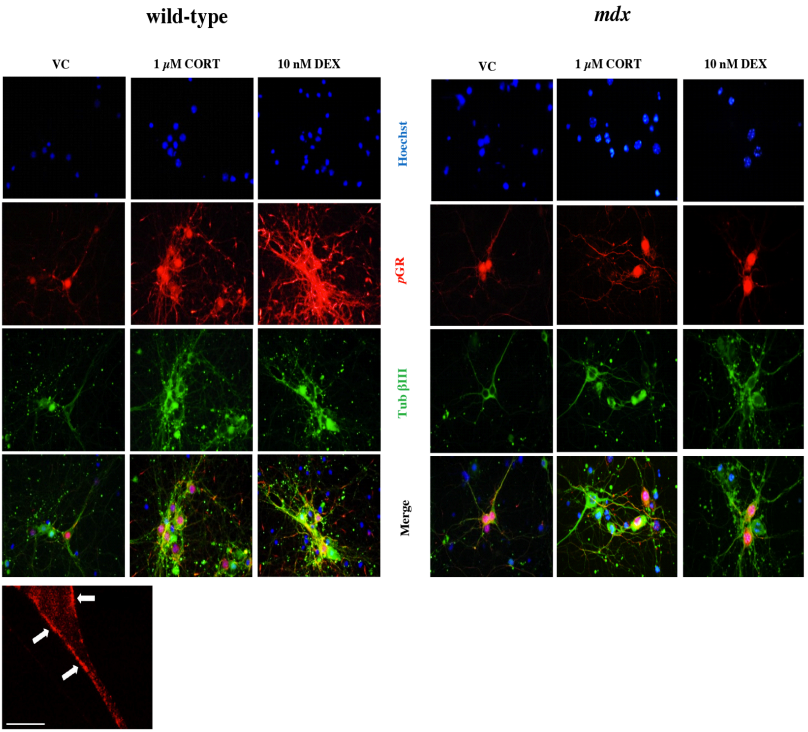

Fig 14. Qualitative analysis of *p*GR intensity of immunolabelling in vehicle, CORT and DEX-treated hippocampal neuron cultures.14DIV wild type and *mdx* mouse hippocampal neurons from E18 embryos are co-immunolabelled for βIII tubulin (Tuj1, green) and *p*GR (red). *p*GR immunofluorescence is localized within cell nuclei, somata and along neurites, often associating with the plasma membrane (arrows in the enlargement), in all experimental conditions: vehicle (VC), 1 µM CORT and 10 nM DEX. The intensity of immunofluorescence increases after both GC treatments; however, this increase in more evident in the wild type mouse neuron cultures compared to those from *mdx* mice. Scale bar: 30 µm; enlargement: 10 µm.

In vivo treatments

In healthy individuals, increase in GC synthesis and release follows the coordinated activation of the neuroendocrine HPA axis and the autonomic nervous system by both emotional and physiological stressors. The ubiquitous expression of GC receptors and the multiple functions of GCs confer this system a central role in the stress response towards homeostasis restoration. Autonomic and limbic circuits (e.g. amygdala, hippocampus) as well as the medial prefrontal cortex, strictly regulate HPA axis activity, as chronic exposure to GCs results in various adverse side effects. In the previous section of the results we showed that hippocampal neurons of *mdx* mice respond differently from wild type to acute CORT treatment *in vitro,* mimicking what has been reported to be a response to chronic exposure. However, if from one side neuron cultures were optimal for investigating, in a clean and controlled manner, fine intracellular responses to GC administration, an *in vivo* approach would give precious information on the structural plasticity to which these neurons undergo while included in their proper circuits. At this aim, adult wild type and *mdx* male mice (6-7 weeks-old) have been subjected to: a) acute treatment with CORT (one intraperitoneal injection of either 40 mg/Kg of CORT) and sacrificed after 90 min; b) acute treatment with CORT and sacrifice after 6 h; c) sub-chronic treatment with DEX (one intraperitoneal injection/day of 100 μg/kg of DEX, for 9 days) and sacrificed 24 h after the last DEX injection. Control mice of both genotypes received acute or sub-chronic injections of the corresponding vehicles. The hippocampi were dissected and processed for molecular biology (analysis of GR and GILZ mRNA levels), biochemistry (analysis of GR, *p*GR, ERK, *p*ERK, Cav1 and Cav2 protein levels). Part of the mice sacrificed after 90 min have also been perfused with opportune fixatives and brains

processed for immunohistochemistry for GR and *p*GR.

The choice to analyse two time points after the acute treatment aimed at uncovering possible differences between short- and long-term responses after acute treatments.

Ninety minutes after acute corticosterone treatment, GR protein levels are differently modulated in wild type and mdx mouse hippocampal neurons

As expected, 90 min after a single CORT injection, the *p*GR protein levels increased significantly compared to control, in both wild type and *mdx* mouse hippocampus, indicating that at this early time point GR has been activated (Fig 15A, A'). However, in *mdx* mouse extracts, *p*GR levels tended to increase also in the vehicle treated hippocampi, although they did not reach statistical significance. On the contrary, in wild type mice GR protein levels remained stable, as expected since 90 min *in vivo* is a time still too short to see eventual genomic effects. Differently, GR protein levels significantly decreased, compared to control, in both vehicle and CORT-treated groups of *mdx* mice (Fig 15B, B'), In addition, levels in these two groups were perfectly comparable, complying what has been reported by the literature on a peculiar susceptibility of *mdx* mice to stressors (178), which in this case could be both injection and the experimenter grasp and manipulation. Interestingly, the possible synergy between endogenous and exogenous GCs in the CORT group does not induce a stronger response compared to the vehicle group, suggesting that the system could be considered as "saturated", similar to sub-chronic or chronic conditions.

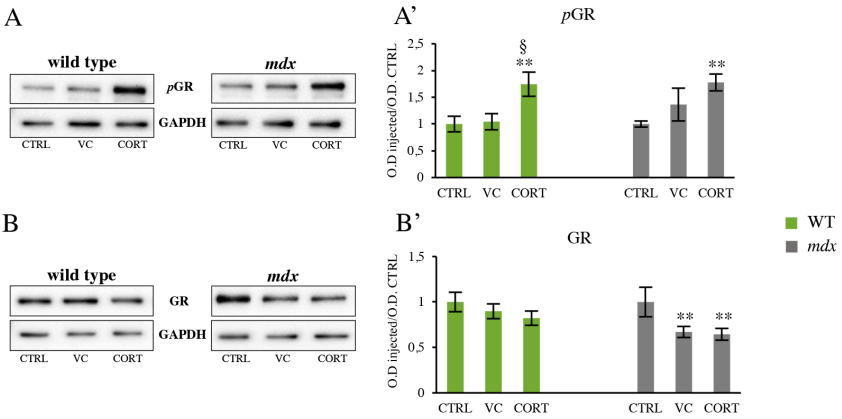

Fig 15. Hippocampal *p*GR and GR protein levels 90 min after acute CORT treatment. A and **B**, representative Western immunoblots of *p*GR and GR protein levels in the hippocampus of wild type and *mdx* mice either untreated (CTRL) or acutely injected with vehicle (VC) or CORT. **A'** and **B'**, Corresponding densitometric analyses. Data are represented as the mean ± SEM of the optical densities (O.D.) of the bands immunopositive for *p*GR and total GR and analysed by the two tail Student's *t*-test. n = 9 independent experiments. **p ≤ 0.01 vs CTRL; §p ≤ 0.05 vs VC.

Modulation of hippocampal GR and GILZ mRNA after 90 min from acute corticosterone treatment are different between wild type and mdx mice

Relative gene expression of GR and GILZ (the protein encoded by Dsip gene) were evaluated, by real-time RT-PCR, in the three experimental conditions, as indication of a genomic response to the acute CORT treatment. GILZ is a transcription factor important in the regulation of inflammatory responses, and is one of the early target genes of the nuclear *p*GR (179).

For this reason, its expression was used, along that of the GR gene, as an indicator of GR-mediate transcriptional activity. In wild type mouse hippocampus, GR mRNA levels did not change significantly, with respect to control, 90 min after either vehicle or CORT injection. Differently, in both experimental groups, GILZ mRNA levels were significantly up-regulated, suggesting an extreme reactivity to treatments (Fig. 16). Conversely, in *mdx* mouse hippocampi, the only difference compared to control animals was a significant reduction in the GR mRNA levels after CORT injection, suggesting an early de-regulation of the GR expression (Fig. 16).

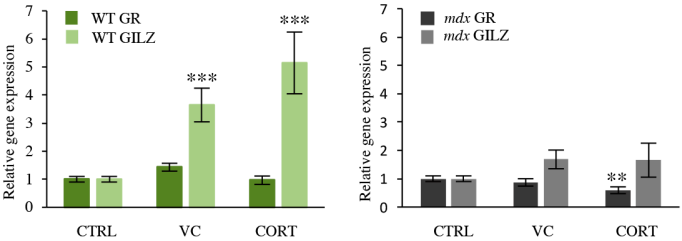

Fig. 16. Hippocampal GR and GILZ mRNA levels 90 min after acute CORT treatment. GR and GILZ mRNA levels were evaluated by real time RT-PCR in the hippocampus of control (CTRL), vehicle (VC) and CORT injected wild type and *mdx* mice. Data are represented as the mean relative expression ± SEM. n = 9 independent experiments. **p ≤ 0.01; ***p ≤ 0.001 *vs* CTRL.

Immunohystochemical analysis of pGR and GR localization

GR and *p*GR localization at the hippocampal levels before and after acute CORT treatment was also investigated by

immunohistochemistry with the immuno-enzymatic peroxidase anti-peroxidase method.

In CTRL conditions, GR immunoreactivity is intense in both wild type and *mdx* mouse hippocampus, with a marked distribution within the DG and CA1 hippocampal regions, while the CA3 region is only faintly labelled (Fig. 17). Ninety minutes after CORT treatment, GR distribution did not change, but the intensity of immunolabelling in the DG appeared lower compared to control, especially in the hippocampus of *mdx* mice. Differently, basal levels of *p*GR immunolabelling in control condition was extremely faint, in both genotypes, with immunopositive cells mainly localized in a zone within the posterior portion of the CA1 region. Ninety minutes after acute CORT administration, the distribution of *p*GR immunopositive neurons did not change, but the intensity of immunolabelling increased visibly in both genotypes, although appeared a bit more intense in the wild type mice (Fig. 17).

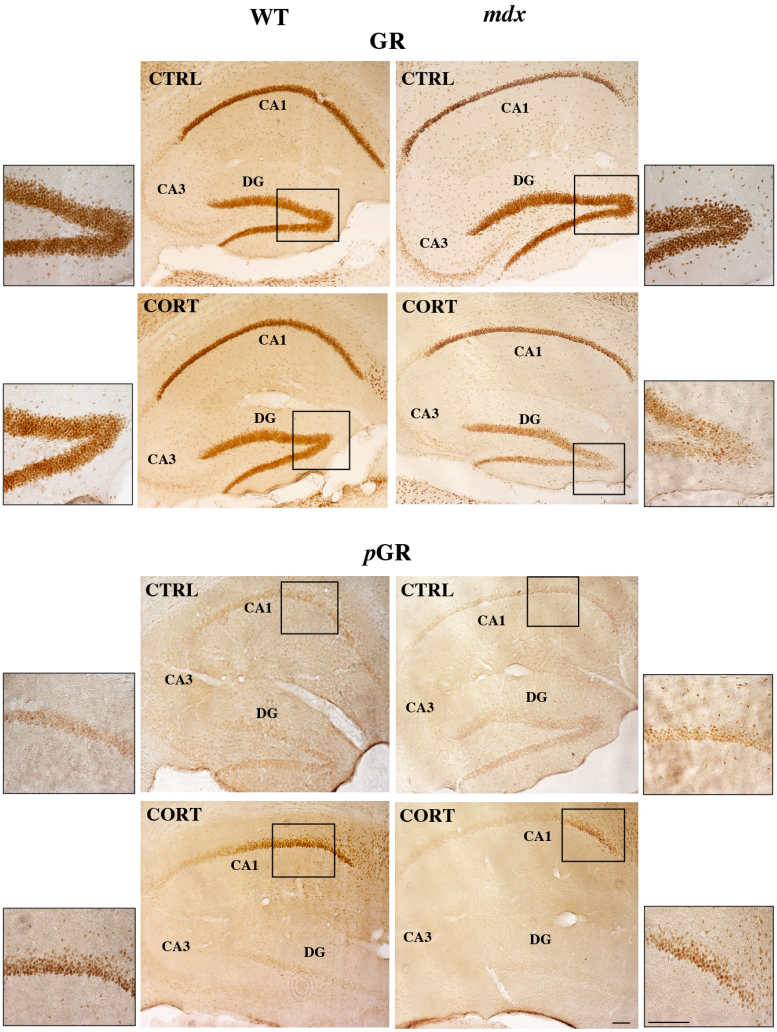

Fig 17: GR and *p*GR immunohistochemistry on mouse brain sagittal sections. GR and *p*GR immunolabelling was performed on control (CTRL) and CORT injected wild type and *mdx* mice by using the PAP method. Scale bar: 100 µM.

Analysis of the levels of proteins involved in the rapid non-genomic signalling mediated by glucocorticoids 90 min after acute corticosterone treatment

As previously mentioned, the rapid non-genomic GR signalling activates cellular signal pathways, active at various levels, including that of gene expression through its interaction with the GR genomic path. More precisely, its activation recruits proteins of the MAPK family, including the activation of ERK1/2, which operate both within the cell cytoplasm and nucleus, where they regulate the expression of target genes (97). This pathway seems to be finely modulated by the mGRs, which interact with caveolins, proteins component of lipid rafts (180) that, in turn, regulate both structure and function of these membrane micro-domains. Cav1 is also important for GR proper activation and for the recruitment of proteins downstream its signalling (97). An interesting aspect is that Cav1 could be stabilized within the membrane by the Dp427-DGC. It was therefore, important to investigate the effects of acute CORT administration also on this non-genomic pathway.

Ninety minutes after a single CORT or vehicle injection, Cav1 level were significantly increased, in CORT experimental group of wild type mice compared to vehicle and control mice. On the contrary, Cav1 protein levels did not significantly change in *mdx* mouse hippocampus, compared to their respective controls (Fig. 18A, A'), suggesting a signal de-regulation, possibly consequent to excessive levels of endogenous GC and/or GR destabilization.

