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THE ROLE OF HISTAMINE H3 RECEPTOR ANTAGONISTS IN MODULATING AUTISTIC BEHAVIORS AND ALTERED CENTRAL INFLAMMATORY RESPONSES IN DIFFERENT MOUSE MODELS OF AUTISM SPECTRUM DISORDER

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This dissertation is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Under the Supervision of Dr. Bassem Shaban Sadek

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Declaration of Original Work

I, Nermin Abdel Wahab Mahmoud Eissa Abdel Zaher the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled *"The Role of Histamine H3 Receptor Antagonists in Modulating Autistic Behaviors and Altered Central Inflammatory Responses in Different Mouse Models of Autism Spectrum Disorder"*, hereby, solemnly declare that this dissertation is my own original research work that has been done and prepared by me under the supervision of Dr. Bassem Shaban Sadek, in the College of Medicine and Health Sciences at UAEU. This work has not previously been presented or published or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my dissertation have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this dissertation.

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Copy 8 of 20

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Abstract

Autistic spectrum disorder (ASD) represents a neurodevelopmental disorder characterized by impairment of social communication and restricted/repetitive behavior patterns or interests. Brain histamine and acetylcholine play a crucial role in cognitive functions. Considering this, the effects of systemic sub-chronic treatment with H3R antagonist DL77 (5, 10, or 15 mg/kg) on autistic-like behavioral parameters, oxidative stress, and neuroinflammation in male Tuck-Ordinary (TO) and C57BL/6 (C57) mice, prenatally exposed to valproic acid (VPA, 500 mg/kg), were investigated. Furthermore, the effects of dual-active H3R antagonist and balanced acetylcholinesterase inhibitor E100 (5, 10, or 15 mg/kg) on autistic-associated abnormalities of VPA- exposed male C57 mice as well as BTBR T+tf/J (BTBR) mice were assessed. The results showed that VPA-exposed mice exhibited significantly lower sociability and social novelty preference compared to VPA-exposed TO and C57 mice pretreated with DL77 (10 mg/kg) or (15 mg/kg), respectively. Moreover, the same doses of DL77 attenuated repetitive/compulsive behaviors of both strain of mice, without appreciable effects on disturbed anxiety and hyperactivity when compared to the reference drug donepezil (1 mg/kg). The amelioration in autistic-like phenotypes by DL77 were accompanied by the attenuation of oxidative stress by increasing glutathione and decreasing malondialdehyde levels, and attenuation of proinflammatory cytokines interleukin-1β, interleukin-6 and tumor necrosis factor-α in brain tissues from VPA-exposed mice. Comparing the results observed for DL77, the dual-active E100 (10 mg/kg) showed significantly higher improvement of autistic behavioral alterations in VPA-exposed C57 mice, and significantly palliated disturbed anxiety levels. In addition, E100 attenuated several pro-inflammatory cytokines and inflammatory mediators through the suppression of upregulated NF-κB signaling. Immunofluorescence analysis showed significant reduction in ionized calcium-binding adaptor molecule-1 increased expression in VPA-exposed C57 mice by E100, demonstrating attenuation of activated microglia. Similarly, oxidative stress status was also mitigated by E100 in brain tissues. The promising effects of E100 on autistic features in C57 mice were further complemented with the results following treatment with E100 (5 mg/kg) in BTBR mice as idiopathic model of ASD. These results provide

evidence that simultaneous modulation of brain histaminergic and cholinergic neurotransmissions may have therapeutic efficacy for core symptoms of ASD. Further preclinical investigations are still necessary to corroborate and expand these observed data.

Keywords: ASD, mouse models, histamine H3 receptor antagonists, autistic-like behaviors, neuroinflammation, oxidative stress.

Title and Abstract (in Arabic)

دور مثبطات مستقبل الهيستامين H3 في تعديل السلوك التوحدي واستجابات الالتهاب المركزي المتغيرة في نماذج فئران مختلفة من اضطراب طيف التوحد

الملخص

يمثل اضطراب طيف التوحد (ASD) اضطرابًا في النمو العصبي ويتميز بضعف التواصل الاجتماعي وأنماط أو اهتمامات سلوكية مقيدة أو متكررة، ويلعب الهستامين و الأسيتيل كولين دوراً حاسماً في الوظائف الإدراكية. وبامعان النظر ، فقد تم فحص آثار المعالجة شبه المزمنة لدى مثبطات مستقبل الهستامين H3R (DL77 5، 10 ، أو 15 ملغ / كلغ) على المعاملات السلوكية الشبيهة بالتوحد ، والإجهاد التأكسدي ، والتهاب الأعصاب في ذكور فئران Tuck-Ordinary (TO) و C57BL/6 (C57) ، التي تعرضت قبل الولادة لحمض فالبرويك (VPA، 500 ملغم / كغم) . كما ايضا تم اختبار وتقييم E100 (5 ، 10 ، أو 15 مجم / كجم) ثنائي الفعالية كمثبطات مستقبل الهستامين H3R وكذلك مثبط الاستيل كولينيستراز على الاعراَض المرتبطة بالتوحد على فئران C57 الذكور و كذلك فئران (BTBR) BTBR T+tf/j.

وقد أكدت النتائج أن الفئران المعالجه بـ VPA قد أظهرت انخفاضًا كبيرًا في التواصل الاجتماعي وتفضيل الوحدة الاجتماعية مقارنة بفئران TO و C57 المعرضة لـ VPA و المعالجة بـ DL77 (10مجم / كجم) أو (15 مجم / كجم) على التوالي. علاوة على ذلك ، فإن نفس جرعات DL77 أدت إلى إنخفاض في السلوكيات المتكررة / القهريّة لكلتا السلالتين ، دون آثار ملحوظة على القلق المضطرب وفرط النشاط عند مقارنتها بالعقار المرجعي دونيبزيل (1 مغ / كغ). وقد رافق التحسن في الأنماط الظاهرية الشبيهة بالتوحد بواسطة DL77 باستعادة الإجهاد التأكسدي عن طريق زيادة الglutathione وانخفاض مستويات malondialdehyde ، وتخفيف السيتوكينات الموالية للالتهابات interleukin-1β، interleukin -6 و Tumor necrosis factor-α على أنسجة المخ لدى الفئران التي تعرضت قبل الولادة ل VPA.

بمقارنة النتائج التي لوحظت في DL77 ، أظهر المركب E100 ثنائي الفعالية (10 مغ / كغ) تحسنا أعلى بكثير في التغيرات السلوكية التوحدية في فئران C57 المعرضة لـ VPA ،

ومستويات القلق المضطربة بشكل ملحوظ. بالإضافة إلى ذلك ، خفف E100 العديد من السيتوكينات المؤيدة للالتهابات و وسطاء الالتهابات من خلال قمع إشارة NF-κB المنتظمة.

أظهر (Immunofluorescence analysis) انخفاضًا كبيرًا في زيادة التعبير مهيء رابط الكالسيوم الأيوني الجزئ-١ في فئران C57 المعرضة لـ VPA ، مما يدل ،لى اثار E100 على توهين الخلايا المجهرية المنشطة. وبالمثل ، تم تخفيف حالة الإجهاد التأكسدي أيضًا عن طريق E100 في أنسجة المخ. وتم فهم الآثار الواعدة لـ E100 على ميزات التوحد في الفئران C57 مع النتائج التي لوحظت مع (5 E100مجم / كجم) في الفئران BTBR كنموذج مجهول السبب من ASD.

توفر هذه النتائج دليلًا على أن التعديل المتزامن للناقلات العصبية الهيستامينية والكولينينية في الدماغ قد يكون له فعالية علاجية للأعراض الأساسية لـ ASD. ولكن ،لا تزال هناك حاجة للمزيد من التجارب والدراسات قبل السريرية لتأكيد وتطوير هذه النتائج التي تم الحصول عليها.

مفاهيم البحث الرئيسية: ASD ، نماذج الفأر ، مثبطات مستقبلات الهستامين H3 ، السلوكيات الشبيهة بالتوحد ، الالتهاب العصبي ، الإجهاد التأكسدي.

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Dedication

To my beloved parents and family

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ASD	Autism Spectrum Disorder
5-HT	Serotonin
ACh	Acetylcholine
AChEI	Acetylcholine esterase inhibitor
AD	Alzheimer's disease
ADHD	Attention deficit hyperactivity disorder
cAMP	cyclic Adenosine Triphosphate
CAT	Catalase
CNS	Central Nervous System
COX	Cyclooxygenase
CPX	Ciproxifan
DA	Dopamine
DAT	Dopamine transporter
DOZ	Donepezil
DOZ	Donepezil
DR	Dopamine Receptor
DS	Dopaminergic System
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
GABA	λ-amino butyric acid

Glu	Glutamate
GPCR	G-protein coupled receptors
GSH	Glutathione
HA	Histamine
HDC	Histidine Decarboxylase
HNMT	Histamine N-methyl Transferase
HRP	Horseradish peroxidase
HS	Histaminergic System
i.p.	Intraperitoneally
Iba-1	Ionized calcium binding adaptor molecule -1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
mAChR	Muscarinic acetylcholine receptor
MDA	Malondialdehyde
nAChR	Nicotinic acetylcholine receptor
NE	Norepinephrine
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NMDA	N-Methyl-D-aspartate
NRXN	Neurexin
PBS	Phosphate buffer Saline
PD	Parkinson’s disease
PKA	Protein Kinase A

PYR	Pyrilamine
RAM	R-alpha methyl histamine
RIPA	Radioimmunoprecipitation
ROS	Reactive Oxygen Species
SCH	Schizophrenia
SCO	Scopolamine
SOD	Super oxide dismutase
SSRI	Selective serotonin reuptake inhibitor
TGF-β	Transforming growth factor beta
TMN	Tuberomammillary Nucleus
TNF-α	Tumor necrosis factor-alpha
TO	Tuck Ordinary
TS	Tourette syndrome
VPA	Valproic Acid
ZOL	Zolantadine

Chapter 1: Introduction

1. 1 Autism Spectrum Disorder

Autistic spectrum disorder (ASD) is a biologically based neurodevelopmental disorder affecting two major core behavioral symptoms, namely impairments in social skills and restricted /repetitive behavioral pattern or interest of ASD patients (Baronio, Castro, Gonchoroski et al., 2015). These core symptoms can be observed before the age of three years and are lasting for the whole lifetime (Andres, 2002). ASD has become a high priority for scientists and health care providers, and has also attracted the public attention because of reported increase in its prevalence (Sheldrick & Carter, 2018; Xu, Strathearn, Liu et al., 2018). The worldwide estimated prevalence of individuals with ASD diagnosis is strikingly high with prevalence varying across numerous studies, but it is estimated that one in 160 children has an ASD worldwide, and it is expected to increase globally (Arvidsson, Gillberg, Lichtenstein et al., 2018). Despite its increasing prevalence, the pathophysiology of ASD is still understood incompletely, and this can be attributed to challenges in identifying suitable animal models and the complexity of the neurobiology in brain function (Nestler & Hyman, 2010). Several lines of evidence suggest that strong genetic and environmental factors raise the occurrence of ASD in childhood (Baronio et al., 2015). Additionally, the heterogenicity of clinical and behavioral symptoms in autistic children is part of the difficulty in understanding the pathophysiology of this disorder, and consequently no specific treatment can be effective to all autistic children. Therefore, subgrouping of children based on their responding to intervention is essential (James, Melnyk, Fuchs et al., 2009). Studying the drug effects on core symptoms in ASD is very challenging, despite advances in early diagnosis and intervention, efficacious reversal of these core

autistic symptoms is still not approved to date. To date, there are no efficient therapeutic interventions that target the core symptoms of ASD, namely social communication impairments and restricted/repetitive behavior (Sheldrick et al., 2018; Xu et al., 2018). However, pharmacological interventions may be used to provide symptomatic control of associated comorbidities but not to treat core deficits (Hanson, Kalish, Bunce et al., 2007; Wong & Smith, 2006). These associated symptoms of ASD include inattention, hyperactivity, anxiety, sleep disturbances, irritability, aggression and self-injuries. Psychiatric drugs are frequently used for treating these symptoms in autistic children (Findling, 2005). Currently, risperidone and aripiprazole are the only two US Food and Drug Administration (FDA) approved drugs for treating behavioral symptoms associated with ASD, mainly for irritability (Matson, Sipes, Fodstad et al., 2011). Targeting ASD core symptoms for complete and effective treatment has been challenging and not yet achieved as mentioned earlier. Therefore, developing drug therapies for improving and perhaps treating the core symptoms of ASD is significantly important, aiming to improve the quality of life for the suffering patients and easing the burden on their families.

1.1.1 Etiology of ASD

ASD is broadly considered to be a multi-factorial disorder that results from genetic as well as non-genetic risk factors. There is cumulative evidence for the involvement of genetic factors in the etiology of ASD, since siblings born in families with ASD are at 35–40% greater risk to develop ASD and with an increase in the current rate of approximately 1% from a rate of 0.05% in 1970s (Stubbs, Henley, & Green, 2016). Moreover, genetic studies revealed that alteration in the developmental pathways of neuronal and axonal structures that are strongly involved in

synaptogenesis emerge from single gene mutations (Chang, Gilman, Chiang et al., 2015; Geschwind, 2011; Voineagu, Wang, Johnston et al., 2011). It is likely that interactions between multiple genes, and variability in expression as a result of epigenetic factors and exposure to environmental factors are responsible for ASD (Muhle, Trentacoste, & Rapin, 2004). In a previous clinical study involving a twin, it was appraised that the risk of developing ASD was 35–40% due to genetic variability, and the remaining 60% was contributed to by prenatal, perinatal, and postnatal environmental factors (Hallmayer, Cleveland, Torres et al., 2011). Accordingly, environmental factors implicated with ASD included prenatal and perinatal complications (Glasson, Bower, Petterson et al., 2004; Maramara, He, & Ming, 2014), birth and neonatal complications (Gardener, Spiegelman, & Buka, 2011; Guinchat, Thorsen, Laurent et al., 2012), viral infection, autoimmune diseases, and exposure to teratogens and maternal anticonvulsants such as valproic acid (VPA) (Kern & Jones, 2006; Kolevzon, Gross, & Reichenberg, 2007). Therefore, an increased understanding of the interface between genetic and environmental factors in the pathogenesis of ASD may lead to an optimized therapeutic strategy.

1.2 Central histamine and histaminergic system (HS)

Histamine and its receptors have long been known as part of immune and gastrointestinal systems but their discovery in the central nervous system (CNS) and their role in behavior, was largely studied in recent years. The possibility of histamine targeting treatments has recently received considerable attention, basically in neurogenerative diseases (Sadek, Saad, Sadeq et al., 2016c). The role of histamine in cognition, sleep, sensory, motor functions and neuroinflammation have attracted considerable interest (Haas, Sergeeva, & Selbach, 2008; Shan, Bao, & Swaab, 2015;

Witkin & Nelson, 2004). Moreover, histamine was suggested through recent evidence to have a role in regulating microglial activation (Dong, Zhang, Zeng et al., 2014; Frick, Rapanelli, Abbasi et al., 2016), cytokine release (Rocha, Pires, Esteves et al., 2014) and migration (Ferreira, Santos, Goncalves et al., 2012). The HS in the CNS consists of cell bodies of histaminergic neurons confined in one area of posterior hypothalamus called tuberomammillary nucleus (TMN), sending projections to spinal cord, brain stem and many telencephalic brain areas (Brown, Stevens, & Haas, 2001; Haas et al., 2008) (Figure 1). Hence, central histamine has been suggested to be a regulator of activity of the "whole brain" (Wada, Inagaki, Yamatodani et al., 1991).

