Received: 29 July 2022 | Revised: 31 January 2023 | Accepted: 1 February 2023

DOI: 10.1111/bph.16056

RESEARCH ARTICLE

Acute intranasal treatment with nerve growth factor limits the onset of traumatic brain injury in young rats

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Funding information
Italian Ministry of Health Grant, Grant/Award Number: RF-2018-12366594

Background and Purpose: Traumatic brain injury (TBI) comprises a primary injury directly induced by impact, which progresses into a secondary injury leading to neuroinflammation, reactive astrogliosis, and cognitive and motor damage. To date, treatment of TBI consists solely of palliative therapies that do not prevent and/or limit the outcomes of secondary damage and only stabilize the deficits. The neurotrophin, nerve growth factor (NGF), delivered to the brain parenchyma following intranasal application, could be a useful means of limiting or improving the outcomes of the secondary injury, as suggested by pre-clinical and clinical data.

Experimental Approach: We evaluated the effect of acute intranasal treatment of young (20-postnatal day) rats, with NGF in a TBI model (weight drop/close head), aggravated by hypoxic complications. Immediately after the trauma, rats were intranasally treated with human recombinant NGF (50 $\mu g \cdot kg^{-1}$), and motor behavioural test, morphometric and biochemical assays were carried out 24 h later.

Key Results: Acute intranasal NGF prevented the onset of TBI-induced motor disabilities, and decreased reactive astrogliosis, microglial activation and IL-1β content, which after TBI develops to the same extent in the impact zone and the hypothalamus.

Conclusion and Implications: Intranasal application of NGF was effective in decreasing the motor dysfunction and neuroinflammation in the brain of young rats in our model of TBI. This work forms an initial pre-clinical evaluation of the potential of early intranasal NGF treatment in preventing and limiting the disabling outcomes of TBI, a clinical condition that remains one of the unsolved problems of paediatric neurology.

KEYWORDS
intranasal delivery, microglia, motor dysfunctions, nerve growth factor, paediatric rat, reactive astrogliosis, traumatic brain injury

Abbreviations: HH, hypercapnic hypoxic; NGF, nerve growth factor; TBI, traumatic brain injury.

1 | INTRODUCTION

Traumatic brain injury (TBI) can compromise neurological functions with long and permanent sequelae. Paediatric and adolescent populations are at high risk of TBI and in the European Union, 1.5 million people are admitted to a hospital and about 57,000 people die as a consequence of TBI, with an average of 25 years of life being lost due to each death (Majdan et al., 2022). TBI occurs following the transient application of a mechanical force to the brain, often resulting in cellular membrane disruption, axonal shearing and damage to the vasculature (Moppett, 2007). As result of this primary mechanical injury, a secondary cascade of molecular and biochemical events commences within minutes, further worsening the damage caused by the primary insult. Secondary injury may include reperfusion injury, excitotoxicity, amyloid beta-peptide (Aβ)-plaque deposition, inflammation, hypoxia, and ischemia, shaping the extent and severity of the impairment (Ladak et al., 2019). Targeting some of these secondary injury processes can play a pivotal role in the prevention of damage-induced cell death and tissue degeneration and thus provide an attempt to offer a resolution to a looming problem that is primarily affecting the paediatric population. To the best of our knowledge, this is the first study that aimed to study the outcomes of TBI in a paediatric rat model.

Nerve growth factor (**NGF**), the first discovered growth factor and a member of the neurotrophin family, has the potential to counteract many of the secondary deleterious effects triggered by TBI. NGF regulates the glial response (Pöyhönen et al., 2019) both by arresting the cell cycle of astrocytes and therefore the process of reactive astrogliosis (Cragnolini et al., 2012) and by stimulating the anti-inflammatory phenotype of microglia and consequently blocking the release of pro-inflammatory cytokines (Rizzi et al., 2018). NGF stimulates angiogenesis and vasculogenesis (Emanueli et al., 2002) by inducing the production of VEGF (Graiani et al., 2004) by neurons, glia and endothelial cells (Nag et al., 2002; Ogunshola et al., 2000), and supporting the proliferation and migration of endothelial cells (Dolle et al., 2005). NGF also improves motor and learning-memory abilities through the improvement of neuroinflammation after transplantation of DOPA-NGF activated tissue engineering microunits in TBI mice (Zhu et al., 2022).

Over the years, NGF has been conveyed to the brain parenchyma in different ways, in both animals and humans (Manni et al., 2021a) but, to date, intranasal application is considered to provide a less invasive, safe and effective mode of administration for delivery of NGF to the brain (Thorne & Frey, 2001). Intranasal application allows NGF to reach the cerebral parenchyma, mainly by diffusing in the perivascular and perineural spaces of the olfactory and trigeminal nerves (Lochhead & Davis, 2019; Lochhead & Thorne, 2012). To date, intranasal NGF treatment on TBI patients has only been attempted 6 months after the trauma, in a child with severe motor and cognitive impairment and yielded improvements in functional and electrophysiological parameters with the concomitant amelioration of clinical and neurological functions (Chiaretti et al., 2017). Other authors have reported that intranasal NGF improved β-amyloid deposition and aquaporins-4-induced oedema and lowered tau phosphorylation in an open-head model of adult TBI in rats (Lv et al., 2013; Tian et al., 2012).

What is already known

- To date, the treatment of traumatic brain injury (TBI) consists only of palliative therapies.
- Intranasal NGF improves functional and electrophysiological parameters in paediatric TBI patients.

What does this study add

- Early intranasal NGF prevented onset of motor coordination and locomotor dysfunctions following TBI in young rats.
- Intranasal NGF also decreased the TBI-induced reactive astrogliosis and neuroinflammation.

What is the clinical significance

- Early intranasal NGF is potentially attractive in preventing/limiting disabling outcomes of TBI in paediatric patients.

In the work described here, the effects of acute intranasal NGF treatment were evaluated in a paediatric rat model of TBI (weight drop/close-head) aggravated by hypoxic complications. Thus, this study aimed to treat the secondary damage by limiting its development into a chronic state, using an animal model that mimics as much as possible the progression of aetiopathological events (mechanical damage followed by hypoxic complications) to which the brain of a child suffering a severe head injury is subjected.

2 | METHODS

2.1 | Animals

Animal care conformed to the Legislation for the protection of animals used for scientific purposes provided by the relevant Italian law and European Union Directive (Italian Legislative Decree 26/2014 and 2010/63/EU) and the International Guiding Principles for Biomedical Research involving animals (Council for the International Organizations of Medical Sciences, Geneva, CH). The experimental procedures were approved by the Veterinary Department of the Italian Ministry of Health (Permit Number: 603/2019-PR). All adequate measures were taken to minimize animal pain or discomfort and all surgery was

performed under anaesthesia. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020).

Twenty-postnatal day (PND) Wistar rats were housed in the maternal home cage and experiments were carried out before weaning. Animals were maintained in a temperature and humidity-controlled room with a 12-h light–dark cycle in the animal facility of Tor Vergata University of Rome. Standard food and water were available ad libitum.

The animals used in this study were not intentionally randomized but arbitrarily selected without taking sex into account for assignment to the experimental groups. Russell et al., (2011) have shown that 20-PND prepubertal rats did not show acute or long-term sex-related neurobehavioral outcomes in a juvenile rat model of TBI. Furthermore, we performed preliminary statistical analyses of our data (not shown), which did not show a 'sex' effect, before pooling data across both sexes. Experimental researchers were blinded in the assessment of the behavioural outcome and throughout the data analysis.

2.2 | Experimental design

Rats were allocated into four different experimental groups as follows: SHAM, SHAM+NGF, TBI/HH, and TBI/HH+NGF. For TBI and post-injury hypercapnic hypoxic (HH) inductions see Section 2.3 and for intranasal NGF delivery to the brain see Section 2.4. Twenty-four hours post injury animals planned for behavioural tasks were tested with the rotarod test ($n = 8$ for each group; see Section 2.5.1) and the open field test (OFT, $n = 10$ for each group; see Section 2.5.2). Animals used for immunofluorescence experiments ($n = 5$ for each group; see Section 2.6) were terminally anaesthetised with ketamine (90 mg kg^{-1}) and medetomidine (0.5 mg kg^{-1}), followed by intracardial perfusion with 4% (w/v) paraformaldehyde in PBS. Brains were then post-fixed overnight and cryoprotected in 30% (w/v) sucrose dissolved in PBS. Analyses of tissue protein content were performed on tissues micro-dissected 24 h post-trauma to evaluate effects of intranasal NGF. Animals ($n = 11$ for each group; see Section 2.7) were killed by decapitation, under light isoflurane anaesthesia, and brain regions of interest (ROIs) were rapidly micro-dissected on ice by a rat brain matrix, snap-frozen in liquid nitrogen, and stored at −80°C. Both immunofluorescence and protein content analyses were carried out by examining brain areas directly or indirectly affected (parietal cortex and hypothalamus: Paxinos coordinates −2/−4) and brain areas that could be functionally and physically interconnected with parietal cortex and constituting the cortico-thalamic-striatal circuit involved in sensorimotor regulation that seems perturbed in the TBI/HH rat model (striatum: Paxinos coordinates +2/0; thalamus: Paxinos coordinates −2/−4). The serum protein content analysis was performed on samples collected 2, 4, 24, 48, 72, and 96 h post-trauma for a preliminary set-up experiment. Whole blood was drawn by intracardiac sampling at the time of killing and left to clot at room temperature for 30 min. The serum was then collected after centrifugation at 3000× *g* for 15 min.