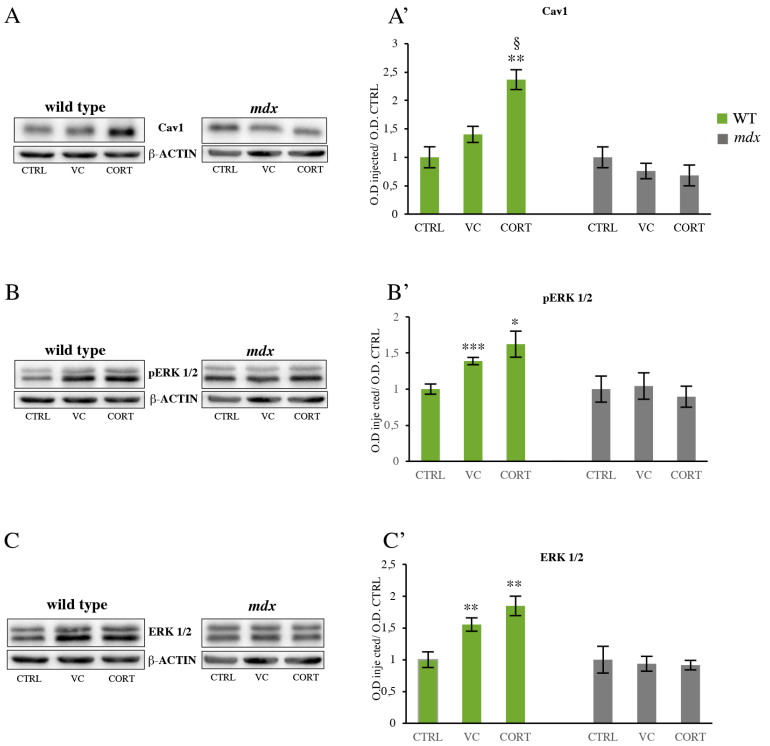

Fig 18. Hippocampal Cav1, ERK and *p*ERK protein levels 90 min after acute CORT treatment. A, **B, C**: representative Western immunoblots of Cav1, *p*ERK and ERK protein levels in the hippocampus of wild type and *mdx* mice either untreated (CTRL), or acutely injected with vehicle (VC) or CORT. **A'**, **B', C'**: corresponding densitometric analyses. Data are represented as the mean ± SEM of the optical densities (O.D.) of the bands immunopositive for each protein and analysed by the two tail Student's *t*-test. n = 5 independent experiments. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001 vs CTRL; §p ≤ 0.05 vs VC.

ERK 1/2 and its phosphorylated form is one of the best-known mediators of the non-genomic mGR pathway. After

acute CORT treatment, a significant increase in *p*ERK and total ERK protein levels were observed only in the wild type mouse hippocampus (Fig. 18B, C), suggesting a prompt activation of this pathway in response to GCs (86). No changes were, instead, observed after treatments (CORT and/or vehicle) neither in the levels of *p*ERK nor on those of total ERK proteins in *mdx* mice (Fig 18B, B', C, C').

Any situation perceived as potentially aversive will stimulate the amygdala-sympathoadrenal and hypothalamic-pituitary-adrenal (HPA) axis to increase plasma and brain levels of glucocorticoids (181). This physiological reaction to stressors results in enhanced arousal, appraisal, autonomic and cognitive performance in response to both prompt threat and vulnerability (182). Nevertheless, in terms of animal assays, a number of variables such as cage density, luminosity, time of the day, noise level, diet, animal handling and manipulation greatly affect sensitivity in stress assays (183, 184). To evaluate the effects of manipulation and injection in both genotypes, we performed one preliminary corticosterone RIA on trunk blood samples from adult male wild type and *mdx* mice either acutely injected with vehicle or not-injected. These very first data suggest an opposite trend between two genotypes: in wild type mice, vehicle injection seems to induce a decrease in CORT levels compared to control (control: 334,29 ng/ml; vehicle: 159,50 ng/ml). Differently, control *mdx* mice have blood CORT levels (104,56 ng/ml) close to those of vehicle injected wild type mice, suggesting a pre-existing "stressful" conditions, probably due to chronic muscles inflammation and functional hippocampal alterations. Vehicle injection increased CORT levels (340,94 ng/ml), suggesting that an increased stressful condition impacts hippocampal negative regulation of the HPA axis, as reported in the literature (110, 111). This very preliminary result, to be

confirmed and statistically analysed, reinforces the observation of an intrinsic high susceptibility of vehicle treatment, especially for *mdx* mice.

Six hours after acute corticosterone treatment, GR protein levels are differently modulated in wild type and mdx mouse hippocampal neurons

As previously stated, GCs induce both a rapid non-genomic response *via* membrane receptors and a slow, persistent genomic response *via* cytoplasmic/nuclear receptors (Fig. 19). Corticosteroid hormone levels in the brain are raised with a delay of approximately 20 min, compared to the rise observed in the plasma (185), and return to baseline after 1-2 h. Based on the data obtained after 90 min from acute CORT administration, we investigated long-term effects after a single CORT injection. At this aim, wild type and *mdx* mice were sacrificed 6 h from acute CORT or vehicle treatment, and brain tissue was prepared for biochemical and molecular analysis.

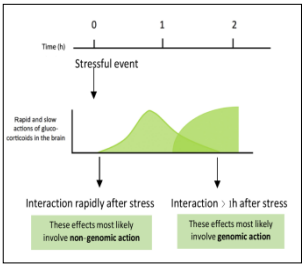

Fig 19. Time course of genomic and non-genomic responses to GCs by mGR and cytoplesmic/nuclear GR Modified from 213

As expected, after 6 h, levels of hippocampal *p*GR for both genotypes were similar to control, in both vehicle and CORT treated groups (Fig. 20A, A'), indicating that at this time receptors are not anymore activated. Differently, GR protein levels increased in the CORT-treated groups of wild type animals, suggesting either a positive control of its synthesis, or a decrease in its degradation, or both. In *mdx* mouse hippocampi, instead, GR protein levels significantly decreased in both CORT and vehicle- treated groups compared

to control (Fig. 20B, B'). This not only strongly suggests a deregulation of protein synthesis, as it occurs in chronic exposures to GCs, but also reinforces the observation of an intrinsic high susceptibility of vehicle-treated mice to stressful events.

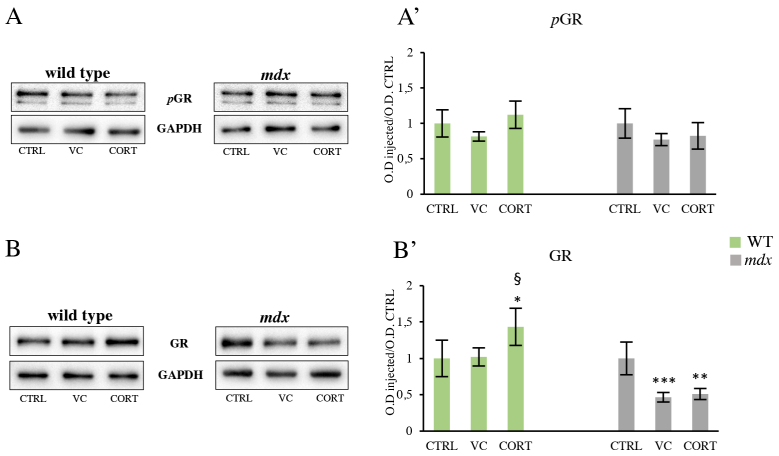

Fig. 20. Hippocampal *p*GR and GR protein levels 6 h after acute CORT treatment. A and **B**, representative Western immunoblots of *p*GR and GR protein levels in the hippocampus of wild type and *mdx* mice, either untreated (CTRL) or acutely injected with vehicle (VC) or CORT. **A'** and **B'**, corresponding densitometric analyses. Data are represented as the mean ± SEM of the optical densities (O.D.) of the bands immunopositive for *p*GR and total GR and analysed by the two tail Student's *t*-test. N = 6 independent experiments. **p ≤ 0.01, ***p ≤ 0.001 *vs* CTRL; §p ≤ 0.05 *vs* VC.

Interestingly, comparing GR basal protein levels between control mice of the two genotypes, we observed they were significantly higher in *mdx* mice (Fig. 21). No differences were, instead observed in the level of GR phosphorylation (not shown). This suggests that, in dystrophic mice, a possible exposure to endogenous GCs, because of muscle degeneration/inflammation, would alter the equilibrium of GC synthesis and/or degradation, determining its accumulation within cells. Since the levels of GR phosphorylation are not different between the two genotypes, large part of this receptor in *mdx* mice is possibly not phosphorylated and, presumably, not functional. However, these aspects need a deeper investigation.

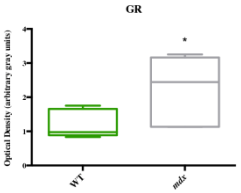

Fig. 21 The box plot compare values of the mean of optical densities (O.D.) of GR the immunopositive bands in wild type and mdx control mice. Data are reprensented as the mean ± SEM of 6 independent experiments

Modulation of hippocampal GR and GILZ mRNA after 6 h from acute corticosterone treatment are different between wild type and mdx mice

After evaluating the effect of long-term exposure to an acute administration of GCs on the level and activation of the GR, te next step was to analyze possible changes in GR transcriptional activity. As shown in Fig. 22, GR mRNA levels in both wild type and *mdx* mouse hippocampi did not change neither in vehicle nor in CORT treated mice, compared to control. Differently, GILZ mRNA significantly increased in both vehicle and CORT experimental groups of the wild type genotype, but remained unchanged in *mdx* mouse hippocampi.

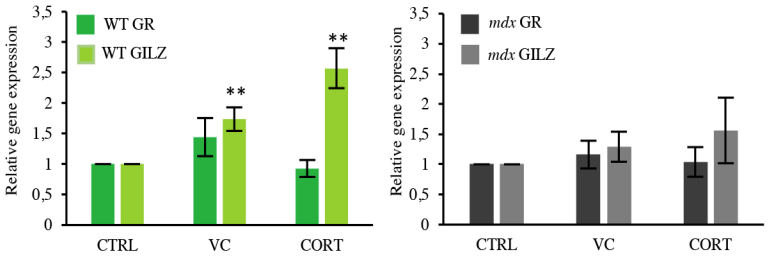

Fig. 22 Hippocampal GR and GILZ mRNA levels 6h after acute CORT treatment. GR and GILZ mRNA levels were evaluated by real time RT-PCR in the hippocampus of control (CTRL), vehicle (VC) and CORT injected wild type and *mdx* mice. Data are represented as the mean relative expression ± SEM. N = 6 independent experiments. **p ≤ 0.01 vs CTRL.

The intersting aspect of this result is that, once again, the modulation of this early target of GR activity is enanched in wild type mice only, in both injected groups (CORT and vehicle). Although these data needs a more accurate evaluation, it appears that, despite changes in GR phosphosylation and/or protein levels, in *mdx* mice the genomic response is not properly triggered, or at least not as much as it occurs in the wild type. This may rely on a defective receptor signaling mechanism/s, which in healthy organisms allows its own regulation.

Analysis of the levels of proteins involved in the rapid non-genomic signalling mediated by glucocorticoids 6 h after acute corticosterone treatment

As reported by Groeneweg (78), CORT inhibits the HPA axis within minutes and promotes rapid adaptive responses to stress. These effects are too rapid to be caused by gene-mediated

activity and do not require protein synthesis. Therefore, as already investigated for the 90 min group of mice, we evaluated the moduation of the non genomic response after 6 h from acute CORT treatment. Again, proteins analyzed were Cav1, *p*ERK and ERK, with the addition of Cav2. This caveolin is primarily associated with the Golgi apparatus, and its function is to chaperone proteins, which need to be inserted into the plasma membrane, including Cav1 (186). As shown in Fig. 23A, A', 6 h after treatment with either vehicle or GC, Cav1 protein levels in the wild type mouse hippocampus increased significantly in the CORT experimental group, correlating with the increased levels of GR previously described. Possibly, large part of the receptor is inserted into the membrane as a response to an acute, but prolonged, exposure to CORT and Cav1 is needed to stabilize lipid raft-associated GRs. In addition, similarly to GR, Cav1 levels in the *mdx* mouse hippocampus decreased, compared to control, in both vehicle and CORT treated experimental groups, supporting the hypothesis of a positive correlation between the amount of Cav and stabilized rceptors. However, it needs to be clarified that Cav in general are not only expressed by neurons, but also at high levels in the endothelial cells of the brain vessels and in astrocytes. This means that the changes observed in these experimental contexts may also include changes in other districts.

Differently from Cav1, in wild type mice Cav2 protein levels did not changes compared to control, after treatments (Fig. 23B, B'), but significantly decreased in both vehicle and CORT groups of *mdx* mice. This suggests alterations also in the mechanism of Cav1 stabilization into the plasma membrane through Cav2, independently from the type of cells.

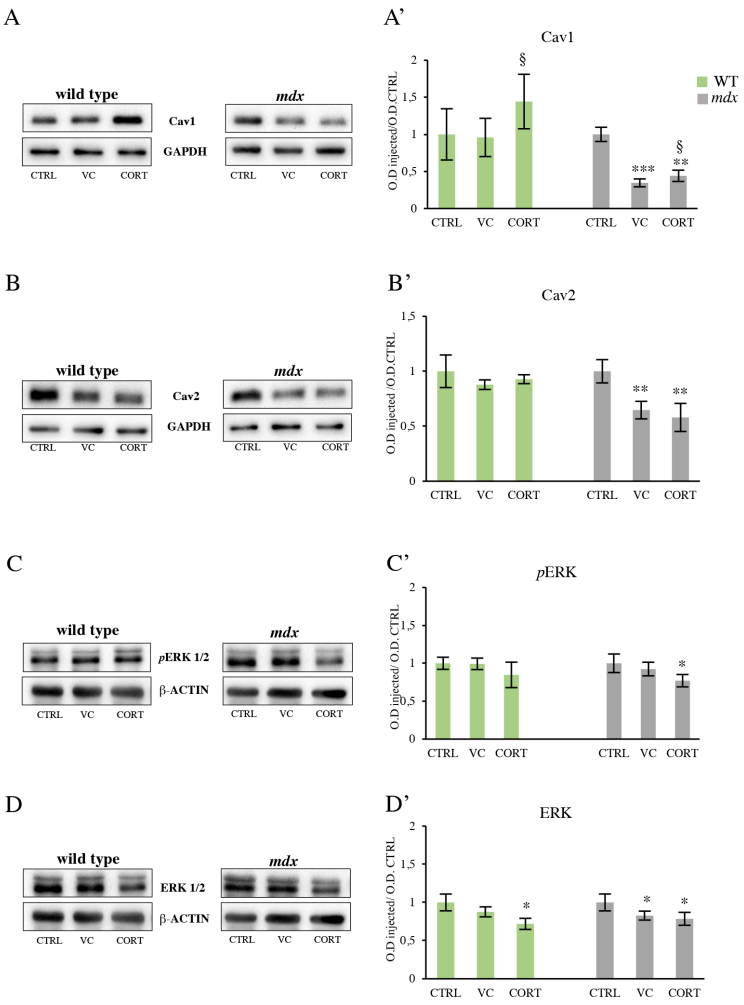

Fig 23. Hippocampal Cav1, Cav2, ERK and *p*ERK protein levels 6 h after acute CORT treatment. A, B, C, D: representative Western immunoblots of Cav1, Cav2, *p*ERK and ERK protein levels in the hippocampus of wild type and *mdx* mice either untreated (CTRL), or acutely

injected with vehicle (VC) or CORT. **A'**, **B', C', D'**: corresponding densitometric analyses. Data are represented as the mean ± SEM of the optical densities (O.D.) of the bands immunopositive for each protein and analysed by the two tail Student's *t*-test. N = 6 independent experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.01 vs CTRL; §p ≤ 0.05 vs VC.

