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Lestaurtinib (CEP-701) modulates the effects of early life hypoxic seizures on cognitive and emotional behaviors in immature rats

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ABSTRACT

Hypoxic encephalopathy of the newborn is a major cause of long-term neurological sequelae. We have previously shown that CEP-701 (lestaurtinib), a drug with an established safety profile in children, attenuates short-term hyperexcitability and tropomyosin-related kinase B (TrkB) receptor activation in a well-established rat model of early life hypoxic seizures (HS). Here, we investigated the potential long-term neuroprotective effects of a post-HS transient CEP-701 treatment. Following exposure to global hypoxia, 10 day old male Sprague–Dawley pups received CEP-701 or its vehicle and were sequentially subjected to the light–dark box test (LDT), forced swim test (FST), open field test (OFT), Morris water maze (MWM), and the modified active avoidance (MAAV) test between postnatal days 24 and 44 (P24-44). Spontaneous seizure activity was assessed by epidural cortical electroencephalography (EEG) between P50 and 100. Neuronal density and glial fibrillary acidic protein (GFAP) levels were evaluated on histological sections in the hippocampus, amygdala, and prefrontal cortex at P100. Vehicle-treated hypoxic rats exhibited significantly increased immobility in the FST compared with controls, and post-HS CEP-701 administration reversed this HS-induced depressive-like behavior ($p < 0.05$). In the MAAV test, CEP-701-treated hypoxic rats were slower at learning both context-cued and tone-signaled shock-avoidance behaviors ($p < 0.05$). All other behavioral outcomes were comparable, and no recurrent seizures, neuronal loss, or increase in GFAP levels were detected in any of the groups. We showed that early life HS predispose to long-lasting depressive-like behaviors, and that these are prevented by CEP-701, likely via TrkB modulation. Future mechanistically more specific studies will further investigate the potential role of TrkB signaling pathway modulation in achieving neuroprotection against neonatal HS, without causing neurodevelopmental adverse effects.

1. Introduction

Neonatal seizures occur in 1–5/1000 newborn [1], most commonly in the setting of hypoxic encephalopathy [2–4], a relatively common brain insult affecting 1–8/1000 live births in developed countries and up to 26/1000 in underdeveloped countries [5]. Despite their often transient nature, substantial clinical and experimental data suggest that early life seizures are associated with long-term neurological consequences [2,6–10], even though separating their effects from those attributable to the inciting brain insult and accompanying lesional

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Abbreviations: DAB, 3,3'-Diaminobenzidine; EEG, electroencephalography; FST, forced swim test; GFAP, glial fibrillary acidic protein; HS, hypoxic seizures; LDT, light–dark box test; LSD, least significant difference; MAAV, modified active avoidance; MWM, Morris water maze; OFT, open field test; P#, postnatal day #; SRS, spontaneous recurrent seizures; SWD, spike–wave discharge; TrkA, tropomyosin-related kinase A; TrkB, tropomyosin-related kinase B; TrkC, tropomyosin-related kinase C; DMSO, Dimethyl sulfoxide.

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burden has been challenging [10–12]. Nevertheless, both clinically and in animal models, neonatal hypoxia, often with accompanying acute seizures, is associated with long-term memory and cognitive deficits [13,14], behavioral disturbances including hyperactivity, anxiety, and depression [7,14,15], as well as later life epilepsy [7,16–19]. Despite the advent of therapeutic hypothermia, improvements in these outcomes have been modest, and neonatal hypoxia remains a major source of long-term neurological complications [17–19] calling for novel therapies.

We have previously shown that the transient systemic administration of CEP-701 (lestaurtinib), an investigational new drug (IND) with an established clinical safety profile in children with cancer [20], attenuates the short-term hyperexcitability that follows hypoxic seizures (HS) in a rat model of neonatal hypoxic encephalopathy [21]. Even though CEP-701, a synthetic derivative of the indolocarbazole K252a, has nonspecific multikinase inhibitory properties, we had provided circumstantial evidence for a CEP-701 mechanism of action via the reversal of early HS-induced hippocampal tropomyosin-related kinase B (TrkB) receptor activation [21]. Multiple other early post-HS molecular and electrophysiological hippocampal changes have been described in this model [7,22]. These early changes are believed to set the stage for a long-term disruption in synaptic plasticity and to be implicated in the emergence of later life hippocampal spontaneous recurrent seizures (SRS) [7,23] and long-term cognitive and emotional deficits [7,14]. Our recently reported early HS-induced imbalance in TrkB signaling [21] might also be a contributor to the detrimental long-term consequences of early life hypoxia. Indeed, derangements in TrkB signaling have been implicated in seizure emergence in multiple other rodent models [24–27], as well as in learning and memory deficits [28,29], and in disturbances in emotional behaviors [30–32]. The exact contribution of TrkB receptor signaling to emotional behavioral regulation remains elusive as both overactivation and underactivation of this receptor have been reported with anxious-like and depressive-like behaviors in rodents [31,32]. Nevertheless, it appears that an imbalance in TrkB signaling and the ensuing derangements in synaptic plasticity may contribute to the emergence of emotional behavioral disturbances including depression [33] and may disrupt emotionally-relevant learning and memory [34].

Given the potential contributions of TrkB signaling to epileptogenesis, learning, and emotional regulation, we hypothesized that the previously reported short-term beneficial effect of its transient post-HS blockade with CEP-701 [21] will preserve normal brain synaptic plasticity and, therefore, attenuate HS-induced long-term cognitive and emotional behavioral deficits and later life SRS. Despite its multiple potential targets, CEP-701 has been used by us and others to modulate TrkB activation because of its known human safety profile [20], and thus its potential for translatability. Here, we continued our clinically translatable work on this drug with promising short-term effects in the HS rat model [21] and investigated its potential sustained long-term effect against HS-induced later life SRS and cognitive and emotional behavioral disturbances in the developing rat brain.

