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DEPRESSION INDUCED BY EARLY-LIFE HYPOXIC SEIZURES: PATHOGENESIS AND MOLECULAR ALTERATIONS

by
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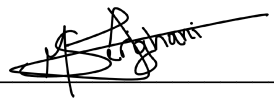

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ABSTRACT
OF THE THESIS OF

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Major: Neuroscience

Title: Depression Induced by Early-life Hypoxic Seizures: Pathogenesis and Molecular Alterations

Background: Children with epilepsy are more likely to develop depression which reduces their quality of life and leads to higher suicide risks in adolescence. The treatment of depression that accompanies epilepsy is understudied and effective therapeutic approaches remain elusive, let alone the fact that some antidepressant drugs have proconvulsant properties, increasing the risk of seizures. This renders the treatment of depression in pediatric patients suffering from epilepsy even more challenging. Thus, improving our understanding of the molecular pathways involved in epilepsy and depression is fundamental to optimize therapeutic strategies. Prior work in our rat model of neonatal hypoxic encephalopathy revealed the emergence of peri-adolescent depressive-like behaviors following early life hypoxic seizures (HS), potentially via HS-induced hippocampal TrkB (tropomyosin-related kinase) receptor pathway dysfunction. Investigating whether the mechanisms of depression induced by early-life HS are similar to those of major depression holds an important translational clinical value. Sertraline, a standard clinically employed antidepressant, is a serotonergic drug that was also shown to exert a serotonin-related modulation of the TrkB pathway in rodent models of major depressive disorders. Here, we aimed to investigate whether sertraline is effective against HS-induced depression and to assess possible molecular alterations mediating HS-induced deficits.

Methods: Hypoxia was induced in postnatal day 10 (P10) rat pups while the rest remained under normoxic conditions. Only pups that experienced 6 seizures or more were included in this study. The rats were then divided into 4 groups: NV (normoxic with vehicle water), HV (hypoxic seizure with vehicle water), NSERT (normoxic with sertraline), and HSERT (hypoxic seizure with sertraline). Sertraline was administered via drinking water between P24 and P30. Depressive-like and anxiety-like behaviors were then investigated in the forced swim test (P25-26) and the open field test (P27), sequentially. Rats were sacrificed at P30. Potential neuronal cell loss and alteration in synaptic plasticity were assessed through immunohistochemistry via Neu-N and synaptophysin (SYP) staining, respectively.

Results: Rats in both HV and HSERT groups had a comparable number of seizures ($p>0.05$). Rats' weight and water consumption were comparable among all groups between P21 and P30 ($p>0.05$). During the first testing day of the forced swim test, the HV group revealed increased immobility when compared to controls (NV) ($p<0.05$).

HSERT immobility percentages were comparable to those of the NV group ($p>0.05$). Nonetheless, during the second testing day, NV and HV groups had a comparable immobility time ($p>0.05$), with a lower immobility percentage observed in HSERT and NSERT groups when compared to NV and HV ($p<0.05$). During the first testing day of the FST, the high activity percentages of HV, NV, and NSERT groups were comparable ($p>0.05$). However, the high activity time of the HSERT group was significantly higher than that of the NSERT group ($p<0.05$). On the second testing day, both NV and HV had comparable high activity percentages ($p>0.05$), while the HSERT group had a high activity percentage significantly higher than that of both NV and HV ($p<0.05$). In the open field test, the cumulative total distance traveled by the HV rats was significantly lower than that of the NV group ($p<0.05$). The HSERT group had a total distance traveled comparable to that of the HV and NSERT groups ($p>0.05$). The HV group traveled a total distance in the open field significantly lower than that of the NV group ($p<0.05$). The HSERT group traveled a total distance significantly lower than that of the NV group ($p<0.05$), but comparable to that of the HV group ($p>0.05$). The NSERT group traveled a total distance significantly lower than that of the NV group ($p<0.05$). HV rats spent significantly more time and traveled less in the periphery when compared to NV ($p<0.05$). The HSERT and HV groups traveled a comparable distance in the periphery ($p>0.05$), but the HSERT group spent more time in the periphery when compared to HV ($p<0.05$). The NSERT group spent more time in the periphery when compared to NV ($p<0.05$). The HV group spent a significantly lower time and traveled a smaller distance in the center when compared to NV ($p<0.05$). The HSERT group spent a significantly lower time and traveled a smaller distance in the center when compared to NV ($p<0.05$). HV and HSERT groups had a similar distance traveled in the center, but the HSERT group spent less time in the center when compared to HV ($p<0.05$). The NSERT group spent a significantly lower time and traveled a smaller distance in the center when compared to NV ($p<0.05$). Preliminary histological data revealed comparable neuronal densities in all groups; however, SYP seems to be overexpressed in the prefrontal cortex and the hippocampus of HV rats in the hilar and CA2-3 regions when compared to NV.

Conclusion: Our early-life hypoxic-seizure model successfully recapitulates depressive-like and anxiety-like behaviors in peri-adolescent rats similar to the behavioral defects observed in pediatric patients who have suffered from perinatal hypoxic-ischemia. Short-term sertraline administration reversed depressive-like behaviors in the treated rats; however, it failed to reverse and exacerbated hypoxic seizure-induced anxiety. These effects on anxiety and depression are commonly observed early in the treatment course with antidepressants. The hypoxic-seizure-associated behavioral deficits seem to be mediated by maladaptive changes in markers of synaptic plasticity. These results confirm cognitive and emotional behavioral derangements correlated with early-life hypoxic seizures highlighting the necessity of treating seizure-associated behavioral comorbidities. Investigating the effects of a longer treatment duration will help determine if sertraline's chronic administration holds anxiolytic effects and will depict its optimal use in children who have suffered from hypoxic seizures.

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ABBREVIATIONS

AC: anterior cingulate cortex

AED: anti-epileptic drug treatment

ASD: autism spectrum disorder

ATP: adenosine triphosphate

BDNF: brain-derived neurotrophic factor

BW: birth weight

CCC: cation chloride cotransporter

COX-2: cyclooxygenase-2

CP: cerebral palsy

CT: computed tomography

DAT: dopamine transporter

dHPC: dorsal hippocampus

GLUT-1: glucose transporter-1

HIE: hypoxic-ischemic encephalopathy

HIF-1α: hypoxia-inducible factor-one alpha

HPA: hypothalamus-pituitary-adrenocortical

HPC: hippocampus

HS: hypoxic seizures

IL: infralimbic cortex

KCC2: potassium chloride co-transporter

MDD: major depressive disorders

mPFC: medial prefrontal cortex

MRI: Magnetic resonance imaging

MSB: maximal seizure burden

mTOR: mammalian target of rapamycin

NAc: nucleus accumbens

NE: neonatal encephalopathy

NET: norepinephrine transporter

NKCC1: sodium-potassium chloride cotransporter

NMDA: N-methyl-aspartate

NR: nucleus reuniens

OCD: obsessive-compulsive disorder

OGD: oxygen-glucose deficiency

PBS: phosphate-buffered saline

PDGF: platelet-derived growth factor

PET: positron emission tomography

PFA: paraformaldehyde

PFC: prefrontal cortex

PL: prelimbic cortex

ROS: reactive oxygen species

SD: sprague dawley

SERT: serotonin transporter

SNRI: serotonin-norepinephrine reuptake inhibitors

SSRI: selective serotonin reuptake inhibitor

SYP: synaptophysin

TCA: tricyclic antidepressant

TRD: treatment-resistant depression

TrkB: tropomyosin receptor kinase B

TSB: total seizure burden

VEGF: vascular endothelial growth factor

vHPC: ventral hippocampus

VTA: ventral tegmental area

CHAPTER I

INTRODUCTION

A. Hypoxic Ischemic Encephalopathy (HIE) of the newborn

Epilepsy affects 1 to 2% of the world's population (1,2). Seizure activity is caused by structural modifications in the brain. It has been hypothesized that a variety of mechanisms participate in the dysregulation of the central nervous system leading to epilepsy, as neuronal degeneration, disturbance in the blood-brain barrier, amygdala dysfunction, alterations in glutamate secretion, oxidative stress, hypoxic episodes, and epigenetics (3-9). Diagnostic and therapeutic applications are challenging, particularly in the case of neonatal seizures (10,11). There is growing proof that neonatal seizures result in detrimental neurodevelopmental outcomes, manifesting in cerebral palsy, psychomotor retardation, and the onset of post-neonatal epilepsy alongside psychiatric symptoms, as depression (12-15). The leading cause of those neonatal seizures is hypoxic-ischemic encephalopathy (7). Treatment of these seizures and their neurocognitive sequelae, like depression, has not been well established. Furthermore, growing evidence is showing that the standardly used antidepressants worsen seizures, lower their threshold, and are ineffective in the treatment of their associated depression. Yet, the possibility of treating depression in epilepsy through selective serotonin reuptake inhibitors (SSRIs) has not been well investigated (16).

1. Disruption of the brain electrical activity in epilepsy

Epilepsy affects 1 to 2% of the world's population (1,2). Seizure activity is caused by structural modifications in the brain. It has been hypothesized that a variety of mechanisms participate in the dysregulation of the central nervous system leading to epilepsy, as neuronal degeneration, disturbance in the blood-brain barrier, amygdala dysfunction, alterations in glutamate secretion, oxidative stress, hypoxic episodes, and epigenetics (3-9). Diagnostic and therapeutic applications are challenging, particularly in the case of neonatal seizures (10,11). There is growing proof that neonatal seizures result in detrimental neurodevelopmental outcomes, manifesting in cerebral palsy, psychomotor retardation, and the onset of post-neonatal epilepsy alongside psychiatric symptoms, as depression (12-15). The leading cause of those neonatal seizures is hypoxic-ischemic encephalopathy (7). Treatment of these seizures and their neurocognitive sequelae, like depression, has not been well established. Furthermore, growing evidence is showing that the standardly used antidepressants worsen seizures, lower their threshold, and are ineffective in the treatment of their associated depression. Yet, the possibility of treating depression in epilepsy through selective serotonin reuptake inhibitors (SSRIs) has not been well investigated (16).

2. Hypoxia is a relatively common condition in newborns with long term sequelae

Neonates subjected to severe brain anoxia during the perinatal period will develop hypoxic-ischemic encephalopathy (HIE) (17). This reduced brain oxygenation can be caused by perinatal asphyxia, placental issues, congenital cardiac diseases, or by different problems immediately after birth, such as respiratory arrest (18) (Fig.1). In

cases of severe hypoxemia, the brain as well as peripheral tissue (as the heart and muscles), will develop an oxygen debt resulting in anaerobic glycolysis, lactic acidosis, and subsequent physiological and neurobehavioral damages (19). Developed countries have a lower incidence of HIE (1 to 8 per 1000 live births), whereas this rate increases to reach 26 per 1000 live births in the developing world (20).

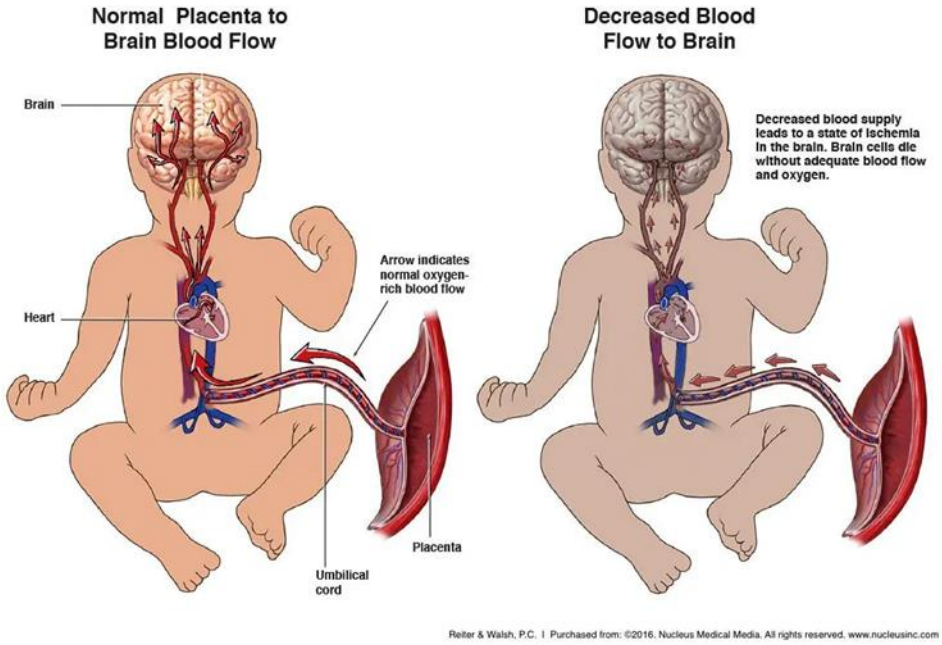

Figure 1. Hypoxic-ischemic encephalopathy of the newborn. The reduced blood flow causes a decreased brain oxygenation leading to ischemia (24).

The underlying cellular mechanisms of this reduced brain oxygenation in HIE involve a variety of defects. This includes oxidative stress, a failure of energy production in mitochondria, and a variation of blood flow and brain tissue metabolism. As a result, necrosis might occur leading in many cases to cerebral palsy (CP) and higher mortality rates (21,22). The resulting CP is characterized by abnormal motor development along with neurobehavioral deficits (23).

a. Clinical diagnosis

Clinical symptoms can be subdivided into acute and chronic. Acute defects include for example seizures and difficulty breathing. Chronic symptoms include mental retardation, learning impairment, depression, cerebral palsy, and epilepsy (25-27). Infant brain injuries develop over time, hours to days post-incident; this provides an important window for neuro-protective applications. A close birth monitoring could help provide a prognosis for this condition: umbilical pH, blood gas check 1h after delivery, brain, and tissue biomarkers monitoring, neuroimaging, Apgar scores, and neurological changes surveillance are useful tools (22). A low Apgar score at 1, 5, and 10 min is correlated with potentially higher mortality risk or chronic motor disabilities (22). Another useful criterion would be the Sarnat and Sarnat scoring criteria for HIE. Lower scores of 1 – 10 characterize infants born with mild HIE, 11-14 moderate HIE whereas higher scores of 15-22 fall into severe cases of HIE (28). Epidemiological studies have shown that higher mortality levels were recorded among infants with severe and moderate HIE, with no death recorded in cases of mild HIE. Additionally, birth weight (BW) plays an important role regarding the survival of neonates: those with higher BW had significantly higher survival rates than infants born with low BW. This complies with previous studies done in other hospitals implying that new strategies to treat low BW should be implemented to increase infants' survival (22,29-31). Nonetheless, diagnosis based on clinical symptoms might be subjective and time-consuming. Additionally, the application of computed tomography (CT) and magnetic resonance imaging (MRI) for HIE diagnosis is controversial due to fear of radioactive exposure and higher cost (32). Thus, new means of diagnosis are to be further investigated.

b. Long-term effects of seizure burden and lesions

The immature brain during neurodevelopment is characterized by enhanced excitation and reduced inhibition (33). Seizures often accompany HIE and are correlated with the development of later-life epilepsy. With hypoxia, those neonatal seizures contribute to energy depletion, cerebral blood flow alteration, and possible neuronal loss (34). A study revealed that the higher the seizure burden is, the worst the neurological outcome. Abnormal developments were recorded among neonates presenting a TSB (total seizure burden) of more than 40 min and an MSB (maximal seizure burden) of more than 13 min regardless of therapeutic hypothermia treatment (34). Infant surviving neonatal seizures suffer from global developmental delay alongside later intellectual disability or cerebral palsy (35). Spastic quadriparesis is the most encountered subtype of cerebral palsy (36). Motor, language, and cognitive functions can be assessed between the age of 24 to 48 months, but for a better motor disability identification, diagnosis at 2 years of age is the best. Cognitive skills and behaviors are best revealed at 6 to 7 years of age (34). The mentioned deficits are mediated by pathophysiological cellular dysfunctions.

The downstream physiological dysregulations resulting from HIE seizures progressively evolve, thus the timing of the treatment is important. Those defects develop over 3 phases. During the first 60 min post-injury, the primary phase consists of partial healing mechanisms. In the latent phase, occurring up to the 48th-hour post-injury, inflammatory responses, oxidative stress, and apoptosis take place in between the 1st and 6th hour (37). Between the 6th and 48th hour, excitatory neurotransmitters and free radicals are released with depletion of phosphate reserves (38). The tertiary phase,

taking place months after ischemia, is as well characterized by apoptosis, in addition to a remodeling of the injured brain areas, and astrogliosis (39). Furthermore, many cellular pathways are altered post-HIE among which BDNF-TrkB, mTOR, as well as other membrane-bound receptors and channels.