Concerning MAPK, *p*ERK levels in the hippocampus of wild type mice tended to decrease following CORT treatment compared to controls, although this modulation did not reach significance due to a high variability. A significance that was, instead, reached in the hippocampus of CORT-injected *mdx* mice (Fig. 23C, C'). Total ERK protein levels, also significantly decreased in both genotypes after CORT (wild type) or both CORT and vehicle (*mdx*) treatments (Fig. 23D, D'). Therefore, non-genomic signaling 6 h after acute CORT treatment shows a general down regulation in *mdx* mouse hippocampi, which was only observed for total ERK in wild type mice.

In vivo sub-chronic treatment with dexamethasone

As already introduced, to date there is not yet a curative therapy for DMD, and dystrophic patients are treated with chronic administrations of GCs to ameliorate the devastating muscular inflammation. Therefore, after evaluating cellular and systemic responses of wild type and dystrophic mouse hippocampus to acute CORT administration, we performed a sub-chronic treatment *in vivo* by using DEX as exogenous GC. This is a powerful synthetic analogue of CORT, largely used in the literature for sub-chronic and chronic treatments (187, 188, 189, 190). Wild type and *mdx* mice were injected daily with a dose of DEX for 9 days. Twenty-four hours after the last DEX injection, mice were sacrificed and the hippocampus analysed by using biochemical assays to evaluate whether and in which direction long-term treatments modulate levels of all proteins

so far taken into consideration for the genomic and non-genomic response to GCs.

GR protein levels are de-regulated in mdx mouse hippocampi compared to wild type after sub-chronic dexamethasone treatment

As in the previous studies, *p*GR and GR protein levels were analysed in control wild type and *mdx* mice and after sub-chronic administration of either vehicle or DEX. As shown in Fig. 24A, A', *p*GR protein levels in the wild type mouse hippocampus significantly increased after DEX treatment. In *mdx* mice, this increase was revealed also in the vehicle treated group, indicating once again the extreme sensibility to stressful events of dystrophic mice. Moreover, on the long period, daily vehicle treatment induced even a stronger response when compared to DEX treatment, suggesting that increasing over a certain limit circulating GCs (endogenous *plus* exogenous) down-regulates somehow the amount of receptor ready to be phosphorylated.

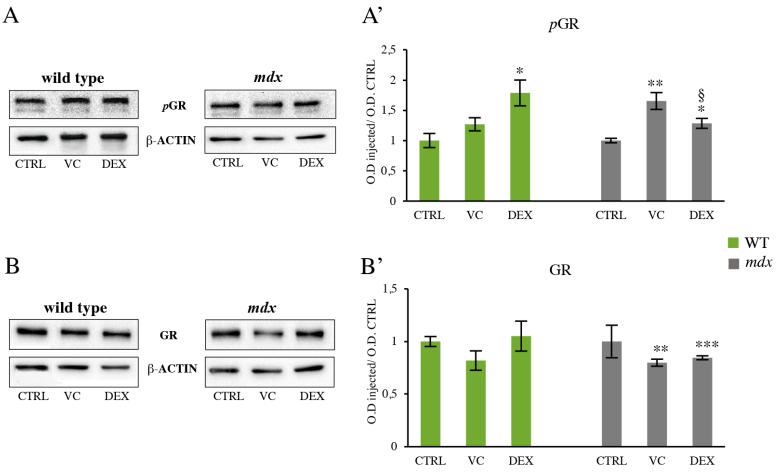

Fig. 24 Hippocampal *p*GR and GR protein levels after sub-chronic DEX treatment. A and **B**, representative Western immunoblots of *p*GR and GR protein levels in the hippocampus of wild type and *mdx* mice, either untreated (CTRL) or sub-chronically injected with vehicle (VC) or CORT. **A'** and **B'**, corresponding densitometric analyses. Data are represented as the mean ± SEM of the optical densities (O.D.) of the bands immunopositive for *p*GR and total GR and analysed by the two tail Student's *t*-test. N = 8 independent experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 vs CTRL; §p ≤ 0.05 vs VC.

At an increase in *p*GR in wild type mice did not correspond an increase in GR protein levels, which remained similar to control in both injected experimental groups (24B, B'). This is a clear indication that persistent engagement of GR does not always positively correlate with the amount of receptor. This aspect is even more dramatic in *mdx* mice, where hippocampal GR protein levels significantly decreased in both vehicle and DEX-treated mice. As it will be discussed later,

chronic exposure to GCs down-regulate GR expression and synthesis, an aspect that is deleterious for the hippocampal control on the HPA axis (110). These results indicate that in dystrophic subjects this cut off line is reached well before wild type individuals.

Modulation of hippocampal GR and GILZ mRNA after sub-chronic DEX treatment are different between wild type and mdx mice

After evaluating the effect of exposure to a sub-chronic administration of GCs on GR protein levels and activation, the next step was to analyze possible changes in the GR transcriptional activity. As shown in Fig. 25 GR mRNA levels in wild type mouse hippocampi did not change neither in vehicle nor in CORT treated mice, compared to control.

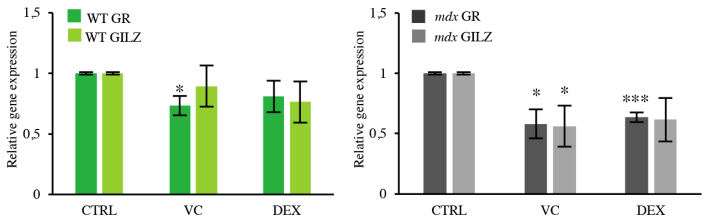

Fig 25. Hippocampal GR and GILZ mRNA levels after sub-chronic DEX treatment. GR and GILZ mRNA levels were evaluated by real time RT-PCR in the hippocampus of control (CTRL), vehicle (VC) and DEX injected wild type and *mdx* mice. Data are represented as the mean relative expression ± SEM. N = 6 independent experiments. *p ≤ 0.05; ***p ≤ 0.001 vs CTRL.

Differently, GR mRNA significantly decreased in both vehicle and DEX *mdx* mouse experimental groups. The interesting aspect of this result is that, in the wild type mouse hippocampus, GILZ mRNA remained unchanged after both DEX and vehicle administration, suggesting a block of this early genomic GR response on long lasting exposures to GCs. GILZ mRNA in *mdx* hippocampi, instead, significantly decreased after both vehicle and DEX treatment.

Analysis of the levels of proteins involved in the rapid non-genomic signalling mediated by glucocorticoids after sub-chronic dexamethasone treatment

Hippocampal protein levels of Cav1, Cav2, *p*ERK and total ERK were analysed after sub-chronic treatment. No changes were observed for any of the analysed protein between control and either vehicle or DEX-injected wild type mice (Fig. 26 A, A'; B, B'; C, C'; D, D'), indicating that over a long period this form of signalling is not active anymore. This is partly true also in *mdx* mice, with exception of Cav2 and total ERK, which significantly increased after both vehicle and DEX treatment, compared to control (Fig. 26 B, B'; D, D'), suggesting an over-production of these proteins, or a down-regulation of their degradation, as symptoms of a deregulated response to GR persistent engagement.

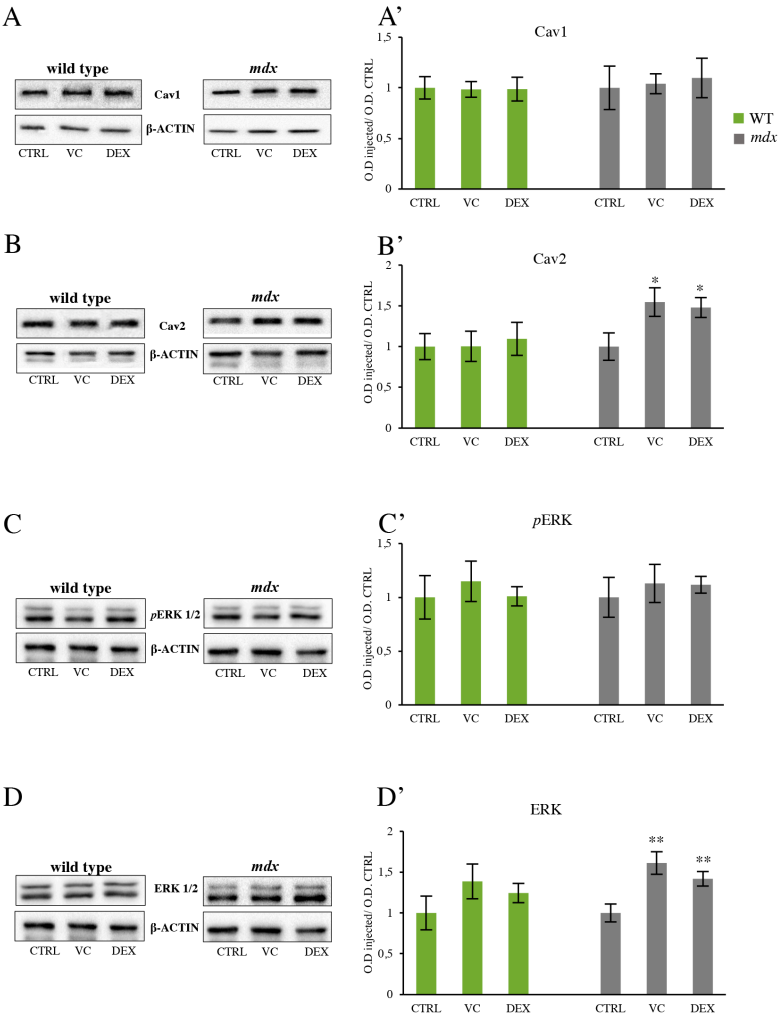

Fig 26: Hippocampal Cav1, Cav2, ERK and *p*ERK protein levels after sub-chronic DEX treatment. A, B, C, D: representative Western immunoblots of Cav1, Cav2, *p*ERK and ERK protein levels in the hippocampus of wild type and *mdx* mice either untreated (CTRL), or acutely injected with vehicle (VC) or CORT. **A', B', C', D'**: corresponding

densitometric analyses. Data are represented as the mean ± SEM of the optical densities (O.D.) of the bands immunopositive for each protein and analysed by the two tail Student's *t*-test. N = 8 independent experiments. *p ≤ 0.05, **p ≤ 0.01, vs CTRL.

Quantitative analysis of the neural precursor cell proliferation in the hippocampal dentate gyrus

Lack of Dp427 causes alterations in proliferation and differentiation processes due to unusual characteristics in neural progenitor cells. In *mdx* mice, alteration of intracellular calcium currents induces alteration in the control of cell cycle (191), and the reduced density of pyramidal cells in the CA1 region of the dorsal hippocampal suggests the presence of neurodegenerative and/or anti-neurogenic processes, which contribute to the onset of cognitive symptoms (192).

The general view about neurogenesis is that stress and stress hormones inhibit adult neurogenesis by inhibiting neural stem cell proliferation. Several studies in the early 1990s showed that administration of adrenal hormones in rats negatively affect the incorporation of 3H-thymidine, while removal of adrenal hormones by adrenalectomy boosters the appearance of 3H-thymidine labelled cells (110), suggesting an inhibitory role for stress-induced GCs in adult neurogenesis. However, the relationship between stress and adult neurogenesis seems far more complex than a simple inhibitory role. For example, running wheel performance and an enriched environment stimulates survival of new born cells and neuronal differentiation in mouse brain, while simultaneously increasing GC levels (193, 194). In addition, an acute 3 h of immobilization stress test, which induces elevation in plasma CORT levels, induce cell proliferation in the DG (195). In turn, hippocampal neurogenesis may facilitate normalization of GC levels after stress (118), suggesting a bi-directional relationship between adult hippocampal neurogenesis and regulation of the

HPA axis. Clearly, the effect of stress and stress hormones on adult neurogenesis is complex. These results can be explained by several factors, including the cell types involved, and the nature and duration of stress. GR expression in progenitor cells and immature neurons (139) also suggests a direct effect of circulating GCs on adult neurogenesis. Such a direct role is further suggested by the fact that GR activity, mRNA and protein levels are tightly regulated by several factors, which also regulate adult neurogenesis. The relationship between GR activity and neurogenesis is graphically shown in Fig. 27 (111): low levels of stress in mice kept in a poor environment, or with a sedentary lifestyle, induce low levels of proliferation and maturation. Controllable stress, like enriched environments, physical activity and learning, coincides with increased levels of GR activation and is associated with increased cell proliferation and correct integration of mature neurons. Too much GR activation, as during uncontrollable stress, negatively affects proliferation and neuronal integration.

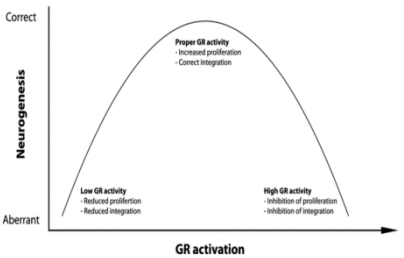

Fig. 27 Schematic representation of the relationship between GR activity and neurogenesis (111).

Based on these data, we evaluated the effects on NPC proliferation in both wild type and *mdx* mouse DG before and after sub-chronic treatment with DEX. The quantitative analysis was performed by labelling proliferating cells with EdU, injected intraperitoneally in the last three days of the DEX treatment.

At first, we evaluated and compared the number of EdU^+ cells in wild type and *mdx* mouse DG. As shown in Fig. 28, the number of proliferating NPS is slightly less in *mdx* mouse hippocampus compared to wild type, although this difference is still far from significance ($p = 0.13$) due to the low number of experiments so far performed ($n = 3$). When comparing the different experimental groups within the same genotype, we observed that in wild type mice the number of EdU^+ cells didn't change in the DG of DEX-treated mice compared to control, but significantly decreased compared to vehicle injected mice (Fig. 29, as also reported by Kim et al. (161). Interestingly, following vehicle injection we observed a significant increase in the number of EdU^+ cells, suggesting that a mild stress can promote cell proliferation, as reported above. Differently, in *mdx* mouse DG, DEX sub-chronic treatment induced a significant increase ($p \leq 0.05$) in the number of EdU^+ cells compared to control mice (Fig. 29). These data strongly suggest a signalling de-regulation of the cell cycle control in *mdx* mouse NPC, which could interfere with the physiological protective mechanism, aiming at reducing cell proliferation in adverse conditions.

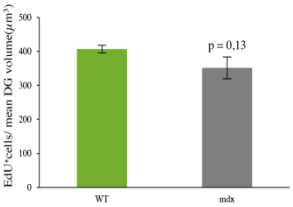

Fig. 28: Quantitative analysis of EdU^+ cell/mean DG volume in wild type and mdx mice. Data were expressed as the mean ± SEM and analyzed by the two tail Student's t-test. n = 3 independent experiments.