2. Materials and methods

2.1. Animals and experimental design

The Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut approved all experiments. Male Sprague–Dawley rats housed with their dams in a temperature-controlled room at 22 °C and maintained on a 12-h light–dark cycle with permanent access to food and water were used in this study. Hypoxic seizures were induced in postnatal day 10 (P10) male rat pups with 15 min of graded global hypoxia (7–4% O2), as previously described [23]. Briefly, in an airtight chamber, oxygen concentration was dropped down by nitrogen gas infusion and maintained at 7% for 8 min, 5% for 6 min, and 4% for 1 min before termination of hypoxia. Given that males and females may have different neurodevelopmental trajectories during the peripubertal age, this study was conducted only on males. We selected pups that had six or more tonic–clonic seizures for inclusion in the study. Each pup received either CEP-701 (HCEP group: hypoxia with CEP-701 treatment) or its vehicle (HV group: hypoxia with vehicle). CEP-701 (3 mg/kg/dose), dissolved in dimethyl sulfoxide (DMSO) (2 mg/ml), was administered intraperitoneally (i.p.), immediately after HS, and 12-h post-HS. This dosing regimen was shown to reverse HS-induced TrkB activation as well as early HS-induced hyperexcitability [21]. Age- and weight-matched control littermates were sham manipulated under normoxic conditions and received CEP-701 (NCEP group: normoxia with CEP-701 treatment) or its vehicle (NV group: normoxia with vehicle). Since CEP-701 was dissolved in DMSO, both HV and NV groups received DMSO vehicle in a volume-matched dose (1.5 ml/kg) to CEP-701 treated rats. In this study, 10 litters were used, and each litter consisted of 8–12 male pups. Every litter was divided into 4 groups assigned to the four treatment arms. Rats were subjected to a battery of behavioral testing panels between P24 and P44 ($n = 20$–24 per group). Continuous long-term electroencephalography (EEG) was initiated following the behavioral panels between P50 and P100 ($n = 10$–12 per group). All rats were sacrificed at P100 for histological analyses, except for a cohort of rats ($n = 2$–3 per group), in which EEG recordings were extended until P140 (Fig. 1). Histological analyses were performed in the amygdala, prefrontal cortex, and hippocampus. Hippocampal pyramidal neuronal density was assessed ($n = 6$ per group). We also performed immunohistochemical staining for glial fibrillary acidic protein (GFAP) ($n = 3$ per group) since it is a marker of neuronal damage expressed in activated astrocytes following stress or injury of the central nervous system [35], especially that increases in GFAP-labeled hippocampal cells have been described in models of acquired seizures [36].

2.2. Cognitive and emotional behavioral panels

We selected a battery of behavioral testing panels to perform between P24 and P44, in order to assess for commonly encountered chronic cognitive and emotional behavioral disturbances following early life hypoxia [7,13–15]. Given the known prominent molecular and electrophysiological hippocampal changes in the employed HS model [21,22], these tests were also tailored to amygdalohippocampal functions [37,38]. The light–dark box test (LDT) and the forced swim test (FST) were performed to assess anxiety-like and depressive-like behaviors. The open field test (OFT) assessed exploratory behaviors, anxiety-like behaviors, and hyperactivity. Visuospatial navigation was evaluated in the Morris water maze (MWM). A modified active avoidance (MAAV) paradigm was employed to test for emotionally-relevant learning. Testing was always started at 9 am. The testing panels were performed in sequence from the least to the most aversive in all animals ($n = 20$–24 per group) as delineated in Fig. 1.

2.2.1. Light–dark box test (P24)

The LDT was conducted in a shuttling box (Coulbourn Instruments, Harvard apparatus, USA) that consists of two equal compartments (H: 34 cm, W: 27 cm, L: 27 cm), communicating via a 9 × 9 cm door located in the middle of a metallic partition wall. The left side is lit and has white walls with visual cues (dices and beads). The right side is dark with black walls. Rats were placed in the dark compartment and allowed to roam for a total duration of 5 min freely. After testing, the apparatus was cleaned with unscented detergent then 70% ethanol and allowed to dry before the next rat was tested. Light–dark box test outcome parameters (total time spent in each compartment and the number of transitions) were obtained using Graphic State 4 software (Coulbourn Instruments, Harvard Apparatus, USA).

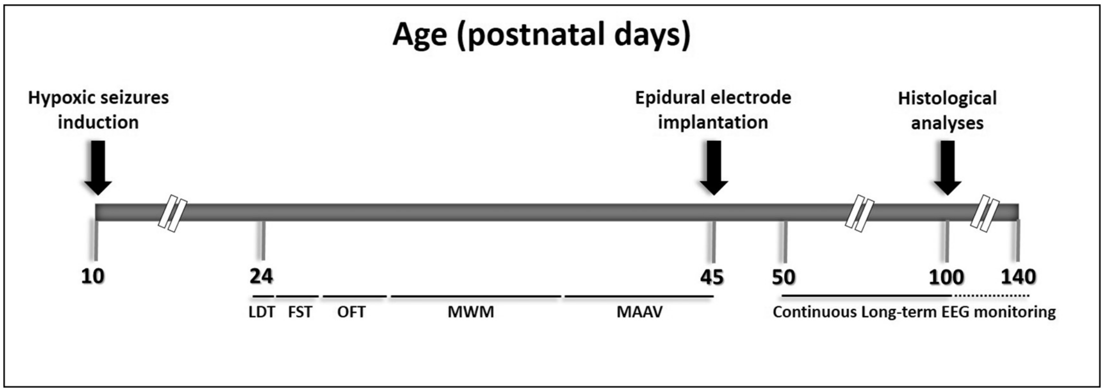

Fig. 1. Schematic study design. Sprague–Dawley rat male pups were exposed to hypoxia-induced neonatal seizures at postnatal day 10 (P10). Behavioral testing was initiated at P24 with the light–dark box test (LDT), followed by the forced swim test (FST) at P25–26, the open field test (OFT) at P27–29, the Morris water maze (MWM) test at P30–37, and the modified active avoidance (MAAV) test at P38–44. Epidural electrodes were surgically implanted at P45, and prolonged continuous EEG recordings were obtained between P50 and 100, and up to P140 in a cohort of rats.