2.3 | Experimental TBI

A modified closed-head weight-drop model (Mychasiuk et al., 2014) was performed as follows: 20-PND male and female rats were lightly anaesthetised with 2% isoflurane in air until the toe pinch response had disappeared. Animals were placed in a prone position on tinfoil below the head injury device so that the skull region between coronal and lambdoid sutures was centred beneath the weight drop path. To establish the TBI, a weight of 420 g was dropped from a height of 100 cm on the rat's skull. The extent of the weight needed to induce a consistent injury was chosen by performing a preliminary experiment in which the damage generated by different weights was evaluated on the rotarod time latency performance 24 h post-injury (Figure S1). For the rotarod protocol, see Section 2.5.1 and Figure S1A, for rotarod time latency see Figure S1B. SHAM rats received isoflurane anaesthesia only, without the trauma procedure. Loss of righting reflex was used as confirmation of injury and the righting reflex latency (time taken for animals to return to a standing position from the supine position following removal of anaesthetic) was measured and used as a marker of injury severity as it reflects the loss of consciousness (Mychasiuk et al., 2014) (Figure S1C). Animals in which loss of consciousness was less than 180 s were not considered. Additional post-traumatic hypoxia, which is common in patients with head injuries, was shown to worsen the TBI-induced diffuse neurological dysfunction (Bramlett et al., 1999; Matsushita et al., 2001). Thus, when the righting reflex was re-established, a post-injury hypercapnic hypoxic (HH) insult was performed. TBI rats were placed for 1 h in a sealed plexiglass chamber containing an atmosphere of 4% CO_2, 8% O_2, and 88% N_2, and then returned to their home cage. The mean mortality in the preparation of the experimental TBI/HH rat model was 10.3%.

2.4 | Intranasal administration for NGF delivery to brain parenchyma

Immediately after the TBI/HH insult, SHAM and TBI/HH rats were subjected to acute intranasal treatment with saline (SHAM and TBI/HH experimental groups) or with human recombinant NGF (SHAM+NGF and TBI/HH+NGF experimental groups). Animals were lightly anaesthetised with 2% isoflurane in air and the head was elevated to keep the nasal cavity upward. About 10 µl of saline or drug (depending on the weight of the rat) was delivered to each nostril using a Hamilton microsyringe and, after the final droplet, the rat's nose was kept pointing upward for 20 s. Each rat received a total amount of 50 µg·kg^{-1} of NGF, intranasally delivered in three rounds with 90 min of inter-treatment interval. That dose of NGF

was chosen to match the published (Chiaretti et al., 2017) and ongoing clinical study on children affected by the outcomes of severe-TBI (Clinical Trials Register 2019-002282-35/IT, 2019).

2.5 | Behavioural tasks

2.5.1 | Rotarod

Post-injury motor coordination and balance were evaluated by a rotarod test. The day before the injury induction, a training session was performed. Rats (n = 8 for each group) were habituated to the rotarod apparatus (Ugo Basile, Italy), rotating at 20 rpm until the rats stayed on the rod without falling for 60 s, and this procedure was repeated three times at 10 min intervals. In the testing session, rats were placed on the rotating rod, accelerating from 20 to 60 rpm within 210 s (Figure S1A), and the process was continued until the rats fell from the rotating cylindrical platform to the floor. The latency of rats falling from the rod was the test's endpoint measure and was recorded. For rats that remained on the platform, the cut-off time was 210 s. Rotarod test was repeated three times with 15 min intervals and the mean of three trials was used.

2.5.2 | Open-field test

The open-field test (OFT) was performed in an open-field box which was made of black-coloured plywood (60 cm in length × 65 cm in width) surrounded by 35 cm high walls. The floor of the arena was divided with light red painted lines. Before OFT, rats (n = 10 for each group) were allowed to acclimatise to the experimental room in their home cages for 30 min to minimize stress. All animals were tested 24 h post-injury. The distance travelled and the average velocity of moving was measured to assess the locomotor activity. The total time of moving, immobility and time spent in the central and peripheral part of the arena was analysed to assess the motivation to explore unsafe areas voluntarily. At the end of every session, the box was thoroughly cleaned using 20% ethanol. Each trial was recorded using a video camcorder (CCD camera ICD-47EG, Ikegami Tsushinki, Tokyo, Japan) positioned above the OFT arena, and the Ethovision 3.0.15 tracking software (Noldus, Wageningen, Netherlands) was used for data acquisition.

2.6 | Immunofluorescence and confocal microscopy

Coronal brain sections (n = 5 rats for each group) were processed for confocal microscopy. Brains were cut on a cryostat and serial 40 µm-thick sections were sampled to cover ROIs corresponding to the parietal cortex and hypothalamus (Paxinos coordinates −2/−4). To cover these ROIs, slices for each rat were analysed spanning 400 µm from each other. Free-floating coronal brain sections were pre-incubated with PBS containing 10% (v/v) donkey serum, 1% (w/v) BSA and 0.3% (v/v) Triton X-100, for 2 h at room temperature before being incubated overnight at 4°C with primary antibodies: mouse GFAP (BD Pharmingen, cat# 556327, RRID:AB_396365) diluted 1:200, rat CD11b (BioRad, cat# MCA711G, RRID:AB_323167) diluted 1:200, rabbit Iba1 (FUJIFILM Wako Pure Chemical Corporation, cat# 019-19741, RRID:AB_839504) diluted 1:200, rabbit ki67 (Abcam, cat# ab16667, RRID:AB_302459) diluted 1:200, goat TrkA (R&D systems, cat# AF175, RRID:AB_354970) diluted 1:100, mouse $p75^{NTR}$ B1 (Santa Cruz Biotechnology, cat# sc-271708, RRID:AB_10714958) diluted 1:200, and rabbit $p75^{NTR}$ H92 (Santa Cruz Biotechnology, cat# sc-5634, RRID:AB_648183) diluted 1:100. Sections were rinsed in PBS and specific secondary antibodies were incubated for 1 h at room temperature. After extensive washes, Hoechst reagent was used for nuclei staining. The immunofluorescence images were captured by a confocal laser-scanning microscope (Leica SP5, Leica Microsystems, Wetzlar, Germany) under sequential mode. For the production of figures, processing was done by using the Adobe Photoshop CS6 software (Adobe Systems Incorporated, San Josè, United States). Image processing was performed using the ImageJ software (https://imagej.nih.go). For the analysis of the $GFAP^+$ cell morphology, for each image (63× magnification), three to five cells were analysed. The background was excluded by applying a threshold setting, which was kept constant for all the analyses. Regions of interest for single-cell were selected and the area, corresponding to the surface occupied by all pixels whose values exceed the defined threshold, and the perimeter was automatically calculated by the software (Figure S7). The intensity distribution was measured, integrated, and normalized to the value of the selected area using the 'integrated density' module. The module 'shape descriptor' was used to analyse the cell circularity (4π area/perimeter^2) and the cell solidity (area/convex area) (Figure S7). The number of processes of cells that had at least one extroflection with a length at least equal to the cell body diameter was manually evaluated and their length was measured. For each image, 3 to 6 cells were selected and the average mean process length for each cell was considered.

2.7 | Protein extraction, western blot and ELISA

A separate set of experiments was performed to collect tissues to be processed for brain protein content analyses. Brain areas corresponding to ROIs were micro-dissected from n = 11 rats for each group and homogenized in ice-cold RIPA lysis buffer at a 1:100 ratio of brain tissue to buffer (w:v), using a pre-cooled glass potter and centrifuged at ~14,000 × g for 10 min at +4°C. The total content of GFAP (R&D System, DuoSet ELISA cat# DY2594-05) and **IL-1β** (R&D System, DuoSet ELISA cat# DY201-05) was measured in tissue extracts accordingly to the manufacturer data sheet and expressed as pg·mg^{-1} of total proteins. The GFAP content was also analysed by SDS-PAGE as previously described (Soligo et al., 2015) by blotting with purified mouse IgG2b anti-GFAP antibody (BD Pharmingen, cat# 556327, RRID:AB_396365) diluted 1:500. Five different sets of samples were run, membranes were scanned by BioRad ChemiDoc™ XRS^+ for analyses, and gel densitometry of GAPDH-normalized

bands was performed on immunoblot images using the ImageJ gel analysis tool. Values were expressed as a percentage of the SHAMs group's mean value. The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology*.

2.8 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology. Operators blinded to the experimental groups' allocation performed statistical analysis for behavioural tests, immunofluorescence and protein ELISAs.

Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software). Statistical analysis was performed without taking sex into account for assignment to experimental groups, because preliminary analyses for the effects of both trauma and intranasal NGF on males and females did not reveal a main or interaction effect related to sex. The Shapiro–Wilk normality test was used to determine if variables followed a normal distribution. Results are presented as means ± SEM (for data with Gaussian distribution) or median ± range (for data with non-Gaussian distribution). An unpaired *t*-test was used to evaluate the latency of the righting reflex on SHAM and TBI/HH rats and one-way ANOVA was performed to evaluate the effect of the different extents of weight needed to induce a consistent injury and the time-course analyses of serum GFAP content. For immunofluorescence analysis and gel densitometry ($n = 5$ rats per experimental group), the Kruskal–Wallis ANOVA on ranks followed by Dunn's post-hoc method was used. To assess the effects of trauma and treatment, on rotarod test ($n = 8$ rats per experimental group), OFT ($n = 10$ rats per experimental group), and ELISA ($n = 11$ rats per experimental group), two-way ANOVA was performed, followed by Bonferroni post-hoc test for multiple comparisons. Post hoc tests were conducted only if F in ANOVA achieved $P < 0.05$. All two-way and Kruskal–Wallis ANOVA data are reported in the Supplementary materials.