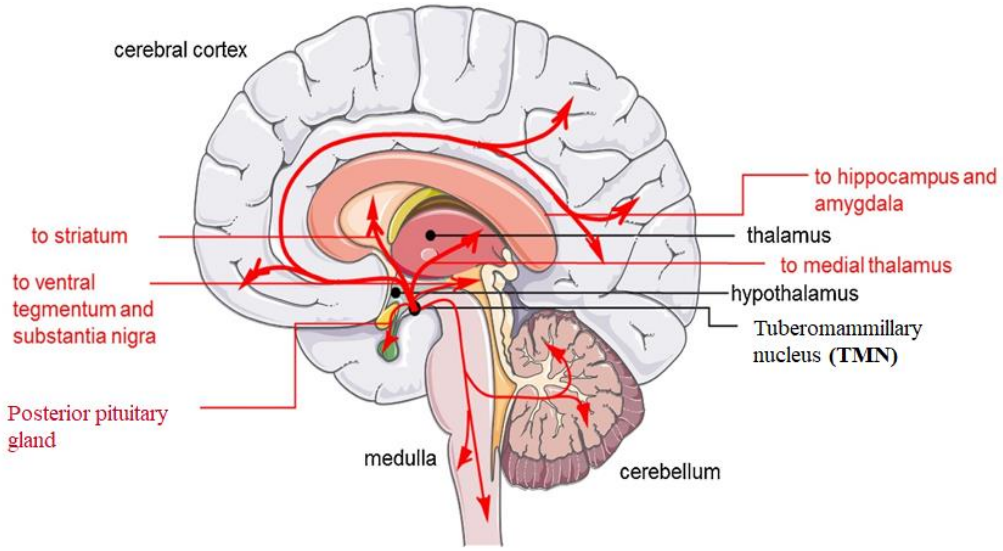


Figure 1: Histaminergic neuronal projections from tuberomammillary nucleus (TMN).

Innervations originates from TMN to all over the CNS from ventral part to the hypothalamus and septum, and from dorsal part to the thalamus, hippocampus, amygdala, and forebrain. There is one descending pathway to the brain stem, cerebellum and spinal cord. Adapted from (Sadek, et al., 2016c).

Histamine is biosynthesized mainly in the TMN, where the dietary amino acid pre-cursor L-histidine is decarboxylated and converted to histamine by a specific

enzyme called histidine decarboxylase (HDC). The central histamine action is inactivated by histamine N-methyltransferase (HNMT) in the extracellular space throughout the brain (Brown et al., 2001; Haas et al., 2008; Prell, Hough, Khandelwal et al., 1996). In the CNS, histamine biosynthesis and release is modulated by H3 auto-receptors through cAMP/PKA dependent pathways (Bongers, Bakker, & Leurs, 2007). Histamine may be able to activate both ligand-operated channels and receptors linked to second messengers. With regard to receptors, the effects of histamine are exerted through activation of four different G-protein coupled receptors (GPCR) subtypes namely H1-H4R, expressed through the brain (Arrang, Devaux, Chodkiewicz et al., 1988; Arrang, Garbarg, & Schwartz, 1983, 1985, 1987; Hill, 1990; Hill, Ganellin, Timmerman et al., 1997; Lovenberg, Roland, Wilson et al., 1999), however, H1-3Rs are highly founded in the brain and H4Rs have extremely low expression through CNS with unclear function (Sadek & Stark, 2016e; Schneider & Seifert, 2016). The H3Rs are predominantly and heterogeneously expressed in the CNS. They are mainly expressed in the basal ganglia, globus pallidus, hippocampus, cerebellum and cortex in humans (Li, Zhu, & Wang, 2014; Martinez-Mir, Pollard, Moreau et al., 1990). As mentioned earlier pre-synaptic H3R are coupled to Gi/Go-proteins and function as auto-receptors that control firing of histamine neurons modulating synthesis and release of histamine with high constitutive activity. In addition, H3Rs function as hetero-receptors and can also regulate the release of other neurotransmitters like dopamine (DA), serotonin (5-HT), norepinephrine (NE), acetylcholine (ACh), λ-amino butyric acid (GABA) and glutamate (Glu) in several brain regions. Thus, blockade of inhibitory histamine H3 auto-receptors reinforces histamine neurotransmission, while antagonism of H3 hetero-receptors accelerates the

corticolimbic liberation of ACh, NE, DA, 5-HT and GABA and Glu (Brown et al., 2001; Haas et al., 2008) (Figure 2). Accordingly, the brain HS and its modulation have been implicated in many brain disorders, suggesting that it may play a critical role in their pathophysiology.

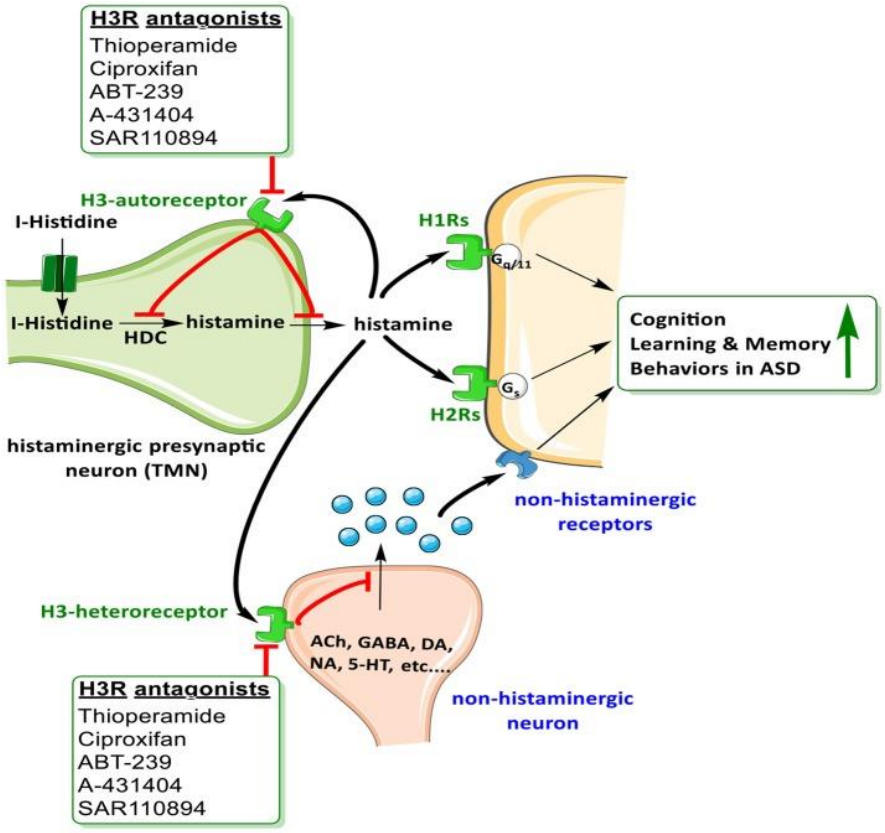


Figure 2: Schematic illustration of histaminergic System (HS).
Possible mechanisms by which histamine H3 receptor antagonists provide various pharmacological effects. Adapted from (Eissa, Al-Houqani, Sadeq et al., 2018).

1.2.1 Histamine receptors

Ash and Schild discovered the H1R in 1966, while studying the effect of antihistamine drugs in the rat uterus and stomach (Ash & Schild, 1966). The three other receptors (H2R, H3R, and H4R) were identified after that. The four receptors are

part of the GPCR superfamily, and they all present with constitutive activity (Bhowmik, Khanam, & Vohora, 2012; Nijmeijer, Leurs, & Vischer, 2010; Schneider, Schnell, Strasser et al., 2010; Threlfell, Exley, Cragg et al., 2008). Activation of these receptors without the presence of agonist was proven by the discovery of constitutively active mutant receptors (Seifert & Wenzel-Seifert, 2002). Ligand binding to a receptor may initiate activity (agonist with positive intrinsic activity) or abolish the effect of an agonist (antagonist with zero intrinsic activity). The agonists stabilize the receptor in active conformation, while the inverse agonists stabilize the receptor in inactive conformation hence, reduces the activity (negative intrinsic activity) (Tao, 2008). Two isoforms of the H3R are highly constitutively active: the wild type and an isoform with a deletion in the third intracellular loop (Bond & Ijzerman, 2006).

H1R is found in different tissues and cells, including smooth muscle, brain, and lymphocytes (Chang, Tran, & Snyder, 1979; Hill & Young, 1978). H1R signal transduction results in activation of phospholipase C, which promotes the inositol triphosphate-dependent release of Ca^{2+} from intracellular stores and diacylglycerol-sensitive activation of protein kinase C (Haas et al., 2008; Smit, Hoffmann, Timmerman et al., 1999). H1R is involved in the modulation of important processes and mice lacking this receptor display different impairments, such as, in spatial memory and in sleep-wake characteristics (Huang, Mochizuki, Qu et al., 2006; Masuoka & Kamei, 2007).

Similarly, as H1R, H2R presents with typical GPCR characteristics. Its activation stimulates adenylyl cyclase leading to cyclic adenosine monophosphate (cAMP) production, a second messenger that has different roles in the cell (Hegstrand, Kanof, & Greengard, 1976). The role of H2R in the CNS is not understood completely,

but basic research reports that H2R is related to the processes of learning and memory, motor control, and thermoregulation (Dai, Kaneko, Kato et al., 2007; Tabarean, Sanchez-Alavez, & Sethi, 2012).

H3R is mainly found in the brain, as reported it regulates food intake, memory, acetylcholine release, and consolidation of fear memories (Cangioli, Baldi, Mannaioni et al., 2002; Gemkow, Davenport, Harich et al., 2009). H3R's activation inhibits cAMP synthesis and activates MAP kinases and the AKT/GSK3β axis (Bakker, Timmerman, & Leurs, 2002; Bongers, Sallmen, Passani et al., 2007; Mariottini, Scartabelli, Bongers et al., 2009). When activated, the receptor inhibits cell firing and decreases the release of histamine by histaminergic neurons, moreover inhibits secretion of norepinephrine, serotonin, and other neurotransmitters by non-histaminergic neurons (Gomez-Ramirez, Ortiz, & Blanco, 2002; Threlfell et al., 2008; Yamasaki, Tamai, & Matsumura, 2001). Recently, mice lacking H3R reported several alterations. They presented with enhanced histaminergic neurotransmission, which led to changes in the behavioral phenotype (Gondard, Anaclet, Akaoka et al., 2013).

H4R was the last histamine receptor to be identified. It is mainly implicated with immune functions, however its presence in the brain, the bone marrow, peripheral blood, spleen, thymus, small intestine, colon, heart, and lung is known (Moya-Garcia, Rodriguez, Morilla et al., 2011; Nakamura, Itadani, Hidaka et al., 2000; Zampeli & Tiligada, 2009). It is involved in modulation of different processes, for example, dendritic cell activity, interleukin (IL) release, and likely regulation of blood brain barrier permeability (Karlstedt, Jin, & Panula, 2013; Mommert, Gschwandtner, Koether et al., 2012; Simon, Laszlo, Lang et al., 2011).

1.2.2 Implication of HS in brain disorders and ASD

The HS has been reported to be involved in several brain disorders such as in Alzheimer's disease (AD), schizophrenia (SCH), sleep disorders, drug dependence, and Parkinson's disease (PD) (Baronio, Gonchoroski, Castro et al., 2014).

In recent studies it was reported that histaminergic signalling abnormalities may contribute to rare diseases such as Tourette syndrome (TS) (Paschou, Fernandez, Sharp et al., 2013). TS was also reported to be among the most prevalently comorbid neurodevelopmental disorders with ASD (Gillberg & Billstedt, 2000), sharing genetic risk factors (Clarke, Lee, & Eapen, 2012; Fernandez, Sanders, Yurkiewicz et al., 2012). Additionally, both conditions share features of neuroinflammation (Kern, Geier, Sykes et al., 2015; Muller, 2007; Theoharides, Tsilioni, Patel et al., 2016), and increased microglia activation (Frick et al., 2016). Histamine plays a role in neuroinflammation (Jutel, Blaser, & Akdis, 2005; Theoharides et al., 2016), and microglia regulation (Dong et al., 2014; Ferreira et al., 2012; Frick et al., 2016; Rocha et al., 2014), suggests the HS may partly mediate the neuroinflammatory phenotype associated with these disorders. A study of TS reported a rare non-sense mutation in HDC, a gene encoding Histidine decarboxylase enzyme that synthesizes histamine from histidine (Karagiannidis, Dehning, Sandor et al., 2013). Other recent studies of ASD also implicate potential involvement of HS in this disorder as de novo deletions overlap in Histamine N-methyl transferase, a gene which encodes the enzyme that inactivates histamine (Griswold, Ma, Cukier et al., 2012; Mulatinho, de Carvalho Serao, Scalco et al., 2012). Furthermore, analysis of gene mapping of rare copy number variant in TS reported significant overlap with those revealed in ASD (Fernandez et

al., 2012). All of these findings and overlaps between the two disorders, raised the possibility of the implication of HS in ASD.

In addition, replicated findings suggested that SCH and ASD may share similar biological pathways demonstrating that both conditions have structural variation at regions 16p11.2 (McCarthy, Makarov, Kirov et al., 2009; Weiss, Shen, Korn et al., 2008), 22q11.2 (Guilmatre, Dubourg, Mosca et al., 2009; Vassos, Collier, Holden et al., 2010) and 1q21.1 (Brunetti-Pierri, Berg, Scaglia et al., 2008; Ikeda, Aleksic, Kirov et al., 2010; Mefford, Muhle, Ostertag et al., 2010; Stefansson, Rujescu, Cichon et al., 2008), which are susceptibility factors for a variety of neurodevelopmental phenotypes.Moreover, copy number variations and deleterious mutations in synaptic organizing proteins including *NRXN1* have been associated with both SCH and ASD (Gauthier, Siddiqui, Huashan et al., 2011). Other recent publications of single-genome wide copy number variation revealed rare variants at *NRXN1* and catenin alpha3 loci suggesting a risk factor overlap with both ASD and SCH. In addition to the genetic overlap between both disorders, they also share behavioral symptoms. Social cognitive impairments are a hallmark behavioral deficit of both ASD and SCH (Couture, Penn, & Roberts, 2006; Meyer, Feldon, & Dammann, 2011). Furthermore, neuroinflammation as a consequence of microglia activation plays an important role in both SCH and ASD (Nakagawa & Chiba, 2016). Considering these evidences, it is suggested that HS dysfunction may be involved in the aetiology of ASD, since both SCH and TS disorders have substantial genetic and symptomatic overlap with ASD.