2.2.2. Forced swim testing (P25–26)

The FST consisted of a 5-minute session followed by another 10-minute session, 24 h later. Cylinders (50 cm in height, 20 cm in diameter) were filled to a depth of 25 cm with 25 °C water. Rats were placed in the cylinders, and their swimming behaviors were video recorded then analyzed for struggling and immobility using the SMART video tracking 3.0 software (Panlab, Harvard apparatus, USA). This software has an activity detection mode that allows the automated detection of the rat motor activity level. These settings were adjusted by the operator such that immobility is defined as lack of movements except for the minimal limb activity required to stay afloat. After testing, rats were placed in a cage on dry towels under a heat lamp, and allowed to dry for 10 min [39].

2.2.3. Open field test (P27–29)

The OFT was conducted over three sessions, 10 min each, performed over three consecutive days using an opaque plexiglas square field (W: 80 cm, L: 80 cm, H: 40 cm). On the first testing day, a single small object (cube) was placed in the middle of the field's floor, then on each of the two subsequent test days, a new object was added (a small ball, then a small bottle). Rats were placed individually in the nearest corner to the most recently added novel object on a given day. The floor surfaces and walls of the apparatus were cleaned with unscented detergent then a 70% alcohol solution between animals. The rats' movements were video recorded then analyzed using SMART software.

2.2.4. Morris water maze (P30–37)

A dark blue circular plastic pool, 150 cm in diameter and 80 cm in height (Coulbourn Instruments, USA), was filled to a depth of 30 cm with 25 °C water. On the first testing day, rats were allowed to swim for 2 min freely. In this test day that fosters habituation, an escape platform was not present. During the spatial acquisition trials on days 2–6, an “invisible platform” was placed 2 cm below the water surface. Rats were placed in the pool, and if they failed to find the platform in 2 min, they were placed on it for 30 s by the operator. Four daily trials were performed with a 30 s intertrial resting period, and 4 immersion landmarks that were equidistant to the platform and their sequence was changed every day. On testing day 7, the probe trial was performed to assess retention of spatial navigation. The platform was removed, and rats were immersed in water at a novel starting position opposite to the quadrant where the platform was located, and were allowed to swim freely for 2 min. On the last day, to assess motor and visual functions, rats were allowed to swim to a visible platform with 4 trials per rat. The MWM experiments were video recorded and analyzed with the automated SMART software.

2.2.5. The modified active avoidance test (P38–44)

We developed the MAAV test to simultaneously assess amygdalohippocampal-dependent learning of contextual and auditory emotional cues, as well as learning of adaptive shock-avoiding shuttling behaviors (Fig. 2). This test is conducted in a standard shuttle box (Coulbourn Instruments, USA) that consists of two equal compartments (H: 34 cm, W: 27 cm, L: 27 cm), communicating via a 9 × 9 cm opening located in the middle of the metallic partition wall. The box is placed in a soundproof isolation cubicle (H: 80 cm, W: 53 cm, L: 53 cm) (Coulbourn Instruments, USA). The box is equipped with a tone generator and infrared beam sensors that detect transitions between the chambers. This seven-day test consisted of one day of habituation followed by 5 days of training and one retention test day. During habituation, rats were allowed to explore the shuttle box covered with white foam panels in

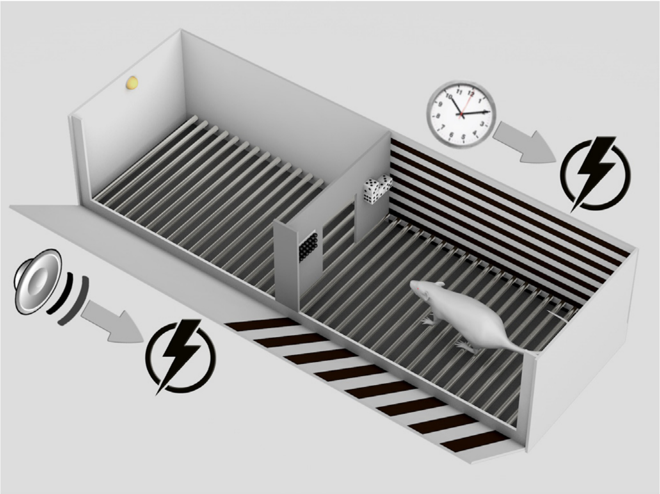

Fig. 2. Schematic representation of the modified active avoidance (MAAV) test. We developed this test by modifying the standard active avoidance shuttle box, to simultaneously assess emotionally-relevant auditory and contextual learning, and the ensuing acquisition of adaptive behavioral avoidance of tone-signaled and context-cued electrical foot-shocks. In the left compartment, the foot-shock is signaled by a tone. In the contextually modified right chamber (black-white wall patterns, and visual cues such as dices and beads), the shock is not signaled by a tone, but is regularly administered every 10 s spent on that side. Shuttling between the 2 compartments prevents an incoming shock (avoidance). Over multiple daily trials, normal rats learn to shuttle between the compartments in order to avoid the electrical shocks.

both compartments for 5 min. During training, a protocol is customized using the Graphic state 4 software so that an incoming electrical foot shock (0.5 mA, 15 s duration) is signaled with a 15 s tone in the left compartment (40 s intertrial rest period on that side) but not in the right one, where an electrical foot shock is delivered every 10 s spent in that compartment. Here, compared to the habituation day, the left chamber remains unchanged but the right chamber is modified (foam plate with black-white strip pattern and visual cues such as dices) for contextual conditioning training. Shuttling through the opening between the two compartments prevents an incoming shock (shock avoidance) or terminate an ongoing one (escape). Cycles in both the left and right chambers are repeated for a total of 30 signaled trials. After training, a two-part retention test was performed on day 6. The first part consisted of two sessions, 2 min each, where the rat was allowed to freely roam in the shuttle box without tone stimuli or shocks in order to assess retention of contextual learning (the compartments' pattern remains as they were on days 1–5). In the second part of the retention subtest, the visual cues were removed from the right chamber, and 30 tone-signaled avoidance trials were delivered in either compartments with an intertrial interval of 30 s. The 30 avoidance trials were followed by 2 min of continuous tone to assess freezing responses.