2.9 | Materials

Human recombinant NGF (**Oxervate** formulation) was kindly provided by Dompè farmaceutici S.p.A., (Milan Italy). Isoflurane and ketamine were supplied by Zoetis Italia srl (Rome, Italy), and medetomidine by Orion Pharma (Milan, Italy).

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander, Fabbro, et al., 2021; Alexander, Kelly, et al., 2021).

3 | RESULTS

To investigate the effects of acute intranasal treatment with NGF (performed immediately after TBI/HH), the TBI/HH rat model was first established and the time window for analysis chosen (Section 3.1). Thus, we analysed the ability of intranasal NGF in preventing and/or limiting the TBI/HH-induced motor coordination dysfunction and locomotor impairment, possibly indicative of functional alterations of cortico-thalamo-striatal circuits (Section 3.2) (Mancini et al., 2021). The effects of intranasal NGF in limiting the outcomes of secondary lesions in the affected area were therefore studied. As secondary injury readouts, markers of astrogliosis (GFAP and astrocyte morphology) and neuroinflammation (microglial activation and IL-1β content) were assessed and the potential direct effect of intranasal NGF on glial cells was investigate (Section 3.3). Finally, we accounted for the analysis of the possible spread of neuroinflammation to the thalamus and striatum, involved in sensorimotor regulation (Figure S2).

3.1 | Progression of functional impairment in TBI/HH rats

On the day of injury, animals weighed 46.86 ± 0.57 g and a significant increase in righting reflex time latency was observed in TBI/HH rats (302 ± 26 s), compared with that in SHAM rats (59 ± 14.1); Figure S1C).

To confirm the progression of TBI/HH-induced functional impairment and to identify the time window suitable for studying the effects of intranasal NGF, preliminary time-course experiments on SHAM and TBI/HH animals were performed (Figures S3 and S4). Rotarod tests were performed 24, 48, 72, and 96 h post-trauma on separate groups of animals (Figure S3A). Two-way ANOVA for time latency revealed main effect for both trauma and time. Multiple comparisons showed a significant decrease in time latency at 24, 48, and 72 h post-trauma in TBI/HH rats compared with SHAMs, which was no longer evident at 96 h post-trauma (Figure S3A, P values in graph). Moreover, the improvement of performance in SHAM rats was observed at early time points (48 vs. 24 h), whereas TBI/HH rats improved their performance slowly (96 vs. 24 h, Figure S3A, P values in table below the graph), although the growth curves of SHAM and TBI/HH rats were comparable (Figure S3B). These results indicated that the trauma makes TBI/HH rats less prone to cope with the motor task in the first 72 h post-damage, whereas 96 h post-trauma their performance reached that of SHAMs. This was supported by the results of the single rotarod sessions (Figure S3C), showing that TBI/HH rats, in contrast to the SHAMs, did not improve their performance in subsequent trials of the same session, up until 96 h post-trauma (Figure S3C, P values in the tables below the graphs). Probably, TBI/HH rats were unable to learn and memorize the motor sequence necessary to take the test, or they suffered from muscle fatigue that made them unable to cope with the test at least up to 72 h post-injury. A strong correlation between time latencies of righting reflex and rotarod was observed (Figure S3D).

Extensive subdural haematoma was observed following the TBI/HH-induction, which remained evident up to 24 h post-trauma

and then gradually reabsorbed in the following hours (Figure S4A). At the same time, the analysis of serum GFAP, a known marker of trauma (Thelin et al., 2017), was carried out to further follow the course of the disease over time (Figure S4B). Serum GFAP significantly increased 2 h post-trauma, peaking at 4 h and decreasing 24 h after trauma (Figure S4B, *P* values in the graph) and then returned to baseline.

Given these results and considering that functional deficits are already evident 24 h post-trauma and persist for at least 72 h, in this work, we focused on an early analysis window. The effects of intranasal NGF, administered immediately after trauma, were then analysed 24 h post-TBI/HH (Figure 1a).

3.2 | Effect of intranasal NGF on TBI/HH-induced impairment of motor coordination and locomotor activity

To confirm the effect of acute intranasal NGF on TBI/HH-induced dysfunctions of motor coordination, balance, and stability, rotarod performances were evaluated 24 h post-trauma (Figure 1b, Table S1). The rotarod time latency was significantly lower in TBI/HH rats when compared with SHAMs, whereas intranasal NGF significantly improved the TBI/HH-impaired performances, which returned to that of SHAM rats (Figure 1b).

Locomotor activity was studied by evaluating the distance travelled, the average velocity, the time of moving, and the time of immobility in the OFT apparatus (Figure 1c–f, Table S1). Two-way ANOVA for all the analysed parameters revealed a main effect for trauma, treatment, and their interaction (Table S1). When compared with SHAMs, TBI/HH rats exhibited significantly lower total distance travelled (Figure 1c), total average velocity (Figure 1d), and total time of moving (Figure 1e), showing instead a higher time of immobility (Figure 1f). The intranasal NGF did not modify the performances of SHAM rats but counteracted the TBI/HH-induced locomotor deficit, re-establishing normal performances (Figure 1c–f).

To assess the motivation to voluntarily explore unsafe areas, the animal performances were evaluated in the central and peripheral parts of the arena (Figure 2, Table S2). Representative images of behaviour in the arena are reported in Figure 2a. In the inner

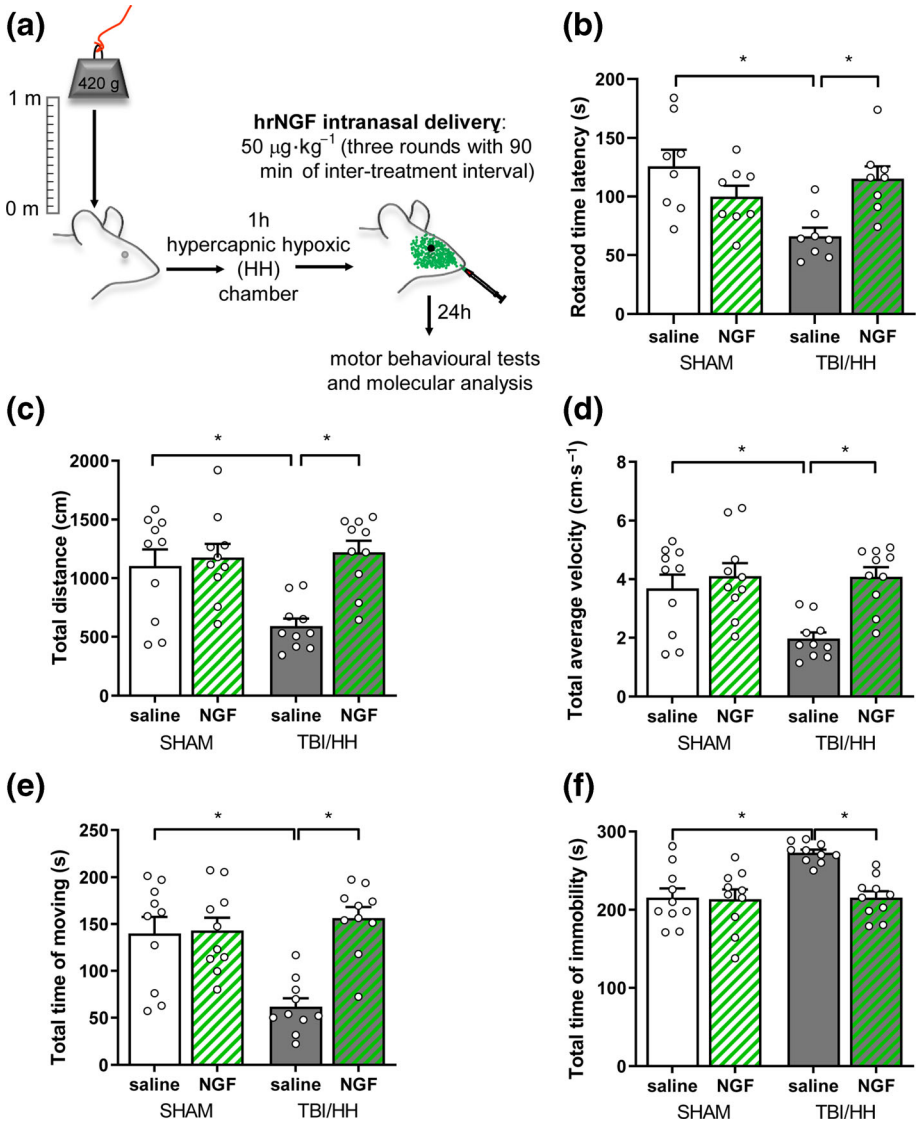

FIGURE 1 Acute treatment with intranasal NGF prevents the establishment of motor disabilities in TBI/HH rats. (a) Experimental design. (b) Immediately after trauma, SHAM and TBI/HH rats were treated with intranasal NGF and 24 h post-TBI/HH, the rotarod time latency to fall was measured. N = 8 animals per experimental group. (c–f) Animals were tested in an open field (OFT) and the total distance travelled (c), the mean total velocity (d), the total time of moving (e), and the total time of immobility (f) were analysed. N = 10 animals per experimental group. The values presented are means ± SEM. *$P<0.05$, significantly different as indicated; two-way ANOVA (see Table S1) followed by Bonferroni multiple comparisons test.

zone, two-way ANOVA for the total distance travelled and total time of moving revealed a main effect for treatment only (Table S2). Although, in the inner zone, the onset of trauma did not alter the performances of TBI/HH rats compared with that of SHAMs, intranasal NGF significantly increased the distance travelled and the time of moving of TBI/HH rats (Figure 2b,f). These apparently unclear results could indicate that intranasal NGF, restoring TBI/HH-impaired motor performance (Figure 1), disclose some trauma-related behaviours (such as those related to motivation and stress), otherwise hidden by locomotor disability. In the outer zone, the behaviour follows that already observed when locomotor activity was studied in the entire arena, such that intranasal NGF restored the overall performances altered by the trauma (Figure 2, Table S2).