3. Molecular pathways alteration and cellular dysfunction

a. Molecular pathways alteration

i. BDNF-TrkB pathway: brain-derived neurotrophic factor- tropomyosin receptor kinase B pathways

Neurotrophin BDNF has an important trophic role and participates in high cognitive functions. It is a major key factor determining the pathophysiology of many brain disorders including neurological diseases, neurodegenerative ones, psychiatric disorders, addiction, and schizophrenic psychosis (40). It plays an important role as well in neurogenesis (41). The fixation of BDNF on its receptor TrkB activates downstream effectors that alter synaptic plasticity and provide strong anti-hypoxic and neuroprotective effects after OGD (oxygen-glucose deficiency) (42-44). Some studies have found that BDNF and TrkB are permanently reduced in the brain regions affected by HIE (45). Thus, the upregulation of BDNF has been perceived as a possible means of treatment after hypoxic events (46). On the other hand, evidence has shown that HS might result in the activation of TrkB pathways. One TrkB isoform, the immature form of hypo-glycosylated TrkB (i-TrkB), is synthesized after episodes of intermittent hypoxia and results in ligand-independent TrkB signaling upon phosphorylation (47). This TrkB pathway activation participates in both acute and chronic epileptogenesis and can be reversed by CEP-701 (lestaurtinib), a drug with an established safety profile in

children suffering from acute myeloid leukemia (48,49). All the above proves that the fine homeostasis of the BDNF/TrkB pathways is of crucial importance in post-hypoxic ischemia. In fact, any dysregulation will result in detrimental effects. A better understanding of this pathway's functions will help develop new therapies aiming to reduce HI-induced physiological dysregulation.

ii. AMPA pathway: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

Glutamate receptors are excessively stimulated following HIE. The 3 major types of ionotropic glutamate receptors, NMDA, AMPA, and kainite receptors, are found in most neurons and glial processes (50). Those ionotropic receptors participate in activity-dependent neuronal plasticity and neuronal excitation for normal tasks involving glutamate as somatosensory functions, learning, and memory which suggests their involvement in HIE detrimental brain damages (51). Hyperactivation of this pathway in epilepsy is correlated with excitotoxicity involved in selective motor neurons death (52-54). Treatment of HS rats with AMPAR antagonists attenuates mossy fiber sprouting in the CA3 region, lowers the frequency of seizures, and reestablishes social novelty preferences (33). This strongly suggests the AMPA receptor's role in epileptogenesis and behavioral alterations.

iii. Cation chloride cotransporter: potassium chloride co-transporter KCC2 and sodium-potassium chloride cotransporter NKCC1

Cation chloride cotransporters (CCC) are differentially expressed along the nervous system in various neural cell types. Sodium potassium chloride cotransporter (NKCC1) and potassium chloride co-transporter (KCC2) are the main two active CCC involved in the accumulation and extrusion of Cl^- respectively. Those receptors are differentially expressed between immature and mature neurons. Recapitulation, reversing the normal developmental shift, might result in epilepsy onset (Fig. 2).

Prenatal hypoxia triggers a sustained KCC2 loss in the developing CA3 hippocampus (55). This KCC2 loss has been associated with cases of chronic epilepsy (55-58). Genetically ablating KKC2 in animal models leads to an increased seizure susceptibility through an impaired Cl^- homeostasis (59). Thus, targeting CCC constitutes a window of action for the development of novel therapeutic agents.

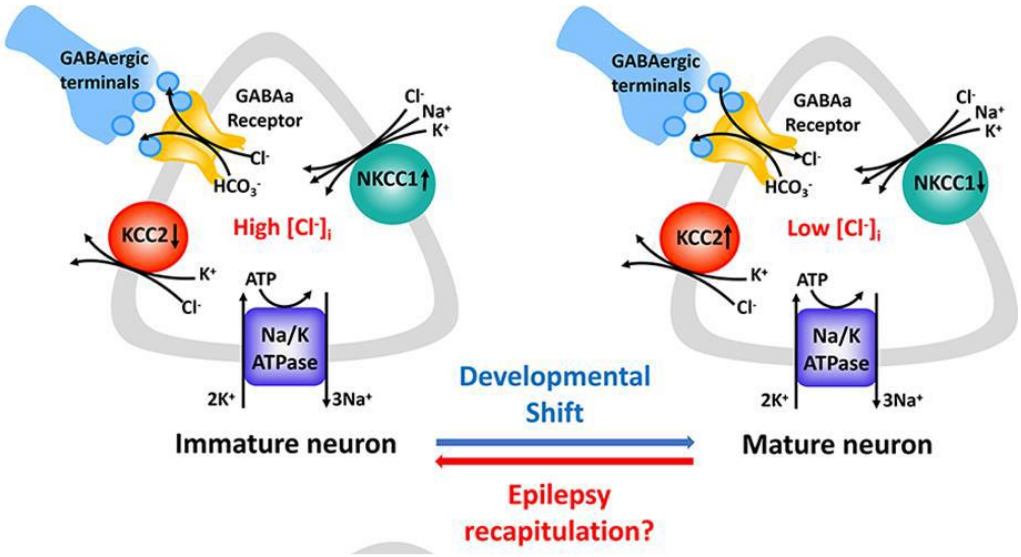

Figure 2. Developmental shift from immature to mature neurons. GABA binding to its GABAa receptors regulates the intraneuronal chloride concentration in immature and mature neurons. Immature neurons have high intracellular Cl- concentration and are characterized by an upregulated NKCC1 expression alongside a reduced expression of KCC2. Conversely, mature neurons exhibit lower intracellular Cl- concentration due to the upregulation of KCC2 and downregulation of NKCC1. Na+/K+ ATPase facilitates Cl- transport by creating an electrochemical gradient of Na+ and K+. When GABA binds to its receptors, ligand-gated Cl- permeable channel open letting Cl- as well as HCO3- out of the cell. The differential expression of NKCC1 and KCC2 among mature and premature neurons is disrupted in epilepsy as mature neurons undergo "recapitulation" disturbing the electrical signaling along the nervous system (60).

iv. mTOR pathway: mammalian target of rapamycin pathway

Stimuli as stress and energy levels deficiencies are sensed by the mTOR (mammalian target of rapamycin) pathway yielding the downstream activation of various cellular mechanisms as angiogenesis, cell growth, apoptosis, and autophagy. mTOR can regulate as well hypoxia-inducible factor-alpha1 (HIF-1α) involved in hypoxic-ischemic sequelae (61,62). Glutamate secretion has been positively correlated with this pathway's activation, through Ca2+-mediated signaling cascades (63). Several studies have associated mTOR hyperactivation with seizure development observed in models of HS rats (33,49,64). This activation plays an important role in neuronal hyperexcitability, synaptic plasticity, and memory consolidation (65,66). A bidirectional interaction between AMPARs and mTOR pathways is observed post-HI (67). Treatment with rapamycin, mTORC1 inhibitor, reverses the increased glutaminergic transmission, reduces seizure, and improves autistic like-behaviors (68). For the above reasons, novel treatments aiming to downregulate mTOR are being further investigated.

b. Cellular dysfunction and oxidative stress post-hypoxic ischemia

Ischemic events lead to an altered cellular function causing the secretion of hypoxia-inducible factors and free radicals, inflammation, and apoptosis. A positive correlation between higher levels of HIF-1α and epilepsy has been observed (69-71)**.** An upregulation of HIF-1α leads in some cases to treatment-resistant epilepsy. The elevated expression of HIF-1α in hypoxia may be caused by insulin, insulin-like growth factor, platelet-derived growth factor (PDGF), epithelial growth factor, and interleukin-1B (72). HIF-1α plays an important gene regulator role in tissues undergoing hypoxia. HIF-1α upregulation results in angiogenesis, glycolysis, recruitment of glucose

transporter 1 (GLUT1), and neuronal accumulation of pyruvate (70,73). This accumulation might decrease intraneuronal pH altering metabolic state (Fig. 3) (73).

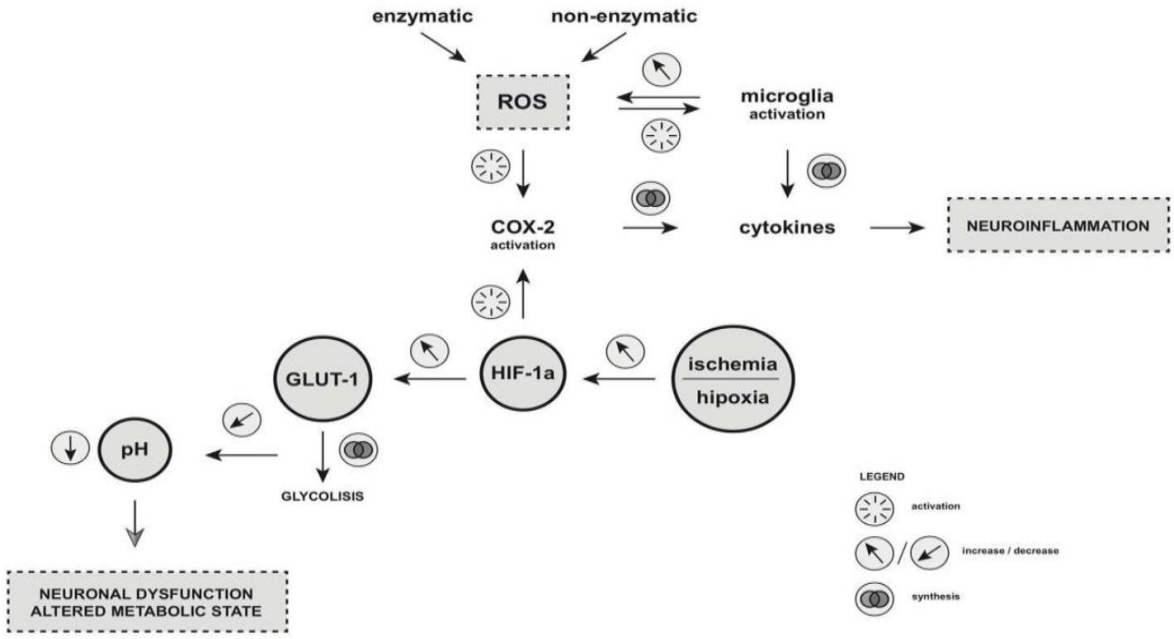

Figure 3. Oxidative stress and hypoxia contribution to epileptogenesis. COX-2 is activated by enzymatic and non-enzymatic pathway of ROS production with the increase HIF-1a under hypoxic condition. This leads to microglial activation and an excessive cytokine production inducing neuroinflammation. The dysregulation of HIF-1a, that usually regulates the glucose metabolism in the central nervous system (CNS) through GLUT-1 synthesis, may result in an accumulation of pyruvate in neuronal cells which is then converted into butyric acid via butyric dehydrogenase. This accumulation of by-products may lower intraneuronal pH yielding dysfunction and altering metabolic state. ROS, reactive oxygen species; COX-2, cyclooxygenase-2; HIF-1a, hypoxia inducible factor-1 alpha; GLUT-1, glucose transporter-1. Illustration by Paulina Szuba (74).

Febrile seizure observed in perinatal ischemia, or post-stroke and transient ischemic attacks could potentially be due to the upregulation of COX-2 and PGE-2 (prostaglandin E2) by the binding of HIF-1α on the hypoxia-responsive element present on the COX2 promoter in DNA (75). This COX-2 activation has been correlated with hypoxia and oxidative stress pathways contributing to the deleterious impact of epilepsy

on the brain (Fig. 3) (76)**.** Further neurological defects in HIE are caused by energy failure and reduced production of the adenosine triphosphate (ATP) due to mitochondrial dysfunction (77). Cytochrome c usually plays an important role in ATP production; however, a brain ischemic event causes oxidative stress inflicting the release of cytochrome c from the intermembrane space of mitochondria into the cytoplasm. This activates caspases and induces apoptosis. Furthermore, hypoxic events lead to intracellular anaerobic glycolysis resulting in the production of excitatory neurotransmitters like glutamate(78,79). The higher expression of glutamate facilitates $Ca2^{+}$ influx into neural cells through both voltage-gated calcium channels and receptor-regulated ones as N-methyl-aspartate [NMDA]. This entails the activation of proteases, lipases, and endonucleases leading to fatty acids release, such as arachidonic acid. The cell's antioxidant capacity is exceeded by the production of reactive oxygen species (ROS) and superoxide free radicals that activate the synthesis of cyclooxygenase and prostaglandin alongside other toxic metabolites (80-82). Additionally, the increased intracellular concentration of Ca^{2+} results in nitric oxide (NO) production by a nitric oxide synthase hypoxia-inducible isoform (iNOS) in microglia, macrophages, and neutrophils (83). Hydroxyl radicals are further produced, because of the activation of NO by superoxide (84). The accumulation of free radicals and glutamate secretion increases inflammatory and immunological responses (85-87). Brain injuries after a HI event manifest particularly through delayed neural cell death. This is mainly mediated by apoptotic pathways, extending from early phases of injury up to weeks; however, in cases of complete energy depletion necrotic cell death takes place (88).

c. Mirco ribonucleic acid altered expressions in hypoxia: possible biomarker and treatment target

EEGs are the main diagnostic tool for epilepsy; however, they present some low specificity (76)**.** It is almost impossible to confirm epileptic seizures only through clinical examinations, except for the case of clonic seizures (89,90). This highlights the need for biomarkers as tools to indicate brain dysfunction, particularly micro ribonucleic acid miRNAs. In addition to their role in cellular processes as differentiation, and apoptosis, key functions of the CNS are regulated by these miRNAs, as neurogenesis, synaptic plasticity, and neuronal development (91,92). Thus, they might be involved in epileptogenesis (93). They are a convenient biomarker because they are easily accessible through minimally invasive biofluid collection in contrast to lumbar puncture. The presence of miRNA in biofluids might be caused by an active secretion during intracellular communication, a passive secretion caused by cellular damage, or a combination of both (94). In a homeostatic state, miRNAs downregulate ROS (95). However, this mechanism is disrupted in epilepsy. Those small non-coding ribonucleic acids alter protein synthesis by interfering with the translation or degradation of messenger RNAs (mRNA), thus modulating gene expressions (96,97). Several studies revealed an altered expression of miRNA after a transient focal cerebral ischemia, in hypoxic patients and patients suffering from temporal lobe epilepsy (98). This affects in turn the expression of HIF-1α (99,100)**.** A bidirectional interaction between miRNA and HIF-1α has been established as well (100,101). Furthermore, those miRNAs can reflect neurobehavioral outcomes of newborns (102). miRNA could play both a role in the pathogenesis as well as in the repair. A study of the downstream targets of miRNA might provide information about the pathways involved, resulting in possible treatment targets (103).

4. Modest efficacy of current treatment strategies

The first line of action after a hypoxic-ischemic event is therapeutic hypothermia directly after birth (104). Yet, alone it is not sufficient as it does not ensure complete neuroprotection. Thus, novel therapeutic strategies are being developed to set new standards of care for the treatment of HIE (25).

a. Current standardly adopted therapeutic approaches

i. Therapeutic hypothermia

Therapeutic hypothermia is the standard clinical care performed to decrease the life-long morbidities of HIE. This treatment acts on the excito-oxidative cascade. It diminishes energy failure and restores the brain concentration of lactic acid, glutamate, and nitric oxide (105). The aim is to achieve whole-body cooling so that the core temperature reaches 33.5°C for 72h (106). Post-treatment, body temperature is increased by 0.5 degrees per hour till the temperature of the esophagus reaches 36.5/37 for 4h (Fig. 4) (107).

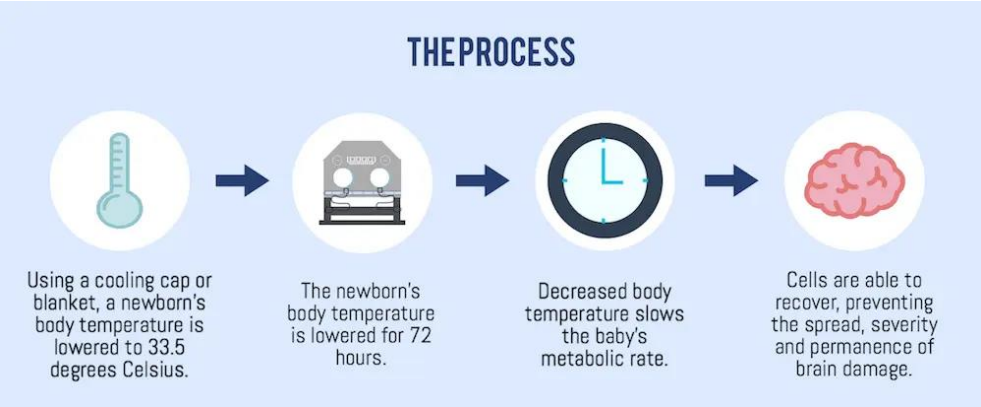

Figure 4. Therapeutic hypothermia process. Selective head cooling or full body cooling is applied to lower body temperature to 33.5 degrees Celsius for 72h. Cooling decreases the newborn's metabolic rate allowing cellular recovery and reducing the severity of brain damages (108).