2.3. Surgical electrode implantation and long-term EEG recording

Epidural electrode surgery was performed at P45 [40]. Rats were anesthetized with an intramuscularly administered mixture of ketamine (60 mg/kg), xylazine (6 mg/kg), and acepromazine (1.25 mg/kg). Once appropriate anesthesia was achieved (lack of signs of pain in response to toe pinching), the rat was placed on a pad, and the hair was shaved with a trimmer from the flat of the nose between the eyes down to the neck. The rat's head was then tightly secured on a stereotaxic frame. Lubricating eye ointment was then applied to prevent drying out and possible eye irritation from the disinfectants. Under sterile conditions (application of iodine then ethanol), an incision was made, and the skull was exposed using a retractor. Five small 1.4 mm holes were made in the skull with a high speed-drill in order to place 5 epidural screw electrodes. These included left and right frontal electrodes (2 mm anterior to, and 3 mm lateral to the bregma), left and right parietal (5 mm posterior to, and 3 mm lateral to the bregma), and one anterior midline reference electrode (6 mm anterior to the bregma) based on the Sherwood and Timiras Atlas of the developing rat brain [41]. The electrode wires were then inserted in a 6-channel pedestal (Plastics One, USA) along with a 6th socket attached to a free wire placed under the skin of the neck serving as the ground electrode. The wires and screws were then covered with dental cement to give the “headset” its final shape. Neomycin powder was applied to the exposed skin edges in contact with the dental cement to prevent postoperative infections. Rats were transferred to single animal EEG cages. Analgesia regimens with paracetamol were administered for 3 days postoperatively (1 mg/ml in drinking water). Following a 5 day postsurgical recovery period, long-term EEG recordings were initiated from P50 to P100 (Xltek, Natus Medical, USA). Two readers blinded to treatment groups reviewed the EEG tracings. In a cohort of rats, EEG were recorded until P140.

2.4. Histological analyses

Rats were sacrificed at P100, and brains were perfused with 4% formaldehyde via the transcardiac route under anesthesia as previously described [42]. Brains were then embedded in paraffin, and 8 μm coronal histological sections were obtained for histological and immunohistochemical analyses. The sections used for hippocampal neuronal density quantification and for hippocampal and amygdalar GFAP staining were selected by visual inspection so that their structural patterns match the coronal sections located at 3.4–3.8 mm posterior to the bregma in the Paxinos and Watson adult rat brain atlas [43]. Coronal sections used to assess possible prefrontal injury via GFAP staining were obtained 4.5–4.9 mm anterior to the bregma as described above. Hippocampal pyramidal neurons were manually counted by an investigator blinded to treatment groups on hematoxylin and eosin (H&E)-stained sections. The average neuronal density of 9 sections per brain was calculated using ImageJ (NIH, USA). For GFAP staining, sections were deparaffinized with xylene and rehydrated through a series of descending grades of alcohol solutions. The sections were then incubated for 60 min in 95 °C citric acid buffer (10 mM, pH = 6) for antigen retrieval and treated for 5 min at room temperature with 3% H_2O_2 to exhaust endogenous peroxidase activity. Following overnight incubation at 4 °C with anti-GFAP antibody (1:1000, EnCor Biotechnology, USA), sections were incubated with peroxidase conjugated antimouse secondary antibody for 1 h at room temperature (Leica Biosystems, UK). Immunohistochemical staining was visualized under the light microscope following application of 3,3'-Diaminobenzidine (DAB) and hematoxilin counterstaining.

2.5. Statistical analyses

Statistical analyses were performed using Prism 7 (GraphPad Software, USA). Results are reported as means ± standard error of the mean (S.E.M.). One-way analysis of variance (ANOVA) was used to analyze data obtained from the LDT, OFT, MAAV (freezing time and the retention subtests), as well as from the MWM probe trial and visual platform tests. Two-way ANOVA for the two factors of treatment group and time of testing was used to analyze data obtained from the FST (immobility per minute), the MAAV (daily shock-avoidance), and from the MWM (daily escape latencies).

3. Results

3.1. Acute hypoxic seizures

All rat pups exposed to hypoxia at P10 experienced acute whole body myoclonic jerks, head bobbing, as well as head and limb tonic–clonic seizures during the 15-minute graded global hypoxia as previously reported in this established model [44]. Out of the 51 pups that experienced acute HS, 47 (92%) had 6 or more tonic–clonic seizures and were included in this study. Seizures were counted by direct observation and then confirmed by reviewing the recorded videos. Hypoxic rats that were destined to receive CEP-701 (HCEP group, n = 24) or its vehicle (HV group, n = 23) experienced a comparable number of acute HS (average seizure number: 8.2 ± 0.4 and 8.5 ± 0.4, respectively; *t*-test; p = 0.54). Rats recovered after undergoing HS and had comparable weights to normoxic rats that received CEP-701 (NCEP group, n = 23) or vehicle (NV group, n = 24) when measured at P24 (64.3 ± 2.2 g for NV, 65.5 ± 2.3 g for HV, 63.8 ± 2.8 g for HCEP, 63.9 ± 1.4 g for NCEP, one-way ANOVA, p = 0.95) and at P90 (477.5 ± 10.7 g for NV, 449.5 ± 11.7 g for HV, 424.2 ± 12.7 g for HCEP, 443.9 ± 18.2 g for NCEP, one-way ANOVA, p = 0.56).

3.2. Anxiety-like behaviors, exploratory behavior, and hyperactivity in the LDT and OFT

First, we assessed the effect of early life HS on activity level, exploratory tendencies, and anxious-like behaviors in closed (LDT) and open environments (OFT). In the LDT, all the groups were comparable in the percentage of time spent in the lit chamber of the box, in the number of transitions between the light and the dark compartments, and in the latency to first transition from the dark to the lit chamber (55.2 ± 1.8%, 13.6 ± 0.6, and 14 ± 2.2 s, respectively for NV; 58.1 ± 1.8%, 12.2 ± 0.7, and 17.4 ± 3.1 s, respectively for HV; 55.9 ± 1.4%, 13.7 ± 0.7, and 14.4 ± 2.8 s, respectively for HCEP; 56.0 ± 2.8%, 13.6 ± 0.7, and 13.9 ± 2.8 s, respectively for NCEP; one-way ANOVA; p = 0.42). There were, therefore, no HSrelated detectable differences in exploratory tendencies or anxious-like behaviors (preference to the dark compartment). In the OFT, there were no statistically significant differences in the total distance traveled by the different groups on any single day or in the cumulative total distance traveled over the 3 testing days (165.78 ± 18.2 m for NV, 158.15 ± 15.1 m for HV, 162.33 ± 14 m for HCEP, 152.43 ± 12.4 m for NCEP, one way ANOVA, $p = 0.95$). All groups spent a comparable duration in object exploration in the center of the open field on each day and cumulatively over the 3 testing days ($p = 0.19$).