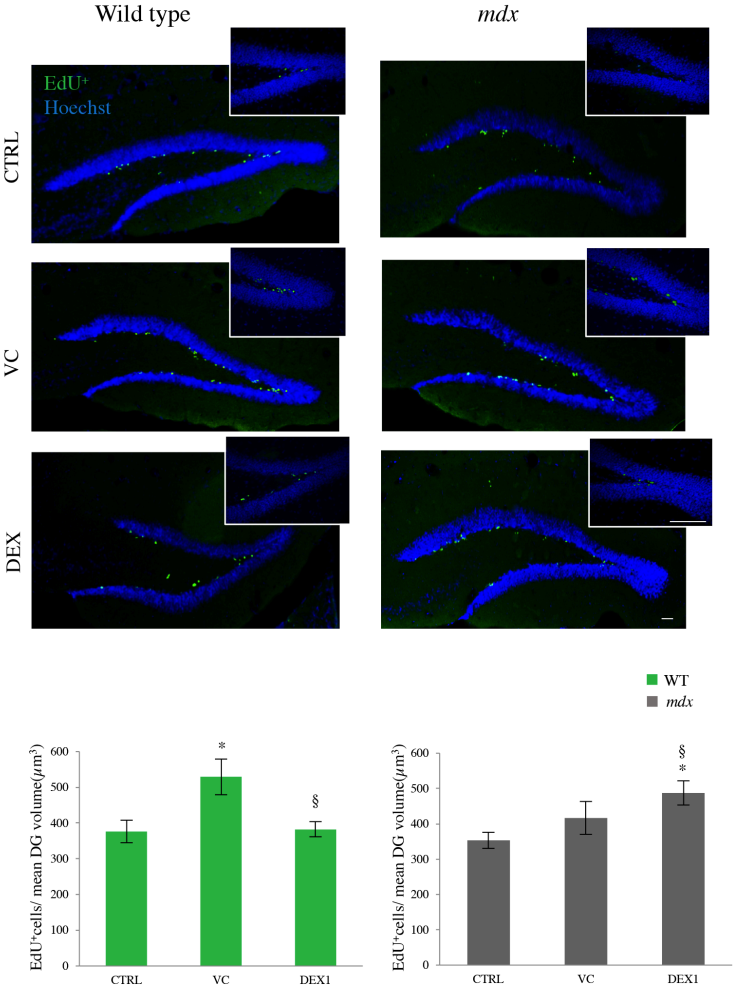

Fig. 29 Quantitative analysis of NPC proliferation at the DG of wild type and *mdx* mice. Proliferating, EdU^{+} (green), NPC are counted over the

entire DG volume of wild type (WT) and *mdx* mice of three experimental groups: control un-injected (CTRL), vehicle injected (VC) and dexamethasone injected (DEX). On the top of the figure, representative pictures, with enlarged insets, showing the EdU^+ cells distribution along the sub-granular zone of the DG. The graphs below report the quantitative analysis obtained by counting EdU^+ cells along the entire DG in serial cryo-sections. Numbers are expressed as the mean ± SEM of EdU^+ cells/mean DG volume and analysed by the Student's *t*-test. N = 4 independent experiments. *$p \leq 0.05$ *vs* CTRL; $^{§}p \leq 0.05$ *vs* VC. Scale bar: Low and high magnifications=100 μM.

Quantitative analysis of apoptotic cells in the hippocampal dentate gyrus

To evaluate whether the differences observed in cell proliferation between the two genotypes also corresponded to changes in cell death, we performed an immunofluorescence quantitative analysis for the presence of the apoptotic marker Casp on brain cryo-sections of wild type and *mdx* mice, before and after sub-chronic treatment with DEX. As shown in Fig. 30 B no changes were observed in the number of apoptotic cells in wild type DG after both VC and DEX treatment. Differently, in *mdx* mice, the number of apoptotic cell death tended to increase, especially in DEX injected mice. However, this increase did not reach significance because due to the low number of experiments so far completed. Interestingly, EdU^+ cells never colocalised with $Casp^+$ cells.

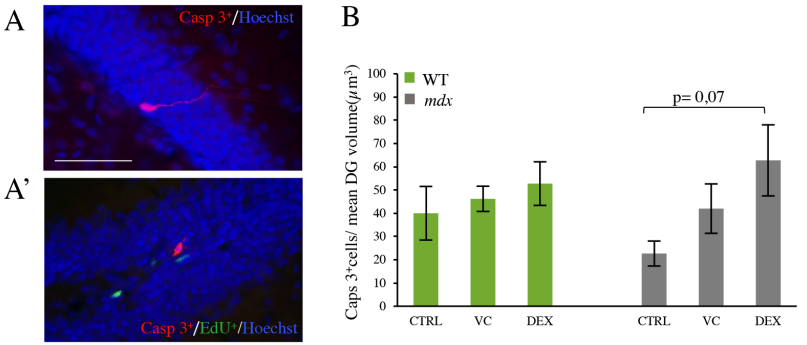

Fig. 30 Quantitative analysis of apoptotic cells at the DG of wild type and *mdx* mice A and **A':** representative images of Casp3$^+$ and EdU$^+$ cells in DG **B** Apoptotic cells are counted over the entire DG volume of wild type (WT) and *mdx* mice of three experimental groups: control un-injected (CTRL), vehicle injected (VC) and dexamethasone injected (DEX). The graph report the quantitative analysis obtained by counting Casp$^+$ cells along the entire DG in serial cryo-sections. Numbers are expressed as the mean ± SEM of Casp$^+$ cells/mean DG volume and analysed by the Student's *t*-test. N = 4 independent experiments. Scale bar =100 μM.

Discussion

Duchenne Muscular Dystrophy (DMD) is a recessive genetic disorder associated to the X-chromosome, which determines loss of the Dp427 dystrophin protein, and characterized by progressive muscular degeneration and non-progressive cognitive deficits, which arise during foetal development (192). Lack of Dp427 and, at various degree, of its shorter isoforms, induces several alterations in both autonomic (51, 52, 54, 55, 56) and central nervous system (1, 196, 197), including the retina (198). To date there is no cure for DMD, although several innovative therapeutic approaches, aiming at reducing the progression of the disease, are applied in clinical protocols (199, 59). However, independently of the therapy adopted, the standard pharmacological treatment is based on the cyclic administration of powerful GC doses, aimed at reducing chronic muscular inflammation. However, a vast literature has demonstrated that prolonged use of high doses of GCs induces severe side effects, also affecting the nervous system. Specifically, in prolonged stressful events, high and persistent levels of CORT have been proposed to be the mediators of detrimental effects, such as memory and cognition impairment, major depression, anxiety disorders, and post-traumatic stress disorder, reduced structural and functional plasticity (61, 200, 135). In physiological conditions, GCs are secreted by the adrenal gland in response to signals from the HPA axis, and represent the hormones by which the body responds to stressful events. Among the brain areas involved in stress response is the hippocampus, the activation of which by GCs, is necessary to de-regulate the HPA axis activity and reduce GC release (176, 201). In DMD patients, as in *mdx* mice, the hippocampus is one of the brain areas mostly affected by the lack of Dp427.

Therefore, the experimental work presented in theses has been aimed at understanding whether hippocampal responses to

either acute of sub-chronic GC administration was different between wild type and dystrophic mice. The parameters so far chosen to unveil this response were: expression, synthesis and localization of the GR, modulation of the GR signalling (both genomic and non-genomic), impact on proliferation of the NPC localized at the DG sub-granular zone, a process reported to be compromised under stress conditions.

The response of cultured hippocampal neurons to acute corticosterone administration is different between wild type and mdx mice

Working with an i*n vitro* system has the advantage to analyse a physiological/pathological response isolated from the *in vivo* contest, rich in background. The first part of this doctoral study, has been cantered to uncover how isolated hippocampal neurons respond to acute (60 min) administration of different CORT concentration: 1 μM and 10 μM.

A first interesting data is that E18 *mdx* mouse hippocampi, the embryonic stage at which hippocampal neuron culture were established, had significantly lower levels of GR compared to wild type, while the level of phosphorylation was comparable. This aspect already indicates that *mdx* mouse hippocampi suffer some long-lasting exposure to endogenous GCs, possibly derived from maternal blood stream as demonstrated by plasma corticosterone measurement. In fact, dystrophic female also experience recurrent muscular degenerations, which in turn increase the levels of endogenous anti-inflammatory molecules, as CORT. Therefore, this down-regulation of GR levels is consistent with the literature, according which increased exposure of placenta to GC, from either maternal or foetal circulation, alters the expression of 11β-HSD2 and of a number of genes involved in its endocrine function. In the foetus, GC overexposure affects almost all the

endocrine systems, which are active during late gestation, including the HPA axis itself (169, 202). If considering that the levels of phosphorylated receptor is similar to that in the wild type mice, we could also speculate that in *mdx* mice a larger proportion, compared to wild type, is active. This aspect may possibly comply with the large body of literature describing the deleterious genetic and epigenetic pre-natal stress effects over foetuses (203, 204).

That the GR response is already "compromised" in *mdx* mouse hippocampal neurons could be also evinced from the different response that hippocampal neurons of the two genotypes have following acute administration of exogenous CORT. While wild type mouse neurons respond as expected, i.e. by increasing receptor phosphorylation, protein level and gene expression compared to control conditions (incubation with vehicle), GR in *mdx* mouse neurons do not undergo to further phosphorylation, their protein levels significantly decrease compared to control conditions and mRNA levels are only slightly up-regulated. Biochemical and molecular biology data were further validated by immunocytochemistry. This response strongly suggests that hippocampal neuron of dystrophic mice respond in a way more similar to an *in vivo* chronic exposure to GCs, in which the hippocampus inhibitory activity over the HPA axis is dramatically reduced or abolished (105, 135). A further important aspect that came out from this experiments is that CORT *per se* induces a dose-dependent degree of neuronal death by apoptosis, evaluated by quantitative Casp 3 immunocytochemistry,

In vivo, wild type and mdx mouse hippocampal neurons respond differently to acute corticosterone treatment

A different response between wild type and *mdx* mice was also obtained following acute CORT administration protocol *in*

vivo. In this case, two groups of mice of both genotypes were injected only once with a dose of either CORT or vehicle and then scarified after 90 min (short-term response) or 6 h (long-term response). The results were compared to their respective un-injected control mice. Expression of hippocampal GR and its activation are key factors in the correct coordination of stress responses, as they determine the sensitivity of this cerebral region to GC and, hence, the direction of its modulation of the HPA axis activity (176, 201). Again, quite similar to what observed in the *in vitro* experiments, 90 min after CORT exposure, both wild type and *mdx* mice respond by significantly increasing levels *p*GR compared to control, indicating the CORT has reached the hippocampus and recognized its receptors. However, a slight increase was also observed in the vehicle-injected *mdx* mouse group, suggesting a peculiar susceptibility of dystrophic mice to stressful conditions (see later in this discussion). Differences between the two genotypes were observed in the total GR protein levels: in the wild type mouse hippocampus their remaine unchanged, as expected since time for new protein synthesis is presumably still too short; differently, those in *mdx* mice decrease significantly in both vehicle and CORT groups. This opens two questions: first, such a specific reduction in protein levels suggests an increase in their degradation and/or a decrease in their normal synthesis. The way for this to occur is that hippocampal neurons perceive this treatment as a further load of GC, which triggers a response typical of "chronic" exposure to stressors (205). This would be in accord to what previously described in the *in vitro* experiments. The second important question is that mice in the vehicle group respond similarly to those who received CORT, opening the interesting question on whether *mdx* mice had a hypersensitivity to experimental treatments, independently from injection of exogenous GCs.

This would mean that mouse manipulation by the experimenter and injections are *per se* capable of inducing a powerful stress response, probably triggered by release of noradrenaline from the autonomic nervous system, activation of amygdala and release of endogenous GCs from the adrenal gland. This hypothesis is consistent with data from a study conducted by Vaillend and Choussenot (178), showing a significant higher response of *mdx* mice, compared to wild type, to different stress-behavioural tests, with high levels of freezing in response to even the simple experimenter grip. This could be related to the fact that the basal CORT levels in wild type and *mdx* mice are different. In addition, coming back to our data, GR protein levels observed in the two injected groups were similar, suggesting that additional CORT does not further increase a systemic response, which could be considered "saturated". It is important to remember that both amygdala and the sympathetic nervous system actively participate in the stress response, and that shortly after stress onset, noradrenaline levels in the baso-lateral amygdala (BLA) are transiently elevated. Corticosteroids reach the same area somewhat later and remain elevated for approximately 1–2 h. Therefore, for a restricted period of time, BLA neurons are exposed to high levels of both hormones. Later on, corticosteroids normalize BLA activity (delayed effect *via* GR), a phase in which higher cognitive controls seem to be restored (206).

Supporting this hypothesis are the results obtained by gene expression analysis: GR expression does not change in wild type mouse hippocampus, coherent with the unchanged levels of GR. Differently, the significant decrease in the CORT group of *mdx* mice suggests an overloading of stressful response, enough to activate a genomic response, that in the case of heavy and prolonged exposures to GCs has been

demonstrated to decrease GR expression (205). An interesting aspect, deserving a deeper analysis, is the significant increase in GILZ gene expression in both vehicle and CORT groups in wild type mice. GILZ is a transcription factor, the expression of which is early regulated by GR activity and that, in turn, modulates the expression of other downstream genes (207, 209). No changes in GILZ mRNA levels were observed in *mdx* mouse hippocampi.

Coherent with all these considerations are the data reporting higher GR protein levels in control *mdx* mouse hippocampus compared to wild type. This means that somehow adult mice are continuously, or recurrently, stimulated; therefore, in the presence of humoral and/or hormonal "overload" a negative feedback mechanism would be triggered.

The results obtained by the immunohystochemical investigation of GR and *p*GR distribution in the hippocampus, before and after CORT administration, were in accord to what described by biochemistry. In addition, GR localization was predominantly in the DG and CA1 regions of the hippocampus, areas implicated in neurogenesis and cognitive function, respectively (208). GR phosphorylation was, instead, more evident in CA1 sub-regions, although a faint and more widespread immunolabelling could be appreciated at higher magnification. Obviously, the amount of *p*GR is relatively lower compared to the total GR and its signal could be below the resolution power of the techniques.

Analysis of some of the parameters of the non-genomic response again highlighted some important differences between the two genotypes. ERK is one of the early downstream proteins in both membrane-bound and cytoplasmic GR signalling, and its activation represents a link between the classical genomic and the rapid non-genomic pathways (209).

Ninety minutes after CORT injection of wild type mice, phosphorylation (activation) of ERK in the hippocampus was significantly higher compared to control and vehicle groups, while it did not change in *mdx* mice, suggesting some impediment in the correct transmission of the fast signalling. Supporting this hypothesis is the lack of changes in protein levels of Cav1, a protein stabilized by the Dp427-DGC, as demonstrated for Cav3 in skeletal muscles (210) and one of the lipid raft proteins thought to be involved in mGR stabilization. The GR-mediated Cav1 activation, which involves its phosphorylation (180), participates in the MAPK-driven intracellular signalling pathway. Therefore, this apparent insensitivity to treatments in dystrophic mice could suggest a further destabilization of Cav1 into the membrane and a non-appropriate downstream signalling to ERK.