Many studies investigate the optimal timing for treatment onset to achieve the best neuroprotective outcomes. It has been shown that treatment should best be done during the latent phase, starting at the 3rd hour after the ischemia (109,110). A study done by Thorsen et al. showed that neonates subjected to hypothermia < 180 min post hypoxia scored better in Bayley Scales of Infant Development II (111). Both, whole-body cooling and selective head cooling significantly improve survival rates, reduce sensorineural disabilities, neuromotor delay, cerebral palsy, and EEG changes, in treated infants in comparison with the control group (112,113). Treatment done as early as 6h after birth significantly improves neurocognitive outcomes and decreases mortality rates (114,115). However, prolonged durations of hypothermia (more than 6h), put children at risk, particularly those with low birth weight without procuring any additional positive outcome (107). Rat animal models of HIE also exhibit a similar sequel (116). In fact, cell death is delayed up to a week- before brain atrophy onset after a brief period of hypothermia of 32°C in P7 rat pups post-injury (117). Additionally, immediate hypothermia after birth increases survival rates of children at 6-7 years old, but with no significant difference in IQ levels compared to the control group (118). Nonetheless, in another study treated groups had significantly scored higher in IQ (more than 85) in comparison with the non-treated group at 6-7 years of age (119). In postnatal day 7 (P7) rat pups models of HIE, the optimal temperature of whole-body cooling lies between 32° and 33.5° C (33.5° being the one admitted in human treatments). It is important to note that cooling pups below 33.5 degrees does not provide additional neuroprotection. Furthermore, intermediate durations of therapeutic hypothermia (~5h) resulted in better neuroprotection than 3 hours in rat pups, but no differences were noted between pups subjected to longer treatment varying from 5 hours to 10 hours of

hypothermia (120). Hypothermia provides its therapeutic effects by upregulating cellular acetylation and suppressing acetyl-CoA, which usually participates in glycolysis, amino-acid catabolism, and ketolysis (121). It additionally reduces the activation of the caspase-3 enzyme, microglia, and NMDA receptors alongside the conservation of membranes integrity (122,123). However, in cases of seizure onset, TH is less effective, since they are a sign of secondary deterioration (124). Additionally, some studies showed that seizures continue to re-occur after the treatment (125). Despite being the first line of action, TH alone is not enough, as 40- 50% of neonates who underwent the treatment develop later neurological complications (104). In fact, therapeutic properties of hypothermia are mostly observable in cases of minor to moderate HIEs whose patients return to normal within 10 days; however, infants suffering from more severe cases of HIE present echo-free space 2 to 3 months' post-ischemia with higher mortality rates (126). This is why an adjuvant for the current treatment is required (127).

ii. Erythropoietin (EPO)

This glycoprotein is synthesized in the fetal liver, and both the liver and kidneys in neonates. Additionally, to its role in erythropoiesis, it has anti-inflammatory, angiogenesis, and neurogenesis properties, particularly that it is being secreted from fetal life (128). A variety of brain cells, as neuronal progenitors, astrocytes, oligodendrocytes, and microglia produce EPO receptors. Hypoxic ischemia results in the activation of EPO signal and EPO receptors in the cerebrospinal fluid (129). EPO-receptor complex limits inflammation and apoptosis (130). In animal studies, the combination of EPO with hypothermia resulted in a lower mortality rate and a reduced cerebral palsy development, while hypothermic treatment on its own did not improve

the development of cerebral palsy in comparison to the group treated with saline solution (131). In mice, EPO was shown to heal sensorimotor functions (132). In humans, EPO decreases as well death rates and reduces basal ganglia lesion in MRI in neonates. It is as well correlated with lower rates of moderate to severe neurodevelopmental disability at 22 months old (133). EPO is a safe and non-toxic treatment adjuvant; however, its precise usage dose is yet to be determined. Some literature states 1000U/kg in the serum to be the optimal concentration for neuroprotection, but further investigations are required (134).

b. Alternative and investigational approaches

Despite the established first-line treatments, it is important to note the important gap between developed and developing countries regarding their availability and the means to provide patients with suitable medical interventions (28). For example, therapeutic cooling is not available in some hospitals (28). This is why alternative treatment strategies that do not require much equipment ought to be further investigated to alleviate the HIE burden and outcomes.

i. Ischemic conditioning

Ischemic conditioning can ensure protection against a lethal ischemic event by putting into action an adaptive process of endogenous protection. This is achieved by inducing sublethal ischemic events. A form of this conditioning is remote ischemic post-conditioning RIPC, where the sub-lethal ischemia is induced at the limb. Ischemic conditioning can be performed remotely from the ischemic targeted organ by putting a cuff on the limb arm or leg that is repeatedly inflated and deflated (135-137). This constitutes a safe and simple means of protection for distant organs such as the brain,

heart, and kidney. This process is observed in tumor growth, where intermittent hypoxia seems to increase tumors resistance to major ischemic events and therapies (138). Previous studies demonstrated that the conditioned sub-lethal ischemia can be applied before the onset of the lethal ischemic event (pre-conditioning), during it, and ahead of reperfusion (per-conditioning), or after the hypoxic episode throughout reperfusion (post-conditioning). However, combining per- and postconditioning significantly increased the neuroprotective outcomes of the treatment (139-141).

The neuroprotective effects of this treatment lie behind its neurochemical basis. Circulating humoral protective factors released after nerve stimulation and limb ischemia play a key role in protecting against ischemic damages. RIPC can reduce hypoxic morbidities by altering mRNA expression that in turn regulate a variety of cellular activities, protein expression, reduces oxidative stress and apoptosis (25,141-146). Moreover, pre-conditioning leads to an early astrocyte differentiation as part of glial cell adaptation playing a key role in neuroprotection (147). Both neonatal and adult brain infarct sizes are reduced by RIPC in animal models and humans (148-150). It is as well accompanied by an important neurological outcome improvement (146,151). Implementing RIPC with TH could help further reduce brain damages and alleviate their detrimental long-term consequence.

ii. Isoflurane

Isoflurane, a volatile anesthetic, can be used as a substitute for ischemic conditioning in adults and neonates (152,153). Neurological damages resulting from a cardiac arrest can be alleviated by isoflurane post- conditioning (154). Isoflurane mostly reduces spatial and memory deficits while barely showing effects on motor functions (155).

iii. Melatonin

Melatonin, synthesized in the pineal gland, has been shown to increase neuroprotection through its antioxidant, anti-inflammatory, and anti-apoptotic properties (156). It can reach various brain regions through its ability to cross physiological barriers and freely diffuse among tissues. By increasing ATP, it regulates apoptosis. Furthermore, melatonin eliminates free radicals by producing antioxidant enzymes. Infants treated with birth hypothermia and melatonin have been shown to have better EEG profiles, fewer white matter abnormalities, better survival, and enhanced neuroprotection in comparison with those only treated with hypothermia at 6 months according to Denver Developmental Screening Test II (157)**.** However, optimal dosage and timing are yet to be determined.

iv. Allopurinol

Allopurinol, and its metabolite oxypurinol, inhibit superoxide radicals by downregulating oxidase enzymes activated during reperfusion (158). Anti-inflammatory properties manifest by reducing neutrophils accumulation. Additionally, it reduces hydroxyl radicals, chelates metal ions, and lowers NO serum concentration (159). Animal studies have proved its neuroprotective effects in rats and revealed that late (24h after ischemia) and reduced doses fail to protect the brain from neuronal damage (160). In moderate cases of hypoxia, survival rates significantly improved at 4-8 years without affecting the Wechsler Preschool and Primary Scales of Intelligence test or the Wechsler Intelligence Scale in the long term (161).

v. Magnesium sulfate

Magnesium sulfate ($MgSO_4$) can be used as an adjuvant neuroprotective treatment or alone. Its mechanism of action is yet to be further investigated; however, it

acts mainly by preventing calcium influx into the cell through a voltage-dependent inhibition of NMDA glutamate receptors (162). Furthermore, secondary inflammation damages are inhibited by the stabilization of the cell membrane and by a reduction of free radicals' formation (163). It accelerates as well the differentiation of oligodendrocytes precursor cells into oligodendrocytes, protecting them from hypoxic damages, thus providing white matter protection (164). Its neuroprotective effects do not apply to differentiated oligodendrocytes, this is why MgSO4 is only prescribed in cases of preterm birth risks and has been shown to lower the risks of cerebral palsy (165). Some studies demonstrated that its combination with hypothermia showed no significant differences between the two treatment groups, while in others increased mortality caused by systemic hypotension was recorded, without being statistically significant (166). Further studies are yet needed to investigate all of the MgSO4 side effects.

vi. Ketogenic diet

HIF-1α results in a glycolysis by-product accumulation (pyruvate converted into butyric acid) lowering the intraneuronal pH. This diet aiming to reduce glucose intake might downregulate the pathways activated by HIF-1α, and enhance substrate conversion in acyl-CoA through beta-oxidation (73). Thus, a ketogenic diet might be beneficial for patients presenting drug-resistant epilepsy (167).

c. AED treatment: Anti-epileptic drug treatment

Clinical neonatal seizures are an emergency and treating them in infants following HIE has many concerns. The main aim of the treatment would be to lessen the seizure burden and reduce future neurodevelopment deficits. However, AED

treatments might exhibit possible risks outweighing their potential beneficial properties. AEDs have been associated with neurotoxicity, apoptosis, alteration of synaptogenesis and myelination, suppression of hyperexcitability, all interfering with normal neonatal brain development (168). Many first-generation AEDs potentially worsen epilepsy comorbidities as ADHD, depression, and anxiety (169). Phenobarbital (first generation AED) might exhibit adverse neuropsychiatric outcomes. Its detrimental effects are further accentuated in HI-infants that usually present disrupted renal functions, in comparison to normothermic ones (64). Second and third-generation AEDs have fewer adverse effects, yet they still contribute to possible adverse outcomes (169). Thus, close monitoring of patients is crucial, and choosing drugs targeting pathways common to both epilepsy and its associated comorbidities would result in the best therapeutic effects.

B. Later life psychiatric sequelae of hypoxic-ischemic encephalopathy (HIE)

1. Comorbidities in epilepsy

Diverse comorbidities are encountered among children with prevalent epilepsy including mental health conditions, neurological and cognitive disorders (170). The relationship between epilepsy and its associated comorbidities is intricate, as one condition could lead to the other. On one hand, epilepsy might lead to anxiety disorder. On the other hand, depression might be a risk factor for epilepsy (Fig. 5 and 6) (171,172). Instead of considering a cause-effect relation, taking notice of common pathophysiological dysregulation in epilepsy and its associated morbidities might enable the treatment of both conditions using the same drug (169). Hippocrates had noted the bidirectional association between depression and epilepsy (173). In both

adults and pediatric patients, psychiatric issues have been reported before the onset of seizures (171,174,175). Major depression is one of the comorbidities that are greatly encountered in people with refractory epilepsy (30 to 50%) (16). In those patients, depression is affecting their life quality more than epileptic episodes. What increases the burden, is that most of them are not screened for depression, which leads to high suicide rates. This depression is not simply due to the disease's social burden or pressure, but it is also caused by a dysfunction in the brain's normal pathways particularly in brain regions related to mood regulation. Individuals with uncontrolled epilepsy are associated with a 5 to 10 times higher prevalence risk of developing depression and suicide in comparison with the rest of the population. Yet, it is important to note the role of personality characteristics, like learned helplessness, in the development of depression while still taking into consideration the functional neuroimaging abnormalities related to depression (16).

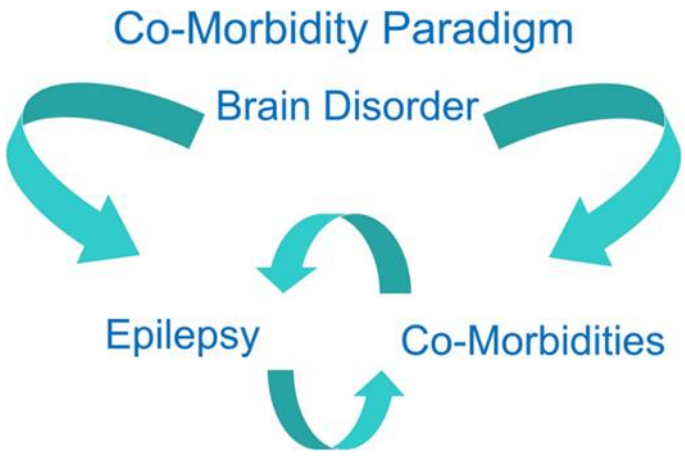

Figure 5. Association between brain disorders, epilepsy and their comorbidities. Brain disorders can prompt epilepsy and other co-morbities. A bidirectional association exists between epilepsy and its comorbidities as one could lead to the other or exacerbate the pre-existing condition (169).

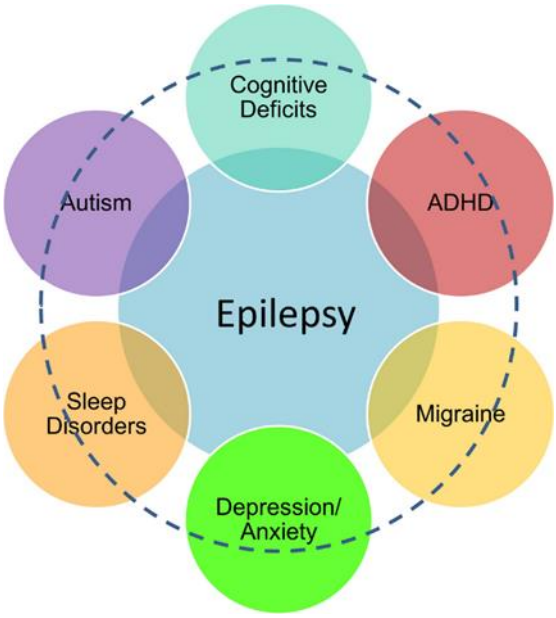

Figure 6. Schematic representation of the relationship between epilepsy and its associated co-morbidities. Children with epilepsy have a higher risk of developing cognitive deficits, autism, sleep disorder, depression and anxiety, migraine, ADHD, and vice versa. The external circle represents the association between all of the morbidities regardless of epilepsy (169).

2. Depression and behavioral problems following hypoxic-ischemic encephalopathy

The long-term outcomes following neonatal encephalopathy (NE) should be approached by acknowledging the dynamism and the interactivity of development. Perinatal brain damages can alter gene expressions which in turn affects behavioral and cognitive dysregulations. Depression and anxiety are behavioral dysregulations highly comorbid with each other and with epilepsy (176). They are the most recorded psychiatric burdens in epilepsy (177-179).

a. <u>Hypoxic ischemia and seizures' effects on the developing brain</u>

The severity of the neonatal insult is a determining factor that dictates its detrimental outcomes. Children suffering from mild NE (neonatal encephalopathy), tend to develop more positive motor and cognitive outcomes making them comparable

to their peers (180). On the other hand, children with severe NE have higher mortality rates and exhibit more severe dysregulations, like cerebral palsy, mental retardation, and cortical visual or auditory impairment (181). Infants suffering from moderate NE exhibit heterogeneity as their school performance is somewhere between that of mild NE and severe NE. Moderate NE children that do not develop cerebral palsy score better but still perform less than those with mild NE and their healthy peers (182). A study showed that infants with moderate NE exhibit more anxious/depressed behaviors and attention problems, while children with mild NE have higher levels of thought problems (181). Additionally, high rates of infants with moderate and severe NE develop autism spectrum disorder (183). A study done by Handel et al. revealed impairment in working memory, verbal and visuospatial long-term memory, and global effect on intelligence in children suffering from NE aged 9 to 10. In those who have developed CP those problems were more pronounced (19). Brain lesions are of interest for the study of behavioral outcomes, as hippocampal volume is significantly reduced in cases of memory deficits and has been correlated with psychiatric disorders. This involves the possibility of developing schizophrenia following perinatal asphyxia, as well as autism (184). Hypoxic-seizure onset in HIE worsens behavioral outcomes. Epidemiological studies revealed that pediatric patients suffering from epilepsy have a risk of 21-60% to develop childhood psychopathologies, a risk at east 3- to 6-fold greater than the normal population (178,185). High rates of behavioral problems are observed in children with epilepsy or chronic conditions involving the central nervous system, in comparison with other children suffering from a chronic condition unrelated to the CNS (186). Up to 50% of children with chronic epilepsy present behavioral problems. Those behavioral changes are affected by the family's environment (187).

Epilepsy results in internalizing problems rather than externalizing them and pediatric depression does not necessarily manifest in sadness (188). Children tend to suffer from anxiousness, irritability, separation anxiety, loss of interest in activities, and suicidal ideation rather than acting out (188-190). Yet, anger and aggression can be observed as well (189,190). Attention problems are very common and can be marked by an academic decline (189,190). In addition to being a debilitating condition, depression can worsen seizure control and is associated with pharmacoresistant epilepsy leading to increased seizure severity, and slower recovery time (191,192). The disruption of molecular pathways contributes to the long-term effects of seizures in patients that manifest in behavioral perturbations.

b. Molecular aspect- serotonin and serotonin receptors

Treating depression in epilepsy has not been well investigated. Let alone the fact that the antidepressants, normally prescribed in the treatment of major depressive disorders, have been shown to worsen seizures, lower their threshold, and are minimally effective for the treatment of epilepsy-related depression (193). Additionally, the effects of depression on the quality of life of patients with epilepsy have been underestimated, thus the main concern of professionals was to attenuate seizures' severity and frequency (194). However, a strong correlation between depression and epilepsy can be established. In fact, limbic brain regions, such as the mesial temporal and prefrontal area, particularly participate in epileptogenesis, and they have a high density of serotonin receptors. Those brain regions coincide with the ones involved in the development of depression related to serotonin receptors density. In humans, visualization of the serotonin (5-HT) receptors, 5-HT1A receptors, via PET scan

(positron emission tomography) reveals a potential pathogenic role of 5-HT in depression and epilepsy (195,196). Some AEDs mechanism of action is as well mediated by increased 5-HT extracellular levels (197-199).