1.2.3 Role of H3R antagonists in CNS diseases and ASD

Exploring the potential role of histamine H3R antagonist in number of CNS diseases like AD, epilepsy, attention deficit hyperactivity disorder (ADHD),

narcolepsy (Baronio et al., 2015.; Baronio et al., 2014; Kasteleijn-Nolst Trenite, Parain, Genton et al., 2013; Savage, Rosenberg, Wolff et al., 2010; Witkin et al., 2004), SCH (Passani & Blandina, 2011) and recently in Tics, TS (Rapanelli & Pittenger, 2016b) and ASD (Baronio et al., 2015), suggest that H3R antagonists may be a potential therapeutic agent for treatment of several symptoms including cognitive impairments. Investigations of H3R antagonist/inverse agonist GSK239512 to assess cognitive enhancing effects showed positive results on memory, attention (Nathan, Boardley, Scott et al., 2013) and displayed improvement in episodic memory in patients with AD (Grove, Harrington, Mahler et al., 2014). Preclinical studies reported that ciproxifan (CPX), an imidazole based H3R antagonist demonstrated improvements in hyperactivity and memory deficits after administration of this drug in a mouse model of AD (Bardgett, Davis, Schultheis et al., 2011). On the other hand, treatment with JNJ-10181457, a selective non-imidazole H3R antagonist reversed cognitive deficits induced by the muscarinic cholinergic antagonist scopolamine (Galici, Boggs, Aluisio et al., 2009), impairments that are commonly featured in ASD. These evidences suggest the potential role of H3R antagonists in rescuing core symptoms of ASD through modulation of cognitive functions. Cognition include self-regulation and social cognition that allows people to appropriately regulate actions related to social issues, and to make plans (Heatherton & Wagner, 2011).

Moreover, recent clinical trials demonstrated the successful effect of pitolisant (Wakix®), an H3R antagonist in treatment of narcolepsy (Baronio et al., 2014). Pitolisant is approved by the European Medicines Agency (EMA) and is the first in class drug to be introduced into clinics. Pitolisant was also suggested to be effective in

epilepsy, which is highly comorbid with ASD (Kasteleijn-Nolst Trenite et al., 2013). In addition, clinical studies revealed the positive effects of H1R and H2R antagonists in children and adolescence with ASD suffering from behavioral and sleep disturbances (Linday, Tsiouris, Cohen et al., 2001; Rossi, Posar, Parmeggiani et al., 1999). Again, accumulated evidence supports the implication of HS in ASD. Additionally, a recent study revealed that impairments in social behavior was ameliorated by H3R antagonists in rodents exposed to phencyclidine (PCP), suggesting its therapeutic value for ASD (Griebel, Pichat, Pruniaux et al., 2012). Based on these findings, Baronio et al. (2015) assessed for the first time the effect of imidazole based H3R antagonist CPX in an animal model of autism induced by prenatal exposure to VPA. The effect of acute administration of CPX (3 mg/kg) 30 minutes before the behavioral test demonstrated efficacy of improving some social impairments and stereotypies in VPA mice. These results suggested that some of the main clinical alterations displayed in ASD could be improved at late time stage, as at adulthood, although at that stage when the tests were carried out many changes had already occurred during brain development and have reached equilibrium. Regardless, a single application of CPX was sufficient to attenuate behavioral deficits (Baronio et al., 2015).

Although several imidazole based H3R antagonists as CPX, showed potency and selectivity in preclinical animal experiments with oral bioavailability (Ligneau, Lin, Vanni-Mercier et al., 1998; Stark, Sadek, Krause et al., 2000), this drug class appeared to have poor CNS penetration and incidence of off-target activity at H4R or other receptors. In addition, imidazole-based agents showed powerful CYP450 isoenzyme inhibitory properties developing many metabolic interactions (Berlin,

Boyce, & Ruiz Mde, 2011; Panula, Chazot, Cowart et al., 2015; Sadek & Stark, 2016e). Consequently, medicinal chemistry efforts developed new chemical entities of various non-imidazole H3R antagonists (Figure 3) with higher affinity and selectivity than the imidazole based H3R antagonist.

DL77, is a novel non-imidazole H3R antagonist that strongly resembles pitosilant in structure (Figure 4) (Sadek, Saad, Latacz et al., 2016b). In previous studies, DL77 showed improvements in cognitive performance by exerting its action through different memory stages, namely acquisition, consolidation, and retrieval (Sadek, Saad, Subramanian et al., 2016d). A very recent preclinical study demonstrated that DL77 ameliorated cognitive deficits induced by the N-methyl-D-aspartate (NMDA) receptor antagonist MK801 in an inhibitory passive avoidance paradigm and in novel object recognition tests in rats (Eissa, Khan, Ojha et al., 2018). These findings demonstrated the potential role of DL77 for treatment of neuropsychiatric disorders associated with cognitive symptoms (Sadek et al., 2016d). As mentioned earlier, social cognitive deficits are a hallmark characteristic of ASD (Couture et al., 2006). Moreover, H3R antagonist DL77 provided promising anticonvulsant activity in experimental epilepsy models (Sadek et al., 2016d). Clinical reports estimated that approximately 20-25% of children with ASD have epilepsy (Woolfenden, Sarkozy, Ridley et al., 2012). It was also reported in a recent population study that 44% of children with ASD were subsequently diagnosed with epilepsy and 54% of children with epilepsy were subsequently diagnosed with ASD (Jokiranta, Sourander, Suominen et al., 2014).

Additionally, several studies demonstrated that H3R antagonists are effective in behavioral impairment and memory deficit in animal model of SCH (Steele,

Minshew, Luna et al., 2007), symptoms which are also found in patients of ASD. However, the preclinical use of numerous non-imidazole-based H3R antagonists, e.g. ABT-239 and A-431404, ameliorated ketamine- and/or MK-801-induced cognitive impairments in experimental rats, demonstrating enhanced results when compared with reference antipsychotics like risperidone or olanzapine (Brown, Whitehead, Basso et al., 2013b).

To this end, based on the accumulation of evidences, the focus on pharmacological evaluation of H3R antagonist DL77 with its high *in-vitro* H3R affinity/selectivity and potent *in-vivo* H3R antagonist activity, on the ASD-like behavioral phenotypes in genetic (BTBR) and nongenetic (VPA induced) animal model of ASD has been proposed.

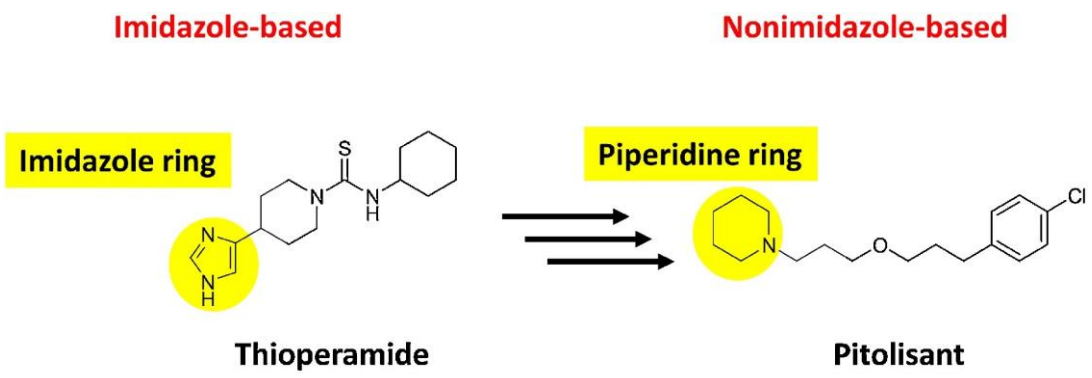


Figure 3: Structural development from imidazole-based to non-imidazole-based Histamine H3 receptor antagonist.
Adapted from (Sadek et al., 2016c).

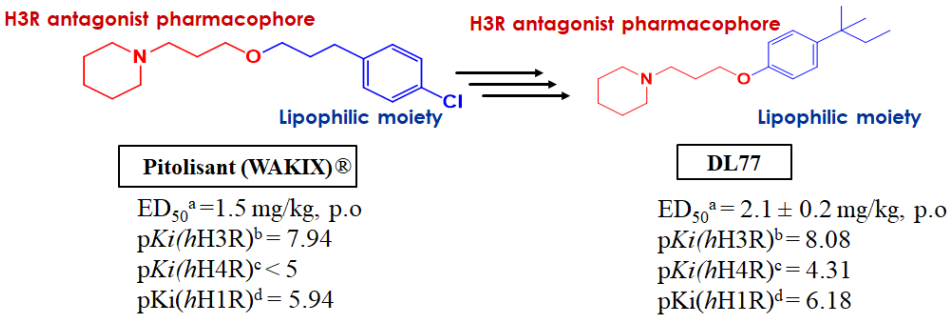


Figure 4: Structural relationship between Pitolisant and DL77.

Showing similar *in vitro* affinities, and *in vivo* antagonist potencies. [a] Central histamine H3R assay *in vivo* after p.o. administration to mice, n=3. [b] [^{125}I] Iodoproxyfan binding assay at human H3R stably expressed in CHO-K1 cells, n=3. [c][^{3}H] Histamine binding assay performed with cell membrane preparation of Sf9 cells transiently expressing the human histamine H4R and co-expressed with Gαi2 and Gβ1γ2 subunits, n=3. [d] [^{3}H] Pyrilamine binding assay performed with cell membrane preparation of CHO-hH1Rcells stably expressing the human H1R, n=3.

Abbreviations: H3R: H3 receptor; p.o.: peroral, CHO: Chinese hamster ovary Adapted from (Sadek et al., 2016b; Sadek et al., 2016d).

1.3 Correlation of neurotransmitter dysfunction in ASD

Research has also focused on the study of neurotransmitters, in search of sensitive and specific markers of ASD as well as potential therapeutic interventions. Several central neurotransmitters (e.g., 5-HT, ACh, DA, GABA and Glu) play a role in initial brain development and the etiology of ASD. Disruption of brain neurotransmitters early during the development phase of the CNS may provide a rational for pharmacological intervention that helps to cure and maybe even preclude some of the severe behavioral symptoms of ASD. Ideally, research in genetics may be able to explain these neurochemical defects at birth, providing possible appropriate insight into medical treatment for infants who are at increased risk for ASD. Growing evidence suggests that a variety of neurotransmitter systems such as ACh, 5-HT, DA, GABA, Glu, and HA are implicated in the onset and progression of ASD -along with genetic and environmental factors discussed below- (Bacchelli, Battaglia, Cameli et al., 2015; Chen, Davis, Guter et al., 2017; Ellenbroek & Ghiabi, 2015; Hellings,

Arnold, & Han, 2017; Hellmer & Nystrom, 2017; Naaijen, Bralten, Poelmans et al., 2017; Nakai, Nagano, Saitow et al., 2017; Paval, 2017; Paval, Rad, Rusu et al., 2017; Shah & Wing, 2006; Wang, Almeida, Spornick et al., 2015) (Figure 5).

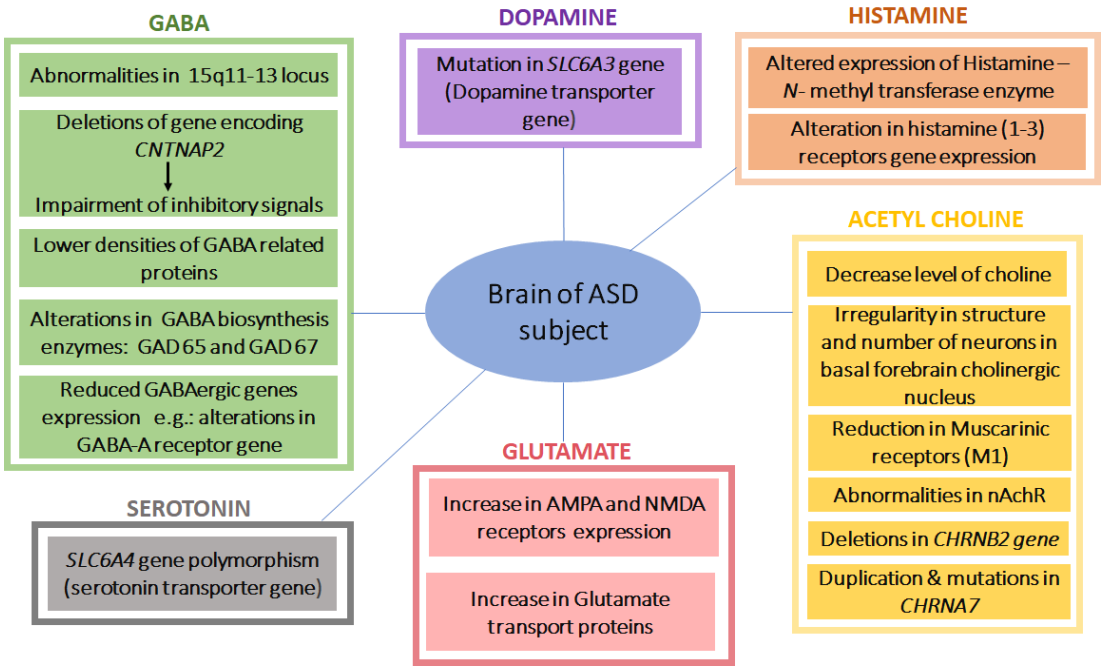


Figure 5: Reported findings in different brains of ASD patients with altered central transmission.
Adapted from (Eissa et al., 2018).

1.3.1 Serotonin

Among all neurotransmitters investigated so far in ASD, 5-HT has motivated the most research efforts and investigations. 5-HT signaling facilitates several neural processes including neurogenesis, cell migration and survival, synaptogenesis, and synaptic plasticity. Interestingly, high 5-HT levels in the blood have been described for up to 45% of tested ASD subjects (Chen, Penagarikano, Belgard et al., 2015; Chen et al., 2017; Ellenbroek, August, & Youn, 2016). Moreover, preclinical investigations using ASD-like animal models reported that hyperserotonemia significantly reduced the motivation for social interest through inhibition of separation distress, potentially

accounting for the social impairments found in ASD individuals (Ellenbroek et al., 2016; Nakai et al., 2017) . Furthermore, 5-HT was found to accumulate mainly in platelets utilizing the specific 5-HT transporter. In line with these findings, genetic studies of linkage stated that the 17q11.2 region containing the 5-HT transporter gene *SLC6A4* polymorphisms appear to be associated with ASD, as Gly56 conversion to Ala56 in the transporter protein resulted in autistic phenotypic features combined with an amplified p38-mitogen-activated protein kinases (MAPK)-sensitive basal phosphorylation process. In addition and in a previous study, higher clearance rates of hippocampal 5-HT were observed and hence hyperserotonemia, which led to a significant hypersensitivity of brain 5-HT (1A) as well as 5-HT (2A) receptors, social impairment and repetitive behavior (Veenstra-VanderWeele, Muller, Iwamoto et al., 2012) (Figure 5).