3.3. Reversal of HS-induced depressive-like behaviors with CEP-701

We also assessed depressive-like behaviors in the FST. On the first testing day of the FST, the percentage of immobility steadily increased as the test progressed, especially in the last 2 min, in all treatment groups. However, there was a divergence in behaviors between the vehicle-treated hypoxic rats and the rest of the groups in the last minute of the test. Quantification of this effect revealed that the HV group spent significantly more immobile time compared with the normoxic rats (% immobility: 20.7 ± 2.8% for HV, 13.4 ± 2.9% for NV; one-way ANOVA; $p < 0.05$). Interestingly, this early life HS-induced decrease in mobility was reversed by CEP-701 treatment in the HCEP group (% immobility: 8.2 ± 1.5%) that exhibited comparable mobility to normoxic controls ($p = 0.13$), and significantly less immobility compared to HV (one-way ANOVA, $p < 0.05$) (Fig. 3). These findings point to long-lasting depressive-like behaviors in the HV group, which was normalized by CEP-701 administration.

3.4. Visuospatial navigation in the MWM

To evaluate visuospatial learning, rats were subjected to the MWM test. During the 5-day spatial acquisition training, escape latencies were comparable among all 4 groups (Fig. 4). In the retention probe trial subtest, all groups also had a similar preference to the probe quadrant.

3.5. Effects of CEP-701 on contextual and auditory learning and memory in the MAAV

Finally, we investigated amygdalohippocampal-dependent learning of emotional cues and adaptive shock-avoiding behaviors in the MAAV. In the 5-day training phase, rats in all groups gradually learned to avoid tone-signaled electrical foot-shock in the left compartment and contextually-cued shocks in the right chamber. However, compared with all other groups, CEP-treated hypoxic rats were significantly slower in acquiring shock-avoiding behaviors in both compartments. However, HCEP rats did ultimately reach their full potential on the last day retention subtests and were comparable to all other groups in contextual preference and tone-signaled shock avoidance (Fig. 5). The latency time to avoid electrical foot-shocks was also statistically comparable among all groups. In addition, there were no significant differences in freezing behaviors during continuous tone exposure in the retention subtest. Early life HS, therefore, did not significantly affect emotionally-relevant contextual and auditory learning. However, the brief post-HS exposure to CEP-701 compounded the effects of HS, leading to chronic deficits in the acquisition of auditory and contextually-cued adaptive shock-avoiding behaviors in the hypoxic rats that received CEP-701.

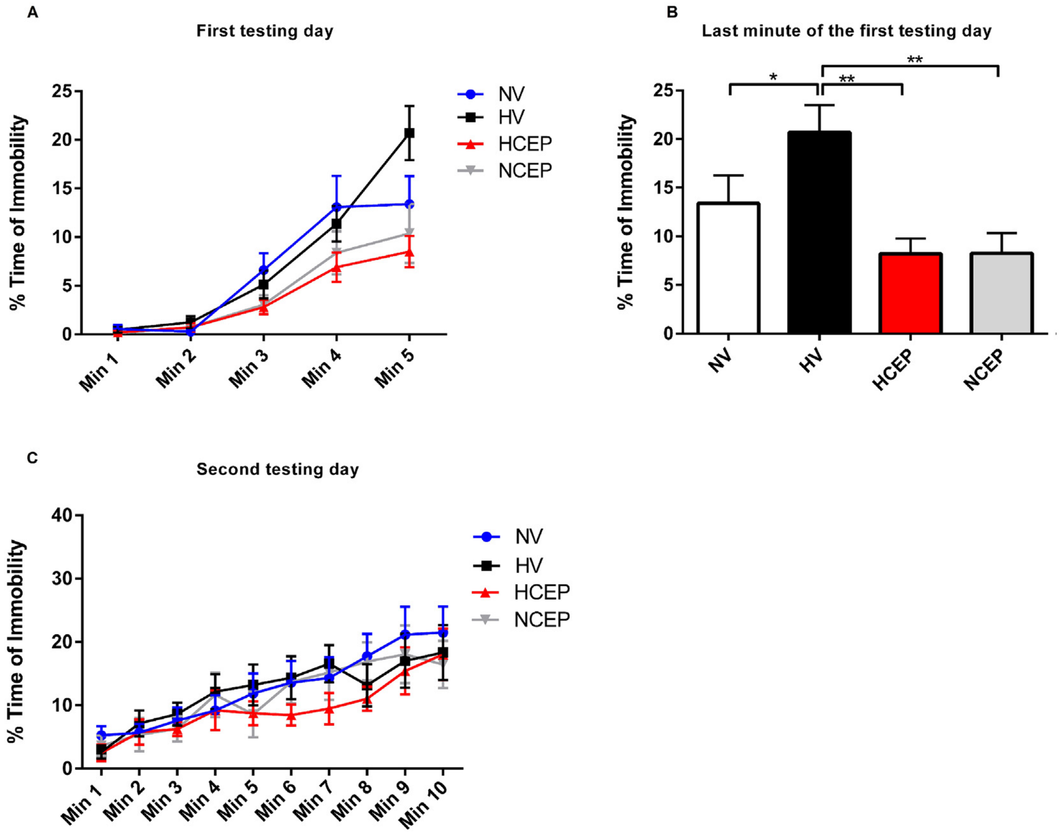

Fig. 3. Reversal of HS-induced depressive-like behavior by CEP-701. Shown are the results of the forced swim test (FST) performed at P25 (day 1) and at P26 (day 2). Panels A and B. The behavior of the HV group diverges from its control group NV with a statistically significant higher immobility time at minute 5 on the first testing day (20.7 ± 2.8% for HV, 13.4 ± 2.9% for NV, one-way ANOVA, $p < 0.05$). CEP-701 reversed this early life HS-induced increase in immobility in the last minute of the testing in the HCEP group (8.2 ± 1.5%; $p < 0.05$) that exhibited significantly less immobility compared with HV ($p < 0.05$), and was comparable to NV ($p = 0.13$) and NCEP (8.3% ± 2; $p = 0.99$). Panel C. On the second day of the test, the percentage of immobility of all groups was comparable.