As expected, six hours after CORT treatment, no more changes in *p*GR were observed, in neither one of the genotypes. Total GR levels, instead, were significantly increased in the wild type mouse hippocampus after CORT administration, as a consequence of early GR activation. Differently, in both vehicle and CORT injected *mdx* mice, GR levels decreased significantly, exactly as observed after 90 min, again arguing in favour of a stressor-saturated system.

The analysis of the non-genomic signalling parameters again identified important differences between the two genotypes. After a longer time from CORT administration, protein levels of Cav1 increased in the wild type mouse hippocampus, suggesting readjustment of newly synthesized receptors into the plasma membrane. The ERK system, instead, was down regulated. A similar silencing was also observed in both vehicle and CORT treated *mdx* mouse hippocampi. However, a powerful decrease was also observed in the levels of Cav1 and Cav2, a protein mainly located within the Golgi

apparatus membrane, working as chaperone in the transport of membrane proteins, including Cav1 (97). One hypothesis we can pursue is that, in mdx mice, Cav1 activity and, hence, protein levels would be decreased by a diminished stabilization into the plasma membrane caused by lack of Dp427 in the DGC. However, a more general consideration is that the caveolins we observe in tissues extract include not only those localized in neurons, but also those expressed by endothelial cells and astrocytes. Therefore, what we observe is possibly a mean of the total levels of Cav within different cell types. Nevertheless, it deserves to be noticed that in *mdx* mice both treatment with vehicle and CORT significantly reduce protein levels of GR, Cav1 and Cav2, suggesting a close connection in the physiology of these proteins.

In vivo, wild type and mdx mouse hippocampal neurons respond differently to dexamethasone sub-chronic treatment

The following experimental set of experiments aimed at uncovering the hippocampal response to prolonged GC treatments, a procedure closer to the therapeutic treatments adopted in DMD. For this procedure, it was used DEX, a powerful analogue of CORT, widely used for long-term studies.

Prolonged DEX treatments kept levels of *p*GR in the hippocampus of wild type mice significantly higher than those in the control, suggesting that receptor activation adjusted to increased levels of GCs. Differently, levels of total GR and of all proteins analysed for the non-genomic signalling were comparable between the three experimental groups, suggesting that constant (one injection/day for 9 days) GR signalling over a prolonged period of time could adjust the "executive" system (GR and proteins of the non-genomic signalling) at basal/physiological levels. Differently, in *mdx* mouse

hippocampi, *p*GR levels increased significantly in both vehicle and DEX groups, reiterating the hypothesis of an over-sensibility of *mdx* mice to manipulation. The higher degree of GR phosphorylation in the vehicle group compared to DEX, may indicate again that the system begins to respond as it was under chronic and repeated stressful waves. As a matter of fact, GR protein levels were significantly decreased in both injected groups compared to control. GR and GILZ gene expression decrease after 9 days of vehicle and DEX administration only in *mdx* mice. Data from the literature have demonstrated that a chronic psychosocial stress, or the administration of high DEX concentrations, induce a reduction in both GR protein and its mRNA levels; however, in wild type condition we observed only a slight tendency to decrease, but this reduction does not reached significance, probably because our sub-chronic treatment represents a time that is still too short to appreciate important changes in gene expression (which are instead observed in chronic treatments). Unlike, an acute stress, or CORT administration, would induce an increase in receptor levels (211).

Dystrophic mice also show differences compared to wild type in the levels of some proteins of the non-genomic signalling. Specifically, while no differences among the three experimental groups were observed in the protein levels of Cav1 and *p*ERK (similar to wild type), those of Cav2 and total ERK increased significantly in both DEX and vehicle groups. This could be a long-term response to protein level adjustments (decreased degradation) in a system that is destabilized compared to the wild type.

Proliferation of neural precursor cells in wild type and mdx mouse hippocampus is differently affected by dexamethasone sub-chronic treatment

Hippocampal NPCs proliferation is a mechanism negatively influenced by stress (201), i.e. chronic GR activation induce an inhibition in NPC proliferation and following adult neurogenesis (161). To analyse how stem cell proliferation in the sub-granular zone of the hippocampal DG is altered in our experimental model, we labelled proliferating cells by EdU and performed a quantitative analysis in control, vehicle-injected and DEX-injected wild type and *mdx* mice. The results reported in this thesis showed significant decrease in NPC proliferation after DEX sub-chronic treatment of wild type mice compared to vehicle. This result is in agreement with other studies on different animal models (161). Interestingly, NPC proliferation after DEX administration did not change compared to control, suggesting inhibition of cell proliferation in stress condition, while it significantly increased after VC treatment. This data suggests that the experimental manipulation itself represents a mild stress for wild type mice that promotes NPC proliferation (111). Differently, in *mdx* mouse hippocampus there was a significant increase in NPC proliferation, compared to the other two groups. Indications are that GC signalling by proliferating hippocampal cells is differently perceived by cells of the two genotypes. The relationship between stress and NPCs proliferation is mediated by GR activation, which in these cells positively and negatively regulates the transcription of important genes for cell cycle control and differentiation (111). However, the modulation by stress of NPCs proliferation is complex and depends on both degree of GR activation and levels of circulating GCs. In fact, a "controllable" stress, like physical activity, is associated with an increase in NPC proliferation, on the contrary, a chronic "uncontrollable" stress,

results in a strong reduction; the discriminating factor between the two events seems to be the degree of GR activation (111). In this work, *mdx* mouse sub-chronic DEX treatment increased GR phosphorylation levels, while reducing hippocampal GR protein levels, suggesting that such a negative regulation could affect other cellular events controlled by GR signalling. The important aspect of these results, however, is that, under stress conditions, healthy wild type mice trigger a typical temporary homeostatic response, which we suppose to be protective of such a delicate mechanism. Differently, in dystrophic mice somehow the steps regulating the incidence of GC on cell cycles are corrupted, actually un-inhibiting a possible ongoing control over cell proliferation in un-injected animals.

Conclusions

The data presented in this thesis indicate that *mdx* mice, one of the most used animal models of DMD, respond differently to stressful events, such as acute or sub-chronic GC administration. Possibly, these differences may stem from a "pre-sensitized" condition, considering that dystrophic subjects are afflicted by continuous cycles of skeletal muscle degeneration, which increase levels of circulating CORT. In addition, reports describe *mdx* mice quite susceptible to different form of stress, as also evidenced by the response to vehicle treatments in this study. What could be, therefore, the link between a dystrophic status and the peculiar response to elevated concentrations of GC? One before all is that the hippocampus (in both DMD patients and *mdx* mice) shows different degrees of structural and functional alterations induced by the lack of Dp427. These physiological alterations could be *per se* a ground of different cell behaviour to stress hormones. Second, Dp427 and the DGC are implicated in the stabilization of cortical cytoskeletal and membrane proteins, among which caveolins. These are also localized into lipid rafts, membrane regions associated to mGR, suggesting that a partial convergence between the two systems may exist. Another aspect is the secondary effect that absence of Dp427 has on the expression of other genes, as demonstrated by us in previous studies. This would further complicate the possible scenario in which stress-related events develop.

It is now proven that DMD is not just a muscle disorder characterized by severe damage of striated muscles, but it is associated with various cognitive disabilities. Although neurological defects are reported in both DMD patients and animal models, they are not considered relevant in the symptomatic picture of DMD, mainly because of premature patient death. To date, therapeutic treatments in clinical trial

potentially increase DMD patient life of about ten years. In this perspective, the onset of neurological symptoms will paradoxically worsen the quality of life. Moreover, very few studies have assessed the mental health of children and adolescent with DMD, although they are at great risk of developing depression and anxiety disorders. Indeed, the conditions of life of a child affected by DMD are very difficult compared to their peers. Soon they lose the ability to walk and are forced to live in a wheelchair. These discomforts, which certainly generate stress, are added to the alterations of specific brain regions, among which hippocampus, which are linked to the lack of Dp427 (and in DMD patients also of other isoforms). To this situation, already complex in itself, are added the chronic and massive treatments with GCs, which worsen the neurological condition of patients and compromise the correct hippocampus function and the systems associated with it (i.e. HPA axis). This generates defects in all those districts and signaling systems that allow our body to respond to stressful events and to restore homeostasis by regulating the levels of circulating GCs.

Importantly, published data to date have not indicated GC-like adverse effects associated with GILZ, suggesting that GILZ exerts immunosuppressive effects that mimic those of GCs but occur via distinct pathways. The similarities and divergence between the effects of GILZ and GC suggest the potential use of GILZ-based therapies in improving GC efficacy while reducing GC metabolic toxicity, and thus the development of new treatment strategies to replace supplement or even replace GC. (212) Further investigation is required to determine whether GILZ induces GC-related adverse metabolic effects. If confirmed, this will support the hypothesis that GILZ therapeutic effects mimic those of GCs but lack GC-like metabolic effects. As a result, GILZ-based gene therapy has

great potential in the therapy of human inflammatory diseases.

In the hope that new therapies could extend the life span of the young DMD patients by reducing and slowing down the muscular degeneration, it is even more important to go deeper in the comprehension of how hippocampal neurons, already affected since embryonic development by DMD, respond to chronic or sub-chronic administrations of GCs.

References

1. Anderson J. L., Head S. I., Rae C. J., Morley W. (2002) Brain function in Duchenne muscular dystrophy, *Brain*, Volume 125, Pages 4–13

2. Sussman, M. (2002). Duchenne muscular dystrophy. *Journal of the American Academy of Orthopaedic Surgeons*, 10(2), 138-151.

3. Ervasti J.M., Campbell K.P. (1991) Membrane organization of the dystrophin-glycoprotein complex, *In Cell*, Volume 66, Pages 1121-1131

4. Koenig M., Hoffman E.P., Bertelson C.J., Monaco A.P., Feener C., L.M. Kunkel (1987) Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals, *In Cell*, Volume 50, Pages 509-517

5. Roberts R. G., Coffey A. J., Bobrow M., David R. Bentley (1993) Exon Structure of the Human Dystrophin Gene, *In Genomics*, Volume 16, Pages 536-538

6. Barbujani G.A., Russo G. A., Danieli A., SpieglerJ W. J., Borkowska I., Hausmanova Petrusewicz (1990) Segregation analysis of 1885 DMD families: significant departure from the expected proportion of sporadic cases *Human Genetics* Volume 84, pp 522–526

7. Blake D.J., Weir A., Newey S.E., Davies K.E. (2002) Function and Genetics of Dystrophin and Dystrophin-Related Proteins in Muscle *Physiological Reviews Published* Vol. 82 2, 291-329

8. Austin R.C., Morris G.E., Howard P.L., Klamut H.J., Ray P.N. (2000) Expression and synthesis of alternatively spliced variants of Dp71 in adult human brain, *In Neuromuscular Disorders*, Volume 10, Issue 3, Pages 187-193

9. Tozawa, T., Itoh, K., Yaoi, T. et al. (2012) The Shortest Isoform of Dystrophin (Dp40) Interacts with a Group of Presynaptic Proteins to Form a Presumptive Novel Complex in the Mouse Brain *Mol Neurobiol* 45: 287

10. Goodwin F., Muntoni F., Dubowitz V., (1997) Epilepsy in Duchenne and Becker muscular dystrophies, In European *Journal of Paediatric Neurology*, Volume 1, Pages 115-119

11. Nico, B., Roncali, L., Mangieri, D., & Ribatti, D. (2005). Blood-brain barrier alterations in MDX mouse, an animal model of the Duchenne muscular dystrophy. *Current neurovascular research*, 2(1), 47-54.

12. Nowak KJ, Davies KE (2004) Duchenne muscular dystrophy and dystrophin: pathogenesis and opportunities for treatment *Third in Molecular Medicine Review Series* 5, 872-876

13. Durbeej M., Campbell K.P. (2002) Muscular dystrophies involving the dystrophin–glycoprotein complex: an overview of current mouse models, *In Current Opinion in Genetics & Development*, Volume 12, Pages 349-361

14. Ehmsen J., Poon E., Davies K. (2002) The dystrophin-associated protein complex *Journal of Cell Science* 2002 115: 2801-2803

15. Waite A., Tinsley C. L., Locke M., Blake D.J. (2009) The neurobiology of the dystrophin-associated glycoprotein complex *Trends in Molecular Medicine* Pages 41:344-359

16. Blake D.J., Kröger S., (2000) The neurobiology of Duchenne muscular dystrophy: learning lessons from muscle? *In Trends in Neurosciences*, Volume 23, Pages 92-99

17. Anderson J.L., Head S.I., Morley J.W. (2012) Duchenne muscular dystrophy and brain function *Muscular Dystrophy*

18. Chamberlain, J. S., Gibbs, R. A., Rainer, J. E., Nguyen, P. N., & Thomas, C. (1988). Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. *Nucleic acids research*, 16(23), 11141-11156.

19. Lidov H.G.W., Byers T.J., Kunkel L.M., (1993) The distribution of dystrophin in the murine central nervous system: An immunocytochemical study, *In Neuroscience*, Volume 54, , Pages 167-187

20. Waite A., Brown S. C., & Blake D. J. (2012). The dystrophin– glycoprotein complex in brain development and disease. *Trends in neurosciences*, *35*(8), 487-496

21. Wersinger E., Bordais A., Schwab Y., Sene A., Bénard R., Alunni V., Sahel JA., Rendon A., Roux MJ., (2011) Reevaluation of Dystrophin Localization in the Mouse *Investigative Ophthalmology & Visual Science* 52:7901-7908

22. Lidov H. G. W., Selig S., Kunkel L.M. (1995) Dp140: a novel 140 kDa CNS transcript from the dystrophin locus, *Human Molecular Genetics*, Volume 4 Pages 329–335

23. Szabó, A., Jancsik, V., Mornet, D., & Kálmán, M. (2004). Immunofluorescence mapping of dystrophin in the rat brain: astrocytes contain the splice variant Dp71f, but this is confined to subpopulations. Anatomy and embryology, 208(6), 463-477.

24. Cyrulnik S.E., Hinton V.J. (2008) Duchenne muscular dystrophy: A cerebellar disorder? *In Neuroscience & Biobehavioral Reviews*, Volume 32, Pages 486-496

25. Craig A.M., Kang Y., (2007) Neurexin–neuroligin signalling in synapse development, *In Current Opinion in Neurobiology*, Volume 17, Pages 43-52

26. Knuesel I., Zuellig R.A., Schaub M.C. and Fritschy JM., (2001) Alterations in dystrophin and utrophin expression parallel the reorganization of GABAergic synapses in a mouse

model of temporal lobe epilepsy *European Journal of Neuroscience* Volume 13 pages 1113–1124

27. Brünig, I., Suter, A., Knuesel, I., Lüscher, B., & Fritschy, J. M. (2002). GABAergic terminals are required for postsynaptic clustering of dystrophin but not of GABAA receptors and gephyrin. *Journal of Neuroscience*, 22(12), 4805-4813.