1.3.2 Dopamine

Dopamine (DA) plays a fundamental role in brain functioning, and the pathophysiological role of dopaminergic system (DS) deficits in ASD is well recognized, with the wide clinical use of antipsychotics that mainly target the D2 receptors (D2Rs) (Baronio et al., 2014; Seeman, 2010) Interestingly, and in a very recent preclinical study, it has been shown that mice with increased dopaminergic neurotransmission in the dorsal striatum via the suppression of dopamine transporter (DAT) expression in substantia nigra neurons or the optogenetic stimulation of the nigro-striatal circuitry exhibited significant deficits in sociability and repetitive behaviors relevant to ASD pathology in several rodent models, while these behavioral changes were blocked by using D1R antagonists (Lee, Kim, Kim et al., 2017). Therefore, D1R agonists produced typical autistic-like behaviors in normal mice or the

genetic knockout (KO) of D2Rs (Lee et al., 2017). Furthermore, the siRNA-mediated inhibition of D2Rs in the dorsal striatum was shown to replicate ASD-like phenotypes in D2R KO mice (Lee et al., 2017). With regard to the DS, genetic studies have demonstrated that mutations of DS-associated genes such as the DAT (Hamilton, Campbell, Sharma et al., 2013), DA receptors (Hettinger, Liu, Schwartz et al., 2008; Qian, Chen, Forssberg et al., 2013), and enzymes of DA biosynthesis (Nguyen, Roth, Kyzar et al., 2014) are implicated in ASD. These studies expanded the evidence of genetically linking between DA transporter and ASD (Bowton, Saunders, Reddy et al., 2014; Hamilton et al., 2013). ASD is strongly associated with a mutation in the DA transporter gene *SLC6A3*, which codes for a protein that contributes to regulation of DA levels in the brain. In fact, DAT is the crucial regulator of DA homeostasis and alteration of this homeostasis as a consequence of DAT dysfunction is associated with ASD and other neuropsychiatric conditions (Hamilton et al., 2013; Hellings et al., 2017; Paval, 2017; Paval et al., 2017). Moreover, it has been found that the dopaminergic fibers arising from the ventral tegmental area (VTA) project to the prefrontal cortex and to regions of the limbic system, such as the nucleus accumbens (NAcc), forming the mesocorticolimbic (MCL) circuit. This circuit was found to be involved in high order brain functions, such as emotional social behavior, reward, motivation and cognition (Paval et al., 2017) (Hellings et al., 2017) (Figure 5). Notably, the DS has been related to behavioral skills belonging to executive functioning, such as analyzing, planning and prioritizing (Baronio et al., 2014; Seeman, 2010). In line with that, children diagnosed with ASD were found to show deficits in tasks linked to executive functioning, including response/selection, planning/working memory and flexibility (Hellings et al., 2017; Paval et al., 2017).

Moreover, the DS has strongly been linked to social behavior, attentional skills and perception, and motor activity, while developmental abnormalities in these areas have all been linked to ASD as well (Hellings et al., 2017; Paval et al., 2017)

1.3.3 GABA and Glutamate

GABAergic as well as glutamatergic systems are also proposed as potential mechanisms for ASD. Consequently, mutations in the respective synaptic proteins would lead to defective neurotransmission at excitatory and inhibitory synapses, leading to disruption of excitatory-inhibitory balance of neurotransmission in postsynaptic neurons, a key mechanism which has strongly been associated with ASD (Jamain, Betancur, Quach et al., 2002; Naaijen et al., 2017). In addition to numerous reports stating the duplication of 15q11-13 locus in ASD populations, a majority of the cases do not show mutations at this locus. However, they do exhibit abnormalities in the expression of protein encoded by 15q11-13 gene, as reported. These results demonstrated the involvement of 15q11-13 locus in ASD, either directly by mutation or indirectly by epigenetic factors (Hogart, Nagarajan, Patzel et al., 2007; Nakai et al., 2017). This site employs numerous genes coding for particular subunits of GABA receptors, namely GABRB3, GABRA5 and GABRG3. There are numerous signalling and scaffolding proteins which are implicated in normal development and GABA synapse function. Therefore, mutations in the genes encoding these proteins consequently result in GABAergic dysfunction, hence inhibitory signalling deficits. For example, deletions in gene encoding a protein contactin-associated protein 2 (CNTNAP2) has been linked to autism (Gregor, Albrecht, Bader et al., 2011; Nord, Roeb, Dickel et al., 2011; Stephan, 2008). Moreover, deficiency in CNTNAP2 results in reduction in GAD1 (Glutamate Decarboxylase 1), parvalbumin and inhibitory

interneurons along with impairments in inhibitory signalling (Penagarikano, Abrahams, Herman et al., 2011; Soghomonian, Zhang, Reprakash et al., 2017). This evidence suggests an association between mutations affecting the function of GABA and ASD. Furthermore, reduced expression of GABAergic genes and lower density of GABA related proteins have been found in brains of ASD patients. Consequently, alterations in GABA-A receptor genes and other genes expressed on GABA interneurons, as well as GABA biosynthesis enzymes (GAD 65 and GAD 67) in the cerebellum and parietal cortex, are strongly implicated in ASD (Coghlan, Horder, Inkster et al., 2012; Fatemi, Halt, Stary et al., 2002).

On the contrary, an increased level in the expression of excitatory Glu receptor AMPA, and of Glu transporter proteins were observed in individuals diagnosed with ASD, with highest expression abnormalities found in the cerebellum (Purcell, Jeon, Zimmerman et al., 2001) (Figure 5). Based on the aforementioned, altered expression of genes related to GABA and/or Glu might be linked to several ASD phenotypic features including cognitive deficits and/or hyperactivity (Jamain et al., 2002; Naaijen et al., 2017). Moreover, abnormalities in both neurotransmitters including an increase in the excitatory Glu and/or decrease in the inhibitory GABA were found to lead to epileptic seizure, a clinical feature which is commonly observed in ASD patients (Ballaban-Gil & Tuchman, 2000; Gillberg, Lundstrom, Fernell et al., 2017; Levisohn, 2007).

1.3.4 Acetylcholine

The brain cholinergic neurotransmission system with ACh plays an essential role in regulating ASD-related behavioral symptoms including attention (Arnold, Burk, Hodgson et al., 2002), cognitive flexibility (Ragozzino, Pal, Unick et al., 1998),

social interaction (Avale, Chabout, Pons et al., 2011) and stereotypical behaviors (Bacchelli et al., 2015; Hellmer et al., 2017; McConville, Sanberg, Fogelson et al., 1992; Wang et al., 2015). Mounting evidence suggests the involvement of cholinergic system dysfunction in the phenotypic outcomes of ASD-related behavioral features, in both humans and animal models - (Karvat & Kimchi, 2014). In ASD patients, there are remarkable abnormalities in the cholinergic system. Anatomically there is irregularity in the number and structure of neurons in a basal forebrain cholinergic nucleus of patients diagnosed with ASD (Kemper & Bauman, 1998). Also, a decrease in the level of choline, the precursor of the neurotransmitter ACh and the agonist for nicotinic-cholinergic receptor (nAChR), was determined in individuals diagnosed with ASD (Friedman, Shaw, Artru et al., 2006), with the severity of ASD related to the decreased level of cytosolic ACh (Sokol, Dunn, Edwards-Brown et al., 2002). Moreover, and by using immunohistochemical analyses, abnormalities in nAChR were observed in several brain regions (e.g. neocortex, cerebellum, thalamus and striatum) of ASD patients, specifically with the major abnormalities being reduction in nAChR subunits and muscarinic receptors (M1 type) (Mukaetova-Ladinska, 2017; Mukaetova-Ladinska, Westwood, & Perry, 2010). Moreover, brain ACh like several other brain neurotransmitters, e.g. DA, 5-HT and GABA, playing a key role in ASD are regulated by different central mechanisms including histamine H3 hetero-receptors (H3Rs), highlighting the potential for HS involvement in ASD. Genetically, studies reported duplications and mutations in *CHRNA7*, the gene encoding the α7nAChR subunit, in ASD patients (Leblond, Heinrich, Delorme et al., 2012), (Mikhail, Lose, Robin et al., 2011), whereas deletion of the *CHRNB2* gene, encoding the β2-subunit of nAChR was observed in other cases (Granon, Faure, & Changeux, 2003).

Furthermore, autism related behavior may be linked to M1 type mAChR inhibition (McCool, Patel, Talati et al., 2008) and cholinergic cell damage (Walker, Diefenbach, & Parikh, 2007) (Figure 5). Consequently, social deficits and repetitive behaviors are the main phenotypic ASD features connected to disruption in cholinergic neurotransmission system (Wang et al., 2015). Also, reduced attention (Arnold et al., 2002), decreased cognitive flexibility (Ragozzino et al., 1998), reduced social communications (Avale et al., 2011) and conventional behaviors have been strongly linked to cholinergic neurotransmission dysfunction (Bacchelli et al., 2015; Hellmer et al., 2017; McConville et al., 1992; Wang et al., 2015).

1.3.5 Histamine

As mentioned earlier, the brain HS was found to display a critical role in cognition, sleep and other neuropsychiatric disorders including SCH and TS that share comorbidity with ASD (Wright, Shin, Rajpurohit et al., 2017). Moreover, in addition to the altered gene expression found for histamine-*N*-methyltransferase (HNMT) enzyme , an enzyme responsible for metabolism of central histamine (HA), gene alteration was also reported for histamine receptor subtypes H1-, H2-, and H3R (Wright et al., 2017). Notably, there has been a rising interest in studying the role of brain HA on behaviors in both physiological conditions and psychiatric diseases, e. g. SCH (Sadek et al., 2016c; Sadek & Stark, 2016e). Interestingly and as revealed in several clinical reports, patients with SCH shared a variety of common symptoms and genetic factors with ASD (Carroll & Owen, 2009; Konstantareas & Hewitt, 2001). Moreover, a significant role for the brain HA has been projected, and a range of several H3R ligands has been developed so far for dual-targeting of both dopaminergic as well as histaminergic neurotransmissions (Baronio et al., 2014; Bishara, 2010).

Furthermore and as discussed above, deficits in regulations of various other neurotransmitters including DA, 5-HT, GABA, and Glu are postulated (Witkin et al., 2004). Therefore, the role of central HA which influences behavior in CNS disorders, may be considered an important pharmacological target for therapeutic purposes (Haas et al., 2008; Naddafi & Mirshafiey, 2013; Shan, Bossers, Luchetti et al., 2012; Tiligada, Kyriakidis, Chazot et al., 2011). There is particular interest in brain H3Rs, as brain H3Rs act as auto-receptors or hetero-receptors and regulate the biosynthesis and release of HA and several other neurotransmitters, which consequently play a role in cognitive and homeostatic processes, as shown in (Figure 2 & 5). Therefore, there is an indirect indication that histaminergic neurotransmission may have a significant role in SCH and that potent and selective H3R antagonists could lead to therapeutic improvements of cognitive symptoms associated with SCH and ASD (Brown, Whitehead, Basso et al., 2013a; Esbenshade, Browman, Bitner et al., 2008; Sadek et al., 2016c; von Coburg, Kottke, Weizel et al., 2009; Witkin et al., 2004).

To date there are scarce literature studies concerning the association of H3R antagonists and treatment of ASD behavioral deficits. Accordingly and in a previous preclinical study, the imidazole-based H3R antagonists, namely thioperamide and ciproxifan, improved impaired prepulse inhibition in an animal model of SCH (Brown et al., 2013a) (Figure 2). Thioperamide and ciproxifan are selective and potent imidazole-based H3R antagonists that are widely used in preclinical animal experiments (Ligneau et al., 1998; Stark et al., 2000). (Brown et al., 2013a). Interestingly, H3R antagonists possess antioxidant activity which could increase their potential clinical use, since oxidative stress is, also, considered as possible mediators of intensified symptoms of SCH and ASD (Mahmood, Khanam, Pillai et al., 2012b).

Moreover, a study considering the effectiveness of the non-imidazole H3R antagonist ABT-288 in the treatment of cognitive deficits linked with SCH revealed that schizophrenic features remained constant during the whole time period of the study, with acceptable safety, tolerability and pharmacokinetic profile of ABT-288 at a 15-fold higher dose and 12-fold higher exposures in subjects with SCH than previously observed in healthy volunteers (Coruzzi, Pozzoli, Adami et al., 2012) (Hsieh, Chandran, Salyers et al., 2010). Moreover, the use of a H3R antagonist ameliorated behavioral impairments in an animal model of SCH, including spatial working memory deficit, an abnormality which also characterizes patients with ASD (Steele et al., 2007). Also, as mentioned previously, a recent preclinical study reported that an acute systemic administration of ciproxifan palliated some sociability impairments and stereotypic behavior in an animal model of VPA-induced ASD in mice (Baronio et al., 2015). However, further research efforts are still necessary to support these initial observations and to achieve enhanced understanding of pathophysiology and therapeutic management of ASD. Based on the aforementioned observations, numerous brain neurotransmitters, e.g. 5-HT, DA, GABA, Glu, ACh, and HA appear to be implicated in the pathophysiology of ASD, since disruption of genes encoding different proteins (receptors and/or catalysing biosynthesis enzymes for neurotransmitters) that affect the respective neurotransmission functionality leads to a variety of phenotypic features of ASD. Interestingly, the above discussed dysfunctions of neurotransmitters, namely 5-HT, DA, GABA, Glu, ACh, and HA were, also, found to be influentially implicated in the clinical outcome features of SCH, a disorder that is comorbid and shares multiple aetiologies and risk factors with ASD (Chisholm, Lin, Abu-Akel et al., 2015; Devor, Andreassen, Wang et al., 2017).

1.4 Current pharmacological intervention

Based on the aforementioned abnormalities in genes as well as various neurotransmitter systems, studying the effects of a given drug on core symptoms in ASD is very challenging. Despite advances in early diagnosis and intervention, efficacious reversal of core autistic symptoms is still not accomplished to date. At the present time there is no definite pharmacological treatment for ASD but treatments for ASD are based on behavioral therapies and the use of highly controlled learning environments. The recent approaches to treatment of ASD set behavioral therapy and atypical language development, as keystone for ASD therapy along with other treatments that tends to ameliorate associated symptoms and not the core deficits (Srinivasan, 2009). The heterogeneity of clinical and behavioral features in children diagnosed with ASD contributes to the difficulty in understanding the pathophysiology of this disorder, and consequently, no specific treatment can be effective for all ASD children. Therefore, subgrouping of children based on responses to intervention is essential (James et al., 2009). As mentioned above, targeting ASD core symptoms for complete and effective treatment has been challenging and not yet achieved, however several pharmacological medications maybe effective in various associated symptoms that often cause significant impairments in ASD (Volkmar, Cook, Pomeroy et al., 1999). These associated symptoms of ASD include inattention, hyperactivity, anxiety, sleep disturbances, irritability, repetitive behavior, aggression and self-injury. Antipsychotics are often used for therapeutic management of ASD symptoms in children (Findling, 2005). Currently, atypical antipsychotics risperidone and aripiprazole are the only two drugs which have so far been approved by FDA for improving behavioral symptoms associated with ASD (Matson et al., 2011), however,

there are several other pharmacological interventions that show effective clinical management of ASD symptoms. The most promising drugs reported for managing behavioral and neurological symptoms of ASD are acting on different brain targets and are summarized in (Table 1). Accordingly, therapeutic benefits have been observed and described with several classes of drugs including selective serotonin reuptake inhibitors (sertraline, citalopram, fluoxetine) for anxiety and repetitive behaviors, psychostimulant (methylphenidate) for hyperactivity, opioid antagonist (naltrexone) for hyperactivity, and atypical antipsychotics (risperidone, olanzapine, clozapine) for temper tantrums, aggression, or self-injurious behavior (Aman, 2004; Kumar, Prakash, Sewal et al., 2012; Moore, Eichner, & Jones, 2004). Notably, numerous candidates of the classes discussed below have progressed to several phases of clinical trials.