The differential gender manifestation of depression between males and females renders depression treatment in epilepsy even more challenging. Women are twice as likely to develop major depressive disorders (MDD) compared to men (200). One of the reasons for this contrast is sex-related dissimilarity in the brain serotonin system. Serotonin levels are 52% lower in adult females than in men. Moreover, females exhibit lower brain serotonin receptors binding and a higher expression of 5-hydroxyindoleaceticacid, a serotonin metabolite, in the cerebrospinal fluid (200). Dopamine and norepinephrine levels vary as well between males and females rat models subjected to hypoxia (200)**.** This dimorphism might explain the difference in responsiveness to treatments in rat models. Taking notice of alteration in neurotransmitters expression in the brain post-hypoxic-seizures might provide better insight into the possibility of treating depression in those patients (16).

c. Effects of hypoxia on the expression of neurotransmitters in the brain

Hypoxia has been shown to lower brain serotonin levels, leading to TRD (treatment-resistant depression) in patients with HIE, MDD, or chronic hypoxic disorder (200). Similarly, in rat models of hypobaric hypoxia, brain serotonin levels are decreased (forebrain and striatum and brainstem) leading to depression (200); however, acute hypoxia results in a whole-brain decrease of serotonin levels (34%) (200). This region-dependent synthesis alteration is not only restricted to serotonin, but it affects brain dopamine and norepinephrine levels as well (200). In fact, the rate-limiting

enzyme of dopamine synthesis (tyrosine hydroxylase TH), and the dopamine to norepinephrine conversion enzyme (dopamine-β-hydroxylase), require molecular oxygen. Housing rats under 10% oxygen for a week results in an increased expression of dopamine and norepinephrine in the frontal cortex, suggesting the involvement of the frontal cortex in mood regulation (200). Additionally, a study performed by Bromfield et al. revealed that higher depression rates are recorded in patients with hypometabolism in their inferior frontal region (201). Similarly, patients suffering from depression without neurological disorder also present the same pattern of hypometabolism (202).

d. The efficacy of antidepressants in the treatment of epilepsy-related depression

Perinatal asphyxia, followed by neonatal encephalopathy severely impairs short and long-term development in its survivors. Adolescent depression being prominent in these children, novel studies have been working on improving the preexisting anti-depressive medications rather than targeting new pathways (194). The prescription of antidepressants for seizure patients has been highly controversial as their administration lowers seizures' threshold (203,204). Different clinical trials aiming to study the efficiency of antidepressants in epilepsy patients have been conducted. Newer generation antidepressants, particularly selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs), exhibit low seizure risks in patients with epilepsy (203,205,206). Second-generation antidepressants, except bupropion, hold an apparent anticonvulsant effect (207). The administration of fluoxetine and citalopram (SSRIs) to non-depressed adults with epilepsy reduces seizure frequency (208-210). In children and adolescents, SSRIs improved depression while worsening seizures in only 2 out of the 36 participants (211). Investigation of SSRIs

efficacy in children and adults with epilepsy revealed that this class of antidepressants does not increase seizure frequency (212). In a study done by Ojemann et al., doxepin (tricyclic antidepressant TCA) reduced seizures in about 79% of patients (15 out of 19), with around 10 % exhibiting an increase in seizures (2 out of 19) (213). A similar study showed that sertraline (SSRI) had increased seizures' severity in only 6% of patients (214). A study performed by Robertson and Trimple comparing the effects of amitriptyline (TCA), nomifesine (norepinephrine-dopamine reuptake inhibitor), and placebo in 42 patients suffering from depression and epilepsy showed that a 6 weeks treatment results in no significant difference among the different groups. An additional 6 weeks treatment duration revealed that patients being treated with nomifesine scored better in the Hamilton Depression Rating Scale, but not in Beck Depression Inventory (215). This treatment-resistant depression (TRD) is highly linked to suicidal behaviors in these patients (200).

e. Selective Serotonin reuptake inhibitors are a safe option for depression treatment in pediatric patients

SSRIs have been shown to have low side effects, low toxicity, and good tolerability by patients, thus their prescription to treat depression in children with epilepsy is safe (216) (200). SSRIs function by blocking serotonin transporter. This leads to an increase in the synaptic serotonin levels, by inhibiting its reuptake (Fig. 7). However, this mechanism of action implies that SSRIs are efficient as long as serotonin levels are moderate to high enough, and those medications might lose their antidepressant properties with low serotonin levels. In fact, mice with low brain serotonin (low levels of tryptophane hydroxylase) exhibit higher depressive-like behaviors in FST compared with mice having normal serotonin levels, both groups

being treated with citalopram or paroxetine (200). However, it is important to note that SSRIs are characterized by different pharmacological profiles, exhibiting different binding affinities to SERT and other monoamine transporters (200). This might alter the efficacy of different SSRIs. Escitalopram (SSRI) binds to SERT with a higher specificity than to the dopamine transporter (DAT) or the norepinephrine transporter (NET), yet it does not improve depressive-like behaviors in rats (200). Similarly, fluoxetine (SSRI) exhibits specificity to serotonin transporters but a minimal action on DAT and NET. As well it does not cause any significant improvement regarding the DLB. Paroxetine (SSRI) and sertraline (SSRI) are characterized with the highest affinity to serotonin transporters and increase swimming in rat models of hypobaric hypoxia (200). However, only sertraline was shown to significantly improve DLB in this rat model. The improvement observed with sertraline lies behind its mechanism of action: in addition to improving the serotonergic transmission, sertraline blocks DAT (200). This blockade of dopamine reuptake alongside that of serotonin might be responsible for the antidepressant properties observed in hypobaric hypoxia models (200). This observation indicates that antidepressants targeting both serotonin transporters and DAT result in better outcomes compared to those only targeting serotonin transporters (200). In addition to alleviating depression, SSRIs inhibit both focal and generalized seizures by increasing extracellular serotonin levels (217,218).

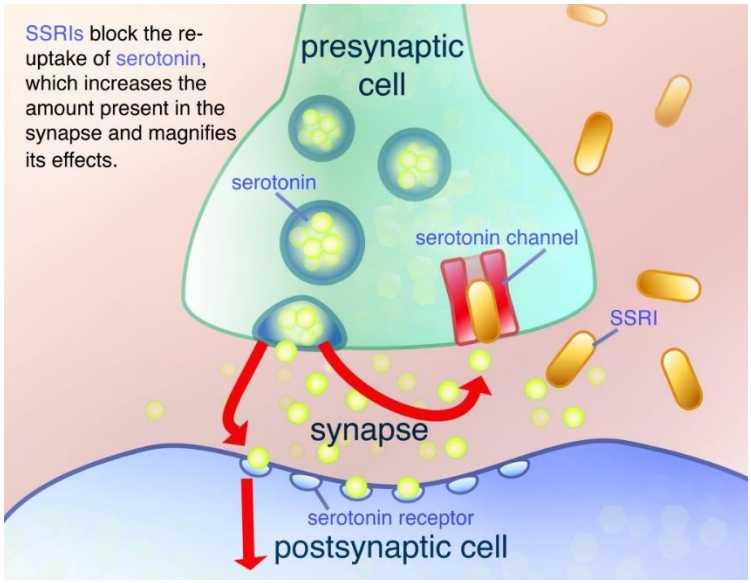

Figure 7. SSRIs mechanism of action. SSRIs block the reuptake of serotonin by binding to the presynaptic serotonin channels resulting in increased extracellular serotonin levels (219).

On another hand, SSRIs might also result in a hypo-dopaminergic state (200). Clinical manifestation of extrapyramidal movement disorder, sexual dysfunction, and mental disorders appear with the use of SSRIs. Those symptoms are alleviated by dopaminergic agonists (196). Thus, a possible explanation for the TRD with the use of fluoxetine, paroxetine, and escitalopram might be the hypo-dopaminergic state resulting from these SSRIs. Not only does it fail to treat depression, but those SSRIs might as well worsen depression by failing to increase serotonergic transmission, alongside their detrimental effects on brain dopamine levels. In fact, SSRIs have been used to treat the increased dopaminergic tones in patients suffering from obsessive-compulsive disorders (OCD) (200)**.** Parkinson's patients also seem to exhibit worse motor symptoms after SSRIs treatment (200)**.** However, sertraline presents lower side effects in comparison to the rest of the SSRIs. Secondary effects such as hypodopaminergic state, increased

plasma prolactin, sexual disorders, and cognitive decline are lower with sertraline in contrast with escitalopram and paroxetine (200)**.** Additionally, in patients with Parkinson's, sertraline has no deleterious effect on the motor system and provides better antidepressant qualities (200). In rats, it can enhance swimming and climbing in FST (200)**.** By improving brain dopaminergic levels, sertraline can thus be considered as one of the safest options of SSRIs to be prescribed for the treatment of depression resulting from hypoxia. Sertraline's effect might be mediated as well by the regulation of certain cellular pathways as the BDNF-TrkB pathway which is involved in depression.

f. BDNF-TrkB pathway involvement in depression and anxiety

Depression and anxiety have different etiologies, but the current behavioral assays do not effectively distinguish them from each other. Behavioral despair and the inability of experiencing pleasure (anhedonia) characterizing depression are controlled by two distinct systems respectively: the brain stress system (hippocampus and hypothalamus-pituitary-adrenocortical HPA axis/ hippocampus-HPA pathway) playing a role in learning and memory and the brain reward system (ventral tegmental area-nucleus accumbens VTA-NAc and VTA-prefrontal cortex pathways) in which the dopaminergic transmission plays an important role in reward and motivation (Fig. 8 and 9).

It seems that BDNF exhibits opposing effects on these 2 systems as an intrahippocampal infusion of BDNF is accompanied by antidepressant effects while it elicits a pro-depressive role in the VTA-NAc reward system (220). Targeting the BDNF-TrkB pathway has been proposed as a possible treatment strategy to alleviate mental outcomes resulting from epilepsy.

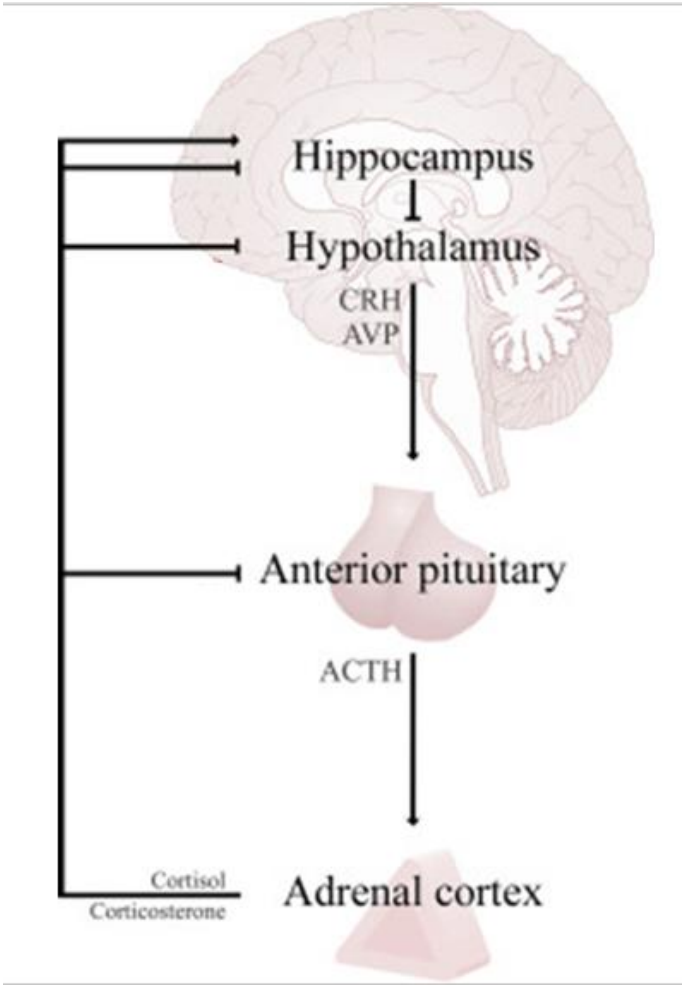

Figure 8. Brain stress axis (hippocampus and hypothalamus-pituitary-adrenocortical HPA axis. In response to a stressful stimuli hypothalamic neurons release corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). This triggers the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. The adrenal cortex subsequently releases of glucocorticoids (cortisol and corticosterone). In addition, the adrenal medulla releases catecholamines (adrenalin and noradrenalin) (not shown). Feedback loops are activated at various levels of the system (adrenal gland, pituitary, hypothalamus, and other brain regions such as the hippocampus and the frontal cortex) to return to homeostatic levels (221).

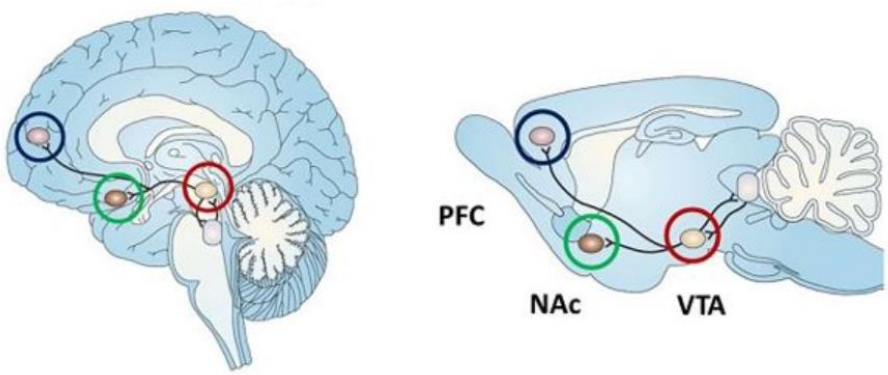

Figure 9. The reward system in humans and rats. The mesolimbic dopamine pathway consists of projections from the ventral tegmental (VTA) area to the nucleus accumbens (NAc) while the mesocortical dopamine pathway consists of projection from VTA to the prefrontal cortex. This system plays a major role in motivation and pleasure. PFC, prefrontal cortex, NAc, nucleus accumbens, VTA, ventral tegmental area (222).

The neurotrophin hypothesis of depression is based on the correlation between antidepressant treatments and the regulation of BDNF. BDNF-TrkB pathways have been shown to take part in the antidepressant effects. The BDNF precursor proBDNF and the BDNF mature form mBDNF can exhibit opposite effects on cellular functions, long-term potentiation (LTP), and long-term depression (LTD), contributing to the dichotomy of BDNF actions on the brain stress and reward axes (hippocampus-HPA pathway and VTA-NAc and VTA-prefrontal cortex pathways). BDNF is being recognized as an important regulator of LTP (long-term potentiation) in the hippocampus. Antidepressants result in the enhancement of hippocampal LTP by promoting mBDNF-TrkB expression. Furthermore, fluoxetine (SSRI) fails to reverse the anxious-like behaviors in BDNFmet/met mice (223). This highlights that the antidepressive effect of SSRI is mediated by BDNF-TrkB signaling. Additionally, LTD (long-term depression) has been suggested to play a role in depressive-like behaviors

since acute stress might induce LTD in the hippocampus, in which LTD is not normally present. A decrease in hippocampal BDNF levels is correlated with stress-induced depressive behaviors and some antidepressants upregulate the expression of BDNF by selectively acting on some of the promoters driving BDNF transcription. Nonetheless, a chronic administration of antidepressants is required for the enhancement of gene expression of both Trk-B and BDNF. On the other hand, the acute use of antidepressants is thought to act by increasing the secretion of BDNF without gene regulation (224). In fact, acute treatment improves FST outcome, while it takes around 21 days (chronic treatment) for BDNF and Ntrk2 mRNA to be upregulated (224). Furthermore, the effects of antidepressants can be observed in postmortem brain tissues where levels of BDNF are lower in patients who have suffered depression but higher in those who were taking antidepressants (225). Depressed patients have a reduced BDNF expression in the hippocampus and prefrontal cortex, yet exhibit an increased BDNF level in the NAc (226-229). However, most studies investigate the levels of total BDNF mRNA or protein without distinguishing its 2 transcripts. Therefore, it remains unclear which of proBDNF or mBDNF is the main mediator of depressive and antidepressive responses. Furthermore, BDNF over-expression might trigger cell death in in-vitro cultured cortical neurons. This necrosis can be reversed by inhibiting TrkB pathways. Additionally, treatment-resistant depression is associated with a higher expression of BDNF in the VTA-NAc pathway, suggesting possible treatment using BDNF-TrkB antagonists (Fig. 10) (230). However, BDNF and its receptors have an important trophic role (40). In BDNF knockout and TrkB-T1 transgenic mice, antidepressant treatment loses its efficacy in the FST where it no longer reduces

immobility time (224). Thus, maintaining homeostatic activation is crucial for normal development and optimal treatment outcomes.