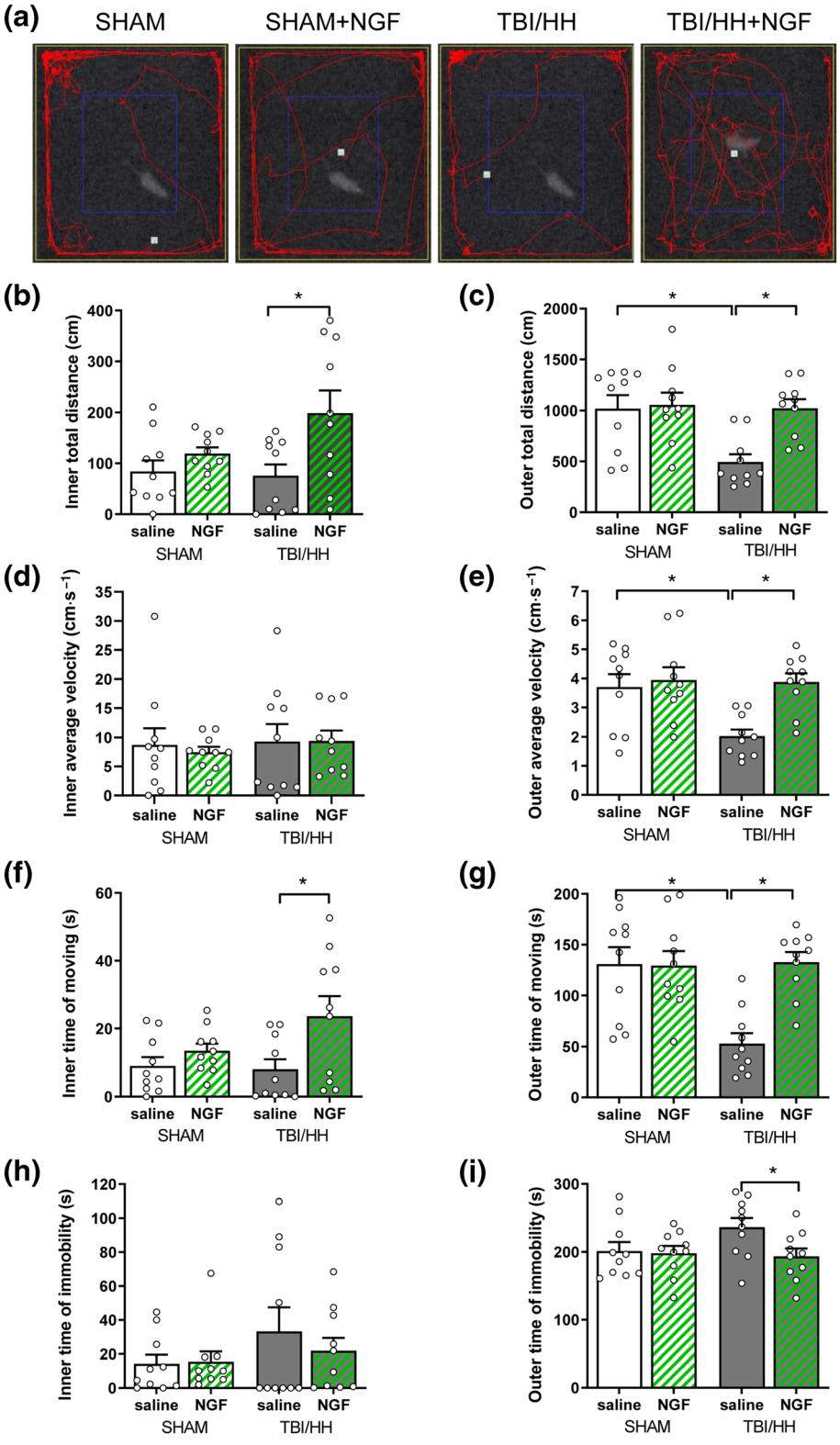

FIGURE 2 Acute treatment with intranasal NGF normalizes the locomotor performances in the open field test (OFT), in TBI/HH rats. (a) Representative images of the motor behaviour of rats in the OFT arena, generated from the tracking software. Total distance travelled in the inner (b) and outer (c) part of the OFT arena was analysed 24 h post-trauma in SHAM and TBI/HH rats treated or not with intranasal NGF. On the same animals the inner (d) and the outer (e) mean total velocity, the inner (f) and the outer (g) total time of moving, and the inner (h) and outer (i) total time of immobility were analysed. N = 10 animals per experimental group. The values presented are means ± SEM. *P<0.05, significantly different as indicated; two-way ANOVA (see Table S2) followed by Bonferroni multiple comparisons test.

3.3 | Effect of TBI/HH and intranasal NGF on neuroinflammation

TBI induces structural and functional alterations of astrocytes that result in reactive astrogliosis (Sofroniew, 2015). To correlate the functional alterations highlighted by motor tests, molecular and morphological analyses were carried out 24 h post-trauma in the cortical area target of the weight drop (Figure 3). The possible modulation of GFAP tissue content by intranasal NGF was evaluated by ELISA (Figure 3a). Two-way ANOVA revealed a main effect for trauma, treatment, and their interaction (Table S3). Intranasal NGF significantly normalized the GFAP content increased by TBI/HH (Figure 3a). Western blot analysis (Figure S5A, Table S4) was consistent with ELISA data. To verify the presence of reactive astrogliosis, immunofluorescence investigations were performed on the parietal cortex and analysed at both longitudinal fissure (Figure 3b–f) and parietal lobe (Figure S6A–E). Marked increase of GFAP staining was evident in TBI/HH rats when compared with SHAMs (Figure 3c,e; Figure S6B, D), which was reduced, in both analysed areas, by intranasal NGF (Figure 3f; Figure S6E). Single-cell analysis showed TBI/HH-induced up-regulation of GFAP intensity at both longitudinal fissure (Figure 3g and Table S5) and parietal lobe (Figure S6F and Table S5) that was normalized after intranasal NGF. Although it is known that the GFAP immunohistochemistry does not label all portions of the astrocyte, only the main stem branches (Sofroniew & Vinters, 2010), morphological evaluation of GFAP$^+$ cells showed an increased cell area covered