1.4.1 Atypical antipsychotics

Clozapine belongs to the class of atypical antipsychotics because it shows capability of binding to 5-HT and DA receptors (Chen, Bedair, McKay et al., 2001; Gobbi & Pulvirenti, 2001; Zuddas, Ledda, Fratta et al., 1996). It is an atypical antipsychotic medication mainly used for SCH that does not improve following use of other antipsychotic medications. In patients with SCH and schizoaffective disorder it may decrease the rate of suicidal behavior. It is possibly more effective than typical antipsychotics and in patients who are resistant to other medications. Clozapine was found to improve hyperactivity and aggression in ASD children, adolescents and adults, but has a limited clinical use because of its hematological safety profile, the potential to lower the seizure threshold in patients, necessitating therapeutic monitoring of patients taking this medication (Chen et al., 2001; Gobbi et al., 2001;

Zuddas et al., 1996). The antipsychotic medication with risperidone is mainly used in ASD patients with SCH, bipolar disorder, and/or irritability symptoms, as this drug has been revealed to be superior to placebo in treating irritability, repetitive behavior, aggression, anxiety, depression and nervousness (McCracken, McGough, Shah et al., 2002; McDougle, Holmes, Carlson et al., 1998). Moreover, risperidone has shown a neuroprotective effect and has enhanced the antioxidant and neuroprotective activity of astroglia cells in brain disorders such as ASD without clinical evidence of extrapyramidal side effects or seizures except mild sedative effects (McCracken et al., 2002; McDougle, Holmes, et al., 1998). However, other side effects with use of risperidone were reported to include increased appetite, fatigue, dizziness and drowsiness (Table 1) (McCracken et al., 2002; McDougle, Holmes, et al., 1998). Aripiprazole is another atypical antipsychotic primarily recommended for the treatment of SCH and bipolar disorder. Other uses include an add-on treatment for major depressive disorder, tic disorders and irritability associated with ASD. It shows a different mechanism of action from those of the other atypical antipsychotics (e.g., clozapine, and risperidone) by acting rather as a partial agonist on the D2Rs and 5-HT1A receptors (Hirsch & Pringsheim, 2016; Marcus, Owen, Kamen et al., 2009; Owen, Sikich, Marcus et al., 2009). However, it displays an antagonist profile at 5-HT2A and 5-HT7 receptors and acts as a partial agonist at the 5-HT2C receptor, both with high affinity (Hirsch et al., 2016; Marcus et al., 2009; Owen et al., 2009). The latter action may be the reason for the minimal weight gain observed during the course of therapy with aripiprazole.

1.4.2 Neurotransmitter reuptake inhibitors

The class of neurotransmitter reuptake inhibitors, e.g. fluoxetine (selective

serotonin reuptake inhibitor) (SSRI) has shown numerous prospective therapeutic benefits, including decreases in rituals, stereotyped and repetitive monotonous behaviors in ASD children and adolescents (DeLong, Ritch, & Burch, 2002; Fatemi, Realmuto, Khan et al., 1998; Hollander, Phillips, Chaplin et al., 2005). Fluoxetine is a SSRI which does not significantly inhibit norepinephrine and dopamine reuptake at therapeutic doses (DeLong et al., 2002; Fatemi et al., 1998; Hollander et al., 2005). It does, however, delay the reuptake of serotonin, resulting in serotonin persisting longer when it is released (DeLong et al., 2002; Fatemi et al., 1998; Hollander et al., 2005). Fluoxetine was found to produce some adverse effects including disinhibition, hypomania, agitation, and hyperactivity (DeLong et al., 2002; Fatemi et al., 1998; Hollander et al., 2005). Fluvoxamine is a drug which functions as a SSRI and σ1 receptor agonist (DeLong et al., 2002; Fatemi et al., 1998; Hollander et al., 2005). Fluvoxamine is used mainly for the treatment of obsessive-compulsive disorder and is also used to treat major depressive disorder and anxiety disorders such as panic disorder and post-traumatic stress disorder. Notably, fluvoxamine is approved to treat social anxiety disorder (DeLong et al., 2002; Fatemi et al., 1998; Hollander et al., 2005). Fluvoxamine has also shown similar potential effects as fluoxetine against autistic disorder (DeLong et al., 2002; Fatemi et al., 1998; Hollander et al., 2005). In a clinical trial, fluvoxamine was found to be well tolerated in ASD adults and it has improved compulsive as well as repetitive behaviors and aggression (DeLong et al., 2002; Fatemi et al., 1998; Hollander et al., 2005). Other SSRIs, including sertraline, paroxetine and escitalopram, showed almost the same potential benefits and adverse effects as compared to fluoxetine and fluvoxamine (Hellings, Kelley, Gabrielli et al., 1996; McDougle, Brodkin, Naylor et al., 1998). Venlafaxine is another SSRI which

has, also, shown improvements of restricted behaviors, decreased interests, social deficits, hyperactivity and communication problems in individuals with ASD (Hellings et al., 1996; McDougle, Brodkin, et al., 1998) (Table 1).

1.4.3 Tricyclic antidepressants

The second-generation tricyclic antidepressant nortriptyline is used in the therapeutic management of major depression and childhood nocturnal enuresis (bedwetting), chronic fatigue syndrome, chronic pain and migraine, and labile affect in some neurological brain disorders (Campbell, Fish, Shapiro et al., 1971; Kurtis, 1966). Clomipramine is another tricyclic antidepressant used for the treatment of obsessive-compulsive disorder, panic disorder, major depressive disorder, and chronic pain (Gordon, State, Nelson et al., 1993; Sanchez, Campbell, Small et al., 1996). Interestingly, nortriptyline has been described to be effective in children with ASD as it improved the hyperactivity, aggressiveness, and ritualized behavior, while imipramine was not well tolerated in ASD children (Campbell et al., 1971; Kurtis, 1966). In a previous clinical trial, 58% of ASD subjects have found clomipramine to be superior to placebo and the antidepressant desipramine in improving ASD symptoms, anger, and compulsive and ritualized behaviors (Gordon et al., 1993; Sanchez et al., 1996). In another clinical study, clomipramine has caused several adverse effects such as sedation and worsening of behaviors like aggression, irritability, and hyperactivity (Gordon et al., 1993; Sanchez et al., 1996) (Table 1).

1.4.4 Anticonvulsants

Lamotrigine, a member of the sodium channel blocking class of anticonvulsants clinically used in the treatment of children diagnosed with epilepsy,

decreased symptoms in approximately 62% of ASD individuals, and no considerable change among placebo-treated and lamotrigine-treated patients was observed in a study comprising 35 patients diagnosed with ASD (Uvebrant & Bauziene, 1994). On the contrary, VPA with its anticonvulsant effect due toblockade of voltage-dependent sodium channels and increased GABA levels in the brain, has shown -as an orphan drug- valuable effects in improving various symptoms and psychiatric comorbidities, e.g. receptive language, affective instability, and aggression, without appreciable clinical effects on core symptoms of ASD (Jobski, Hofer, Hoffmann et al., 2017; Uvebrant et al., 1994). Notably, VPA has been reported to be an inhibitor for histone deacetylase (HDAC), an enzyme which plays –together with other HDACs- an essential regulating role in gene transcription and phenotypic differentiation (Balasubramaniyan, Boddeke, Bakels et al., 2006; Chomiak, Turner, & Hu, 2013; Hsieh & Gage, 2004, 2005; Jessberger, Nakashima, Clemenson et al., 2007). Accordingly, numerous studies reported that specific expression forms of HDAC1 and HDAC2, which are categorized as class I of HDACs, in the murine brain are existent at various developmental ages with HDAC1 expressed in neural stem cells/progenitors and glia, and HDAC2 being upregulated in postmitotic neuroblasts and various but not in fully differentiated glia (Chomiak et al., 2013). Therefore, modulation of HDAC in diverse cell types and at several maturational time points may possibly lead to the observation of intensely diverse clinical outcomes and may explain why HDAC inhibition (for instance with VPA) in adulthood leads to improvement of ASD-like features in animals exposed prenatally to VPA (Chomiak et al., 2013). In previous studies, in utero exposure to VPA in mice induced ASD-like behavioral social interaction deficits, anxiety and spatial learning incapacity (Kataoka, Takuma, Hara et

al., 2013). However, all these behavioral impairments were ameliorated following chronic (5-weeks) treatment with VPA (30 mg/kg/d, i.p.) (Kataoka et al., 2013; Takuma, Hara, Kataoka et al., 2014), suggesting that dose (300-600 mg/kg for acute induction of ASD or 30 mg/kg for chronic treatment goals) or time point (prenatal or 8-week old animals) of using VPA determines whether it is ASD-inducing or palliating cognitive dysfunction (Takuma et al., 2014). VPA-induced ASD in rodents will be discussed in more details under section Non-genetic Animal Models of ASD. Moreover, the anticonvulsant drug levetiracetam was found to be valuable in decreasing hyperactivity, impulsivity, aggression, and emotional lability (Rugino & Samsock, 2002) (Table 1). These clinical observations for several antiepileptic drugs demonstrate that the prevalence of psychopharmacotherapy and polypharmacy in ASD patients is considerable, which is directed to the treatment of non-core ASD symptoms and psychiatric comorbidities, with a lack of pharmacological treatment options for ASD core symptoms.

1.4.5 Glutamate antagonists

Levels of glutamate have been found to be excessively increased in post-mortem brain samples of some ASD individuals (Chez, Burton, Dowling et al., 2007; Owley, Salt, Guter et al., 2006). Numerous studies have publicized the effectiveness of several glutamate antagonists, e.g. amantadine and memantine, in ASD patients (Chez, Burton, et al., 2007; Owley et al., 2006). In a controlled clinical trial, amantadine showed improving effects on hyperactive behavior and inappropriate speech in ASD children (Chez, Burton, et al., 2007; Owley et al., 2006). Also, the clinical use of memantine in the treatment of ASD individuals has shown therapeutic progress with regard to memory, hyperactivity, irritability, language, social behavior

and self-stimulatory behavior (Chez, Burton, et al., 2007; Owley et al., 2006) (Table 1).

1.4.6 Acetylcholinesterase inhibitors

Dysfunction of brain cholinergic neurotransmission has been described in several patients diagnosed with ASD (Chez, Aimonovitch, Buchanan et al., 2004; Hardan & Handen, 2002; Nicolson, Craven-Thuss, & Smith, 2006; Niederhofer, Staffen, & Mair, 2002; Perry, Lee, Martin-Ruiz et al., 2001). Therefore, acetylcholinesterase inhibitor (AChEI) e.g. rivastigmine, donepezil, and galantamine, have in many studies been investigated for the use in ASD children (Chez et al., 2004; Hardan et al., 2002; Nicolson et al., 2006; Niederhofer et al., 2002). Interestingly, the clinical application of rivastigmine in ASD children significantly relieved overall ASD behaviors, however, several adverse effects including nausea, diarrhoea, hyperactivity and irritability were reported (Chez et al., 2004; Hardan et al., 2002; Nicolson et al., 2006; Niederhofer et al., 2002). Among AChEI, donepezil has shown capability to improve irritability and hyperactivity of ASD children (Chez et al., 2004; Hardan et al., 2002; Nicolson et al., 2006; Niederhofer et al., 2002). Moreover, galantamine produced substantial improvements in hyperactivity, irritability, social withdrawal, inappropriate speech, attention deficiency, and reduction in anger in children diagnosed with ASD (Chez et al., 2004; Hardan et al., 2002; Nicolson et al., 2006; Niederhofer et al., 2002) (Table 1). These improvements observed for several AChEI strongly support the hypothesis that enhancing cholinergic neurotransmission in ASD results in positive therapeutic effects.

1.4.7 Psychostimulants

Methylphenidate, the most commonly known CNS stimulant widely used in the therapeutic management of ADHD and narcolepsy, is commonly indicated for autistic children and adolescents (Di Martino, Melis, Cianchetti et al., 2004; Handen, Johnson, & Lubetsky, 2000; Jahromi, Kasari, McCracken et al., 2009; Kim, Shonka, French et al., 2017). Methylphenidate mainly acts as a norepinephrine-dopamine reuptake inhibitor. In numerous controlled studies, methylphenidate palliated several behavior ASD features including impulsivity, attention deficiency, and hyperactivity, but it correspondingly exhibited some initial adverse effects such as aggression, anorexia, and increased wakefulness (insomnia) (Di Martino et al., 2004; Handen et al., 2000; Jahromi et al., 2009; S. J. Kim et al., 2017) (Table 1).

1.4.8 Adrenergic alpha $(\alpha)_2$ receptor agonists

Oral or transdermal administration of selective centrally acting α_2 adrenergic agonist, e.g. clonidine, have revealed to improve mood instability, hyperactive behavior, , aggressiveness and nervousness in ASD individuals (Fankhauser, Karumanchi, German et al., 1992; Ming, Gordon, Kang et al., 2008). Clonidine is a drug used to treat high blood pressure, ADHD, anxiety disorders, tic disorders, withdrawal (from either alcohol, opioids, or smoking), migraine, and certain pain conditions, with largely tolerable adverse effects (Fankhauser et al., 1992; Ming et al., 2008). Also, previous clinical trials carried out with clonidine in ASD subjects have demonstrated clinical effectiveness and safety profile in ASD and related brain disorders (Fankhauser et al., 1992; Ming et al., 2008). Moreover, a retrospective study revealed that the use of guanfacine, a selective α_2 adrenergic receptor agonist used in

the treatment of ADHD, anxiety, and hypertension, was accompanied with improvements in insomnia, attention deficiency, hyperactivity and tics (Boellner, Pennick, Fiske et al., 2007; Posey, Puntney, Sasher et al., 2004; Scahill, Aman, McDougle et al., 2006). However, the most common adverse effects observed with guanfacine were mood alteration fatigue, blurred vision, and headache (Boellner et al., 2007; Posey et al., 2004; Scahill et al., 2006) (Table 1).