28. Albrecht D.E., Froehner S.C. (2002) Syntrophins and Dystrobrevins: Defining the Dystrophin Scaffold at Synapses *Neurosignals* 11:123–129

29. Vaillend C., & Billard J. M. (2002). Facilitated CA1 hippocampal synaptic plasticity in dystrophin-deficient mice: Role for GABAA receptors? *Hippocampus*, *12*(6), 713-717

30. Kueh, S., Head, S. and Morley, J. (2008), GABAA receptor expression and inhibitory post-synaptic currents in cerebellar Purkinje cells in dystrophin-deficient *mdx* mice. *Clinical and Experimental Pharmacology and Physiology*, 35: 207–210

31. Carretta, D., Santarelli, M., Vanni, D., Carrai, R., Sbriccoli, A., Pinto, F., & Minciacchi, D. (2001). The organisation of spinal projecting brainstem neurons in an animal model of muscular dystrophy: a retrograde tracing study on mdx mutant mice. *Brain research*, *895*(1), 213-222.

32. Jagadha V., Becker L.E, (1988), Brain morphology in Duchenne muscular dystrophy: A Golgi study, *In Pediatric Neurology*, Volume 4, Pages 87-92

33. Sogos V., Ennas M. G., Mussini I., Gremo F., (1997) Effect of dystrophin antisense oligonucleotides on cultured human neurons, *In Neurochemistry International*, Volume 31, Pages 447-457

34. Miranda R., Sebrie C., Degrouard J., Gillet B., Jaillard D., Laroche S., & Vaillend C. (2008). Reorganization of inhibitory synapses and increased PSD length of perforated

excitatory synapses in hippocampal area CA1 of dystrophin-deficient mdx mice. *Cerebral Cortex*, *19*(4), 876-888

35. Miranda R., Nudel U., Laroche S., & Vaillend C. (2011). Altered presynaptic ultrastructure in excitatory hippocampal synapses of mice lacking dystrophins Dp427 or Dp71. *Neurobiology of disease*, *43*(1), 134- 141.

36. Knuesel I., Mastrocola M., Zuellig R. A., Bornhauser B., Schaub M. C., & Fritschy J. M. (1999). Altered synaptic clustering of $GABA_A$ receptors in mice lacking dystrophin (*mdx* mice). *European Journal of Neuroscience*, *11*(12), 4457-4462.

37. Sekiguchi M., Zushida K., Yoshida M., Maekawa., Kamichi S., Yoshida M., Sahara Y., Yuasa., Takeda S., Wada K. A, (2009) A deficit of brain dystrophin impairs specific amygdala GABAergic transmission and enhances defensive behaviour in mice, *Brain,* Volume 132, Pages 124–135

38. Coccurello, R., Castellano, C., Paggi, P., Mele, A., & Oliverio, A. (2002). Genetically dystrophic *mdx*/*mdx* mice exhibit decreased response to nicotine in passive avoidance. *NeuroReport*, *13*(9), 1219-1222.

39. Bresolin N., Castelli E., Comi G.P., Felisari G., Bardoni A., Perani D., Grassi F., Turconi A., Mazzucchelli F., Gallotti D., Moggio M., Prelle A., Ausenda C., Fazio G., Scarlato G., (1994) Cognitive impairment in Duchenne muscular dystrophy, *In Neuromuscular Disorders*, Volume 4, 1994 Pages 359-369

40. Vaillend C., Rendon A., Misslin R. et al. Behav Genet (1995) Influence of dystrophin-gene mutation onmdx mouse behavior. I. Retention deficits at long delays in spontaneous alternation and bar-pressing tasks *Behavior Genetics* 25: 569

41. Vaillend C., Billard J.-M., Claudepierre T., Rendon A., Dutar P., Ungerer A., (1998) Spatial discrimination learning

and CA1 hippocampal synaptic plasticity in mdx and mdx3cv mice lacking dystrophin gene products, *In Neuroscience*, Volume 86, Pages 53-66

42. Mehler, M. F., Haas, K. Z., Kessler, J. A., & Stanton, P. K. (1992). Enhanced sensitivity of hippocampal pyramidal neurons from mdx mice to hypoxia-induced loss of synaptic transmission. *Proceedings of the National Academy of Sciences*, 89(6), 2461-2465.

43. Vaillend, C., Mason, S. E., Cuttle, M. F., & Alger, B. E. (2002). Mechanisms of neuronal hyperexcitability caused by partial inhibition of Na+-K+-ATPases in the rat CA1 hippocampal region. *Journal of Neurophysiology*, 88(6), 2963-2978.

44. Levi, S., Grady, R.M., Henry, M.D., Campbell, K.P., Sanes, J.R., Craig, A.M., 2002. Dystroglycan is selectively associated with inhibitory GABAergic synapses but is dispensable for their differentiation. *J. Neurosci.* 22, 4274–4285.

45. Duchenne G. B. (1868) Recherches sur la paralysie musculaire pseudohypertrophique ou paralysie myo-sclërosique. I. Symptomatologie. 11: 179 *PNAS* 89 (6) 2461-2465

46. Cotton S., Voudouris N., & Greenwood K. (2001). Intelligence and Duchenne muscular dystrophy: Full-Scale, Verbal, and Performance intelligence quotients. *Developmental Medicine & Child Neurology*, 43(7), 497-501.

47. Snow W.M., Anderson J.E., Jakobson L.S., (2013) Neuropsychological and neurobehavioral functioning in Duchenne muscular dystrophy: A review, *In Neuroscience & Biobehavioral Reviews*, Volume 37, Pages 743-752

48. de Brouwer APM., Nabuurs SB., Verhaart IEC., Oudakker AR., Hordijk R., Yntema HG., Hordijk-Hos JM., Voesenek K., de Vries BBA., van Essen T., Chen W., Hu H.,

Chelly J., den Dunnen JT., Kalscheuer VM., Aartsma-Rus AM., Hamel BCJ., van Bokhoven H., Kleefstra T., (2014) A 3-base pair deletion, c.9711_9713del, in DMD results in intellectual disability without muscular dystrophy *Eur J Hum Genet* 4:480-485

49. Taylor PJ, Betts GA, Maroulis S, Gilissen C, Pedersen RL, Mowat DR, et al. (2010) Dystrophin Gene Mutation Location and the Risk of Cognitive Impairment in Duchenne Muscular Dystrophy. *PLoS ONE* 5(1)

50. Yotsukura, M., Sasaki, K., Kachi, E., Sasaki, A., Ishihara, T., & Ishikawa, K. (1995). Circadian rhythm and variability of heart rate in Duchenne-type progressive muscular dystrophy. *The American journal of cardiology*, 76(12), 947-951.

51. De Stefano M. E., Leone L., Lombardi L., & Paggi P. (2005). Lack of dystrophin leads to the selective loss of superior cervical ganglion neurons projecting to muscular targets in genetically dystrophic *mdx* mice. *Neurobiology of disease*, *20*(3), 929-942.

52. Lombardi L., De Stefano M. E., & Paggi P. (2008). Components of the NGF signaling complex are altered in *mdx* mouse superior cervical ganglion and its target organs. *Neurobiology of disease*, *32*(3), 402-411.

53. Lombardi L., Persiconi I., Gallo A., Hoogenraad C. C., & De Stefano M. E. (2017). NGF-dependent axon growth and regeneration are altered in sympathetic neurons of dystrophic mdx mice. *Molecular and Cellular Neuroscience*, *80*, 1-17

54. Zaccaria ML, De Stefano ME, Gitti C, Perucci TC, Paggi P (2000) Selective reduction in the nicotinic acetylcholine receptor and dystroglycan at the postsynaptic apparatus of *mdx* mouse superior cervical ganglion. *J Neuropathol Exp Neurol* 59:103-112

55. Di Angelantonio S., De Stefano M. E., Piccioni A., Lombardi L., Gotti C., & Paggi P. (2011). Lack of dystrophin functionally affects α3β2/β4- nicotinic acethylcholine receptors in sympathetic neurons of dystrophic *mdx* mice. *Neurobiology of disease*, *41*(2), 528-537

56. Licursi V., Caiello I., Lombardi L., De Stefano M. E., Negri R. and Paggi P. (2012), Lack of dystrophin in mdx mice modulates the expression of genes involved in neuron survival and differentiation. *European Journal of Neuroscience*, 35: 691–701.

57. Irwin, A. N., & Herink, M. C. (2017). Eteplirsen for the treatment of Duchenne muscular dystrophy: Quality of evidence concerns an alternative viewpoint. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*.

58. Lamb, M. M. (2016). Corticosteroids affect growth patterns in boys with DMD. *Reactions*, *1597*, 8-16.

59. Reinig, A. M., Mirzaei, S., & Berlau, D. J. (2017). Advances in the Treatment of Duchenne Muscular Dystrophy: New and Emerging Pharmacotherapies. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, 37(4), 492-499.

60. Cain, D. W., & Cidlowski, J. A. (2015). Specificity and sensitivity of glucocorticoid signaling in health and disease. *Best Practice & Research Clinical Endocrinology & Metabolism*, *29*(4), 545-556.

61. de Kloet E.R., Joëls M. & Holsboer F., (2005) Stress and the brain: from adaptation to disease *Nature Reviews Neuroscience* 6, 463-475

62. Jeanneteau F., Chao M.V., (2013) Are BDNF and glucocorticoid activities calibrated? *In Neuroscience*, Volume *239,* Pages 173-195

63. Jeanneteau, F. D., Lambert, W. M., Ismaili, N., Bath, K. G., Lee, F. S., Garabedian, M. J., & Chao, M. V. (2012).

BDNF and glucocorticoids regulate corticotrophin-releasing hormone (CRH) homeostasis in the hypothalamus. *Proceedings of the National Academy of Sciences*, 109(4), 1305-1310.

64. Stavreva D.A., Wiench M., John S., Conway-Campbell B.L., McKenna M.A., Pooley J.R., Johnson T.A., Voss T.C., Lightman S.L. & Hager G.L. (2009) Ultradian hormone stimulation induces glucocorticoid receptor-mediated pulses of gene transcription *Nature Cell Biology* 11, 1093–1102

65. Georges, A. B., Benayoun, B. A., Caburet, S., & Veitia, R. A. (2010). Generic binding sites, generic DNA-binding domains: where does specific promoter recognition come from? *The FASEB Journal*, 24(2), 346-356.

66. Pratt W. B., Toft D.O.; (1997) Steroid Receptor Interactions with Heat Shock Protein and Immunophilin Chaperones, *Endocrine Reviews*, Volume 18, Pages 306–360

67. Wang Z., Frederick J., and Garabedian M.J. (2002) Deciphering the Phosphorylation "Code" of the Glucocorticoid Receptor *in vivo*. *J. Biol. Chem* 277: 26573 -26580.

68. Chen W., Dang T., Blind R. D., Wang Z., Cavasotto C. N., Hittelman A. B., Garabedian M. J. (2008). Glucocorticoid receptor phosphorylation differentially affects target gene expression. *Molecular Endocrinology*, *22*(8), 1754-1766.

69. Freedman, N. D., & Yamamoto, K. R. (2004). Importin 7 and importin α/importin β are nuclear import receptors for the glucocorticoid receptor. *Molecular biology of the cell*, 15(5), 2276-2286.

70. Rogatsky, I., Wang, J. C., Derynck, M. K., Nonaka, D. F., Khodabakhsh, D. B., Haqq, C. M., & Yamamoto, K. R. (2003). Target-specific utilization of transcriptional regulatory surfaces by the glucocorticoid receptor. *Proceedings of the National Academy of Sciences*, 100(24), 13845-13850.

71. Soundararajan, R., Wang, J., Melters, D., & Pearce, D. (2007). Differential activities of glucocorticoid-induced leucine zipper protein isoforms. *Journal of Biological Chemistry*, 282(50), 36303-36313.

72. Ayroldi, E., Zollo, O., Bastianelli, A., Marchetti, C., Agostini, M., Di Virgilio, R., and Riccardi, C. (2007) GILZ mediates the antiproliferative activity of glucocorticoids by negative regulation of Ras 116ignalling. *J. Clin. Invest.* 117**,** 1605–1615

73. Ayroldi, E., Migliorati, G., Bruscoli, S., Marchetti, C., Zollo, O., Cannarile, L., D'Adamio, F., and Riccardi, C. (2001) Modulation of T-cell activation by the glucocorticoid-induced leucine zipper factor via inhibition of nuclear factor-B. *Blood* 98**,** 743–753

74. Ayroldi, E., & Riccardi, C. (2009). Glucocorticoid-induced leucine zipper (GILZ): a new important mediator of glucocorticoid action. *The FASEB Journal*, 23(11), 3649-3658.

75. Lösel, R., & Wehling, M. (2003). Nongenomic actions of steroid hormones. *Nature Reviews* Molecular Cell Biology, 4(1), 46-55.

76. Strehl, C., Gaber, T., Löwenberg, M., Hommes, D. W., Verhaar, A. P., Schellmann, S., & Scheffold, A. (2011). Origin and functional activity of the membrane-bound glucocorticoid receptor. *Arthritis & Rheumatology*, 63(12), 3779-3788.

77. Oakley RH., John A. Cidlowski, (2013) The biology of the glucocorticoid receptor: New signaling mechanisms in health and disease, *In Journal of Allergy and Clinical*

78. Groeneweg FL, Karst H, de Kloet ER, Joëls M (2012) Mineralocorticoid and glucocorticoid receptors at the neuronal membrane, regulators of non-genomic corticosteroid signalling. *Mol Cell Endocrinol*. 350(2):299-309

79. Tasker, J. G., Di, S., & Malcher-Lopes, R. (2006). Rapid glucocorticoid signaling via membrane-associated receptors. *Endocrinology*, 147(12), 5549-5556.

80. Xiao, L., Qi, A., & Chen, Y. (2005). Cultured embryonic hippocampal neurons deficient in glucocorticoid (GC) receptor: a novel model for studying nongenomic effects of GC in the neural system. *Endocrinology*, 146(9), 4036-4041.

81. Malcher-Lopes, R., Di, S., Marcheselli, V. S., Weng, F. J., Stuart, C. T., Bazan, N. G., & Tasker, J. G. (2006). Opposing crosstalk between leptin and glucocorticoids rapidly modulates synaptic excitation via endocannabinoid release. *Journal of neuroscience*, 26(24), 6643-6650.

82. Liu, C., Zhou, J., Zhang, L. D., Wang, Y. X., Kang, Z. M., Chen, Y. Z., & Jiang, C. L. (2007). Rapid inhibitory effect of corticosterone on histamine release from rat peritoneal mast cells. *Hormone and metabolic research*, 39(04), 273-277.

83. Abrami, L., Fivaz, M., Kobayashi, T., Kinoshita, T., Parton, R. G., & van der Goot, F. G. (2001). Cross-talk between caveolae and glycosylphosphatidylinositol-rich domains. *Journal of Biological Chemistry*, 276(33), 30729-30736.

84. Razandi, M., Oh, P., Pedram, A., Schnitzer, J., & Levin, E. R. (2002). ERs associate with and regulate the production of caveolin: implications for signaling and cellular actions. *Molecular Endocrinology*, 16(1), 100-115.

85. Okamoto, T., Schlegel, A., Scherer, P. E., & Lisanti, M. P. (1998). Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. *Journal of Biological Chemistry*, 273(10), 5419-5422.