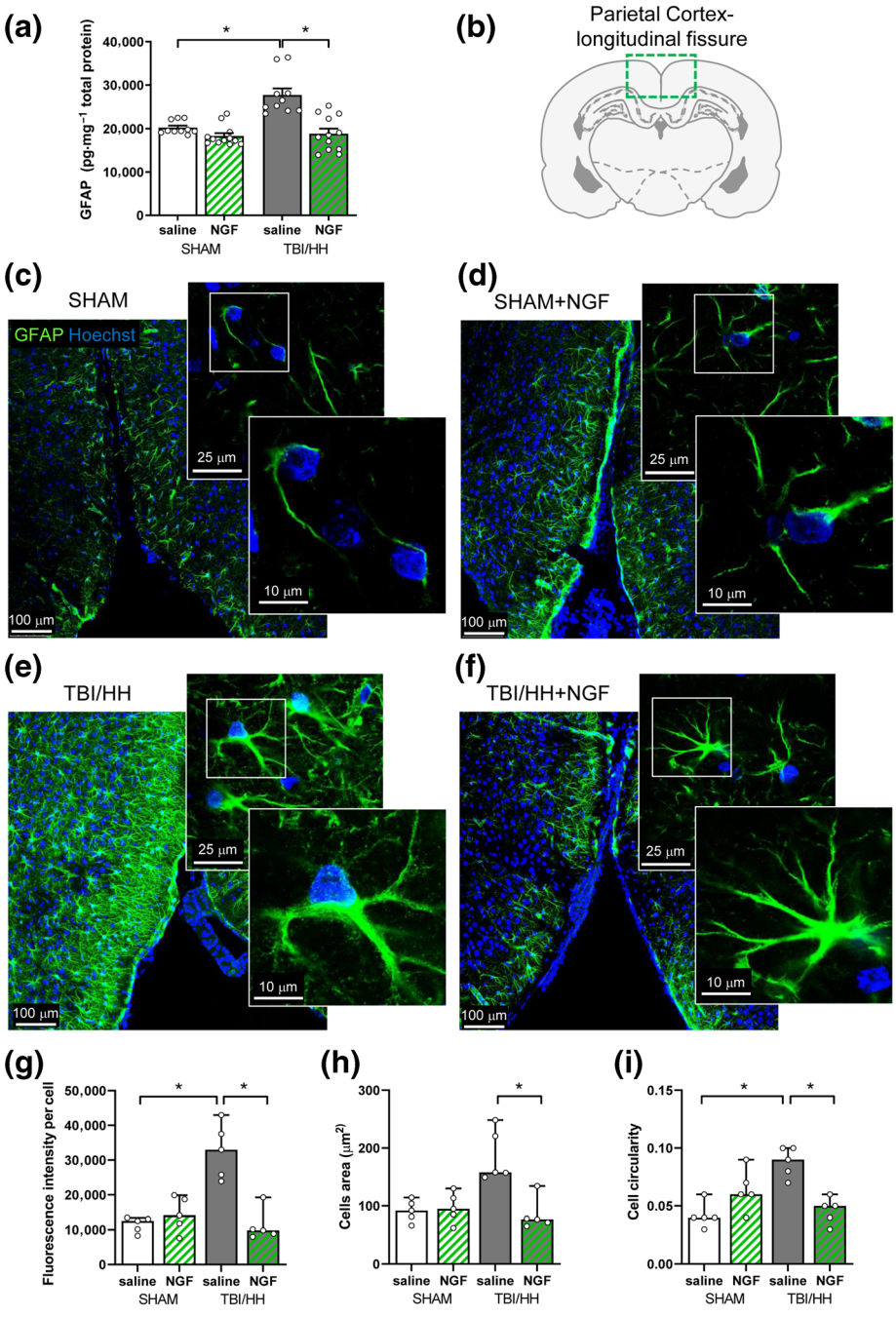

FIGURE 3 Acute treatment with intranasal NGF prevents the onset of reactive astrogliosis in the impact zone of TBI/HH rats. (a) The GFAP content in the impact zone of SHAM and TBI/HH rats treated or not with intranasal NGF was evaluated by ELISA. N = 11 animals per experimental group. The values presented are means ± SEM. *P<0.05, significantly different as indicated; two-way ANOVA (see Table S3) followed by Bonferroni multiple comparisons test. (b) Graphical sketch showing the brain area analysed in the subsequent immunofluorescence and morphological study (shown in panels c–i). Representative images (20×) of immunostaining for GFAP (green) and nuclei (blue) in the impact zone of SHAM (c), SHAM+NGF (d), TBI/HH (e), and TBI/HH+NGF (f) rats. The insets represent high magnification images; scale bars are shown in the images. (g) GFAP fluorescence intensity measured for single cells was obtained by ImageJ 'integrated density' module. (h) The mean cell area was automatically calculated by ImageJ software on the same selected cell. (i) Cell circularity was calculated by ImageJ 'shape descriptor' module. N = 5 animals/experimental group, three images for each animal and 3 to 5 cells for image were analysed. The values presented are medians ± range. *P<0.05, significantly different as indicated; Kruskal–Wallis ANOVA on ranks (see Table S5) followed by Dunn's post-hoc test.

by GFAP labelling in both regions of TBI/HH rats that was reduced after intranasal NGF (Figure 3h, Figure S6G). This result probably reflects a TBI/HH-induced cellular hypertrophy, as confirmed by increased astrocytic circularity (Figure 3i, Figure S6H), cell solidity (Figure S7B,7E), and decreased process length (Figure S7C, F). In contrast, the number of processes for single-cell (Figure S7D, G) was not altered as result of trauma. The significant effect of intranasal NGF in normalizing the morphology of astrocytes was observed for all the parameters affected by the trauma (Figures 3i, S6H, and S7B,C,E,F).

Acceleration/deceleration forces to the brain within the skull often generate TBI-induced hypothalamic–pituitary-neuroendocrine dysfunction (Rowe et al., 2016). To confirm the presence of reactive astrogliosis in the hypothalamus and the possible modulating effect of intranasal NGF, GFAP tissue content and GFAP$^+$ cells morphology were evaluated (Figure 4). Twenty-four hours after trauma, two-way ANOVA revealed a main effect for trauma and trauma/treatment interaction (Table S3). Intranasal NGF normalized the TBI-increased GFAP content in the hypothalamus (Figure 4a), as confirmed by immunofluorescence investigations (Figure 4b–f). The single-cell GFAP labelling was increased in TBI/HH rats compared with SHAMs and normalized by intranasal NGF (Figure 4g, Table S5). Furthermore, intranasal NGF normalized the increased cell area covered by GFAP labelling (Figure 4h) and the TBI/HH-decreased processes length (Figure S7J). On the contrary, intranasal NGF did not affect the TBI/HH-increased cell circularity (Figure 4i) and cell solidity (Figure S7K).

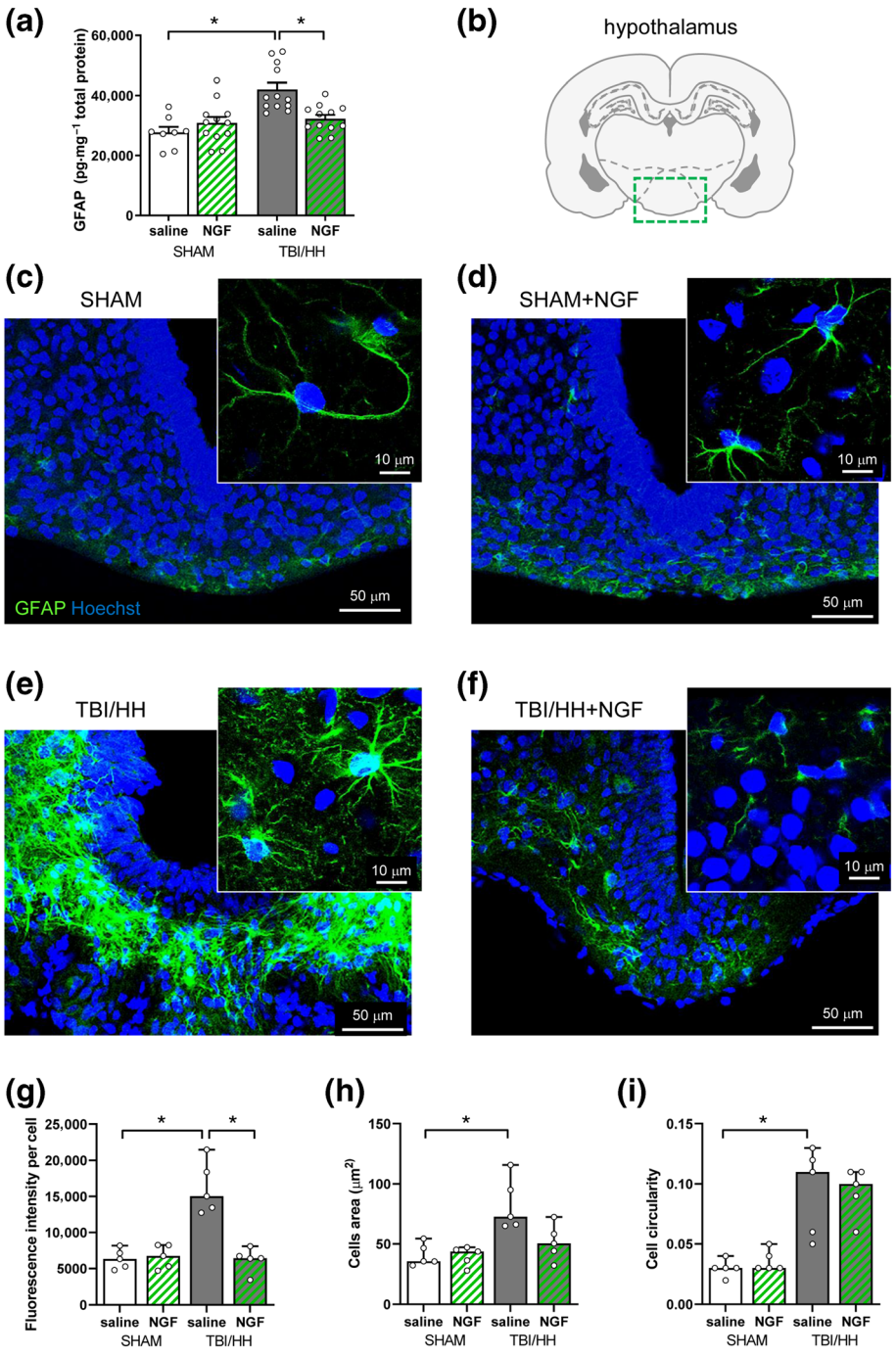

FIGURE 4 Acute treatment with intranasal NGF prevents the onset of reactive astrogliosis in the hypothalamus of TBI/HH rats. (a) The GFAP content in the impact zone of SHAM and TBI/HH rats, treated or not, with intranasal NGF was evaluated by ELISA. N = 11 animals per experimental group. The values presented are means ± SEM. *P<0.05, significantly different as indicated; two-way ANOVA (see Table S3) followed by Bonferroni multiple comparisons test. (b) Graphical sketch showing the brain area analysed in the subsequent immunofluorescence and morphological study (depicted in panels c–i). Representative images (20×) of immunostaining for GFAP (green) and nuclei (blue) in the hypothalamus of SHAM (c), SHAM+NGF (d), TBI/HH (e), and TBI/HH+NGF (f) rats. The insets represent high magnification images; scale bars are reported in the images. (g) GFAP fluorescence intensity measured for single cells was obtained by ImageJ 'integrated density' module. (h) The mean cell area was automatically calculated by ImageJ software. (i) Cell circularity was calculated by ImageJ 'shape descriptor' module. N = 5 animals per experimental group, three images for each animals, and three to five cells for image were analysed. The values presented are medians ± range. *P<0.05, significantly different as indicated; Kruskal–Wallis ANOVA on ranks (see Table S5) followed by Dunn's post-hoc test.