1.4.9 Opiate antagonists

Based on the reputed role of endogenous opioids such as β-endorphin and encephalin in the regulation of social behavior, the opiate antagonist naltrexone has been assessed in ASD (Bouvard, Leboyer, Launay et al., 1995; Clifford, Dissanayake, Bui et al., 2007; Elchaar, Maisch, Augusto et al., 2006; Kolmen, Feldman, Handen et al., 1995; Panksepp & Lensing, 1991). The results observed in numerous studies for naltrexone showed that it might be able to treat behavioral aberrations perceived in ASD patients and induced by dysfunction of the brain opioid system (Bouvard et al., 1995; Clifford et al., 2007; Elchaar et al., 2006; Kolmen et al., 1995; Panksepp et al., 1991). Moreover, numerous studies revealed the significant improvements of various behavioral symptoms obtained with the use of naltrexone in ASD children (Bouvard et al., 1995; Clifford et al., 2007; Elchaar et al., 2006; Kolmen et al., 1995; Panksepp et al., 1991). Furthermore, these studies reported that naltrexone treatment provided substantial enhancements in self-injurious behavior, hyperactivity, social withdrawal, agitation and irritability in ASD patients (Bouvard et al., 1995; Clifford et al., 2007; Elchaar et al., 2006; Kolmen et al., 1995; Panksepp et al., 1991) (Table 1).

The aforementioned described drugs acting at different targets are used to therapeutically manage the behavioral as well as psychiatric symptoms of ASD.

However, several brain regions are altered in ASD individuals, resulting in loss of neuronal function, and behavioral and sensory impairments, including inattention, hyperactivity, mood fluctuations, aggressiveness, agitation, social deficits and repetitive and restricted behavior. In the brain, the plasticity of brain tissue, nerve connections and balance of several neurotransmitters are all implicated in pathophysiology of ASD individuals. Apart from environmental factors, other pathological conditions such as autoimmunity, chronic neuroinflammation, oxidative stress, mitochondrial dysfunction are involved in etiopathogenesis of ASD (Kumar et al., 2012).

Up until now, there is no approved drug existing in the market, which is specific for treating symptoms associated with ASD, however, preclinical and clinical research and development are in progress to find new therapeutic entities. Currently, there are several candidates that successfully passed different clinical phases of drug development, with different pharmacological targets to modulate ASD behavioral and neurological symptoms**.** Interestingly, 10% of these clinical candidates are currently in phase 1, 46% have already progressed to phase 2, 27% advanced to phase 3, and 15% reached to phase 4 of clinical development, whereas 2% are currently of preclinical interest (Ruhela, Prakash, & Medhi, 2015)

Table 1: Selected medications of different classes that are currently used to manage different symptoms of ASD

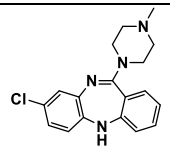
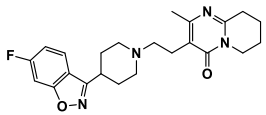
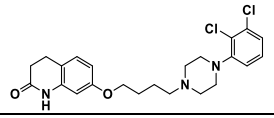
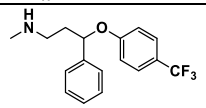
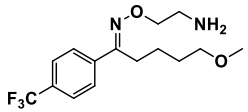
Class	Medication	Structure	Pharmacological effects	Side effects	Reference
Atypical Antipsychotics	Clozapine		Improves hyperactivity and aggression in ASD patients	Requires patient's haematological safety monitoring and it lowers seizure threshold	(Chen et al., 2001; Gobbi et al., 2001; Sahoo, Padhy, Singla et al., 2017; Zuddas et al., 1996)
	Risperidone		Reduces irritability, repetitive behavior, aggression, anxiety, and depression & nervousness. It shows neuroprotective activity, modulates astroglia function, and increases the brain antioxidant activity.	Mild sedation, increased appetite, fatigue, dizziness, drowsiness, tremor, and constipation	(Hara, Ago, Taruta et al., 2017; McCracken et al., 2002; McDougle, Holmes, et al., 1998)
	Aripiprazole		Reduces autistic symptoms in children such as irritability, stereotypy, and hyperactivity	Fatigue, vomiting, weight gain, tremor, and extrapyramidal symptoms	(Hirsch et al., 2016; Marcus et al., 2009; Owen et al., 2009)
Selective Serotonin Reuptake inhibitor (SSRI)	Fluoxetine		Reduces stereotyped and repetitive behavior in children and adolescents with ASD.	Hypomania. agitation, and hyperactivity	(DeLong et al., 2002; Fatemi et al., 1998; Hendriksen, Klinkenberg, Collin et al., 2016; Hollander et al., 2005)
	Fluvoxamine		Improves compulsive repetitive behaviors, aggression	Irritability, and increase risk of suicidal ideas	(Brown, Eum, Cook et al., 2017; Howes, Rogdaki, Findon et al., 2018; Lee, Kim, Kim et al., 2018; Martin, Koenig, Anderson et al., 2003; McDougle, Naylor, Cohen et al., 1996)

Table 1: Selected medications of different classes that are currently used to manage different symptoms of ASD *(continued)*

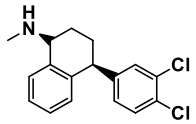
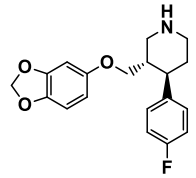
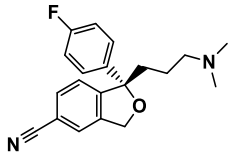
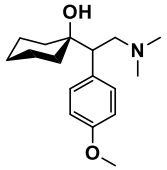
Class	Medication	Structure	Pharmacological effects	Side effects	References
Selective Serotonin Reuptake inhibitor (SSRI) (*Continued)*	Sertraline		All SSRIs possess similar effects to fluoxetine and fluvoxamine. Sertraline shows improvements in repetitive and disruptive behavior in adults with ASD, paroxetine reduced aggression, and escitalopram shows improvements in irritability, stereotypy, hyperactivity and inappropriate speech.		(AlOlaby, Sweha, Silva et al., 2017; Hellings et al., 1996; McDougle, Brodkin, et al., 1998)
	Paroxetine				(Davanzo, Belin, Widawski et al., 1998; Hellings, Reed, Cain et al., 2015)
	Escitalopram				(Brown et al., 2017; Owley, Walton, Salt et al., 2005; Viktorin, Uher, Reichenberg et al., 2017)
	Venlafaxine		Improves restricted behavior & interest, social and communication deficits, and hyperactivity	Irritability, and increase risk of suicidal idea	(Carminati, Deriaz, & Bertschy, 2006; Carminati, Gerber, Darbellay et al., 2016)

Table 1: Selected medications of different classes that are currently used to manage different symptoms of ASD *(continued)*

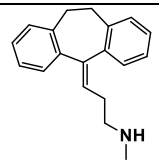
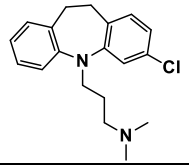
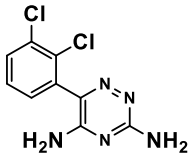
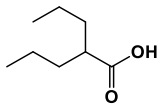
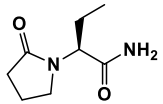
Class	Medication	Structure	Pharmacological effects	Side effects	Reference
Tricyclic Antidepressant	Nortriptyline		Improves hyperactivity and aggressiveness in autistic children	Sedation, increase in aggression, irritability and hyperactivity	(Campbell et al., 1971; Hong, Lee, Han et al., 2017; Kurtis, 1966)
	Clomipramine		Improves anger and compulsive and ritualized behavior		(Gordon et al., 1993; Hong et al., 2017; Sanchez et al., 1996)
Anticonvulsants	Lamotrigine		Improves overall autistic symptoms	Life-threatening skin reactions, including Stevens-Johnson syndrome, drug reaction with eosinophilia and systemic symptoms, and toxic epidermal necrolysis	(Jobski et al., 2017; Uvebrant et al., 1994)
	Valproic acid		Improves receptive language, affective instability, aggression, and social skills	Irritability, weight gain, anxiety	(Anagnostou, Esposito, Soorya et al., 2006; Hollander, Chaplin, Soorya et al., 2010; Hollander, Soorya, Wasserman et al., 2006; Jobski et al., 2017)
	Levetiracetam		Decreases symptoms such as hyperactivity, impulsivity, aggression, and affective lability	CNS effects such as somnolence, decreased energy, headache, dizziness, mood swings and coordination difficulties	(Jobski et al., 2017; Rugino et al., 2002)

Table 1: Selected medications of different classes that are currently used to manage different symptoms of ASD *(continued)*

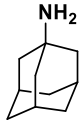
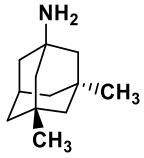
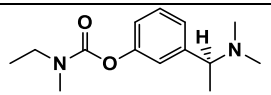
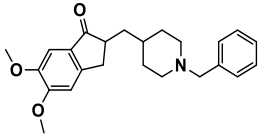
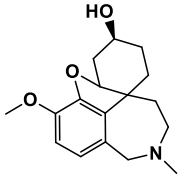
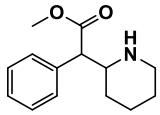
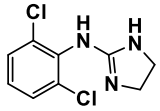
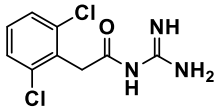
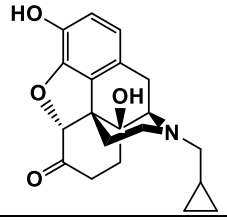
Class	Medication	Structure	Pharmacological effects	Side effects	Reference
Glutamate antagonist	Amantadine		Improves hyperactivity and speech disturbance	Nervousness, anxiety, agitation, insomnia, difficulty in concentrating, and exacerbations of pre-existing seizure disorders and psychiatric symptoms in patients with SCH or PD	(King, Wright, Handen et al., 2001; Naaijen et al., 2017)
	Memantine		Improves memory, hyperactivity, irritability, social behavior and communication, and self-stimulatory behavior	Few autistic individuals experienced worsening of autistic symptoms	(Chez, Burton, et al., 2007; Naaijen et al., 2017; Owley et al., 2006; Vorstman, Parr, Moreno-De-Luca et al., 2017)
Acetylcholine esterase inhibitors	Rivastigmine		Improves overall autistic behavior	Nausea, diarrhoea, hyperactivity, and irritability	(Vorstman et al., 2017)
	Donepezil		Improves irritability and hyperactivity	Nausea and vomiting, decreased appetite and weight loss	(Hardan et al., 2002; Vorstman et al., 2017)
	Galantamine		Improves several autistic symptoms in children such as irritability, hyperactivity, social interaction deficits, inappropriate speech, loss of attention, and anger		(Nicolson et al., 2006; Niederhofer et al., 2002; Vorstman et al., 2017)

Table 1: Selected medications of different classes that are currently used to manage different symptoms of ASD *(continued)*

Class	Medication	Structure	Pharmacological effects	Side effects	Reference
Psychostimulant	Methylphenidate		Improves several autistic behavioral symptoms in children and adolescents such as hyperactivity, impulsivity, attention, social communication, and self-regulation	Anorexia, aggression, and insomnia	(Di Martino et al., 2004; Handen et al., 2000; Jahromi et al., 2009; S. J. Kim et al., 2017)
Adrenergic α_2 receptor agonists	Clonidine		Improves hyperactivity, mood fluctuation, aggressiveness and agitation, sleeping pattern and night time awakenings	Sedation, dry mouth, and hypotension	(Fankhauser et al., 1992; Ming et al., 2008; Nash & Carter, 2016)
	Guanfacine		Improves attention, hyperactivity, and tics	Insomnia, fatigue, blurred vision, mood instability, sedation, constipation, irritability, and aggression.	(Boellner et al., 2007; Nash et al., 2016; Posey et al., 2004; Scahill et al., 2006)
Opiate antagonist	Naltrexone		Improves, hyperactivity, irritability, self-injuries. However, ineffective in social deficits.	Gastrointestinal complaints such as diarrhoea and abdominal cramping.	(Bouvard et al., 1995; Clifford et al., 2007; Elchaar et al., 2006; Kolmen et al., 1995; Nash et al., 2016; Panksepp et al., 1991)

1.5 Animal models of ASD

For the understanding of the aetiology and pathogenesis of any human disease, e.g. ASD, experimental rodent models are of substantial meaning. Modelling of human neuropsychiatric diseases with animals is very challenging due to the multifaceted nature of these disorders, combined with the absence of effective diagnostic biomarkers and objective tests for accurate diagnosis (Nestler et al., 2010). Moreover, neuropsychiatric diseases, such as ASD, are multifactorial with genetic, medical and neurodevelopmental conditions associated with the disease (Masi, DeMayo, Glozier et al., 2017; Medline Â, 2016). Generally, there are two types of rodent models for ASD, namely the genetic and non-genetic animal models. Accordingly, ASD genetic animal models are highly applicable when they reproduce the ASD features that are existent in an individual human genetic disease such as tuberous sclerosis and fragile X syndrome. Therefore, the ASD genetic models induced in mice should be established on an identified genetic cause of a disease, reflect key aspects of the human symptoms and respond to pharmacotherapies that are operational in the human diseases (Chadman, Yang, & Crawley, 2009).

1.5.1 Genetic animal models of ASD

A considerable advancement in detecting the genetic basis of several human diseases has been achieved by geneticists. These achievements include AD, Huntington's disease, and some forms of breast cancer. However, more difficulties were associated with the detection of genetic factors for multifaceted diseases such as SCH, diabetes, bipolar disorder, and also ASD (Fregeac, Colleaux, & Nguyen, 2016; Hu, Ehli, & Boomsma, 2017; Narahari, Hussain, & Sreeram, 2017; Wei, Yuan, Liu et al., 2015). In the case of ASD, extensive research efforts elucidated underlying

mechanisms of the disorder and concluded that these mechanisms might include dysfunctions of neuronal microRNAs (miRNAs), which are regulators of gene expression, and which have gained much attention in pathophysiology of various psychiatric illnesses (Andolina, Di Segni, & Ventura, 2017). Accordingly, about half of all identified miRNAs in humans are expressed in the CNS and exhibit modulatory functions crucial for numerous biological processes associated to the development of the CNS (Hu et al., 2017). Consequently, disruptions in miRNA biogenesis and miRNA-target interaction have been linked with CNS diseases, including ASD (Hu et al., 2017). Moreover, three miRNA (miRNA-7, miRNA-9, and miRNA-106b) were found to be associated with neurodegenerative diseases and only one, namely miRNA-9, with intellectual disability (Doxakis, 2010; Y. Hu et al., 2017; Wang, Liu, Zong et al., 2010; Xu, Zong, Li et al., 2011). Furthermore, it was recognized that the intellectual disability in Down syndrome which is a neurodevelopmental disorder, was caused by an extra-copy of chromosome 21 (Lejeune, Gautier, & Turpin, 1959). However, it is now being firmly established that deletions in the chromosome 21 are commonly discovered in some patients with intellectual disability including ASD (Hogart, Wu, LaSalle et al., 2010). Similarly, increased risk of delayed development, intellectual disability and neurological as well as psychiatric problems associated with ASD, SCH, epilepsy and hypotonia was found to be the result of micro deletions in the long (q) arm of the chromosome in a region designated q21.1 (Knight, Regan, Nicod et al., 1999; Mefford, Cooper, Zerr et al., 2009; Ravnan, Tepperberg, Papenhausen et al., 2006). Moreover, chromosome 15 has been described to be as one of several chromosomes enriched in segmental low copy repeats or duplicons (Bailey, Gu, Clark et al., 2002). These duplicons were revealed to strongly contribute to a