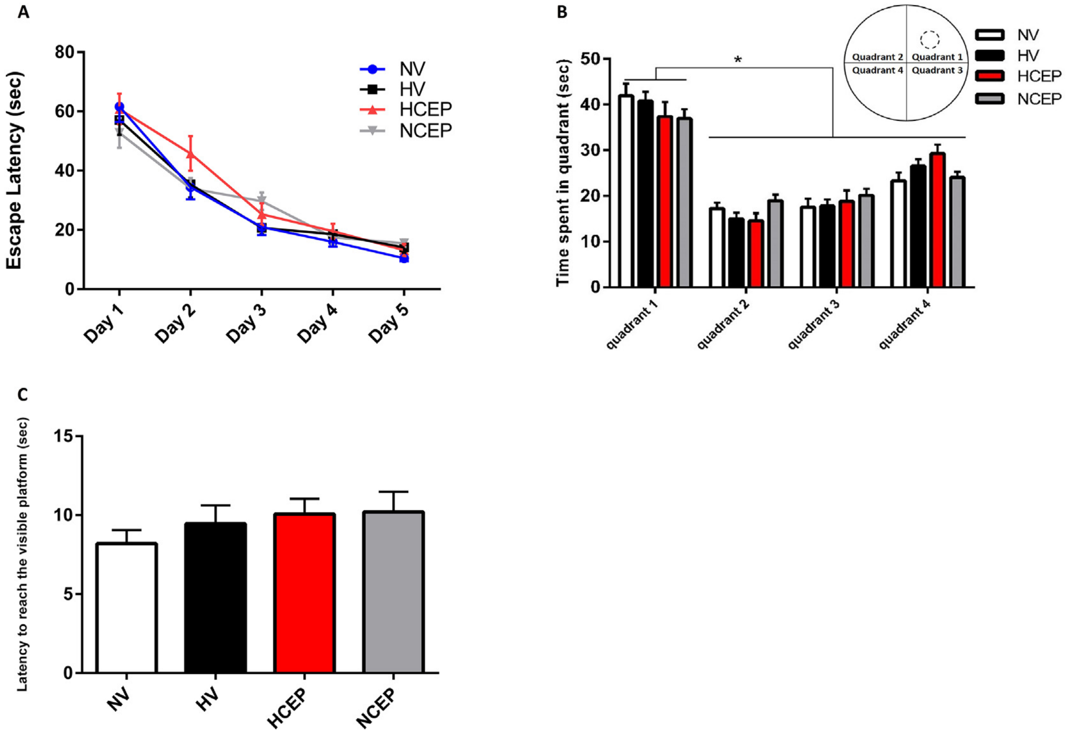

Fig. 4. Morris water maze place learning and probe trial results. Panel A. Two-way repeated measures ANOVA with post hoc Fisher least significant difference (LSD) revealed that all groups had a similar performance in learning the place of the platform on all 5 testing days as measured by comparable average escape latencies. Panel B. The dashed circle in the water maze diagram corresponds to the previous location of the platform. Two-way ANOVA with post hoc Fisher LSD revealed that all groups spent significantly more time in quadrant 1 compared with quadrants 2, 3, and 4 (*$p < 0.05$ for quadrant 1 versus all other quadrants in paired comparisons), with no effect for HS or treatment. Panel C. All the rats had comparable latency in reaching the visible platform indicating comparable visual and motor functions in all groups.

3.6. Long-term EEG and histological analyses

No SRS were detected in any of the four groups ($n = 10$–12 per group) during the recording period between P50 and 100. Given the potential time-dependence of seizure emergence, we prolonged the recordings in a cohort of rats ($n = 2$–3 per group) until the age of P140, but no seizures were detected (Supplementary Fig. 1). Since our HS model is mainly characterized by hippocampal molecular and electrophysiological changes [7,22,23], we assessed hippocampal pyramidal neuronal density on H&E-stained sections. There were no statistically significant differences between hippocampal pyramidal neuronal densities among all treatment groups (362.9 ± 11.2 cell/mm^2 for NV, 363 ± 5.5 cell/mm^2 for HV, 356.3 ± 13.2 cell/mm^2 for HCEP, 344.2 ± 7.7 cell/mm^2 for NCEP, one-way ANOVA, $n = 6$ per group, $p = 0.44$). Hippocampal GFAP staining did not reveal detectable differences between the groups (Supplementary Fig. 2). Moreover, given the prominent depressive-like behavioral sequelae of HS, GFAP and H&E staining of the amygdala and prefrontal cortex were also performed but there was no detectable evidence of overt neuronal loss or astrogliosis.

4. Discussion

4.1. Reversal of HS-induced long-term depressive-like behaviors with transient post-HS CEP-701 treatment

In this study, we aimed at investigating whether the previously shown short-term neuroprotective effects of CEP-701 [21], a drug with an established clinical safety profile in children [20], are sustained against the long-term sequelae of early life HS. We found that CEP-701 reverses HS-induced chronic behavioral deficits. Compared with normoxic controls, vehicle-treated rats that experienced early life HS exhibited decreased struggling behaviors in the FST. A transient post-HS treatment with CEP-701 reversed this HS-induced behavioral change to normal patterns. Even though the pattern of struggling in the FST can be affected by hyperactivity as well as by deficits in hippocampal learning as we have previously shown [45], the herein measured increased immobility was interpreted in the context of results obtained from other complementary panels, as a depressive-like behavior. Indeed, the HS-induced decreased mobility in the FST is unlikely to be secondary to reduced activity levels as there were no differences in activity levels between the normoxic and hypoxic rats in the OFT. This study, therefore, reveals that transient early life HS predispose to long-term depressive-like behaviors, and demonstrates the possibility of modulating a clinically relevant HS-induced long-term behavioral deficit with a brief transient post-HS treatment.