Glial responses to TBI are not isolated but integrated and coordinated. Astrocytic-microglial crosstalk is well described and mediated by cytokine release (Mira et al., 2021). Microglial activation leads to the release of mediators such as the pro-inflammatory IL-1β (Clark et al., 2019) that can trigger reactive astrogliosis and to be in turn released by reactive astrocytes (Sofroniew, 2015). Thus, the effects of intranasal NGF were evaluated on the possible TBI-induced alteration of Iba1 expression, an established marker of activated microglia (Karve et al., 2016), and IL-1β content in the cortical impact zone and hypothalamus (Figure 5, S8, Table S3, S6). Twenty-four hours after trauma, Iba1 staining was increased in TBI/HH rats compared with SHAMs in both the impact zone (Figure 5a,c) and the hypothalamus (Figure S8A,C) and normalized by intranasal NGF (Figures 5d and S8D). Non-parametric ANOVA on single-cell fluorescence intensity revealed a significant effect on both regions (Table S6). In the impact area, the Iba1 expression was significantly decreased by intranasal NGF in TBI/HH rats (Figure 5e) whereas a non-significant tendency to decrease was observed in the hypothalamus (Figure 5f). Two-way ANOVA for IL-1β content in the impact zone revealed a main effect for trauma, treatment, and their interaction, whereas in the hypothalamus, a main effect for trauma and trauma/treatment interaction was observed (Table S3). In both impact zone (Figure 5g) and hypothalamus (Figure 5h), the TBI/HH-increased IL-1β content was normalized by intranasal NGF, restoring IL-1β levels to those of SHAM rats.

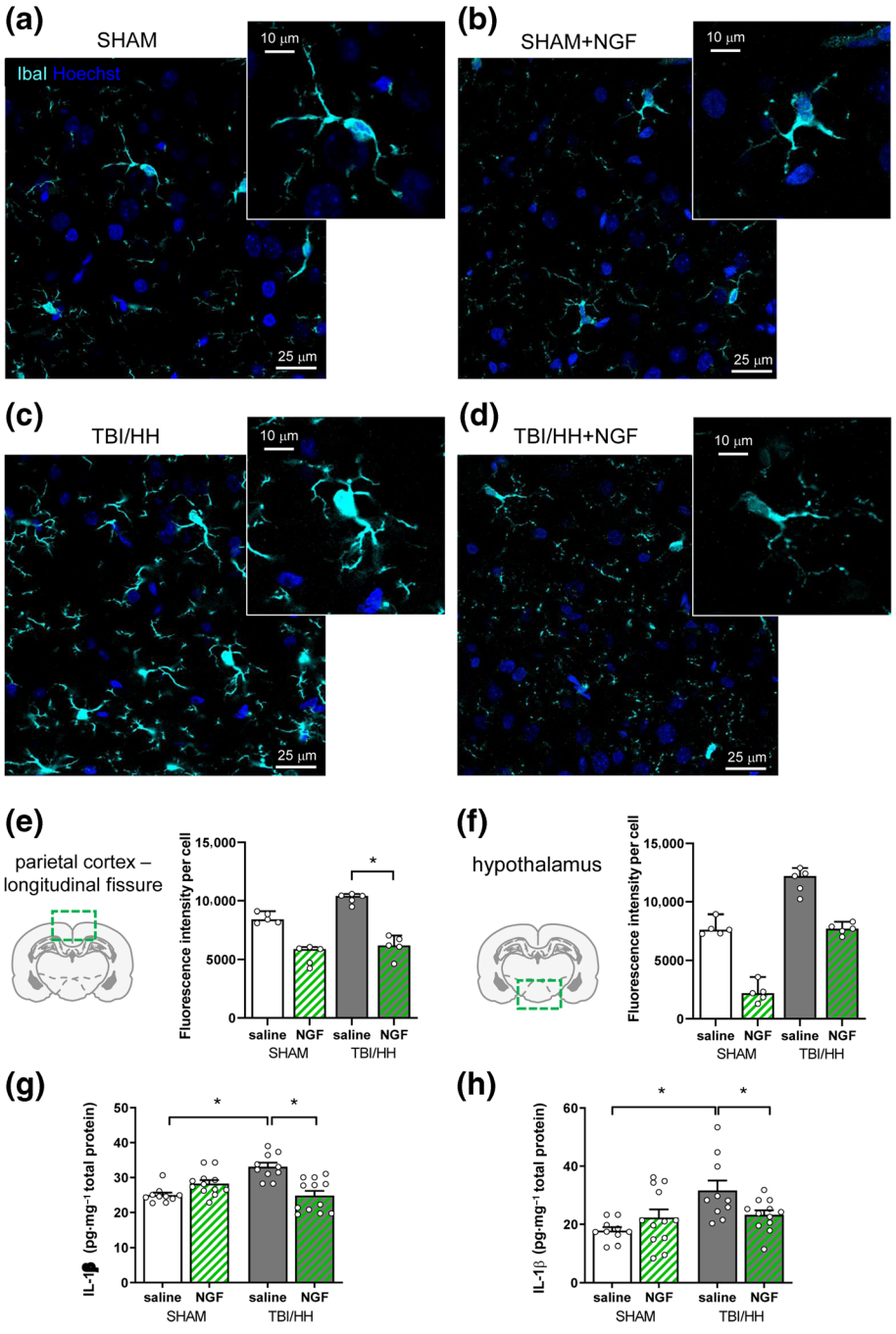

FIGURE 5 Acute treatment with intranasal NGF prevents the onset of microglial activation and the IL-1β content in the impact zone and the hypothalamus of TBI/HH rats. Representative images (63×) of immunostaining for Iba1 (cyan) and nuclei (blue) in the longitudinal fissure of parietal cortex of SHAM (a), SHAM +NGF (b), TBI/HH (c), and TBI/HH+NGF (d) rats. The insets represent high magnification images; scale bars are reported in the images. Iba1 fluorescence intensity measured for single cells in the impact zone (e) and hypothalamus (f) was obtained by ImageJ 'integrated density' module. N = 5 animals/experimental group, three images for each animals and 3 to 5 cells for image were analysed. The values presented are medians ± range. *P<0.05, significantly different as indicated; Kruskal–Wallis ANOVA on ranks (see Table S6) followed by Dunn's post-hoc test. The IL-1β content in the parietal cortex (g) and hypothalamus (h) of SHAM and TBI/HH rats treated or not with intranasal NGF was evaluated by ELISA. N = 11 animals per experimental group. The values presented are means ± SEM. *P<0.05, significantly different as indicated; two-way ANOVA (see Table S3) followed by Bonferroni multiple comparisons test.

Overall, the analysis on glial phenotypes involved in reactive neuroinflammation after the trauma suggests that intranasal NGF may interfere with the progression of the brain damage by dampening the astrocytes- and microglia-mediated neuroinflammation. The modulation of the IL-1β content by intranasal NGF may therefore be related to the direct effect of the treatment on these cell populations. Thus, to verify whether after the trauma the observed TBI-induced alterations of astrocytes and microglia were due to their proliferation and whether intranasal NGF may interfere with such pathological alteration of cell cycle regulation, Ki67 immunostaining was performed in GFAP$^+$ and CD11b$^+$ (as marker of total microglial population) cells (Figure 6, Figure S9). In both the impact zone and the hypothalamus, the number of cells stained for Ki67 was increased by the trauma and normalized after intranasal NGF (Figure 6a–d, Figure S9A–D). Non-parametric ANOVA revealed a significant effect for the percentage of Ki67$^+$ cells on both the total GFAP$^+$ and CD11b$^+$ cells (Table S6). Both in the impact zone and hypothalamus, intranasal NGF normalized the percentage of Ki67/GFAP (Figure 6e,g) and Ki67/CD11b (Figure 6f,h) double-positive cells. These results suggest that intranasal NGF may interfere with the progression of brain damage after TBI, limiting the pathological proliferation and possibly the activation (as demonstrated by its effects on the tissue content of IL-1β) of glial cells, major players of the onset and development of neuroinflammation.

To investigate the hypothesis that astrocytes and/or microglia may be direct cellular targets of exogenous NGF, the pattern of NGF receptors was analysed on GFAP$^+$ and CD11b$^+$ cells (Figure S10). These pilot data revealed that GFAP$^+$ cells may directly respond to intranasal NGF as they express **TrkA** (Figure S10A) and p75^{NTR} (Figure S10B), though the receptor modulation did not change following TBI/HH and/or treatment with intranasal NGF. In contrast, Iba1$^+$ microglia did not express either receptor (Figure S10C), suggesting a primary effect of intranasal NGF on astrocytes and an indirect effect on microglia, likely to arise from glial crosstalk.