mechanism in which low copy repeats facilitated misalignment during meiosis I, leading to unequal recombination of nonallelic homologous and developing a sequence of common cut-off point along the 15q11.2-q13 region (Christian, Fantes, Mewborn et al., 1999; Robinson, Bernasconi, Mutirangura et al., 1993; Robinson, Binkert, Gine et al., 1993; Robinson, Spiegel, & Schinzel, 1993). Previous reports suggested, also, several cases of ASD with cytogenetic abnormalities in the 15q11–13 region (Cook, Courchesne, Cox et al., 1998), and the frequency of cytogenetic abnormalities in this region was found to be around 0-3% in ASD, and in fact, the most frequent cytogenetic abnormality in this population (Lord, Cook, Leventhal et al., 2000). Moreover, it has been reported that the paternal inheritance of a cytogenetical abnormality leads to a normal phenotype; maternal inheritance leads to autism or atypical autism (Tabet, Verloes, Pilorge et al., 2015). Furthermore, previous efforts revealed that 16p13.1 micro duplication syndrome is caused by interstitial duplications encompassing 16p13.1, which is a risk factor for a range of neuropsychiatric disorders, including ASD, intellectual disability, SCH, and ADHD (Grozeva, Conrad, Barnes et al., 2012; Mefford et al., 2009; Ramalingam, Zhou, Fiedler et al., 2011; Ullmann, Turner, Kirchhoff et al., 2007). There are, also, monogenic diseases that have been associated with ASD and display its characteristic features: tuberous sclerosis, fragile X syndrome, Rett syndrome and neurofibromatosis 1 (Hulbert & Jiang, 2016; Wetmore & Garner, 2010). Accordingly, single gene mutation gave rise to several human syndromes leading to an increase in the risk for developing ASD. Moreover, autistic traits are also connected to copy-number variants that lead to inheritance of maternal 15q11–13 duplication, resulting in Prader-Willi syndrome (Ogata, Ihara, Murakami et al., 2014). Furthermore, the developed strategies for the identification of genetic

variants has led to the description of novel syndromic forms of ASD and facilitated an understanding of the relationship between genetic traits and phenotype. Consequently, these genetic dissimilarities identified so far, along with the newly developed strategies for genetic engineering, improved the expansion of genetic animal models for ASD. Accordingly, mice are the major animal model for ASD due to their genetic manipulability and their capacity to show behavioral deficits associated with ASD features (Chen et al., 2015). Moreover, construct as well as face validity of mice ASD animal models together with the availability of well-established techniques to manipulate their genome and study brain function at numerous levels of analysis contribute to the overall validity of mouse models (Hulbert et al., 2016). Also, mice as mammals are genetically and biologically similar to humans, however, their rapid reproduction and accelerated development allow for the testing of large numbers of animals at a relatively low cost (Hulbert et al., 2016). Accordingly, animal models for genes of a syndromic disorder were commonly found to present autistic traits including Fragile X (FMR1) with social interaction deficits, hyperactivity, and cognitive impairments (Bhattacharya, Kaphzan, Alvarez-Dieppa et al., 2012; Y. Hu et al., 2017; Hulbert et al., 2016; Ronesi, Collins, Hays et al., 2012; Willem Verhoeven, 2011), Rett syndrome and MECP2 mutations with the resulted repetitive and stereotypic/restricted behaviors, abnormal gait and reduced anxiety, decreased pain and normal olfactory discrimination (Chao, Chen, Samaco et al., 2010; Samaco, McGraw, Ward et al., 2013). Other common syndromic disorder with ASD phenotype include tuberous sclerosis TSC1 or TSC2 with social interaction deficits and repetitive/restricted behavior or interest (Chao et al., 2010; Willem Verhoeven, 2011), Timothy syndrome (TS) CACNA1C with impairments in social interactions, repetitive/stereotypic

behaviors, and increased fear conditioning (Ergaz, Weinstein-Fudim, & Ornoy, 2016) and Phelan-McDermid syndrome with restricted interest as well as cognitive and motor deficits (Ergaz et al., 2016; Giza, Urbanski, Prestori et al., 2010) In addition to PTEN mutations and Cortical dysplasia focal epilepsy with deficits in social interactions, restricted interest, sensory sensitivity, elevated anxiety, and seizures (Scott-Van Zeeland, Abrahams, Alvarez-Retuerto et al., 2010)(Cook & Scherer, 2008; LaSalle, Reiter, & Chamberlain, 2015; Piochon, Kloth, Grasselli et al., 2015). All behavioral features of the above described syndromes are associated with ASD. Numerous inbred mouse strains show face validity as ASD models in addition to the genetically modified animal models of ASD. As such inbred strains display robust and well-replicated social deficits and repetitive behaviors (Kazdoba, Leach, Yang et al., 2016). These inbred strains are found to display idiopathic autism, as their ASD-relevant behaviors are not caused by known genetic mutations (Kazdoba et al., 2016). For instance and while assessing sociability, inbred strains like A/J, BALB/cByJ (BALB), BTBR T+Itpr3tf/J (BTBR), C58/J (C58), and 129S1/SvImJ mice showed lack of sociability, as compared to inbred mouse strains with high sociability, such as C57BL/6J (B6) and FVB/NJ mice (Brodkin, 2007; Y. Hu et al., 2017; Kazdoba et al., 2016; McFarlane, Kusek, Yang et al., 2008; Moy, Nadler, Poe et al., 2008; Moy, Nadler, Young et al., 2007). Moreover, it has been shown that several mouse strains, e.g. BTBR and C58, also demonstrate explicit motoric stereotypies or repetitive behaviors, such as jumping, digging, and high levels of self-grooming and marble burying (Kazdoba et al., 2016; Pasciuto, Borrie, Kanellopoulos et al., 2015).

1.5.2 Non-genetic animal models of ASD

Environmental animal models are induced by prenatal exposure of pregnant

animals to certain chemical compounds, infections, or inflammations. The environmental inducing factor is only of significance if the chemicals used in the animal model cause the same effects in humans. Accordingly, the antiepileptic drug, VPA was found to significantly increase the ASD degree in offspring of treated mothers (Christianson, Chesler, & Kromberg, 1994; Moore, Turnpenny, Quinn et al., 2000), and similarly showed to induce ASD-like behaviors in mice and rats (Anshu, Nair, Kumaresan et al., 2017; Nicolini & Fahnestock, 2017; Rodier, Ingram, Tisdale et al., 1997). Therefore, the ASD-like animal model induced by in utero exposure to the anticonvulsant drug VPA has been well established and recognized to study ASD features (Nicolini et al., 2017). Moreover, the immunomodulatory drug thalidomide, which was used by pregnant women in the 1950s, was revealed to be associated with a marked escalation in the incidence of ASD in their offspring (Stromland, Nordin, Miller et al., 1994). Furthermore, several reports revealed that methylmercury, which is formed from inorganic mercury by the action of microbes that live in aquatic systems including lakes, rivers, wetlands, sediments and soils, exposure during childhood caused a variety of neuropsychological abnormalities, e.g. memory, attention and language, (Davidson, Cory-Slechta, Thurston et al., 2011; Falluel-Morel, Sokolowski, Sisti et al., 2007; Grandjean, Budtz-Jorgensen, White et al., 1999). Also, a previous study has shown that acute systemic administration methylmercury during developmental phases elicited hippocampal cell death, reductions in neurogenesis, and severe learning impairments (Grandjean et al., 1999). Thimerosal was a widely used preservative in numerous biological and drug products since 1930s, including many vaccines. It is a mercury-containing organic compound which contains ethyl mercury, a compound supposed to be the cause of several adverse neurodevelopmental deficits,

including ASD (Hviid, Stellfeld, Wohlfahrt et al., 2003). Also, a previous study in which 10,000 cases have been examined showed that there is a significant association between maternal viral infection in the first trimester and ASD in the offspring (Atladottir, Thorsen, Ostergaard et al., 2010). Indeed, when animals were exposed to the maternal immune activation with polyinosine: cytosine (poly I:C) at embryonic day 9.5, the offspring displayed histological and behavioral abnormalities that resembled ASD (Garay, Hsiao, Patterson et al., 2013; Hsiao, McBride, Chow et al., 2012; Shi, Smith, Malkova et al., 2009). On behavioral level, these animals demonstrated impaired sociability, communication differences and increased repetitive stereotyped behaviors and had smaller brain sizes at birth, followed by macroencephaly in adulthood. In line with these observations, it has been shown that viral infections in the mother can prompt substantial changes in the immune system in both mother and fetus, leading to long-term epigenetic alterations in the offspring (Kong, Frigge, Masson et al., 2012). Consequently, the timing of immune insults during developmental phase may be one source of the heterogeneity in the phenotypic features observed in ASD (Kong et al., 2012). Accordingly, male rats treated with thalidomide/VPA demonstrated immunological changes such as decreased splenocyte proliferative response to mitogenic stimulation, lower thymus weight, and decreased interferon (IFN)-γ/IL-10 ratio in peritoneal macrophages, whereas female rats in this study failed to show many of these behavioral and immunological changes, indicating that sex-specific responses to some environmental factors might play a significant role on the phenotypic outcomes of several ASD features (Schneider, Roman, Basta-Kaim et al., 2008). Also and subsequent to viral infection, the immune response was found to produce various cytokines, such as (IL)-1, -2 and -6 which modulate the release of

several monoamines such as 5-HT in the hippocampus and other brain regions (Libbey, Sweeten, McMahon et al., 2005). Indeed, maternal infection was found to lead to elevated levels of cytokines and chemokines including IL-1β, IL-6, IL-8, and IL-12p40 in the plasma of ASD children, and such increases were reported to be associated with more reduced communication skills and abnormal behaviors (Patterson, 2012). Also, the offspring of infected animal mothers given poly (I:C) exhibited both ASD-like behavioral and neuropathological deficits as described above (Gadad, Hewitson, Young et al., 2013; Garay et al., 2013; Hsiao et al., 2012; Patterson, 2012; Shi et al., 2009). Based on the aforementioned results, environmental influences both *during* and *after* pregnancy can significantly influence the immune system and the developing nervous system to play a role in constructing several neurodevelopmental disorders including ASD.

It should be stressed that experimental animal models (genetically manipulated animal models, animal models obtained by destruction of certain CNS areas, and animal models obtained by using maternal factors) are indispensable for exploring the pathophysiologic causes of brain disorders, e.g. ASD, although they do not reflect the entire state of ASD disease. Moreover, animal models (mostly rodents) are widely used to study the development of cortical neurocircuit, genetic analysis and molecular mechanisms underlying ASD, and the palliative effects of newly developed drugs on core as well as associated symptoms of ASD. Furthermore, genetic as well as non-genetic rodent models have the capability of being the major objects of pharmaceutical industry in testing drug efficacy, dosage and acute as well as (sub)chronic toxicology.

1.6 Neuroinflammation and ASD

Neuroinflammation is a response that involves neurons, microglia and macroglia, which are cells that are present in the CNS (Bradl & Hohlfeld, 2003; Carson, Doose, Melchior et al., 2006). Neuroinflammation has been reported to be an important feature of many neurodegenerative disease and neuropsychiatric conditions such as multiple sclerosis, AD, PD, narcolepsy and ASD (Carson, Thrash, & Walter, 2006; Frick et al., 2016). Implication of the role of inflammation in aetiology of ASD was based on evidence and medical history of autistic individuals that show signs of neuroinflammation, altered inflammatory responses and immune abnormalities throughout life. This is confirmed by increasing clinical and experimental evidence that links immune and inflammatory alterations with the pathogenesis of ASD (Lucchina & Depino, 2014). Moreover, post mortem studies have supported this evidence and have shown neuroinflammation in several brain regions of patients with ASD (Vargas, Nascimbene, Krishnan et al., 2005). Microglia as the brain's resident inflammatory cells have critical role in mediating neuroinflammation and regulation of brain development and homeostasis. In fact, they play critical role in defence and tissue repair. Microglia activation is the first sign of neuroinflammation, and abnormalities in microglia have been implicated in autism (Carson et al., 2006; Frick et al., 2016). After being activated they may cause neuronal dysfunction and cell death (neurodegenerative role). Activated microglia roundup, proliferate, migrate, phagocytes, present antigens to T-cells, release a variety of oxidants such as reactive oxygen species (ROS), and activate several genes and proteins, such as inducible nitric oxide synthase (iNOS), cyclooxygenase1 (COX1), cyclooxygenase2 (COX2), and variety of proinflammatory cytokines including IL-1β, tumor necrosis factor alpha

(TNF-α) (Figure 6), and that is what is notably observed in autism (Monnet-Tschudi, Defaux, Braissant et al., 2011). In chronic neuroinflammation, microglia remain activated for long periods, with continuous release of cytokines and neurotoxic molecules that contribute to loss of synaptic connections and neuronal death (Lyman, Lloyd, Ji et al., 2014). Chronic or excessive neuroinflammation is diagnosed in ASD (Kern et al., 2015), this observed chronic glia activation and altered inflammatory function maybe partly responsible for the observed behavioral features in ASD, as chronic peripheral inflammation and abnormal inflammatory responses in the brain may lead to cognitive dysfunction (Lucchina et al., 2014).

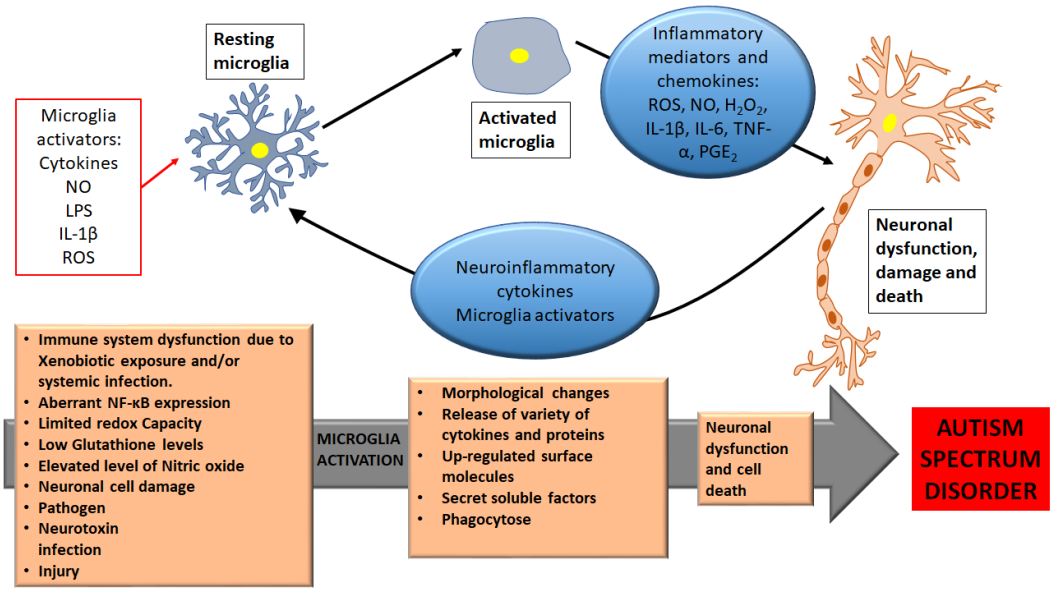


Figure 6: Schematic depiction of microglia activation neuronal cell death.
Neuroinflammatory proteins and cytokines due to microglia activation by genetic and different environmental activators, leading to neuron dysfunctions and cell death. Adapted from (Shabab, Khanabdali, Moghadamtousi et al., 2017).