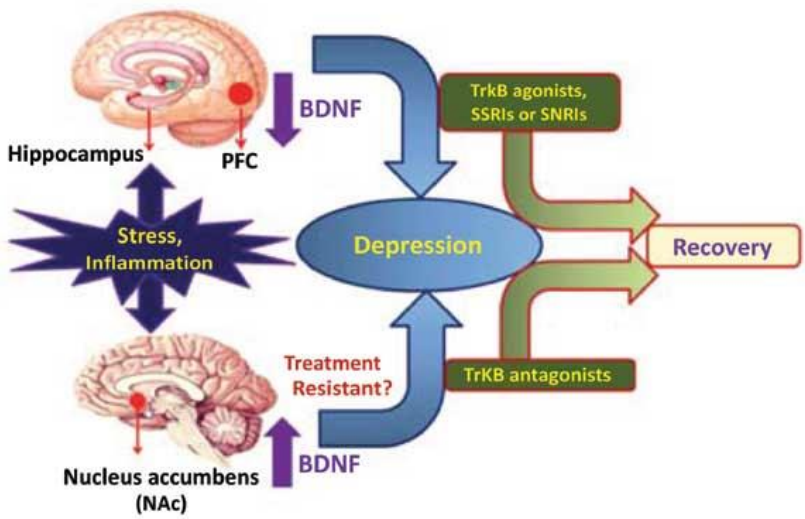

Figure 10. Schematic representation of bdnf-trkb regulation in depression. In preclinical studies, inflammation downregulates BDNF in the hippocampus and prefrontal cortex but upregulates it in the NAc leading to depressive-like behaviors in rodents. For such a phenotype, TrkB agonists and antidepressants, as SSRIs and SNRIs, could effectively treat depression. Nonetheless, treatment resistant depression is correlated with a and increased BDNF expression in the VTA-NAc pathway. In this case, TrkB antagonists are better treatment option (231).

3. Hippocampal and prefrontal cortex's contribution to cognitive and emotional regulation

a. The Hippocampus

The hippocampus is a component of the limbic system that plays a critical role in the association and processing of various information crucial for daily life functions (Fig. 11). It is a bilateral structure located within the anterior part of the medial temporal lobe, present in humans and other mammals. Each hippocampus can be divided into 3 anatomical areas: the cornu ammonis (CA1-CA3), the dentate gyrus, and

the subiculum (232). A hippocampal cross-section reveals several molecular layers (called strata) from the outer in as follows: stratum lacunosum-moleculare, stratum radiatum, stratum lucidum, stratum pyramidale, and stratum oriens. The hippocampus is a highly connected structure exhibiting abundant interconnectivity with different cortical and subcortical regions (233,234). A variety of behavioral and cognitive processes are regulated by the hippocampus including, but not limited to, memory, spatial navigation, emotional processing, stress regulation, and decision making (235). Sensory information of the surrounding environment is incorporated by the hippocampus into configurational neural representations (236). The ventral hippocampus in rodents, corresponding to the anterior part in humans, acquaints for coarse global representation. On the other hand, the dorsal hippocampus, corresponding to the posterior hippocampus in humans, is in charge of fine-grained, local representation (237,238).

Given that the creation of stable associations requires Hebbian plasticity between neurons (LTP and LTD), it has been suggested that the hippocampus generates interconnected maps of physical and mental space (239,240). Furthermore, growing evidence has been emphasizing hippocampal role in pattern separation and approach-avoidance conflict processing in anxiety-provoking situations (241,242). All of these functions require interconnectivity with other brain structures including the limbic system and the prefrontal cortex (Fig. 11).

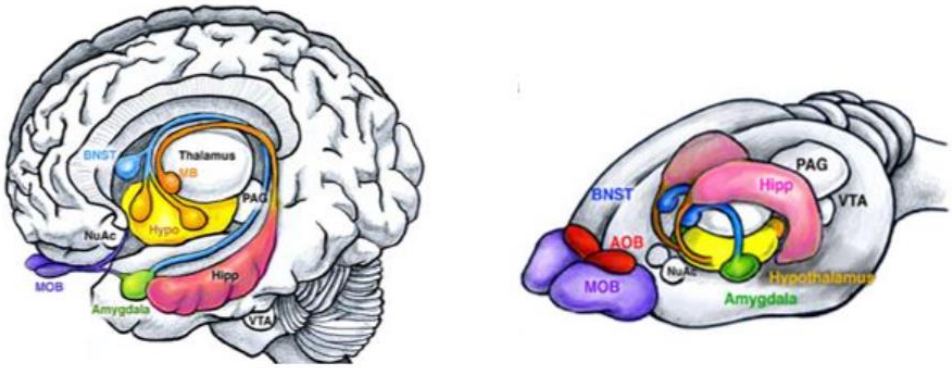

Figure 11. Parts of the limbic system and reward system in humans and rats. The colored regions represent the limbic system that controls innate behaviors. Projections form the reward circuit to the limbic system control learned and intentional behaviors. BNST, bed nucleus of stria terminalis; Hipp, hippocampus; Hypo, hypothalamus; MB, mamillary bodies; PAG, periaqueductal gray; NuAc, nucleus accumbens; VTA, ventral tegmental area; MOB, main olfactory bulbs; AOB, accessory olfactory bulbs (rat only) (222).

b. The prefrontal cortex

The prefrontal cortex PFC, in contrast to the hippocampus, is a more phylogenetically evolved structure. It is mostly developed in primates, particularly humans, and is crucial for emotional regulation and higher-order cognitive processes. In primates, the PFC can be divided into distinct anatomical and functional regions: a dorsolateral division and a ventromedial division. The dorsolateral division is responsible for cognitive functions as executive control, attention, and working memory. In contrast, the ventromedial (or orbitomedial) structure acquaints for emotional and motivational regulation (243,244). In rodents, the PFC can be divided into medial, lateral, and ventral subdivisions, each of which in turn consists of several subregions (245). The medial PFC (mPFC) is associated with cognitive function as working memory, attentional shifting, and the regulation of emotional responses (246-248). The dorsal rodent mPFC, corresponding to the dorsolateral primate PFC, is

responsible for cognitive function, in contrast to the ventral rodent mPFC, corresponding to the ventral primate mPFC, that is involved in emotional behaviors (245,249-252). These functions require a linkage with various brain regions including the hippocampus.

c. Hippocampal and prefrontal cortex interconnections

The PFC and the hippocampus are linked via several direct and indirect pathways (Fig. 12). The hippocampus, most importantly the ventral hippocampus (vHPC), sends output excitatory glutaminergic projections to the PFC, primarily to the mPFC, in both rodents and primates (250,251,253-255). In mice, monosynaptic projections originate from the anterior cingulate (AC) portion of the mPFC and terminate in the CA1 and CA3 regions of the dorsal hippocampus dHPC. In addition to these monosynaptic projections, indirect bidirectional connections exist between the PFC and the hippocampus via relay structures like the nucleus reuniens (NR) of the thalamus (Fig. 12) (246,256).

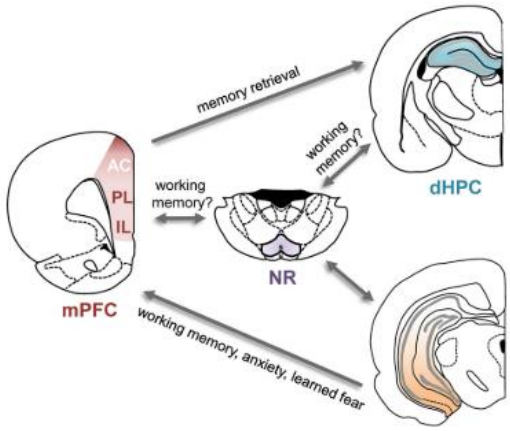

Figure 12. Direct and indirect connections between the prefrontal cortex (PFC) and hippocampus and their functional roles. Schematic of the direct and some of the indirect connections between the hippocampus and PFC. Arrows indicate direction of the projections. AC, anterior cingulate cortex; dHPC, dorsal hippocampus; IL, infralimbic cortex; mPFC, medial prefrontal cortex; NR, nucleus reuniens; PL, prelimbic cortex; vHPC, ventral hippocampus (257).

4. Synaptic plasticity involvement in cognitive and behavioral regulation

In response to perturbations, neuronal excitability is maintained at a relatively stable level via compensatory changes in synaptic plasticity. This modification in synaptic plasticity is of crucial importance, particularly during the developmental and learning-related changes, as it stabilizes neuronal firing and prevents abnormal neuronal activity (258). A good indicator of neuronal synaptic plasticity is synaptophysin. This calcium-binding integral membrane glycoprotein is the most abundant molecule in the neuronal synaptic regions (259,260). Synaptophysin plays a key role in the regulation of the synaptic vesicles cycle rather than directly influencing the release of neurotransmitters (260). This is done by ensuring effective retrieval and trafficking of synaptobrevin-II (SybII) that plays an essential role in vesicles docking and exocytosis at the presynaptic membrane level (260). The regulation of the synaptic vesicles cycle is particularly important in the early developing brain in which neuronal circuits undergo intense activity (260). A dysregulated expression of synaptophysin is observed in a variety of mental and central nervous system disorders. Synaptophysin is downregulated in patients suffering from bipolar disorder and in the hippocampi of Alzheimer's patients (261). Furthermore, mutations in SYP encoding genes have been recorded in patients presenting intellectual difficulties with or without any associated epilepsy (262). Synaptophysin homeostasis is thus crucial to maintain an equilibrium between excitation and inhibition ensuring a regulated neuronal circuitry activation.

C. Animal models for depression and hypoxic seizure studies

1. The Hypoxic Seizure model: model selection and hypoxia induction

Hypoxia can be induced at different stages: intrauterine, perinatal, and neonatal hypoxia models have been described (263). Hypoxia ischemia can be achieved in sheep through maternal hypoxia, or through the occlusion of the fetal umbilical cord, but the high cost of these animal models and their large size make this protocol inconvenient (264).

In the neonatal rat brain, HIE can be induced through the ligation of one common carotid artery, followed by systemic hypoxia. The latter is achieved by a reduction of the ambient oxygen to 8% using nitrogen. It is of great value to note that postnatal day 7 rat brains are phenotypically similar to that of the human fetus in its 3rd trimester (265). This age falls into a developmental period of enhanced excitability and synaptic plasticity similar to that observed in the human neonatal period between 32-40 gestational weeks (266).

In our laboratory, hypoxic seizure Sprague Dawley rat models are obtained by putting P9 or P10 pups in an airtight chamber, in which oxygen concentration will be gradually decreased, through N2 gas infusion, from 20.9 % (ambient) to 7% for 8 min, then 5% for 6 min, to finally reach 4% for 1 min. This hypoxia induction lasts for a total of 15 min, and allows the induction of tonic-clonic seizures in P10 pups. Such a hypoxic model does not result in morphological brain damages in the prefrontal cortex, limbic structures, and does not trigger seizure reoccurrence. This allows the study of less severe cases of hypoxia. Within the absence of anatomical changes, the behavioral outcome observed could be caused by synaptic reorganization involving downstream pathways dysregulation, as TrkB (49). A similar HS model revealed the absence of cell

death; however, the persisting increase in excitability in the adult hippocampus suggests that permanent alterations in hippocampal synaptic plasticity mediate the epileptogenic effect of hypoxia (266).

2. Emotional Behavior Testing: Depressive-like Behaviors

A variety of tests can be adapted to assess emotional behavioral disturbance in pups and to validate the efficacy of novel therapies in achieving neuroprotection after brain insults. A previous study done by our team investigating the effects of CEP-701 on cognitive and behavioral outcomes in immature rats revealed that the most efficient test in assessing behavioral changes was the forced swim test (FST) and the open field test (OFT). The FST allows the assessment of depressive-like behaviors by measuring the immobility percentages. The OFT helps to assess anxiety-like behaviors, in addition to exploratory behaviors and hyperactivity by measuring the time spent and distance traveled in different field areas with the different objects presented to the rat. One test alone is not sufficient, as it might be misleading, anxiety might manifest as hyperactivity which in turn will affect the FST outcomes. Thus, an OFT is crucial to distinguish hyper-active from non-hyperactive behaviors (49).

CHAPTER II

AIMS AND HYPOTHESIS

Aim 1: To investigate the long-term effect of early-life hypoxic seizures (HS) on depression and anxiety-like behaviors in peri-adolescent rats.

Hypothesis 1: Rats that undergo early-life hypoxic seizures will have increased depression and anxiety-like behaviors in peri-adolescence age.

Aim 2: To investigate whether HS-induced depressive-like behaviors are reversed by Sertraline (Zoloft), a clinically employed standard anti-depressive medication.

Hypothesis 2: HS-induced depressive-like behaviors will be reversed by Sertraline.

Aim 3: To determine neuronal cell loss post hypoxic seizures.

Hypothesis 3: Rats that undergo hypoxic seizures will not present neuronal cell loss despite behavioral changes.

Aim 4: To investigate whether hypoxic seizures result in synaptic plasticity alterations.

Hypothesis 4: Rats that undergo hypoxic seizures will manifest a maladaptive alteration in synaptic plasticity markers.

CHAPTER III

MATERIALS AND METHODS

A. Animals and experimental design

The Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut approved all experiments. Eighty male Sprague Dawley rats housed in a temperature-controlled room and maintained on a 12-hour light-dark cycle with permanent access to food and water were used in this study. Rats were subjected to hypoxia at postnatal 10 (P10) or sham-treated under normoxic conditions. Rats were then divided into 4 groups: the NV group (normoxic with vehicle, n=18), the HV group (hypoxic seizure with vehicle, n=17), the NSERT group (normoxic with sertraline, n=19), and the HSERT group (hypoxic seizure with sertraline, n=19). Following hypoxic seizures induction only pups having 6 or more tonic-clonic seizures were included in the study. Rats were then administered sertraline via drinking water at a dose of 5 mg/kg/day from P24 till P30 and subjected to a battery of behavioral testing panels (Fig. 13), then sacrificed at postnatal day 30 (P30) for histological analyses, including hippocampal pyramidal neuronal density and hippocampal protein levels of markers of synaptic plasticity, synaptophysin (SYP).

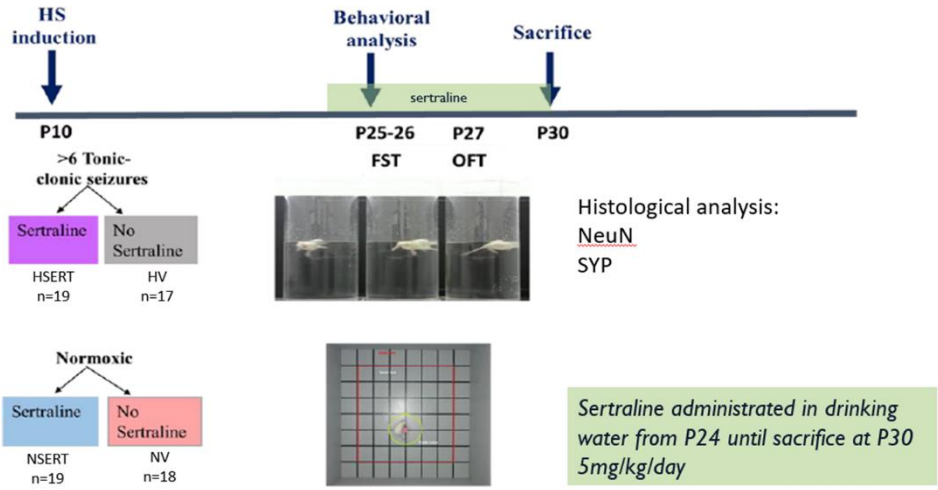

Figure 13. Experimental design of the project. Hypoxic seizures were induced in postnatal day 10 (P10) rat pups while the rest remained under normoxic conditions. Then, the rats were assigned to 4 groups by administering sertraline via drinking water: HSERT (hypoxic seizure with sertraline, n=19), HV (hypoxic seizure with vehicle, n=17), NSERT (normoxic with sertraline, n=19), and NV (normoxic with vehicle, n=18). Depressive-like and anxiety-like behaviors were then investigated in the forced swim test (P25-26) and the open field test (P27), sequentially. Rats were sacrificed at P30. Potential neuronal damage and alteration in synaptic plasticity were assessed through immunohistochemistry via Neu-N and synaptophysin staining, respectively. NV group (normoxic with vehicle, n=18), HV group (hypoxic seizure with vehicle, n=17), NSERT group (normoxic with sertraline, n=19), HSERT group (hypoxic seizure with sertraline, n=19).