The observed neuro-motor alterations may be indicative of functional alterations of the cortico-thalamo-striatal circuits, described as essential for locomotor activity and movement execution (Mancini et al., 2021). Pathological activation of striatal glia could influence the synaptic bases of network functioning, leading to behavioural abnormalities during neurological disorder (Mancini et al., 2021). Thus, we attempted to perform a preliminary analysis on the effect of intranasal NGF on the functioning of this specific circuit, verifying the possible development of neuroinflammation in the thalamic and striatal areas of rats traumatized and/or treated with intranasal NGF. Two-way ANOVA for GFAP tissue content revealed a main effect for trauma in the thalamus (Table S3), although the post-hoc analysis did not demonstrate significant differences among experimental groups (Figure 7a). At the same time, in the striatum, a main effect for trauma and trauma/treatment interaction was revealed (Table S3). Indeed, GFAP tissue content was found significantly increased after the trauma and intranasal NGF limited this TBI/HH-boosted GFAP up-regulation (Figure 7b). On the other hands, analyses of IL-1β tissue content revealed a main effect for trauma and trauma/treatment interaction in the thalamus (Table S3), where the TBI/HH-induced

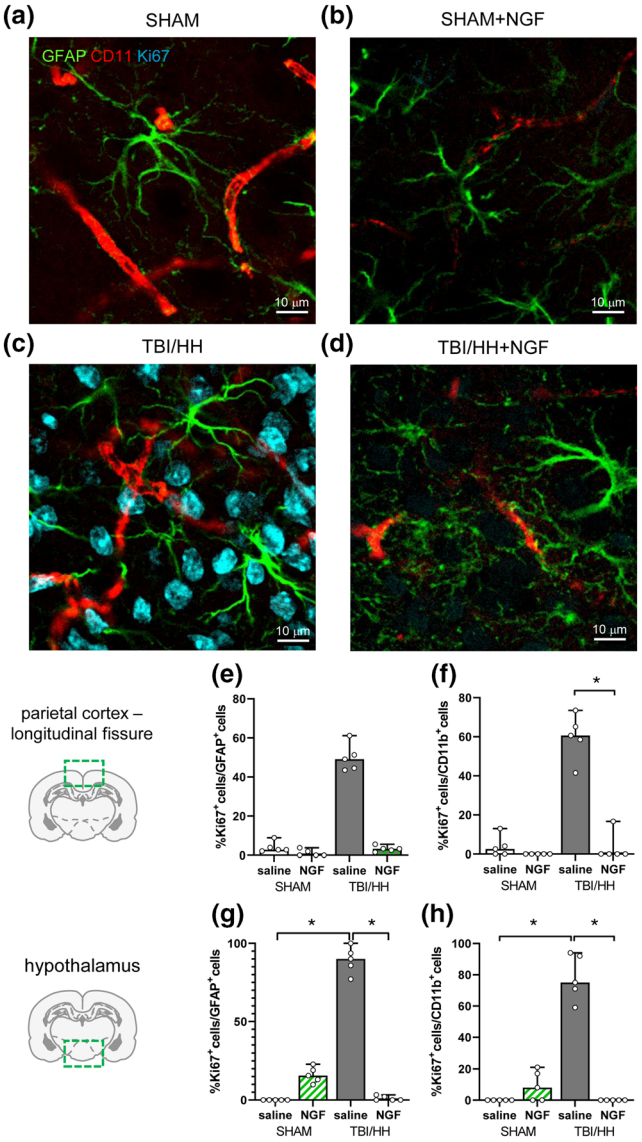

FIGURE 6 Acute treatment with intranasal NGF limits the astrocytic and microglial proliferation induced by TBI/HH. Representative images of immunostaining for GFAP (green), CD11b (red) and Ki67 (cyan) in the longitudinal fissure of parietal cortex of SHAM (a), SHAM+NGF (b), TBI/HH (c), and TBI/HH+NGF (d) rats. Percentage of Ki67$^+$ cells on the total GFAP$^+$ (e) and CD11b$^+$ cells (f) in the impacted parietal cortex, as shown in the corresponding graphical sketch. Percentage of Ki67$^+$ cells on the total GFAP$^+$ (e) and CD11b$^+$ cells (f) in the hypothalamus as shown in the corresponding graphical sketch. N = 5 animals/experimental group. The values presented are medians ± range. *P<0.05, significantly different as indicated; Kruskal–Wallis ANOVA on ranks (see Table S6) followed by Dunn's post-hoc test.

increment of IL-1β was significantly counteracted by intranasal NGF (Figure 7c), but not in the striatum (Figure 7d, Table S3).

4 | DISCUSSION

In this work, we have studied the effects of acute treatment with intranasal NGF on some of the pathological alterations developing in

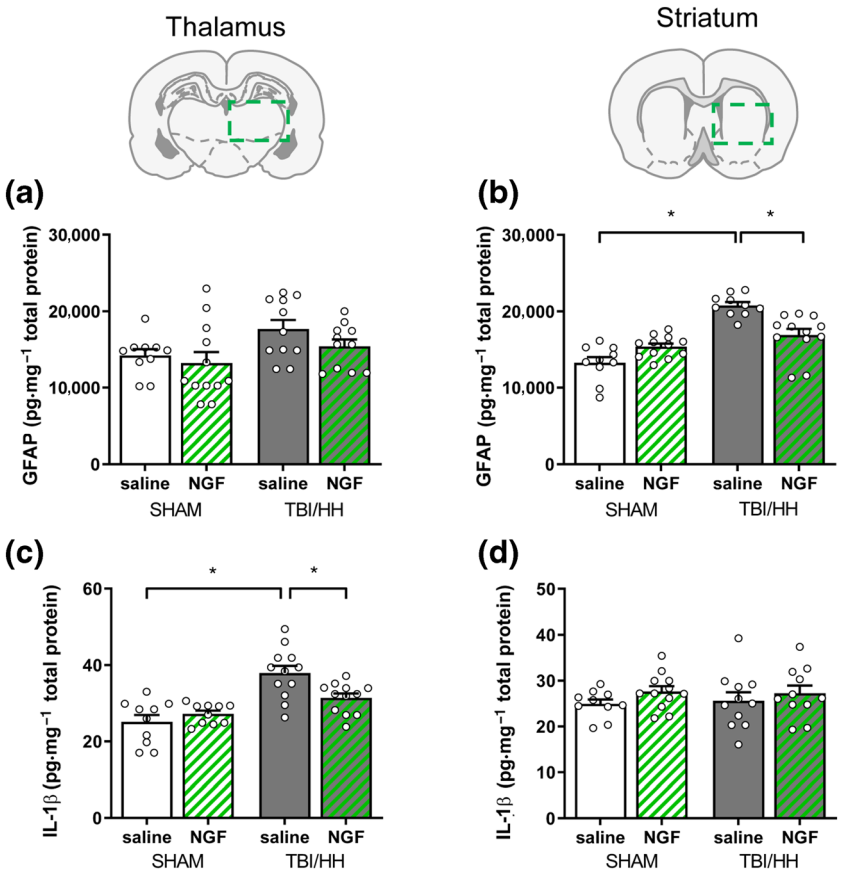

FIGURE 7 Acute treatment with intranasal NGF limits the increase of GFAP content in the striatum and IL-1β content in the thalamus of TBI/HH rats. The GFAP content in the thalamus (a) and striatum (b), and the IL-1β content in the thalamus (c), and striatum (d) of SHAM and TBI/HH rats treated or not with intranasal NGF was evaluated by ELISA. In the graphical sketches shown above the relative graphs, the brain area dissected and analysed is highlighted. N = 11 animals per experimental group. The values presented are means ± SEM. *$P<0.05$, significantly different as indicated; two-way ANOVA (see Table S3) followed by Bonferroni multiple comparisons test.

rat brain after severe TBI. First, we set up an experimental model of TBI/HH in young rats that could encompass both the primary and secondary damage observed in a human paediatric population with severe TBI. Then, we studied the effects of intranasal NGF on parameters indicative of different pathological components and known to be altered following TBI. This approach is functional to an initial evaluation of the potential of early treatment with intranasal NGF in preventing and limiting the disabling outcomes of a clinical condition that represents one of the greatest unsolved problems in neurology.

The results indicate that 24 h post-TBI/HH, the acute treatment with intranasal NGF prevents the establishment of motor disabilities related to balance, motor coordination, and locomotor activity, ensuring performance similar to normal. The effect of intranasal NGF on such functional alterations is consistent with its ability to modulate parameters closely related to trauma, such as reactive astrogliosis, proliferation of microglia, and neuroinflammation. In the cortical impact zone and the hypothalamus, intranasal NGF reduced the GFAP content, limiting astrocytic hypertrophy and proliferation, infiltration of microglia, and the levels of the pro-inflammatory cytokine IL-1β. The propagation of injury from the impact zone to functionally and physically related brain areas shows a different trend depending on the parameter analysed. Astrogliosis is detected not only in the impact zone and the hypothalamus but also in the striatum, whereas IL-1β content is altered in the thalamus. Acute intranasal NGFtreatment normalizes such TBI/HH-induced alterations, suggesting its clear and promising role in preventing and/or limiting the outcomes of TBI.