4.2. Potential role for TrkB-mediated aberrant synaptic reorganization in HS-induced long-term depressive-like behaviors

Emotional behavioral disturbances in periadolescent children are known sequelae of early life hypoxia [13] and common comorbidities of the pediatric epilepsies [46]. Far from being subtle accompaniments, they tend to impact quality of life at times more drastically then seizures themselves, especially depression that is associated with a high risk of suicide [47,48]. Successfully treating these comorbidities is often challenging because of their etiologically heterogeneous underlying mechanisms that include frequent seizures, interictal spikes, lesional burden, and antiseizure-medications. Here, we show that transient early life HS can result in chronic depressive-like behaviors in the absence of overt structural changes in the prefrontal cortex or limbic structures, and in the absence of recurrent later life SRS. It is therefore likely that these observed long-term behavioral deficits are the result of HS-induced chronic aberrant synaptic reorganization, potentially involving an imbalance in TrkB receptor signaling. Indeed, the reversal of the behavioral deficits in the HCEP group with a CEP-701 dosing

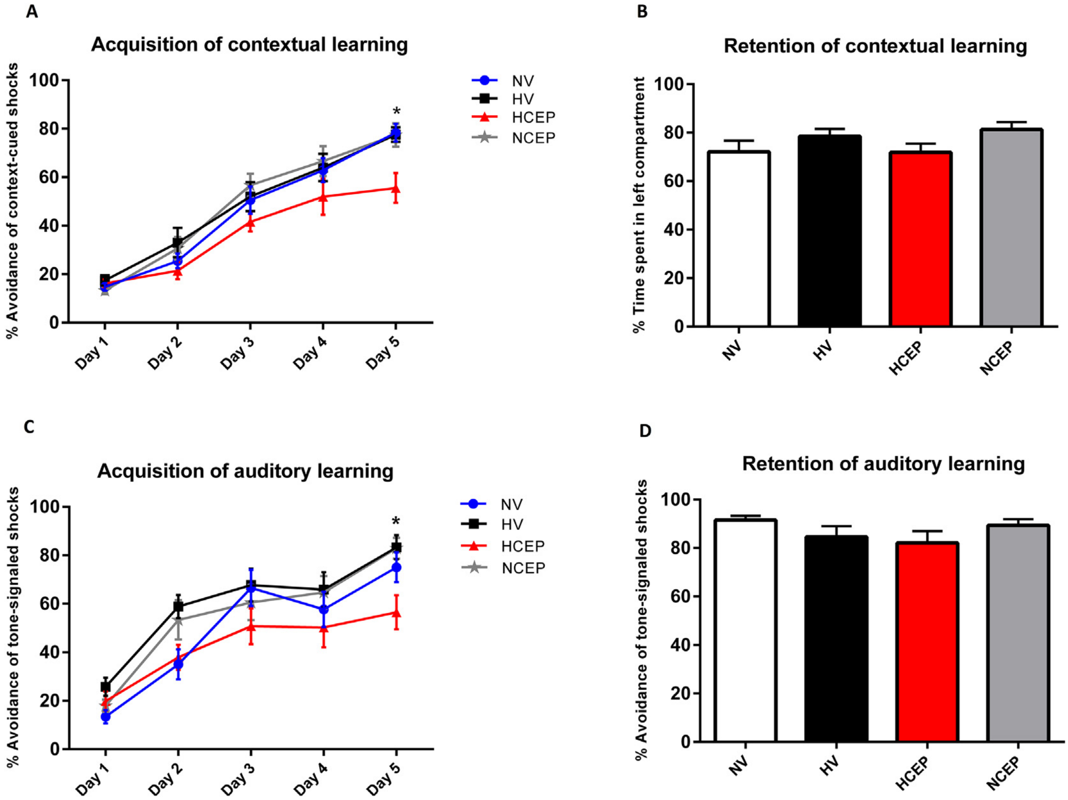

Fig. 5. The modified active avoidance (MAAV) test. Panel A. Shown is the learning curve of electrical foot-shock avoidance in the right contextually modified compartment over the 5 training days. The HCEP group was significantly slower in the incremental acquisition of shock avoiding behaviors, and this effect was statistically significant on the 5th testing day (two-way ANOVA, $p < 0.05$). Panel B. In the context retention subtest, all the groups had a similar preference to the left compartment when allowed to freely roam on day 6, as measured by the percentage of time spent on the left side (72.2 ± 4.5% for NV, 78.5 ± 3% for HV, 71.9 ± 3.6% for HCEP, 81.3 ± 3.1% for NCEP, one-way ANOVA, $p = 0.18$), and the number of transitions between the two compartments (5.5 ± 0.7 for NV, 6.9 ± 0.9 for HV, 6.4 ± 0.8% for HCEP, 5.4 ± 0.8% for NCEP, one-way ANOVA, $p = 0.53$). Panel C. Shown is the learning curve of tone-signaled electrical foot-shock avoidance in the left compartment over the 5 training days. The HCEP group was significantly slower in the incremental acquisition of shock avoiding behaviors, and this effect was statistically significant on the 5th testing day (two-way ANOVA, $p < 0.05$). Panel D. In the auditory learning retention subtest on day 6, all groups, including HCEP, were comparable in reaching their full potential.

regimen known to modulate TrkB activation [21] suggests a role for an HS-induced TrkB pathway imbalance in their emergence. The observed neuroprotective effect of CEP-701 cannot be due to a potential anticonvulsant effect against the initial HS as both the HCEP and HV groups had a comparable original burden of HS given the post-HS treatment paradigm. Even though CEP-701 acts on multiple molecular targets including Janus kinase 2 (JAK2) and tropomyosin-related kinase receptors A and C (TrkA and TrkC), it was used to target TrkB in human studies because of its favorable safety profile and lack of alternatives. However, unlike any of the other potential targets of CEP-701, TrkB is the only target that was shown to play a key role in rodent models of seizure and emotional disturbances [21,31,32,49], including mood disorders that accompany chemoconvulsant-induced limbic seizures [27]. However, given the lack of target specificity of CEP-701, more mechanistic studies are needed to support the herein presented circumstantial and biological plausibility-related evidence and to confirm that HS-induced long-term emotional behavioral changes were reversed via the modulation of HS-induced maladaptive TrkB activation.