B. Hypoxia induction and inclusion criteria

To induce HS, P10 male Sprague Dawley (SD) rats, placed on a heating pad regulated at a temperature of 34°C, were exposed to 15 min of graded global hypoxia in an airtight chamber into which nitrogen (N2) was rapidly infused. The oxygen concentration was maintained at 7% for 8 minutes, 5% for 6 minutes, and 4% for 1 minute. Hypoxia induced myoclonic jerks, followed by tonic-clonic head and limb movements. The total number of tonic-clonic movements during hypoxia were recorded

for each pup. Only animals that had 6 or more tonic-clonic seizures were included in the study.

C. Sertraline administration

Sertraline (SERT) (Pfizer, AUBMC pharmacy) 50 mg tablet was crushed and diluted in water. SERT was administered orally at a dose of 5.0 mg/kg/day in the animals' drinking water, starting P24 and continuing until the end of experiments (P30). The SERT solution was prepared using drinking water as a vehicle. Liquid consumption was controlled (with calibrated bottles) and monitored every day from P22 till P30. Weight was monitored starting the weaning day (from P21 till P30). The drug administration was adjusted based on liquid consumption and animal weight. Freshly prepared solutions were provided. Normal drinking water was given to control animals. During this period, the rats were kept in groups of 2 to 3 animals with unrestricted access to food.

D. Behavioral testing

Rats belonging to the 4 groups described above were weaned and separated from their mother at P21 followed by behavioral testing 4 days after, during which the animals were handled several times. To assess the cognitive and emotional behavioral derangements at the peri-adolescent age (P24-P45), rats of all treatment groups were subjected to behavioral panels including the forced swim test (FST) and the open field test (OFT). The order of the test was selected as previously mentioned so none of the tests' outcomes interferes with the other.

1. Forced swim test for the assessment of depressive-like behaviors

Rats were individually placed in a transparent cylinder (50 cm in height, 20 cm in diameter) filled with water reaching a 30 cm height (Fig. 14). The water temperature was regulated at 25-27 °C and was changed between animals. The FST consists of two 10-minute sessions 24 hours apart. Immobility behavior was measured to assess depressive-like behaviors and is defined as the state where the animals are making only minimal limbs movements required to stay afloat. High activity levels were measured as well to assess struggling and are defined as the state where all four limbs are in motions with the front feet breaking through the surface of the water. The duration (P25-P26) rats spent immobile was measured using an automated feature, the smart software version 3 (Panlab/Harvard Apparatus) which tracks the global activity of tested animals. At the end of each test, the water in the cylinders was changed and rats were allowed to dry under a heat lamp in a cage covered with an absorbent towel for 10 min.

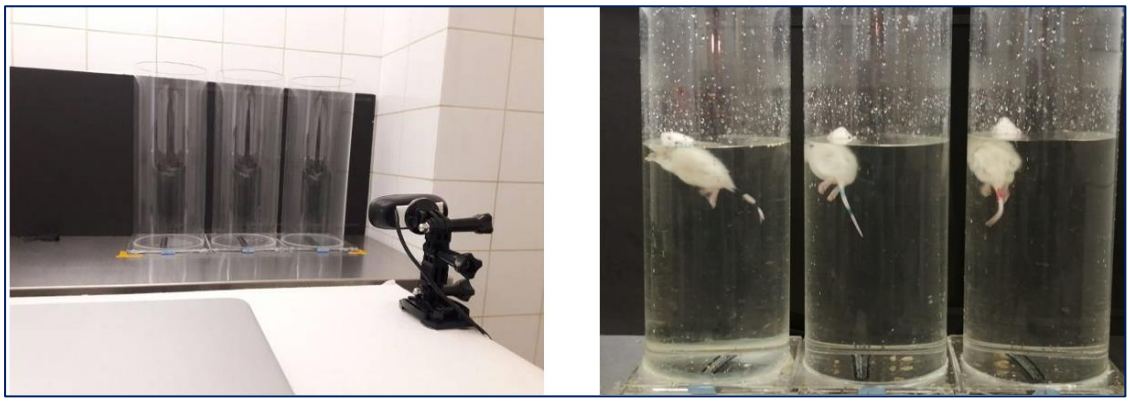

Figure 14. Forced swim test experimental setup. Rats were placed in 3 plexiglass cylinders filled with 30 cm depth of water. The experiment was video recorded.

2. Open filed test for the assessment of anxiety-like behaviors

The OFT was conducted over three sessions, five minutes each, performed over one day using an opaque plexiglass square field (W 80 cm, L 80 cm, H 40 cm) (Fig. 15). On the first testing session, a single small object (cube), was placed in the middle of the field's floor, then on each of the two subsequent sessions, 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 session. 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 video tracking 3.0 software (Panlab, Harvard apparatus, USA).

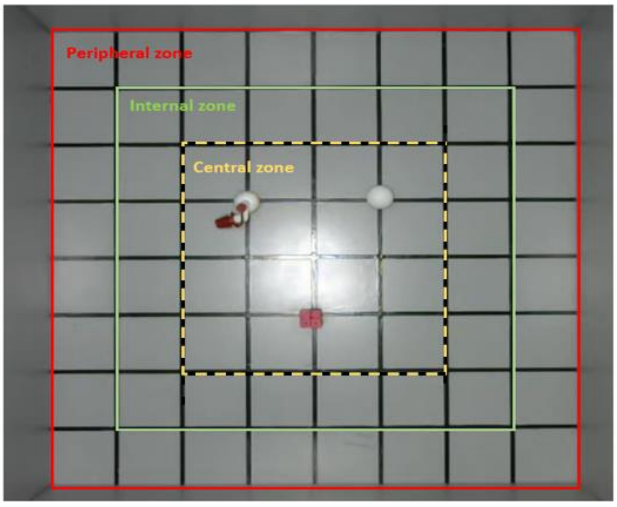

Figure 15. Open field zones. This picture represents all 3 objects put during the 3rd testing session.The central zone contains all the objects. The internal zone includes all the area within the green square (it includes the central zone). The peripheral zone is only restricted to the area present between the red and green squares.

E. Euthanasia and cardiac perfusion

To perform various microscopic examinations on brain slices, brains were perfused with 4% depolymerized paraformaldehyde (PFA) in phosphate-buffered saline

(PBS) (pH=7.4). The procedure starts with general anesthesia with an intramuscularly administered mixture of ketamine (80 mg/kg), xylazine (10 mg/kg). Once anesthesia is achieved (unresponsiveness to noxious stimuli), the animal was placed on its back. Pins were used to restrain the limbs and to prevent accidental or fluid-induced shifting during the procedure. An incision in the abdomen was made just below the diaphragm. The diaphragm was then removed to access the rib cage. Two vertical cuts were made along the sides of the rib cage, allowing it to be lifted, exposing the heart. A needle was then inserted into the left ventricle and PBS (1X) solution was pumped slowly into the animal. Immediately after insertion of the needle in the left ventricle and just before starting the pump, the right atrium was cut open to allow fluid to drain. Once all the blood has been flushed from the animal, the brain was fixed by PFA. Brains were collected and stored in PFA for 48 hours, then put in a 30% sucrose solution at 4 degrees till they sank (around 1 week) before paraffin embedding. Coronal sections of 8 µm in thickness were obtained for histological and immunohistochemistry analyses, namely hippocampal and prefrontal cortex neuronal density quantification and Syp staining.

F. Histological study and analysis

Coronal sections of 8 µm in thickness were obtained for histological and immunohistochemical analyses. Sections were selected by visual inspection to match the structural pattern of hippocampal coronal sections located 1.9-2.8 mm posterior to the Bregma and prefrontal section located 3.6 to 4.6 mm anterior to the Bregma of P21 rats in the Stereotaxic Atlas of the Developing Rat Brain. This histological landmark was chosen based on the involvement of the prefrontal cortex in mood regulation and

the role of the hippocampus in pattern separation especially in anxiety-provoking situations (242).

1. Immunohistochemistry

Immunohistochemistry was performed on brain sections of a sample of 1-2 rats from each group. Slides were first deparaffinized with xylene and rehydrated through a series of descending grades of alcohol solutions. Slides were incubated for 60 minutes in 90 °C sodium citrate buffer (10 mM, pH = 6) for antigen retrieval and then treated for five min at room temperature with 3% H2O2 to neutralize endogenous peroxidase. Slides were incubated overnight at 4 °C with primary antibody solutions of the following antibodies: anti-NeuN (MAB377; dilution 1:100, Millipore), and anti-Syp (sc-12737; dilution 1:500, Santa Cruz Biotechnology). Sections were then incubated with peroxidase-conjugated anti-mouse secondary antibody for one hour at room temperature (Leica Biosystems, UK). Immunohistochemical staining was visualized under the light microscope following the application of 3,3'-diaminobenzidine (DAB) and hematoxylin counterstaining. The average neuronal density and the optical density of Syp stained sections were visually inspected by an investigator blinded to treatment groups.

2. Image analyses

Immunostained brain sections were imaged using the uSCOPE (uScope MXII, USA) machine. Rats' hippocampi were divided for histological analyses into 3 areas of interest each. The CA1-subiculum and CA2-CA3 areas were determined by extending an imaginary line from the tip of the dentate gyrus to the distal border of the

hippocampus passing through the tip of the dentate inner blade. The dentate hilar zone border was determined by a line extending from the tip of the inner blade to the outer blade's border (Fig. 16). For prefrontal sections, the anterior cingulate, prelimbic, and infralimbic cortices were selected for analysis (Fig. 17). NeuN positive cells number and SYP optical density were assessed visually (3 sections per brain, 1 brain pe group) by an investigator blinded to treatment groups.

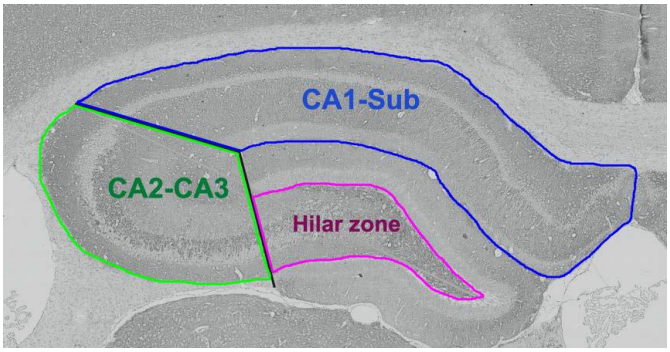

Figure 16. Hippocampal areas division for analyses. Rat's dorsal hippocampus was divided into three areas of interest: CA1-subiculum (blue), CA2-CA3 (green), and dentate hilar zone (purple) to analyze for potential neuronal loss with NeuN staining and alterations in hippocampal synaptic plasticity with Syp staining.

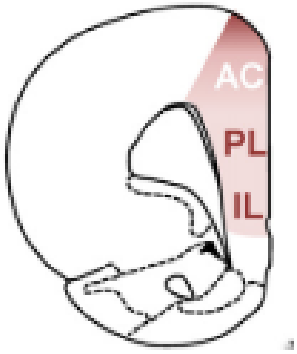

Figure 17. Prefrontal cortex areas division for analysis. Rat's prefrontal cortex was divided into three areas of interest: infralimbic (IL), prelimbic (PL), and anterior cingulate cortex (AC) to analyze for potential neuronal loss with NeuN staining, and alterations in hippocampal synaptic plasticity with SYP staining.

G. Statistical analyses

All data analyses were performed using Prism 8 (GraphPad Software, USA). Animals were randomized to treatment groups before seizure induction or any data analysis. Unless otherwise stated, data are presented in graphs as mean ± standard error of the mean (SEM). The FST was analyzed using two-way analysis of variance (ANOVA) with repeated measures followed by post hoc Fisher least significant difference (LSD) test, whereas OFT data were analyzed using a one-way ANOVA with post hoc Fisher LSD test. A p-value of less than 0.05 was considered statistically significant.

CHAPTER IV

RESULTS

A. Acute hypoxic seizures count

Among the rats that underwent hypoxia, only the ones having 6 seizures, or more, were selected for the study. Hypoxic rats were divided into two treatment groups: one drinking vehicle water, HV, and the second one drinking water with sertraline, HSERT. A comparable number of seizures was recorded between the two groups (Fig. 18). Weights and water consumption were comparable between all rats on all days after weaning ($p>0.05$) (Fig. 19 and 20).

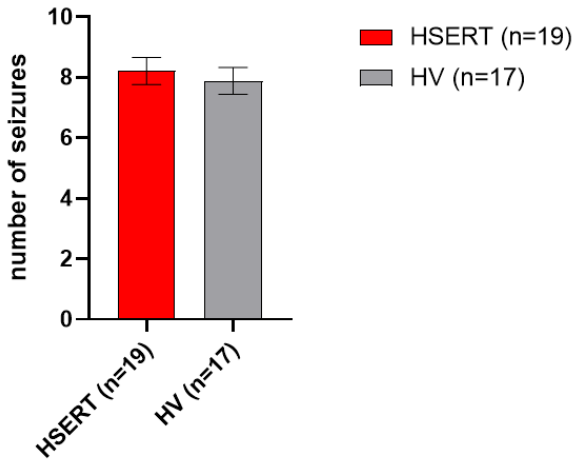

Figure 18. The acute number of seizures recorded in hypoxic rats. Unpaired t-test revealed a comparable seizure number between the two groups subjected to hypoxia ($p>0.05$). HV group (hypoxic seizure with vehicle, n=17), HSERT group (hypoxic seizure with sertraline, n=19).

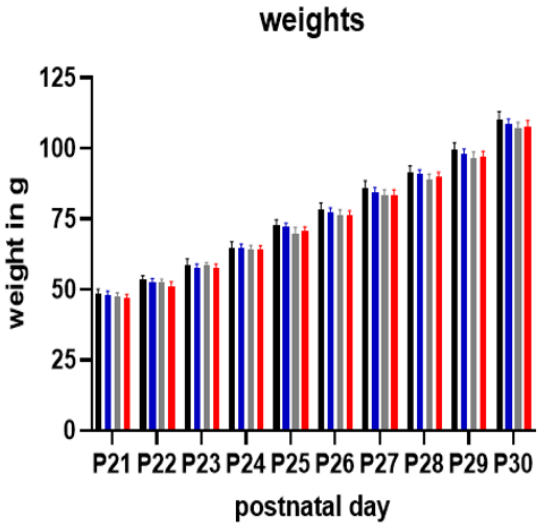

Figure 19. Weight monitoring after weaning. Two-way repeated-measures ANOVA with post hoc Fisher's least significant difference (LSD) revealed that all groups had a significant increase in weight from P21 to P30 ($p<0.05$) with comparable weights between all treatment groups from P21 to P30 ($p>0.05$, two-way repeated-measures ANOVA with post hoc Fisher's LSD). NV group (normoxic with vehicle, n=18), HV group (hypoxic seizure with vehicle, n=17), NSERT group (normoxic with sertraline, n=19), HSERT group (hypoxic seizure with sertraline, n=19).

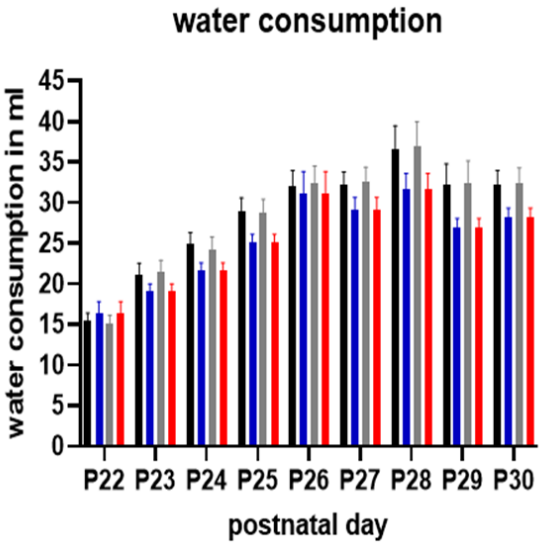

Figure 20. Water consumption monitoring after weaning. Two-way repeated-measures ANOVA with post hoc Fisher's least significant difference (LSD) ANOVA revealed a comparable water consumption among all treatment groups from P22 to P30 ($p> 0.05$). NV group (normoxic with vehicle, n=18), HV group (hypoxic seizure with vehicle, n=17), NSERT group (normoxic with sertraline, n=19), HSERT group (hypoxic seizure with sertraline, n=19).

B. Forced Swim Test

Depressive-like behaviors following early life hypoxic brain insult were investigated by performing the FST at P25-26. An earlier increase in immobility percentage in rats is considered to reflect depressive-like behaviors. During the first testing day, the HV group exhibited a significantly higher immobility time when compared to the NV and HSERT groups, while HSERT rats' immobility time was comparable to that of NV and NSERT. Both NV and NSERT had comparable immobility times throughout the test. However, the HSERT high activity percentage was higher than that of the NSERT group. On the second testing day, the HV group immobility time was comparable to that of the NV group. Sertraline-treated groups, NSERT and HSERT, exhibited immobility time lower than that of the NV group. Furthermore, the HSERT groups had a higher high activity time when compared to both NV and HV groups. However, at the last testing minute, both the immobility and high activity time of all treatment groups were comparable (Fig. 21, 22, 23, and 24).