86. Samarasinghe, R. A., Di Maio, R., Volonte, D., Galbiati, F., Lewis, M., Romero, G., & DeFranco, D. B. (2011). Nongenomic glucocorticoid receptor action regulates

gap junction intercellular communication and neural progenitor cell proliferation. *Proceedings of the National Academy of Sciences*, 108(40), 16657-16662.

87. Hammes, S. R., & Levin, E. R. (2011). Minireview: recent advances in extranuclear steroid receptor actions. *Endocrinology*, 152(12), 4489-4495.

88. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., & Woodgett, J. R. (1994). The stress-activated protein kinase subfamily of c-Jun kinases. *Nature*, 369(6476), 156-160.

Immunology, Volume 132, Pages 1033-1044

89. Blenis, J. (1993). Signal transduction via the MAP kinases: proceed at your own RSK. *Proceedings of the National Academy of Sciences*, 90(13), 5889-5892

90. Seger, R., & Krebs, E. G. (1995). The MAPK signaling cascade. *The FASEB journal*, 9(9), 726-735.

91. Traverse, S., Gomez, N., Paterson, H., Marshall, C., & Cohen, P. (1992). Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. *Biochemical Journal*, 288(2), 351-355.

92. Nguyen, T. T., Scimeca, J. C., Filloux, C., Peraldi, P., Carpentier, J. L., & Van Obberghen, E. (1993). Co-regulation of the mitogen-activated protein kinase, extracellular signal-regulated kinase 1, and the 90-kDa ribosomal S6 kinase in PC12 cells. Distinct effects of the neurotrophic factor, nerve growth factor, and the mitogenic factor, epidermal growth factor. *Journal of Biological Chemistry*, 268(13), 9803-9810.

93. Davis, R. J. (1995). Transcriptional regulation by MAP kinases. *Molecular reproduction and development*, 42(4), 459-467.

94. Qiu, J., Wang, P., Jing, Q., Zhang, W., Li, X., Zhong, Y., & Chen, Y. (2001). Rapid activation of ERK1/2 mitogen-activated protein kinase by corticosterone in PC12 cells. *Biochemical and biophysical research communications*, 287(4), 1017-1024.

95. Schlegel, A., Wang, C., Katzenellenbogen, B. S., Pestell, R. G., & Lisanti, M. P. (1999). Caveolin-1 Potentiates Estrogen Receptor α (ERα) Signaling caveolin-1 drives ligand-independent nuclear translocation and activation of ERα. *Journal of Biological Chemistry*, 274(47), 33551-33556.

96. Jain, S., Li, Y., Kumar, A., & Sehgal, P. B. (2005). Transcriptional signalling from membrane raft-associated glucocorticoid receptor. *Biochemical and biophysical research communications*, 336(1), 3-8.

97. Matthews, L., Berry, A., Ohanian, V., Ohanian, J., Garside, H., & Ray, D. (2008). Caveolin mediates rapid glucocorticoid effects and couples glucocorticoid action to the antiproliferative program. *Molecular endocrinology*, 22(6), 1320-1330.

98. Zhu, P., Baek, S. H., Bourk, E. M., Ohgi, K. A., Garcia-Bassets, I., Sanjo, H., & Rose, D. W. (2006). Macrophage/cancer cell interactions mediate hormone resistance by a nuclear receptor derepression pathway. *Cell*, 124(3), 615-629.

99. McEwen, B. S., & Sapolsky, R. M. (1995). Stress and cognitive function. *Current opinion in neurobiology*, 5(2), 205-216.

100. Kim, J. J., & Diamond, D. M. (2002). The stressed hippocampus, synaptic plasticity and lost memories. *Nature Reviews Neuroscience,* 3(6), 453-462.

101. Kim, J. J., Lee, H. J., Welday, A. C., Song, E., Cho, J., Sharp, P. E., & Blair, H. T. (2007). Stress-induced alterations in hippocampal plasticity, place cells, and spatial

memory. *Proceedings of the National Academy of Sciences*, 104(46), 18297-18302.

102. 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(17), 1554.

103. Conrad, C. D., Magariños, A. M., LeDoux, J. E., & McEwen, B. S. (1999). Repeated restraint stress facilitates fear conditioning independently of causing hippocampal CA3 dendritic atrophy. *Behavioral neuroscience*, 113, 902.

104. Schoenfeld, T. J., & Gould, E. (2012). Stress, stress hormones, and adult neurogenesis. *Experimental neurology*, 233(1), 12-21. *Science* Vol. 239:1416.

105. Wang, Q., Van Heerikhuize, J., Aronica, E., Kawata, M., Seress, L., Joels, M., & Lucassen, P. J. (2013). Glucocorticoid receptor protein expression in human hippocampus; stability with age. *Neurobiology of aging*, 34(6), 1662-1673.

106. Sapolsky, R. M. (2000). Stress hormones: good and bad. *Neurobiology of disease*, 7(5), 540-542.

107. ter Heegde, F., De Rijk, R. H., & Vinkers, C. H. (2015). The brain mineralocorticoid receptor and stress resilience. *Psychoneuroendocrinology*, 52, 92-110.

108. Sterner, E. Y., & Kalynchuk, L. E. (2010). Behavioral and neurobiological consequences of prolonged glucocorticoid exposure in rats: relevance to depression. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 34(5), 777-790

109. McEwen, B. S., Eiland, L., Hunter, R. G., & Miller, M. M. (2012). Stress and anxiety: structural plasticity and epigenetic regulation as a consequence of stress. *Neuropharmacology*, 62(1), 3-12.

110. Kim, E. J., Pellman, B., & Kim, J. J. (2015). Stress effects on the hippocampus: a critical review. *Learning & Memory*, 22(9), 411-416.

111. Saaltink, D. J., & Vreugdenhil, E. (2014). Stress, glucocorticoid receptors, and adult neurogenesis: a balance between excitation and inhibition? *Cellular and Molecular Life Sciences*, 71(13), 2499-2515.

112. de Kloet, E. R., Oitzl, M. S., & Joëls, M. (1999). Stress and cognition: are corticosteroids good or bad guys? *Trends in neurosciences*, 22(10), 422-426.

113. Groeneweg, F. L., Karst, H., de Kloet, E. R., & Joëls, M. (2011). Rapid non-genomic effects of corticosteroids and their role in the central stress response. *Journal of endocrinology*, 209(2), 153-167.

114. Buckingham, J. C., Solito, E., John, C., Tierney, T., Taylor, A., Flower, R., & Morris, J. (2003). Annexin 1: a paracrine/juxtacrine mediator of glucorticoid action in the neuroendocrine system. *Cell biochemistry and function*, 21(3), 217-221.

115. Mamalaki, E., Kvetnansky, R., Brady, L. S., Gold, P. W., & Herkenham, M. (1992). Repeated Immobilization Stress Alters Tyrosine Hydroxylase, Corticotropin-Releasing Hormone and Corticosteroid Receptor Messenger Ribonucleic Acid Levels in Rat Brain. *Journal of neuroendocrinology*, 4(6), 689-699.

116. Cameron, H. A., & Gould, E. (1994). Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus. *Neuroscience*, 61(2), 203-209.

117. Mirescu, C., & Gould, E. (2006). Stress and adult neurogenesis. *Hippocampus*, 16(3), 233-238.

118. Snyder, J. S., Soumier, A., Brewer, M., Pickel, J., & Cameron, H. A. (2011). Adult hippocampal neurogenesis

buffers stress responses and depressive behaviour. *Nature*, 476(7361), 458-461.

119. Nemeroff, C. B., & Vale, W. W. (2005). The neurobiology of depression: inroads to treatment and new drug discovery. *The Journal of clinical psychiatry*, 66, 5-13.

120. Surget, A., Tanti, A., Leonardo, E. D., Laugeray, A., Rainer, Q., Touma, C., & Belzung, C. (2011). Antidepressants recruit new neurons to improve stress response regulation. *Molecular psychiatry*, 16(12), 1177-1188.

121. Kempermann, G. & Ehninger, D., (2008). Neurogenesis in the adult hippocampus. *Cell and tissue research*, 331(1), 243-250.

122. Eriksson, P. S., Perfilieva, E., Björk-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A., & Gage, F. H. (1998). Neurogenesis in the adult human hippocampus. *Nature medicine*, 4(11), 1313-1317.

123. Spalding, K. L., Bergmann, O., Alkass, K., Bernard, S., Salehpour, M., Huttner, H. B., & Possnert, G. (2013). Dynamics of hippocampal neurogenesis in adult humans. *Cell*, 153(6), 1219-1227.

124. Bielefeld, P., Vliet, E. A., Gorter, J. A., Lucassen, P. J., & Fitzsimons, C. P. (2014). Different subsets of newborn granule cells: a possible role in epileptogenesis? *European Journal of Neuroscience*, 39(1), 1-11.

125. Jessberger, S., & Gage, F. H. (2014). Adult neurogenesis: bridging the gap between mice and humans. *Trends in cell biology*, 24(10), 558-563.

126. Oomen, C. A., Bekinschtein, P., Kent, B. A., Saksida, L. M., & Bussey, T. J. (2014). Adult hippocampal neurogenesis and its role in cognition. *Wiley Interdisciplinary Reviews: Cognitive Science*, 5(5), 573-587.

127. Abrous, D. N., & Wojtowicz, J. M. (2015). Interaction between neurogenesis and hippocampal memory

system: new vistas. *Cold Spring Harbor perspectives in biology*, 7(6), a018952.

128. Cho, K. O., Lybrand, Z. R., Ito, N., Brulet, R., Tafacory, F., Zhang, L., & Scharfman, H. E. (2015). Aberrant hippocampal neurogenesis contributes to epilepsy and associated cognitive decline. *Nature communications*, 6.

129. Anacker, C., & Pariante, C. M. (2011). Can adult neurogenesis buffer stress responses and depressive behaviour? *Molecular psychiatry*.

130. Lucassen, P. J., Fitzsimons, C. P., Korosi, A., Joels, M., Belzung, C., & Abrous, D. N. (2013a). Stressing new neurons into depression? Adult neurogenesis, major depression, stress. *Molecular psychiatry*, 18(4), 396-398.

131. Lucassen, P. J., Naninck, E. F., van Goudoever, J. B., Fitzsimons, C., Joels, M., & Korosi, A. (2013b). Perinatal programming of adult hippocampal structure and function; emerging roles of stress, nutrition and epigenetics. *Trends in neurosciences,* 36(11), 621-631.

132. Czéh, B., Michaelis, T., Watanabe, T., Frahm, J., de Biurrun, G., van Kampen, M., & Fuchs, E. (2001). Stress-induced changes in cerebral metabolites, hippocampal volume, and cell proliferation are prevented by antidepressant treatment with tianeptine. *Proceedings of the National Academy of Sciences*, 98(22), 12796-12801.

133. Czéh, B., Welt, T., Fischer, A. K., Erhardt, A., Schmitt, W., Müller, M. B., & Keck, M. E. (2002). Chronic psychosocial stress and concomitant repetitive transcranial magnetic stimulation: effects on stress hormone levels and adult hippocampal neurogenesis. *Biological psychiatry*, 52(11), 1057-1065.

134. Czéh, B., Fuchs, E., Wiborg, O., & Simon, M. (2016). Animal models of major depression and their clinical

implications. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 64, 293-310.

135. Lucassen, P. J., Pruessner, J., Sousa, N., Almeida, O. F., Van Dam, A. M., Rajkowska, G., & Czéh, B. (2014). *Neuropathology of stress*. *Acta neuropathologica*, 127(1), 109-135.

136. Ehninger, D., & Kempermann, G. (2006). Paradoxical effects of learning the Morris water maze on adult hippocampal neurogenesis in mice may be explained by a combination of stress and physical activity. *Genes, Brain and Behavior*, 5(1), 29-39.

137. Fitzsimons, C. P., Van Hooijdonk, L. W. A., Schouten, M., Zalachoras, I., Brinks, V., Zheng, T., & Verhaagen, J. (2013). Knockdown of the glucocorticoid receptor alters functional integration of newborn neurons in the adult hippocampus and impairs fear-motivated 124ignallin. *Molecular psychiatry*, 18(9), 993-1005.

138. Ekdahl, C. T. (2012). Microglial activation–tuning and pruning adult neurogenesis. Frontiers in pharmacology, 3. Enhanced sensitivity of hippocampal pyramidal neurons from *mdx* mice to hypoxia-induced loss of synaptic transmission *Human Molecular Genetics*, Volume 4, Pages 329–335

139. Garcia, A., Steiner, B., Kronenberg, G., Bick-Sander, A., & Kempermann, G. (2004). Age-dependent expression of glucocorticoid-and mineralocorticoid receptors on neural precursor cell populations in the adult murine hippocampus. *Aging cell*, 3(6), 363-371.

140. Fitzsimons, C. P., Ahmed, S., Wittevrongel, C. F., Schouten, T. G., Dijkmans, T. F., Scheenen, W. J., & Vreugdenhil, E. (2008). The microtubule-associated protein doublecortin-like regulates the transport of the glucocorticoid receptor in neuronal progenitor cells. *Molecular Endocrinology*, 22(2), 248-262.

141. Weaver, I. C., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., & Meaney, M. J. (2004). Epigenetic programming by maternal behavior. *Nature neuroscience*, 7(8), 847-854.

142. Szyf, M., Weaver, I. C., Champagne, F. A., Diorio, J., & Meaney, M. J. (2005). Maternal programming of steroid receptor expression and phenotype through DNA methylation in the rat. *Frontiers in neuroendocrinology*, 26(3), 139-162.

143. Meaney, M. J., Szyf, M., & Seckl, J. R. (2007). Epigenetic mechanisms of perinatal programming of hypothalamic-pituitary-adrenal function and health. *Trends in molecular medicine*, 13(7), 269-277.

144. Zhang, T. Y., Labonté, B., Wen, X. L., Turecki, G., & Meaney, M. J. (2013). Epigenetic mechanisms for the early environmental regulation of hippocampal glucocorticoid receptor gene expression in rodents and humans. *Neuropsychopharmacology,* 38(1), 111-123.

145. Mirescu, C., Peters, J. D., & Gould, E. (2004). Early life experience alters response of adult neurogenesis to stress. *Nature neuroscience*, 7(8), 841-846

146. Mak, G. K., Antle, M. C., Dyck, R. H., & Weiss, S. (2013). Bi-parental care contributes to sexually dimorphic neural cell genesis in the adult mammalian brain. *PloS one,* 8(5), e62701.

147. Leuner, B., Kozorovitskiy, Y., Gross, C. G., & Gould, E. (2007). Diminished adult neurogenesis in the marmoset brain precedes old age. *Proceedings of the National Academy of Sciences*, 104(43), 17169-17173.

148. Murphy, E. K., Spencer, R. L., Sipe, K. J., & Herman, J. P. (2002). Decrements in nuclear glucocorticoid receptor (GR) protein levels and DNA binding in aged rat hippocampus. *Endocrinology*, 143(4), 1362-1370.

149. Mizoguchi, K., Ikeda, R., Shoji, H., Tanaka, Y., Maruyama, W., & Tabira, T. (2009). Aging attenuates glucocorticoid negative feedback in rat brain. *Neuroscience,* 159(1), 259-270.