4.3. Adverse long-term effects of CEP-701 on emotionally-relevant auditory and contextual learning in a novel behavioral testing paradigm

We are also reporting here the results of the MAAV test, a novel test for simultaneously assessing emotionally-relevant contextual and auditory-cued instrumental active avoidance. We developed this test by modifying the standard active avoidance shuttle box apparatus and experimental protocol. This allowed the successful concomitant testing of auditory and contextual emotional cues learning, and the acquisition of learned adaptive shock-avoiding behaviors as evidenced by the simultaneous gradual incremental daily increases over the 5 training days in the rate of successful avoidance of both tone-signaled and context-cued shocks. However, similarly to the MWM test, HS did not affect the outcomes of the MAAV, in line with previous reports on passive avoidance testing following early life hypoxia [50]. Interestingly, despite performing similarly to the other groups in the retention subtest of the MAAV, the CEP-701 treated hypoxic rats were slower in reaching their full potential during the daily acquisition trials. In this test of emotionally relevant learning that recruits the amygdalohippocampal circuitry, CEP-701 compounded the effects of early life HS. Given the established role of the TrkB signaling pathway in emotionally-relevant learning [34], it is likely that the attenuation of its post-HS activation contributed to this long-term CEP-701-related neurodevelopmental adverse effect on the rate of learning acquisition, even though CEP-701 did not have a similar negative effect in normoxic rats. Chronic medication adverse effects on learning have been reported with many drugs routinely used in the neonatal period including the most commonly used one, phenobarbital [51]. The fact that the mechanisms of learning, memory, and behavioral adaptability are similar to those involved in seizure propagation not only explains why cognitive and emotional behavioral deficits are the most common comorbidities of the epilepsies, but also explains why pharmacologically targeting seizure mechanisms, even with brief transient drug exposures, may result in chronic cognitive and behavioral deficits. This is particularly true when drugs are administered during the critical neonatal period that corresponds to the maximal brain growth spurt [52].

4.4. Relevance of strain, species, and hypoxia severity on long-term behavioral sequelae in rodent hypoxia models

In our study, there were no detectable HS-induced deficits in the MWM test. Interestingly, MWM deficits have been reported in HS rat models with relatively severe hypoxic insults accompanied by prominent hippocampal neuronal loss [14], but not in milder nonlesional HS models like ours [53]. This test may not be sensitive enough for the nonlesional hippocampal molecular and electrophysiological disturbances previously described in our HS model [7,21,22], in concordance with emerging literature suggesting that genetically-induced functional hippocampal alterations may not affect visuospatial navigation in the MWM [54]. Along those same lines, deficits were not detected in the LDT and the OFT. Hyperactivity in the OFT has been described in the periadolescent and adult ages following acute HS in Wistar rats [55] but not in the Long–Evans [56] or Sprague–Dawley [14] strains. Adult anxiety-like changes in the LDT were also reported in a mouse model of early life hypoxia [56]. Our results, in the context of these data, suggest that hyperactivity and anxiety-like rodent behavioral responses following early life HS are possibly age-, strain-, and species-dependent. Interestingly, this work also points to a possible strain difference between Sprague–Dawley and Long–Evans rats in their susceptibility to develop later life SRS following early life HS. Prolonged continuous EEG recordings performed in our rodent EEG monitoring unit between P50 and 100 and up to P140 in some rats did not reveal spikes, sharp waves, or SRS. On the other hand, up to 100% of Long–Evans rats have been reported to develop SRS during this age timeframe when subjected to the same herein employed protocol for hypoxia induction [7,23]. Of note, the described SRS have a peculiar spike–wave discharge (SWD) morphology and frequency that is seen in normoxic Long–Evans control rats [7]. While an electrographic SWD signature shared between injured and control rats points to an inherited electrographic pattern rather than to an acquired injury-related one, recent work has even challenged the epileptic nature of these discharges and suggested that they may represent a nonepileptic behavioral state-related normal pattern [57].

In summary, we have characterized the long-term emotional behavioral and cognitive effects of early life HS in a relatively mild nonlesional model that lacks chronic SRS. This allowed us to conclude that transient early life HS, in and of themselves, predispose to chronic depressive-like behaviors likely via inducing aberrant synaptic reorganization [9]. Existing literature suggests that post-HS synaptic reorganization is the result of seizure activity and not hypoxia alone [58]. The observed modulatory role of CEP-701 circumstantially implicates TrkB signaling in these post-HS culprit molecular synaptic alterations. The mixed positive and negative effects of this drug may be the result of its modulation of multiple TrkB downstream pathways with various adaptive and maladaptive roles [59]. From a translational end, not only does the clinical relevance of this work lies in providing a proof of concept that a postinsult treatment paradigm can be successful against detrimental chronic HS-induced depressive-like behaviors, but it has a direct translational value given the established relative human safety of CEP-701 [20]. Nevertheless, in addition to the nonspecificity of CEP-701, limitations of this study also include the use of only one dosing regimen, and the potential inapplicability of its conclusions to females as it only included male pups. Future studies that include females, other drug dosing regimens, and more mechanistic studies are warranted to further investigate the potential application of TrkB modulation as a neuroprotective strategy following neonatal HS. Moreover, the mechanistic dissection of the likely mixed adaptive and maladaptive roles of the various TrkB downstream pathways may pave the way for potentially more selective TrkB modulatory strategies that can achieve neuroprotection without causing adverse neurodevelopmental effects; a clinical objective that has been notoriously challenging in the critical neonatal period [51,52].

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Conflict of interest

None of the authors has any conflict of interest to disclose.

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