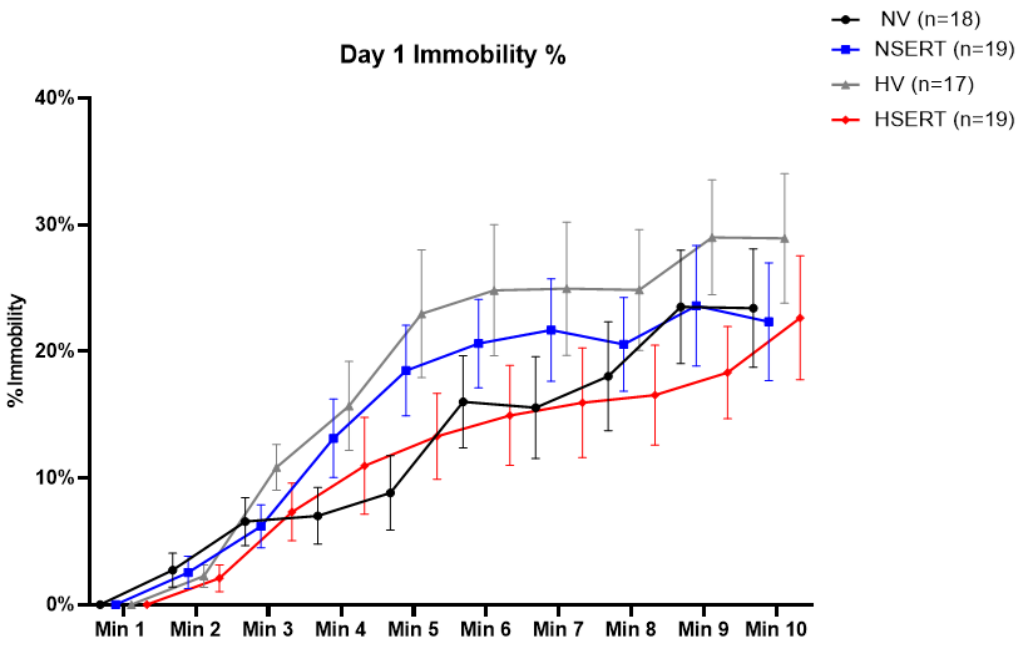

Figure 21. Immobility percentages during the first testing day of the forced swim test. Two-way repeated-measures ANOVA with post hoc Fisher's least significant difference (LSD) revealed that all treatment groups exhibit a significant increase in their immobility percentage from minute 1 to 10 ($p<0.05$, two-way repeated-measures ANOVA with post hoc Fisher's LSD). The behavior of the HV group diverged from its control group NV, with HV having a higher immobility percentage starting minute 2 that persists throughout the test and reaches a statistical significance at minute 5 ($p<0.05$, two-way repeated-measures ANOVA with post hoc Fisher's LSD). The immobility percentage of the HSERT group diverged from that of HV starting minute 2 and persists during the whole test with HSERT reaching a statistically significant lower immobility percentage at min 9 when compared to HV ($p<0.05$, two-way repeated-measures ANOVA with post hoc Fisher's LSD) but comparable to that of NV and NSERT ($p>0.05$, two-way repeated-measures ANOVA with post hoc Fisher's LSD). On the last testing minute, the immobility time of all groups was comparable ($p>0.05$, two-way repeated-measures ANOVA with post hoc Fisher's LSD). NV group (normoxic with vehicle, n=18), HV group (hypoxic seizure with vehicle, n=17), NSERT group (normoxic with sertraline, n=19), HSERT group (hypoxic seizure with sertraline, n=19).

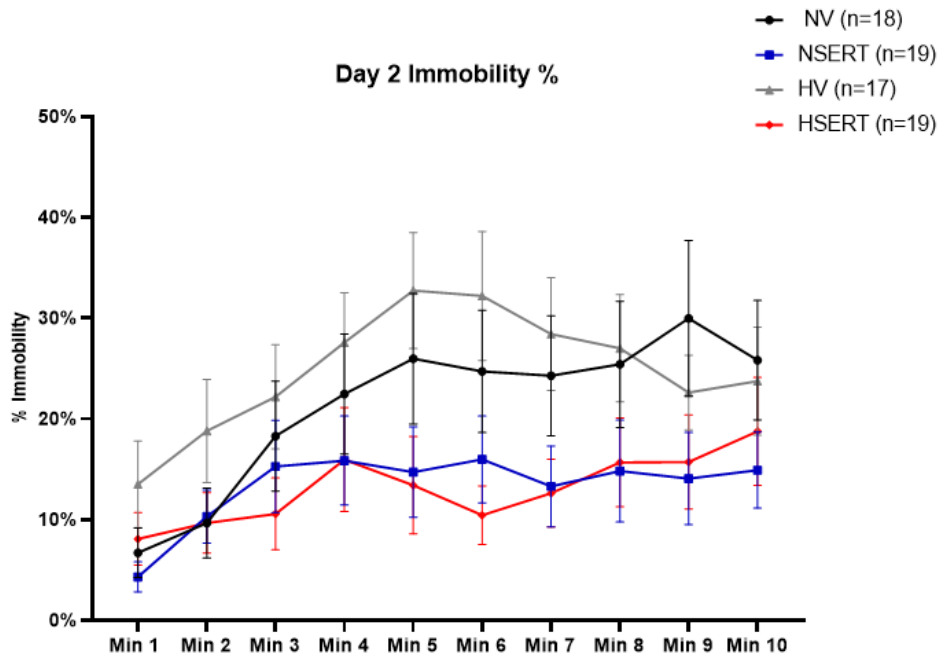

Figure 22. Immobility percentages during the second testing day of the forced swim test. Two-way repeated-measures ANOVA with post hoc Fisher's least significant difference (LSD) revealed that on the second testing day the HV group immobility percentage was comparable to that of the NV group ($p>0.05$). Additionally, the HV group had a higher immobility time in contrast to both HSERT and NSERT groups, reaching significance at minutes 5,6, and 7 ($p<0.05$, two-way repeated-measures ANOVA with post hoc Fisher's LSD). Both sertraline treated groups (NSERT and HSERT) presented similar immobility times ($p>0.05$, two-way repeated-measures ANOVA with post hoc Fisher's LSD), with lower immobility percentages when compared to the NV group. A statistically significant lower immobility percentage was recorded in HSERT when compared to NV at minutes 6 and 9, and in NSERT when compared to NV at min 9 ($p<0.05$, two-way repeated-measures ANOVA with post hoc Fisher's LSD). On the last testing minute, the immobility time of all groups was comparable ($p>0.05$, two-way repeated-measures ANOVA with post hoc Fisher's LSD). NV group (normoxic with vehicle, n=18), HV group (hypoxic seizure with vehicle, n=17), NSERT group (normoxic with sertraline, n=19), HSERT group (hypoxic seizure with sertraline, n=19).

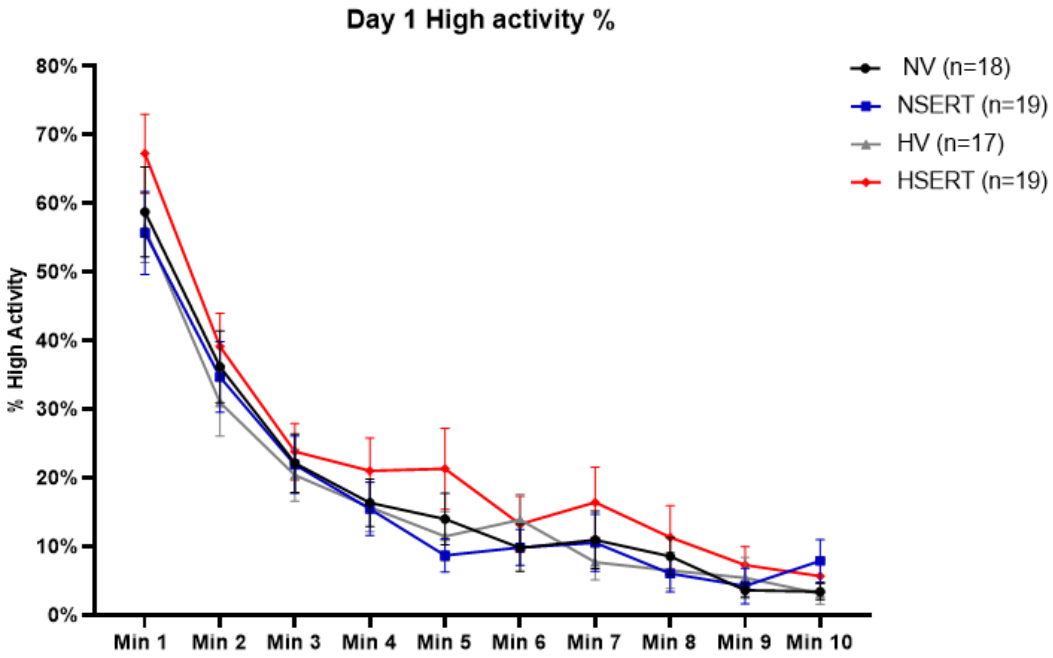

Figure 23. High activity percentages during the first testing day of the forced swim test. During the first testing day, two-way repeated-measures ANOVA with post hoc Fisher's least significant difference (LSD) revealed that all groups had a significant decrease in their high activity from minute 1 till minute 10 ($p<0.05$). The HSERT group had a high activity percentage exceeding that of the NSERT group reaching significance at minutes 1 and 5 ($p<0.05$, two-way repeated-measures ANOVA with post hoc Fisher's LSD). The NV, NSERT, and HV groups had comparable high activity percentages throughout the first testing day ($p>0.05$, two-way repeated-measures ANOVA with post hoc Fisher's LSD). NV group (normoxic with vehicle, n=18), HV group (hypoxic seizure with vehicle, n=17), NSERT group (normoxic with sertraline, n=19), HSERT group (hypoxic seizure with sertraline, n=19).

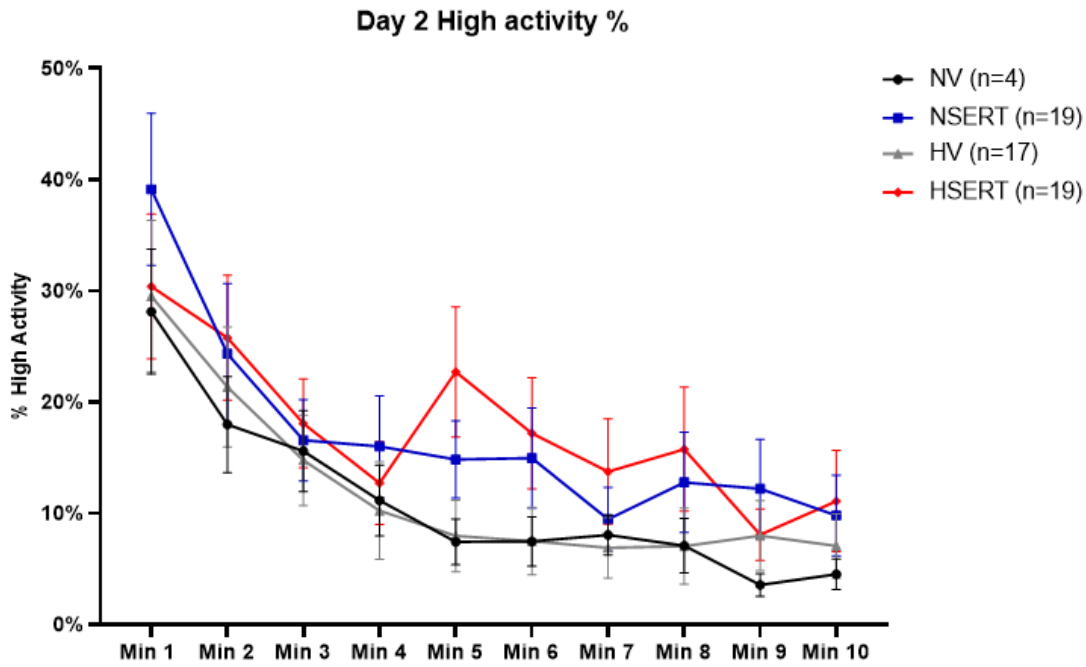

Figure 24. High activity percentages during the second testing day of the forced swim test. During the second testing day, two-way repeated-measures ANOVA with post hoc Fisher's least significant difference (LSD) revealed that all groups had a significant decrease in their high activity from minute 1 till minute 10 ($p<0.05$). The HSERT group had a high activity time higher than that of NV and HV groups reaching significance at minute 5 ($p<0.05$, two-way repeated-measures ANOVA with post hoc Fisher's LSD). The NV, NSERT, and HV had a comparable high activity level during the whole test ($p>0.05$, two-way repeated-measures ANOVA with post hoc Fisher's LSD). NV group (normoxic with vehicle, n=18), HV group (hypoxic seizure with vehicle, n=17), NSERT group (normoxic with sertraline, n=19), HSERT group (hypoxic seizure with sertraline, n=19).

C. Open Field Test

The open-field test is widely used to assess hyperactivity, anxiety-like and exploratory behaviors via the measurement of the total distance traveled by the rat (level of hyperactivity), the time spent in different zones (anxious rat spend more time in the outer fields), and the exploratory behavior (time spent in the inner zone next to objects).

The total distance traveled by the HV group during all 3 sessions was significantly lower than that of the NV group ($p<0.05$). The HSERT group had a total distance traveled significantly lower than that of the NV group ($p<0.05$) but comparable to that of the HV ($p>0.05$). NSERT rats had a total distance traveled significantly lower than that of the NV group ($p<0.05$) but comparable to that of the HV ($p>0.05$) (Fig. 25).

The HV group spent a statistically significant higher time in the periphery with a statistically significant lower distance traveled when compared to the NV groups ($p<0.05$). The HSERT group spent significantly more time in the periphery when compared to NV, HV, and NSERT groups ($p<0.05$). The HSERT groups traveled a total distance in the periphery significantly lower than the NV group ($p<0.05$). Furthermore, the total time spent in the periphery by the NSERT group was significantly higher than that of the NV group ($p>0.05$), but the total distance traveled in the periphery by the NSERT group was comparable to that of both NV and HV groups ($p>0.05$) (Fig. 26).

The distance traveled in the center and the time spent in the center by both HV and HSERT groups was significantly lower than that of the NV ($p<0.05$). Furthermore, the time spent in the center by the HSERT group was significantly lower than that of the HV groups ($p<0.05$) with a total distance traveled in the center comparable to the HV group ($p>0.05$). The time and the distance traveled in the center by the NSERT group

were lower than that of NV ($p<0.05$) and comparable to that of the HV group ($p>0.05$) (Fig. 27).

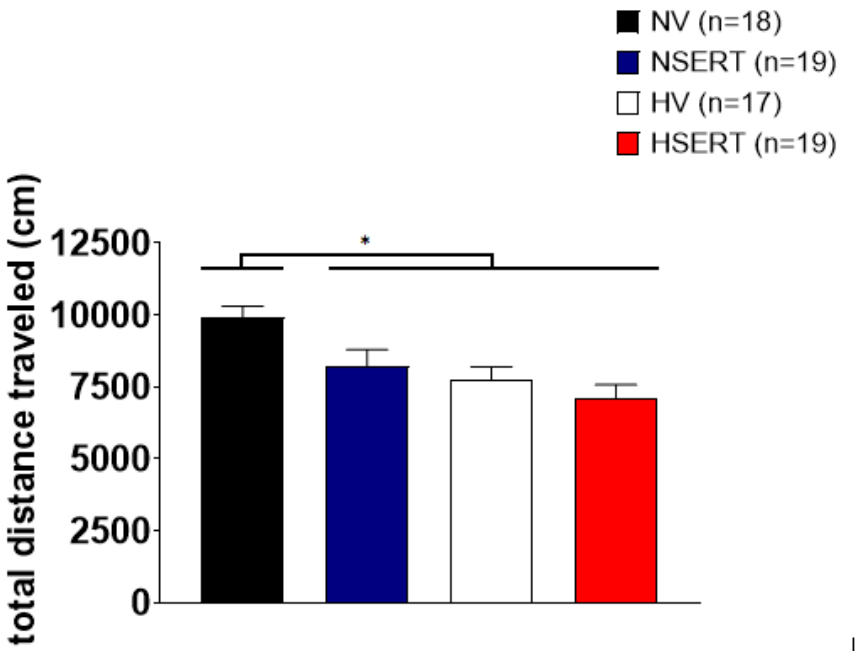

Figure 25. Total distance traveled during all the 3 sessions by the different groups. One-way ANOVA with post hoc Fisher's LSD revealed that the total distance traveled by the HV group during all 3 sessions was significantly lower than that of the NV group ($p<0.05$). The HSERT group had a total distance traveled significantly lower than that of the NV group ($p<0.05$, one-way ANOVA with post hoc Fisher's LSD) but comparable to that of the HV ($p>0.05$, one-way ANOVA with post hoc Fisher's LSD). NSERT rats had a total distance traveled significantly lower than that of the NV group ($p<0.05$, one-way ANOVA with post hoc Fisher's LSD) but comparable to that of the HV($p>0.05$, one-way ANOVA with post hoc Fisher's LSD). NV group (normoxic with vehicle, n=18), HV group (hypoxic seizure with vehicle, n=17), NSERT group (normoxic with sertraline, n=19), HSERT group (hypoxic seizure with sertraline, n=19).