150. Tanapat, P., Galea, L. A., & Gould, E. (1998). Stress inhibits the proliferation of granule cell precursors in the developing dentate gyrus. *International Journal of Developmental Neuroscience*, 16(3), 235-239.

151. Gould, E., & Tanapat, P. (1999). Stress and hippocampal neurogenesis. *Biological psychiatry*, 46(11), 1472-1479.

152. Avishai-Eliner, S., Gilles, E. E., Eghbal-Ahmadi, M., Bar-El, Y., & Baram, T. Z. (2001). Altered Regulation of Gene and Protein Expression of Hypothalamic-Pituitary-Adrenal Axis Components in an Immature Rat Model of Chronic Stress. *Journal of neuroendocrinology*, 13(9), 799-807.

153. Gould, E., McEwen, B. S., Tanapat, P., Galea, L. A., & Fuchs, E. (1997). Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. *Journal of Neuroscience*, 17(7), 2492-2498.

154. Slotkin, T. A., Zhang, J., McCook, E. C., & Seidler, F. J. (1998). Glucocorticoid administration alters nuclear transcription factors in fetal rat brain: implications for the use of antenatal steroids. *Developmental brain research*, 111(1), 11-24.

155. Takahashi, L. K., & Goh, C. S. (1998). Glucocorticoid facilitation of cholinergic development in the rat hippocampus. *Neuroscience*, 83(4), 1145-1153.

156. Coe, C. L., Kramer, M., Czéh, B., Gould, E., Reeves, A. J., Kirschbaum, C., & Fuchs, E. (2003). Prenatal stress diminishes neurogenesis in the dentate gyrus of juvenile rhesus monkeys. *Biological psychiatry*, 54(10), 1025-1034.

157. Ambrogini, P., Orsini, L., Mancini, C., Ferri, P., Barbanti, I., & Cuppini, R. (2002). Persistently high corticosterone levels but not normal circadian fluctuations of the hormone affect cell proliferation in the adult rat dentate gyrus. *Neuroendocrinology*, 76(6), 366-372.

158. Haynes, L. E., Lendon, C. L., Barber, D. J., & Mitchell, I. J. (2003). 17β-oestradiol attenuates dexamethasone-induced lethal and sublethal neuronal damage in the striatum and hippocampus. *Neuroscience,* 120(3), 799-806.

159. Crochemore, C., Michaelidis, T.M., Fischer, D., Loeffler, J. P., & Almeida, O.F. (2002). Enhancement of p53 activity and inhibition of neural cell proliferation by glucocorticoid receptor activation. *The FASEB journal*, 16(8), 761-770.

160. Son, G. H., Geum, D., Jung, H., & Kim, K. (2001). Glucocorticoid inhibits growth factor-induced differentiation of hippocampal progenitor HiB5 cells. *Journal of neurochemistry*, 79(5), 1013-1021.

161. Kim, J. B., Ju, J. Y., Kim, J. H., Kim, T. Y., Yang, B. H., Lee, Y. S., & Son, H. (2004). Dexamethasone inhibits proliferation of adult hippocampal neurogenesis in vivo and in vitro. *Brain research,* 1027(1), 1-10.

162. Yehuda R (2009) Status of glucorticoids alterations in post-traumatic stress disorder. *Ann N Y Acad Sci* 1179: 56-69

163. Pichavant C, Aartsma-Rus A, Clemens PR, Davies KE, Dickson G, Takeda S, Wilton SD, Wolff JA, Wooddell CI, Xiao X, Tremblay JP (2011) Current status of pharmaceutical and genetic therapeutic approaches to treat DMD. *Mol Ther* 19:830-840

164. Howell, M. P., & Muglia, L. J. (2006). Effects of genetically altered brain glucocorticoid receptor action on

signallin and adrenal axis regulation in mice. *Frontiers in neuroendocrinology*, 27(3), 275-284.

165. Lehmann, M. L., Brachman, R. A., Martinowich, K., Schloesser, R. J., & Herkenham, M. (2013). Glucocorticoids orchestrate divergent effects on mood through adult neurogenesis. *Journal of Neuroscience*, 33(7), 2961-2972.

166. McEwen, B. S. (2009). The brain is the central organ of stress and adaptation. *Neuroimage*, 47(3), 911.

167. Deuschle, M., Gotthardt, U., Schweiger, U., Weber, B., Körner, A., Schmider, J., & Heuser, I. (1997). With aging in humans the activity of the hypothalamus-pituitary-adrenal system increases and its diurnal amplitude flattens. *Life sciences*, 61(22), 2239-2246.

168. De Filippis, B., Ricceri, L., Fuso, A., & Laviola, G. (2013). Neonatal exposure to low dose corticosterone persistently modulates hippocampal mineralocorticoid receptor expression and improves locomotor/exploratory signalling a mouse model of Rett syndrome. *Neuropharmacology*, *68*, 174-183.

169. Fowden AL & Forhead AJ (2009). Hormones as epigenetic signals in developmental programming. *Exp Physiol* 94, 607–625.

170. Huang C-CJ, Shih M-CM, Hsu N-C, Chien Y & Chung B (2012). Fetal glucocorticoid synthesis is required for development of the fetal adrenal medulla and hypothalamic feedback suppression. *Endocrinology* 153, 4749–4756.

171. Chapman K, Holmes M & Seckl JR (2013). 11β-Hydroxysteroid dehydrogenase: intracellular gate-keepers of tissue glucocorticoid action. *Physiol Rev* 93, 1139–1206.

172. Fowden AL, Li J & Forhead AJ (1998). Glucocorticoids and the preparation for life after birth: are there long-term consequences of the life insurance? *Proc Nutr Soc* 57, 113–122.

173. Moisiadis VG & Matthews SG (2014). Glucocorticoids and fetal programming part 1: outcomes. *Nat Rev Endocrinol* 10, 391–402.

174. Atkinson HC & Waddell BJ 1995 The hypothalamic–pituitary–adrenal axis in rat pregnancy and lactation: circadian variation and interrelationship of plasma adrenocorticotrophin and corticosterone. *Endocrinology* 136 512–520.

175. Boyle, M. P., Brewer, J. A., Funatsu, M., Wozniak, D. F., Tsien, J. Z., Izumi, Y., & Muglia, L. J. (2005). Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes in adrenal axis regulation and behavior. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(2), 473-478.

176. Dong, Y., Poellinger, L., Gustafsson, J. Å., & Okret, S. (1988). Regulation of glucocorticoid receptor expression: evidence for transcriptional and posttranslational mechanisms. *Molecular Endocrinology*, 2(12), 1256- 1264.

177. Yu, I. T., Lee, S. H., Lee, Y. S., & Son, H. (2004). Differential effects of corticosterone and dexamethasone on hippocampal neurogenesis in vitro. *Biochemical and biophysical research communications*, *317*(2), 484-490.

178. Vaillend, C., & Chaussenot, R. (2017). Relationships linking emotional, motor, cognitive and GABAergic dysfunctions in dystrophin-deficient mdx mice. *Human molecular genetics*, *26*(6), 1041-1055.

180. Peffer, M. E., Chandran, U. R., Luthra, S., Volonte, D., Galbiati, F., Garabedian, M. J., & DeFranco, D. B. (2014). Caveolin-1 regulates genomic action of the glucocorticoid receptor in neural stem cells. *Molecular and cellular biology*, 34(14), 2611-2623.

181. Chrousos GP. (2000) Stress, chronic inflammation, and emotional and physical well-being: concurrent effects and

chronic sequelae. *J Allergy Clin Immunol*; 106:S275–291.

182. Rhudy JL (2009). The importance of emotional processes in the modulation of pain. *Pain*; 146:233–4.

183. Chesler EJ, Wilson SG, Lariviere WR, Rodriguez-Zas SL, Mogil JS. (2002a) Identification and ranking of genetic and labo- ratory environment factors influencing a behavioral trait, thermal nociception, via computational analysis of a large data archive. *Neurosci Biobehav* Rev; 26:907– 23.

184. Chesler EJ, Wilson SG, Lariviere WR, Rodriguez-Zas SL, Mogil JS. (2002b) Influences of laboratory environment on behavior. *Nat Neurosci*; 5:1101–2.

185. Droste, S. K., de Groote, L., Atkinson, H. C., Lightman, S. L., Reul, J. M., and Linthorst, A. C. (2008). Corticosterone levels in the brain show a distinct ultradian rhythm but a delayed response to forced swim stress. *Endocrinology* 149, 3244–3253.

186. Spencer, A., Yu, L., Guili, V., Reynaud, F., Ding, Y., Ma, J., & Falk, J. (2017). Nerve Growth Factor Signaling from Membrane Microdomains to the Nucleus: Differential Regulation by Caveolins. *International journal of molecular sciences*, 18(4), 693

187. Cole, M. A., Kim, P. J., Kalman, B. A., & Spencer, R. L. (2000). Dexamethasone suppression of corticosteroid secretion: evaluation of the site of action by receptor measures and functional studies. *Psychoneuroendocrinology*, *25*(2), 151-167.

188. Ago, Y., Arikawa, S., Yata, M., Yano, K., Abe, M., Takuma, K., & Matsuda, T. (2008). Antidepressant-like effects of the glucocorticoid receptor antagonist RU-43044 are associated with changes in prefrontal dopamine in mouse models of depression. *Neuropharmacology*, *55*(8), 1355-1363.

189. Harris, B. N., Saltzman, W., de Jong, T. R., & Milnes, M. R. (2012). Hypothalamic–pituitary–adrenal (HPA)

axis function in the California mouse (Peromyscus californicus): changes in baseline activity, reactivity, and fecal excretion of glucocorticoids across the diurnal cycle. *General and comparative endocrinology*, *179*(3), 436-450.

190. Wróbel, A., Serefko, A., Wlaź, P., & Poleszak, E. (2014). The depressogenic-like effect of acute and chronic treatment with dexamethasone and its influence on the activity of antidepressant drugs in the forced swim test in adult mice. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *54*, 243-248.

191. Mehler M. F. (2000). Brain dystrophin, neurogenetics and mental retardation. *Brain Research Reviews*, *32*(1), 277-307.

192. Miranda, R., Laroche, S., & Vaillend, C. (2016). Reduced neuronal density in the CA1 anterodorsal hippocampus of the mdx mouse. *Neuromuscular Disorders*, *26*(11), 775-781.

193. van Praag H, Kempermann G, Gage FH (1999) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat Neurosci* 2:266–270

194. Snyder JS, Glover LR, Sanzone KM et al (2009) The effects of exercise and stress on the survival and maturation of adult-gen- erated granule cells. *Hippocampus* 19:898–906

195. Kirby ED, Muroy SE, Sun WG et al (2013) Acute stress enhances adult rat hippocampal neurogenesis and activation of newborn neurons via secreted astrocytic FGF2. *Elife* 2: e00362.

196. Chaussenot, R., Edeline, J. M., Le Bec, B., El Massioui, N., Laroche, S., & Vaillend, C. (2015). Cognitive dysfunction in the dystrophin-deficient mouse model of Duchenne muscular dystrophy: A reappraisal from sensory to executive processes. *Neurobiology of learning and memory*,

124, 111-122.

197. Remmelink, E., Aartsma-Rus, A., Smit, A. B., Verhage, M., Loos, M., & van Putten, M. (2016). Cognitive flexibility deficits in a mouse model for the absence of full-length dystrophin. *Genes, Brain and Behavior*, 15(6), 558-567.

198. Dalloz, C., Sarig, R., Fort, P., Yaffe, D., Bordais, A., Pannicke, T., & Nudel, U. (2003). Targeted inactivation of dystrophin gene product Dp71: phenotypic impact in mouse retina. *Human Molecular Genetics*, 12(13), 1543-1554.

199. Niks, E. H., & Aartsma-Rus, A. (2017). Exon skipping: a first in class strategy for Duchenne muscular dystrophy. *Expert opinion on biological therapy*, 17(2), 225-236.

200. Holsboer, F., & Ising, M. (2010). Stress hormone regulation: biological role and translation into therapy. *Annual review of psychology*, 61, 81-109.

201. Lucassen, P. J., Meerlo, P., Naylor, A. S., Van Dam, A. M., Dayer, A. G., Fuchs, E., ... & Czeh, B. (2010). Regulation of adult neurogenesis by stress, sleep disruption, exercise and inflammation: Implications for depression and antidepressant action. *European Neuropsychopharmacology*, 20(1), 1-17.

202. Fowden, A. L., & Forhead, A. J. (2015). Glucocorticoids as regulatory signals during intrauterine development. *Experimental physiology*, 100(12), 1477-1487.

203. Nicolaides, N. C., Kyratzi, E., Lamprokostopoulou, A., Chrousos, G. P., & Charmandari, E. (2015). Stress, the stress system and the role of glucocorticoids. *Neuroimmunomodulation*, 22(1-2), 6-19.

204. Walker, D. J., & Spencer, K. A. (2017). Glucocorticoid programming of neuroimmune function. *General and comparative endocrinology*.

205. Chiba, S., Numakawa, T., Ninomiya, M., Richards,

M. C., Wakabayashi, C., & Kunugi, H. (2012). Chronic restraint stress causes anxiety-and depression-like behaviours, downregulates glucocorticoid receptor expression, and attenuates glutamate release induced by brain-derived neurotrophic factor in the prefrontal cortex. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 39(1), 112-119.

206. Joëls, M., Fernandez, G., & Roozendaal, B. (2011). Stress and emotional memory: a matter of timing. *Trends in cognitive sciences*, 15(6), 280-288.

207. Sacta, M. A., Chinenov, Y., & Rogatsky, I. (2016). Glucocorticoid signaling: an update from a genomic perspective. *Annual review of physiology*, 78, 155-180.

208. Joëls, M. (2009). Stress, the hippocampus, and epilepsy. *Epilepsia*, 50(4), 586-597.

209. Garabedian, M. J., Harris, C. A., & Jeanneteau, F. (2017). Glucocorticoid receptor action in metabolic and neuronal function. F1000Research, 6.

210. Sotgia, F., Lee, J. K., Das, K., Bedford, M., Petrucci, T. C., Macioce, P., & Lisanti, M. P. (2000). Caveolin-3 directly interacts with the C-terminal tail of β-Dystroglycan identification of a central WW-like domain within caveolin family members. *Journal of Biological Chemistry*, 275(48), 38048-38058.

211. Füchsl, A. M., & Reber, S. O. (2016). Chronic psychosocial stress and negative feedback inhibition: enhanced hippocampal glucocorticoid signalling despite lower cytoplasmic GR expression. *PloS one*, 11(4), e0153164.

212. Cheng, Q., Morand, E., & Yang, Y. H. (2014). Development of novel treatment strategies for inflammatory diseases-similarities and divergence between glucocorticoids and GILZ. *Frontiers in pharmacology*, 5.

213. Krugers, H. J., Karst, H., & Joels, M. (2012). Interactions between noradrenaline and corticosteroids in the brain: from electrical activity to cognitive performance. *Frontiers in Cellular Neuroscience*, *6*.