Animal models have been developed to replicate various aspects of human TBI, in both adult (Xiong et al., 2013) and young rats (Kochanek et al., 2017). Although some of these afford a high level of precision and reproducibility, they foresee preventive craniotomy and/or generate focal damage. In an attempt to investigate the effects of intranasal NGF treatment in the paediatric population with severe-TBI, we developed a model in 20-day-old rats that, due to the characteristics of the lesions, may well represent the real clinical setting in TBI. The weight-drop TBI model, previously developed in adult rats (Foda & Marmarou, 1994), produces diffuse brain injury, without the need for preventive craniotomy. By modulating the entity of the weight and the height of the fall, we generated a paediatric, closed-head TBI model characterized by diffuse damage, subdural hematoma, and motor impairment, without causing skull fractures. Additional post-traumatic hypoxia that exacerbates secondary brain damage (Yan et al., 2011), is likely to mimic the hypoxia/reperfusion injury that often follows severe TBI (Yan et al., 2014).

Nose-to-brain passage of NGF may occur either by intracellular or extracellular pathways, the latter mediated by the trigeminal and olfactory fibres that enter the brain, potentially distributing this neurotrophin throughout the whole cerebral parenchyma (Lochhead & Davis, 2019; Manni et al., 2021b). Intranasal NGF delivered to the rat brain parenchyma reaches the olfactory bulb, cortex, cerebellum, brain stem, hippocampus, and amygdala in high concentrations, 30–45 min following administration (Chen et al., 1998). Bioavailability studies show that the elimination half-life of intranasal NGF in rat

hippocampus was 0.45 ± 0.08 h (Vaka et al., 2009). Based on this evidences, we gave treatment with intranasal NGF in three successive rounds with 90 min of inter-treatment delay, starting immediately after the TBI/HH induction, to maintain relevant concentrations of exogenous NGF in the brain parenchyma, at least for the first 4–6 h post-trauma.

Moreover, we delivered a dose of NGF analogous to that we are using in the clinical study on children affected by the outcomes of severe-TBI (EudraCT Nr. 2019-002282-35). Unlike the clinical trial, in which children are treated only after cognitive, motor, and neurosensory deficits have been stabilized, in this preclinical study, we delivered NGF immediately after the TBI/HH induction. The aim, indeed, was to investigate the possibility that intranasal NGF might limit the onset of brain damage and its subsequent development into a chronic injury. Preliminary experiments showed that by 90 min after the trauma, an effective lack of motor coordination was evident and that one single intranasal administration (16.7 $\mu g \cdot kg^{-1}$) was sufficient to limit the progression of motor disability (data not shown). This evidence reinforces the idea that prompt treatment with intranasal NGF may be essential to limit the progression of TBI-related disabilities and suggests that dose-response experiments are needed to identify the minimum dose of drug capable of ensuring pharmacological efficacy. In this article, the temporal window of analysis was instead chosen to be sufficiently distant from the trauma to have full-blown motor dysfunction but not so distant as to hide the putative effects of intranasal NGF with the spontaneous recovery of rats.

Our data indicate that intranasal NGF can interfere with TBI/HH-induced loss of balance and motor coordination, detected by the rotarod test, improving motor learning levels that are impaired at least up to 72 h post-injury. Rotarod performance engages corticostriatal inputs that are associated with increased activity both in associative and sensorimotor inputs (Kupferschmidt et al., 2017). This data reinforces the evidence that motor skill learning, essential for optimizing behaviour and impaired following TBI (Ding et al., 2001), may be modulated by intranasal NGF. Our results suggest that a mechanistic study of possible pharmacological targets of NGF at the striatal level is warranted. Our preliminary pharmacokinetic data indicate that significant levels of intranasal NGF are detectable in the rat striatum (manuscript in preparation). At the striatal level, one of the targets of NGF could be cholinergic interneurons (ChIN), expressing TrkA receptors both during development and in adulthood (Holtzman et al., 1995; Steininger et al., 1993). Although, to the best of our knowledge, there are no data showing dysfunction of cholinergic interneurons in TBI, alterations of the brain cholinergic systems have been reported as a consequence of TBI (Arciniegas, 2003; Pepeu & Grazia Giovannini, 2017; Shin & Dixon, 2015). Furthermore, striatal cholinergic dysfunction is associated with various forms of dystonia (Yalcin-Cakmakli et al., 2018), which is a common long-term neurological outcome of TBI (Silver & Lux, 1994). It is therefore possible that intranasal NGF improved motor behaviour in TBI by acting directly on NGF-responsive striatal neuronal populations or indirectly by regulating the striatal microenvironment, for example, by improving TBI-induced astrogliosis. Our data on GFAP accumulation in the striatum 24 hours post-trauma, that was blocked by intranasal NGF is supportive of the latter possibility.

Intranasal NGF limited the increase in tissue GFAP content and the onset of reactive astrogliosis in the impact zone. This early effect could lie in the ability of NGF to favour the withdrawal of astrocytes from the cell cycle by blocking them in the G1-phase, possibly limiting astrogliosis and the formation of the glial scar (Cragnolini et al., 2012). Supporting such data on astrogliosis, we also found increased levels of pro-inflammatory IL-1β, increased proliferation and activation of microglia, overall compatible with a general early post-traumatic neuroinflammation, that was corrected by intranasal NGF. These results correlate with the evidence that intranasal NGF delivered in adult rats after TBI, promoted an anti-inflammatory response, decreasing brain IL-1β and **TNF-α**, and limiting the activation of NF-κB (Lv et al., 2013). Our recent work on the link between the endogenous NGF system and the development of chronic peripheral inflammation (Farina et al., 2022), suggests that interfering with the balance between different forms of NGF (mature, as distinct from precursor) may indeed affect the development of inflammatory processes. An in-depth analysis of endogenous NGF/proNGF activity and regulation after TBI is therefore warranted, because our therapeutic intervention may shift the balance of different forms of NGF toward the mature moiety, positively affecting both neuroinflammation and glial phenotype (Barcelona et al., 2016; Cheng et al., 2020; Rizzi et al., 2018).

Intranasal NGF limited the astrogliosis and activation of microglia, increased by TBI/HH,-also at hypothalamic levels. TBI patients suffer from transient or permanent pituitary insufficiency, probably due to direct trauma to the hypothalamus or pituitary gland (Gasco et al., 2021). Hypothalamic–pituitary-neuroendocrine dysfunction induced by TBI is believed to depend on acceleration/deceleration forces to the brain within the skull (Rowe et al., 2016), which could degenerate into the onset of hypothalamic astrogliosis. Furthermore, a direct link between post-TBI hypopituitarism, reactive astrogliosis, and neuroinflammation has also been suggested (Kasturi & Stein, 2009; Molaie & Maguire, 2018), as well as a possible effect of icv-NGF on hypothalamic–pituitary function (Luo et al., 2018). Our preliminary pharmacokinetic data indicate that intranasal NGF soon after administration reaches the hypothalamus (manuscript in preparation). Therefore, a more in-depth study would be necessary to investigate the contribution of intranasal NGF on TBI/HH-induced dysregulation of hypothalamic functions and the central regulation of autonomic outflow.

In conclusion, data presented in our first work on the effect of intranasal NGF in a paediatric TBI model, demonstrate that the proposed pharmacological treatment, characterized by the immediacy of post-trauma intervention, is clearly effective in maintaining normal motor skills and in preventing and/or limiting the development of pathological features peculiar to TBI, such as astrogliosis and neuroinflammation. These preclinical data should open a line of research, directing toward an acute intranasal NGF treatment for children hospitalized for severe TBI, that could be associated with the primary treatments in Intensive Care Units.

AUTHOR CONTRIBUTIONS

Luigi Manni: Conceptualization (lead); investigation (equal); funding acquisition (equal); writing—original draft (lead); writing—review & editing (lead). **Eleonora Leotta:** Investigation (equal); formal analysis (equal). **Ilia Mollica:** Investigation (supporting). **Annalucia Serafino:** Conceptualization (supporting). **Annabella Pignataro:** Investigation (supporting). **Illari Salvatori:** Investigation (supporting). **Giorgio Conti:** Funding acquisition (equal); writing—review & editing (supporting). **Antonio Chiaretti:** Funding acquisition (equal); writing—review & editing (supporting). **Marzia Soligo:** Conceptualization (lead); investigation (lead); formal analysis (lead); supervision (equal); writing—original draft (lead); writing—review & editing (lead).

ACKNOWLEDGEMENTS

This work was funded by the Italian Ministry of Health Grant: RF-2018-12366594 'Nerve growth factor in pediatric severe traumatic brain injury: translational and clinical studies on a candidate biomarker and therapeutic drug'. The authors are grateful to Dompé Farmaceutici SpA for providing the human recombinant NGF. The authors acknowledge the valuable technical assistance of Sig. Mauro Fabiani (Institute of Translational Pharmacology - CNR). Open Access Funding provided by Consiglio Nazionale delle Ricerche within the CRUI-CARE Agreement.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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How to cite this article: Manni, L., Leotta, E., Mollica, I., Serafino, A., Pignataro, A., Salvatori, I., Conti, G., Chiaretti, A., & Soligo, M. (2023). Acute intranasal treatment with nerve growth factor limits the onset of traumatic brain injury in young rats. *British Journal of Pharmacology*, *180*(15), 1949–1964. https://doi.org/10.1111/bph.16056