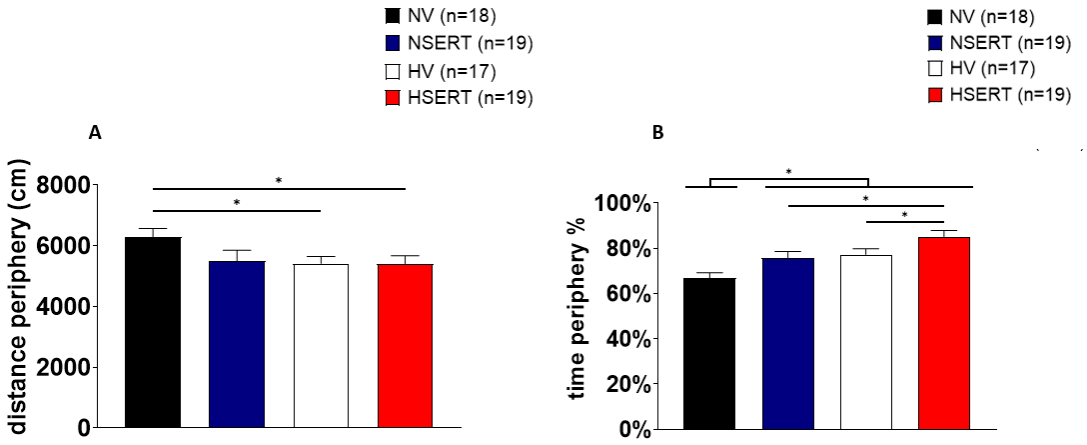

Figure 26. Total time spent and traveled in the periphery. A) Total distance traveled in the periphery during all 3 sessions by the different groups. One-way ANOVA with post hoc Fisher's LSD showed a statistically significant lower distance traveled by the HV group when compared to the NV group ($p<0.05$) but comparable to both NSERT and HV groups ($P>0.05$). The HSERT group traveled a total distance in the periphery significantly lower than that of the NV group ($p<0.05$, one-way ANOVA with post hoc Fisher's LSD) but comparable to both HV and NSERT groups ($p>0.05$, one-way ANOVA with post hoc Fisher's LSD). The NSERT group traveled a total distance in the periphery significantly lower than that of the NV group ($p<0.05$, one-way ANOVA with post hoc Fisher's LSD) but comparable to both HV and HSERT groups ($p>0.05$, one-way ANOVA with post hoc Fisher's LSD)**. B) Total time spent in the periphery during all 3 sessions by the different groups.** One-way ANOVA with post hoc Fisher's LSD showed a significantly higher time spent in the periphery by the HV group when compared to the NV group ($p<0.05$). The HSERT group spent a total time in the periphery significantly higher than that of the NV, HV, and NSERT groups ($p<0.05$, one-way ANOVA with post hoc Fisher's LSD. The NSERT group spent a total time in the periphery significantly higher than that of the NV group ($p<0.05$, one-way ANOVA with post hoc Fisher's LSD) but comparable to the HV group ($p>0.05$, one-way ANOVA with post hoc Fisher's LSD). HSERT group (hypoxic seizure with sertraline, n=19), HV group (hypoxic seizure with vehicle, n=17), NSERT group (normoxic with sertraline, n=19), NV group (normoxic with vehicle, n=18).

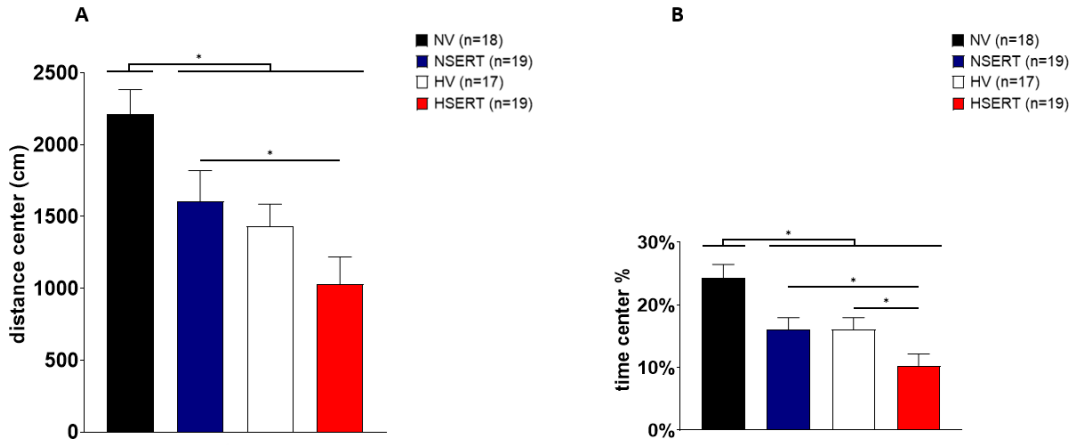

Figure 27. Total time spent and traveled in the center. A) Total distance traveled in the center (next to objects) during all 3 sessions by the different groups. One-way ANOVA showed a statistically significant lower distance traveled in the center by the HV group when compared to the NV group ($P<0.05$, one-way ANOVA with post hoc Fisher's LSD). The total distance traveled in the center by the HSERT group was significantly lower than that of both NV and NSERT groups ($p<0.05$, one-way ANOVA with post hoc Fisher's LSD) but comparable to that of the HV group ($p>0.05$, one-way ANOVA with post hoc Fisher's LSD). The total distance traveled by the NSERT group was significantly lower than that of the NV group ($p<0.05$, one-way ANOVA with post hoc Fisher's LSD) but comparable to that of the HV group ($p>0.05$, one-way ANOVA with post hoc Fisher's LSD). **B) Total time spent in the center (next to objects) during all 3 sessions by the different groups.** One-way ANOVA with post hoc Fisher's LSD showed a statistically significant lower time spent in the center by the HV group when compared to the NV group ($p<0.05$, one-way ANOVA with post hoc Fisher's LSD). The HSERT group spent a total time in the center significantly lower than that of NV, HV, and NSERT groups ($p<0.05$, one-way ANOVA with post hoc Fisher's LSD). The NSERT group spent a total time in the center significantly lower than that of the NV group ($p<0.05$, one-way ANOVA with post hoc Fisher's LSD) but comparable to that of the HV group ($p>0.05$, one-way ANOVA with post hoc Fisher's LSD). NV group (normoxic with vehicle, n=18), HV group (hypoxic seizure with vehicle, n=17), NSERT group (normoxic with sertraline, n=19), HSERT group (hypoxic seizure with sertraline, n=19).

D. Histological Analysis

1. Hippocampal and prefrontal cortex neuronal cell count (NeuN)

Our preliminary histological data, via visual inspection of the hippocampus and prefrontal cortex, revealed a comparable neuronal density between all groups (Fig. 28).

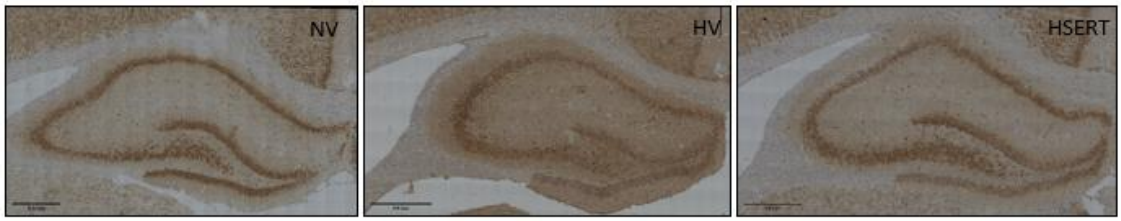

Figure 28. Hippocampal pyramidal neurons density. Visual inspection reveals comparable hippocampal neuronal cell densities among the different groups. NV group (normoxic with vehicle), HV group (hypoxic seizure with vehicle), HSERT group (hypoxic seizure with sertraline).

2. Hippocampal and prefrontal cortex synaptic plasticity marker (Syp)

Our preliminary data suggest an increased expression of synaptophysin in the hilar region and CA2-3 region of the hippocampus of the HV group when compared to the rest of the groups. As for the prefrontal cortex, the HV group presented a higher SYP expression in comparison to the NV group with the NV group having the lowest SYP expression when compared to the rest of the groups (Fig. 29)

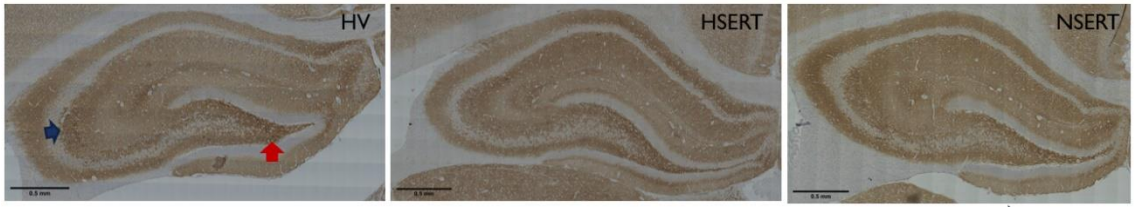

Figure 29. Alteration in hippocampal synaptic plasticity marker, synaptophysin. Visual inspection reveals higher expression of synaptophysin in the hilar region (red arrow) and the CA2-3 region (blue arrow) of the HV group. HV group (hypoxic seizure with vehicle), NSERT group (normoxic with sertraline), HSERT group (hypoxic seizure with sertraline).

CHAPTER V

DISCUSSION

In this study, we show that sertraline can reverse the hypoxic seizure-induced depressive-like behavior. We also confirm that early-life hypoxic seizures result in depressive-like and anxious-like behaviors during peri-adolescence. Children who have suffered from perinatal asphyxia, followed by neonatal hypoxic seizure exhibit impaired short and long-term developmental outcomes, with depression being the most prominent of them. Thus, increasing interest in optimizing therapeutic strategies through a better understanding of the molecular pathways involved in epilepsy and depression has been emerging. We also report preliminary proof of disrupted synaptic homeostasis mediating the molecular alteration in the brain post hypoxic seizures.

In our study, early life HS led to depressive-like behaviors, evidenced by increased immobility and decreased struggling during the FST, that were effectively reversed by sertraline. This complies with previous studies done in our lab (49). ThE protective effect of sertraline against peri-adolescent mood disorders was conferred by a daily administration of the medication starting the peri-adolescent age (P24) following an early brain insult during infancy. Treating hypoxic-seizure-associated comorbidities is crucial for adolescent patients as depression had been shown to highly deteriorate their quality of life (16). SSRIs can be safely used in pediatric patients suffering from epilepsy and this type of antidepressant has been correlated with lower seizure incidence and exhibits anticonvulsant properties (207). Nonetheless, its effectiveness in treating seizure-associated depression remained elusive. Even within the emergence of epilepsy, patients suffer more from seizures' deleterious impact on their mental health more than they do suffer from epileptic episodes. Furthermore, a bidirectional

association exists between seizures and depression. Thus, acting on alleviating one condition might attenuate the severity of the other (171,172). Other studies have revealed the same effect of sertraline, increased swimming and climbing in the FST, in a rat model of hypobaric hypoxia and another model bred for a stress-induced reduction in motor activity in the swim test (268). Better antidepressant properties have been recorded with the administration of sertraline versus that of paroxetine, despite both belonging to the SSRIs family. In fact, sertraline can block dopamine reuptake by targeting the dopamine transporters (200). This suggests that antidepressants that target both dopamine and serotonin transporters are the most suitable treatment option. Furthermore, sertraline's anti-depressive effect can be mediated by a regulation of BDNF and TrkB levels. However, a chronic administration is required to alter gene expression (224). Comparable behaviors of HV and NV rats observed during the second testing day might be caused by rats' habituation to the test. The increased swimming and struggling observed in sertraline are not caused by sertraline-linked hyperactivity but rather sertraline-linked increased anxiety. In fact, the OFT reveals that sertraline administration did not result in any hyperactivity since the NV group had the highest total distance traveled. The OFT highlights anxiety-like behaviors in hypoxic rats that can be worsened by the administration of sertraline. A longer time spent in the peripheral region versus the central regions is considered an indicator of anxious-like behaviors. The absence of anxiolytic effects of sertraline might be caused by the short duration of the treatment (treatment onset one day before behavioral testing). This, reveals that despite the efficiency of SSRIs in alleviating seizure burden and reducing seizure reoccurrence, their prescription to young patients should be done while taking into consideration their potential anxiogenic secondary effect. It has been reported that

acute treatment with SSRIs worsens anxiety-like behaviors (269). This might be mediated by an altered function of the 5-HTergic system (269). Furthermore, a chronic administration of the medication is required to alter the genetic expression of the BDNF-TrkB pathways that in turn regulate behavior (224). Nevertheless, secondary effects such as hypodopaminergic state and cognitive decline are lower with sertraline in contrast with other types of SSRIs (200)**.** Therefore, SSRI can be considered as one of the best options to attenuate HS-induced depression, yet optimal treatment duration ought to be further investigated to determine possible anxiolytic properties.

Moreover, the absence of neuronal cell loss, revealed by comparable neuronal densities, suggests that the behavioral perturbations might be mediated by other molecular alterations. This comparable neuronal density was as well observed in our previous studies (49). Potential cellular mechanisms underlying the observed behavioral deficits possibly include disruption of synaptic plasticity. Our preliminary data revealed a higher expression of synaptophysin, a synaptic plasticity marker, in the hippocampus and prefrontal cortex of rats that underwent hypoxic seizures when compared to the normoxic ones. SYP is present in approximately all neurons and is considered to be a specific marker for synaptic terminals (270). This protein plays an important role in regulating learning, memory (LTP), and intellectual dysregulation in patients suffering from epilepsy (262,275-277). Contrary to our results, a reduction in medial prefrontal cortex mPFC SYP levels was observed in other models of early-life stress and neonatal hypoxic ischemia (271,272). However, the mentioned models of hypoxia, in contrast to ours, did not induce any seizure. Other models of hypoxic seizures have revealed a decrease in the SYP expression restricted to the hippocampal CA1 region. Furthermore, both a decrease in SYP and BDNF positive areas are observed in the hippocampus of

rats subjected to hypoxic ischemia (273). Nonetheless, in previous studies, our model has revealed an increased TrkB activation, underlying an increased BDNF expression (274). This hippocampal BDNF overexpression facilitates LTP possibly explaining the SYP overexpression in rats that underwent hypoxic seizures (223). It becomes evident that a disruption in synaptic plasticity is correlated with behavioral comorbidities observed in hypoxic seizures. More precise quantification of synaptic plasticity markers and BDNF-TrkB pathways activation is required for a better understanding of the underlying molecular mechanisms. This will allow the precise development and optimization of therapeutic applications.

CHAPTER VI

LIMITATIONS AND FUTURE PERSPECTIVE

This work provides valid evidence of sertraline's effectiveness in treating depressive-like behaviors in peri-adolescent rats following early-life hypoxic seizures and highlights the possible anxiogenic secondary effect of short-term treatment. The study, however, has some limitations that can be traced back to time and financial restraints during the COVID-19 pandemic. Typically, histological studies require numbers not less than 3-5 animals per group with 3 sections per brain to be analyzed using the ImageJ software. This ensures sufficiently objective and powered data to establish precise statistics. Due to technical difficulties with the imaging microscope that went out of service, we were not able to get representative pictures for all groups, nor enough pictures to perform any statistical analysis. Hence, our histological outcomes are still preliminary.

Ongoing experiments in our laboratory aim to further investigate hippocampal and prefrontal cortex molecular alterations including histological analysis, miRNA assays, and western-blot testing. Furthermore, our study only included male SD rats. Future work intends to evaluate behavioral outcomes in female rats, as a differential gender manifestation of depression has been reported throughout the literature.

The treatment of hypoxia-induced depression needs to be further investigated due to its harmful effects on the brain and the quality of life of its survivor. Further research is needed to confirm the long-term repercussion of sertraline on behavioral outcomes and support the assumption that SSRIs are the anti-depressant of choice to be prescribed in pediatric patients.

CHAPTER VII

CONCLUSION

Here we demonstrated sertraline's effectiveness in reversing depressive-like behaviors. Our hypoxic-seizure model successfully recapitulates depressive-like and anxiety-like behaviors in peri-adolescent rats similar to the behavioral defects observed in pediatric patients who have suffered from perinatal hypoxic-ischemia. However, the short-term treatment did not improve anxious-like behaviors. The molecular mechanisms mediating these behavioral disturbances possibly include alteration in synaptic plasticity markers and BDNF-TrkB pathway dysfunctions. These results highlight the necessity of treating epilepsy-associated behavioral comorbidities. Investigating the effects of a longer treatment duration will help determine if sertraline's chronic administration holds late-onset anxiolytic effects and will depict its optimal use in children who have suffered from early-life hypoxic seizures.

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