During pregnancy, both environmental and genetic risk factors may affect inflammatory response of new-borns, hence altering postnatal brain development (Adams-Chapman & Stoll, 2006). These genetic and environmental factors can

directly elicit chronic neuroinflammation which in turn may modulate neuronal function and immune response through glia activation, or directly through affecting neuronal function (Depino, 2013) (Figure 7). VPA as an environmental risk factor showed activation in different brain regions, with evidence of long-lasting glia activation in hippocampus and cerebellum (Lucchina et al., 2014). Hippocampus (Depino, Lucchina, & Pitossi, 2011) and cerebellum (DeLorey, Sahbaie, Hashemi et al., 2008; Martin, Goldowitz, & Mittleman, 2010) are two brain regions linked to autism-related behavior, namely social interaction and repetitive behaviors. Additionally, several studies showed that cerebellar inflammation may alter social behavior in adult mice as cerebellum is involved in executive and cognitive functions (Koziol, Budding, Andreasen et al., 2014; Lucchina et al., 2014; Shi et al., 2009; Wang, Kloth, & Badura, 2014). Furthermore, evidence suggests that astrocyte and microglia activation in cortex and cerebellum increase expression of cytokines including IL-6, TNF-α, MCP-1, TGF-β1, IFNγ, IL-8 and other associated genes involved with the immune response in different brain regions of autistic subjects (Chez, Dowling, Patel et al., 2007; Chez & Guido-Estrada, 2010; Garbett, Ebert, Mitchell et al., 2008; Li, Chauhan, Sheikh et al., 2009; Vargas et al., 2005). Alternatively, both these environmental and genetic factors, could chronically alter immune response through increasing production of free radicals, which consequently activate glia cells, increasing inflammatory response, affecting neurons, mediating clinical symptoms of autism (Depino, 2013). These findings suggest that neuroinflammation may contribute to ASD behavioral effect, hence, controlling microglia activation and inhibiting cytokine and free radical production would be a therapeutic strategy for treating ASD.

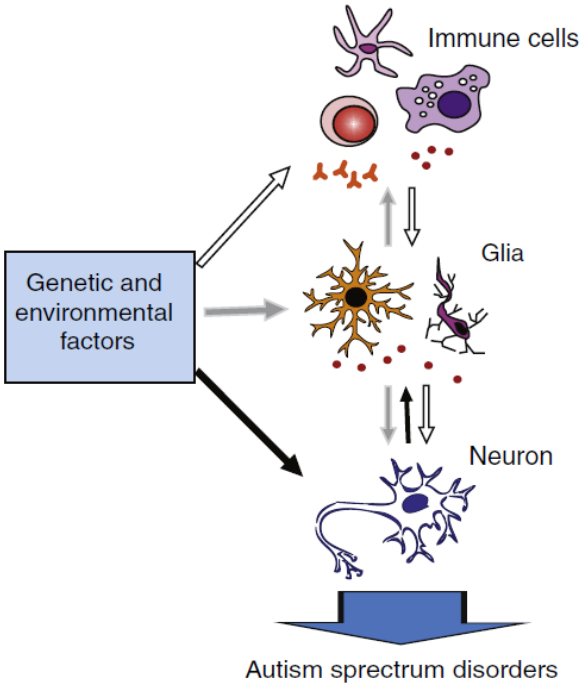


Figure 7: Effect of genetic and environmental factors on neuronal dysfunction and immune response modulating ASD symptoms.
All possibilities contributing to ASD through glia activation (grey arrow), or through directly altering peripheral immune cells (white arrows) which in turn activates glia affecting the neuronal function (black arrows). Adapted from (Depino, 2013).

The emergence that the HS may be implicated in ASD and may contribute in ameliorating its core symptoms, necessitates further research to investigate what role the HS may or may not have in enhanced neuroinflammation in ASD. Possible role of central histamine as regulator of neuroinflammatory processes has received scant attention, but lately the potential importance of HA regulation of microglia (Rocha et al., 2014) was highlighted by the fact that both HA and microglial dysregulation are implicated in neuropsychiatric disease states such as TS and a range of neurodegenerative syndromes. Recent finding of microglial abnormalities in the HDC knockout mouse, a validated model of the pathophysiology of TS (Baldan, Williams, Gallezot et al., 2014), further emphasizes the importance of understanding HA's role in regulating microglial function, especially that TS and ASD have a high degree of

overlap. An *in vitro* study demonstrated that all known histamine receptors are expressed on microglia cells (Ferreira et al., 2012). Another experimental study suggested a role for histamine in the modulation of microglial inflammatory response, demonstrating a dual role of histamine in neuroinflammation regulation, mediated by activated microglia, through modulating cell recruitment and proinflammatory cytokine release, as IL-1β and TNF-α (Ferreira et al., 2012).

A growing understanding of histamine has shed light on the possibility of targeting histamine for therapeutic use to treat CNS disorders associated with microglia-derived inflammation. A very recent study demonstrated the dual role of histamine in the modulation of microglial responses, suggesting that while histamine per se can induce microglia injurious effects, oppositely under inflammatory context, it can inhibit them (Barata-Antunes, Cristovao, Pires et al., 2017a), opening a new perspective for the therapeutic use of histamine to improve inflammation-associated processes in disorders associated with microglia- derived inflammation. The role of H3R antagonist in stimulating the synthesis and release of histamine in brain, as mentioned earlier, suggest that therapeutic use of H3R antagonists may ameliorate neuroinflammation and consequently, improve ASD behavioral symptoms. Moreover, the antioxidant effect of H3R antagonist, as demonstrated by (Mahmood et al., 2012b; Mani, Jaafar, Azahan et al., 2017) strongly suggest that H3R antagonist may have therapeutic potential in the management of ASD.

1.7 Aims and Objectives

Based on all aforementioned evidences and results from our lab, H3 receptor (H3R) antagonists are considered potential therapeutic agents for the management of different brain disorders associated with cognitive impairments. Accordingly, the

hypothesis of my PhD research project is that targeting the brain histaminergic system using H3R antagonists may be new therapeutic agents for reversing core deficits of ASD, using different mouse models of ASD.

The primary focus of this study is to investigate the effects of sub-chronic treatment of non-imidazole H3R antagonists (DL77) on autistic-like animal's behaviors, mainly sociability and social novelty preference deficits, repetitive/ compulsive-like behaviors, abnormal anxiety, hyperactivity through a battery of standard behavioral tests. Another objective of the research is to study the role and implications of the histaminergic system in neuroinflammation and oxidative stress levels, which have been associated with ASD as mentioned earlier. This is approached by determining the effects of DL77 on levels of neuroinflammatory and oxidative stress markers using various experimental approaches in mice brain tissues.

Finally, accumulation of evidence as mentioned above suggested the implicates a variety of neurotransmitter systems such as ACh, 5-HT, DA, GABA, Glu, and HA in the onset and progression of ASD. In view of that, recent advances of developing novel agents with multiple pharmacological effects have become a promising strategy for treatment of diseases of a multifactorial nature, such as ASD. Therefore, we were interested to investigate the effects of novel dual-active ligand E100 with histamine H3 receptor (H3R) antagonist affinity (hH3R Ki = 203 nM) and acetylcholine esterase inhibitory effect (EeAChE IC50 = 1.93µM and EqBuChE IC50 = 1.64 µM) on autistic-like behavioral parameters, oxidative stress, and neuroinflammation in male C57BL/6 mouse model of ASD induced by prenatal exposure to valproic acid (VPA, 500 mg/kg, i.p.) and in BTBR mice.

Chapter 2: Methods

2.1 Animals

Three mice strains at 8 weeks of age were used in this study, Tuck-Ordinary (TO) mice (Harlan, UK), C57BL/6 (C57) and BTBR T+tf/J (BTBR) mice (Jackson Laboratory, Bar Harbor, USA). BTBR is a genetic animal model of autism. It is an inbred strain that naturally exhibits several behavioral symptoms of autism. All mice were bred in the local central animal facility of the College of Medicine and Health Sciences, United Arab Emirates University (Bastaki, Abdulrazzaq, Shafiullah et al., 2018). They were housed in plastic cages under a standard light/dark cycle (12 h dark/light cycle, lights at 6 a.m.) at a constant temperature of 22–25°C, with free access to tap water and a standard rodent chow diet.

2.2 Establishing VPA animal model of autism

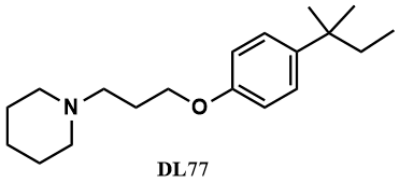
Mice were kept for mating. Female mice were observed daily and placed in a separate cage when pregnancy was confirmed by the presence of a vaginal plug. This day was considered embryonic day 0 (E0). On E12.5, pregnant females were i.p. injected i.p with VPA 500 mg/kg (Sigma-Aldrich, St. Louis, MO, USA) dissolved in isotonic 0.9% sodium chloride solution, and control mice were injected with saline, as previously described (Kataoka et al., 2013; Takuma et al., 2014). The day of delivery was defined as postnatal day 0 (P0). All offspring were weaned, sex-grouped (5–6 mice/cage) at P 21. Pups from VPA-exposed mothers were considered as the VPA mouse model of autism and were used for experiments at8 weeks of age. On the other hand, pups for mothers exposed to saline were used as control mice. TO and C57 VPA

mouse model of autism were developed by this method and all the tests were carried out on both strains.

2.3 Drugs and chemicals

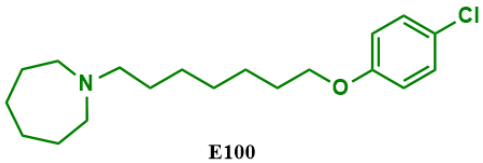
Two H3R antagonists were used in this study. DL77 [1-(3-(4-*tert*-pentylphenoxy) propyl) piperidine] and E100 [1-(7-(4-chlorophenoxy) heptyl) homo-piperidine] (Figure 8). Both compounds were designed and synthesized in the Department of Technology and Biotechnology of Drugs Krakow, Poland, according to a previously described procedure (Kuder, Łażewska, Latacz et al., 2016; Lazewska, Jonczyk, Bajda et al., 2016; Lazewska, Ligneau, Schwartz et al., 2006) and were provided to us. Sodium valproate (VPA) (500 mg/kg, i.p.), donepezil hydrochloride (DOZ) (1 mg/kg, i.p.) (reference drug), the H3R agonist R-α methyl histamine (RAM) (10 mg/kg, i.p.), the brain-penetrant H1R antagonist pyrilamine (PYR) (10 mg/kg, i.p.), the brain-penetrant H2R antagonist zolantadine (ZOL) (10 mg/kg, i.p.), Scopolamine hydrochloride (SCO) (0.3 mg/kg, i.p.), LPS (lipopolysaccharide from *E. coli* serotype 0111:B4), and the assay kit for reduced glutathione (GSH), rabbit anti-cyclo-oxygenase2 (COX2), rabbit anti-inducible nitric oxide synthase (iNOS) antibodies was purchased from Sigma-Aldrich (St. Louis, MO, USA). The lipid peroxidation assay kit for estimation of malondialdehyde (MDA) was obtained from North West Life Science (Vancouver, WA, USA). The assay kits for superoxide dismutase (SOD) and catalase (CAT) were purchased from Cayman chemical (Ann Arbor, MI, USA). For estimation of levels of proinflammatory cytokines IL-1β, IL-6, TNF-α and TGF-β commercially available ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). Rabbit anti- nuclear transcription factor – kappa B p65 (NF-κB p65) antibody was purchased from Abcam, (Cambridge, MA, USA).

Anti-actin antibody was obtained from MERCK (Millipore, USA), while the goat anti-mouse and goat anti rabbit secondary immunoglobulin G antibodies conjugated to horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-ionized calcium-binding adaptor molecule-1 (Iba-1) polyclonal rabbit was purchased from Wako Chemicals (Richmond, VA, USA). Alexa Fluor 488- conjugated secondary goat anti-rabbit antibodies were purchased from Life Technologies (Grand Island, NY, USA). Protease and phosphatase inhibitors were obtained from Thermo Scientific (Waltham, MA, USA). The Pierce® BCA Protein Assay Reagent Kit and the SuperSignal® West Pico PLUS chemiluminescence substrate kit were purchased from Thermo Fisher Scientific Inc. (Rockford, IL, USA). Mini-Protean TGX® precast electrophoresis gels were obtained from BIO-RAD (Bio-Rad Laboratories Inc, USA). Vectashield®, fluorescent mounting media was procured from Vector laboratories (Burlingame, CA, USA). Acetylcholinesterase activity colorimetric assay kit was purchased from BioVision (Milpitas, CA, USA). All the reagents used in the study were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, US). Except for E100, which was dissolved in 1% aqueous solution of Tween 80, all drugs were dissolved in isotonic saline solution. All mice were i.p. injected at a volume of 10 ml/kg adjusted to body weight, and all doses are expressed in terms of the free base.



DL77

K_i (*h*H3R)[a] = = 8.4 ± 1.3 nM
K_i (*h*H1R)[b] = 896 ± 96 nM
K_i (*h*H4R)[c] = 55,990 ± 38,820 nM
ED_{50}[d] = 2.1 ± 0.2 mg/kg, p.o.



E100

K_i (*h*H3R)[a] = 203 nM
K_i (*h*H1R)[b] > 1000 nM
K_i (*h*H4R)[c] > 10000 nM
IC_{50} *Ee*AChE[e] = 1.93 µM
IC_{50} *Eq*BChE[f] = 1.64 µM

Figure 8: Chemical structure and *in vitro* affinities of DL77 and E100 with regard to *h*H1R, *h*H3R, *h*H4R, *in-vivo* potency of DL77; and *in vitro* data of E100 against EeAChE, and EqBChE.

([a]) [^{125}I] Iodoproxyfan binding assay at human H3R stably expressed in CHO-K1 cells, n=3 for DL77 and [^{3}H] N^{α}-Methylhistamine binding assay performed with cell membrane preparation of HEK-*h*H3 cells stably expressing the human for H3R, n= 3 for E100, as reported previously (Lazewska et al., 2006) **([b])** [^{3}H] Pyrilamine binding assay performed as previously reported, with cell membrane preparation of CHO-*h*H1R cells stably expressing the human H1R; n=3 (Schibli & Schubiger, 2002; Schlotter, Boeckler, Hubner et al., 2005). **([c])** [^{3}H] Histamine binding assay performed as reported previously, with cell membrane preparation of Sf9 cells transiently expressing the human H4R and co-expressed with $G_{\alpha i2}$ and β1γ2 subunits; n=3 (Meier, Apelt, Reichert et al., 2001). **([d])** Central histamine H3R assay in vivo after po administration to mice, n=3 (Lazewska et al., 2006). *h*H1R: human histamine H1 receptor; **([e and f])** E100 comparable inhibitory potency for both cholinesterases (EeAChE, and EqBChE) in low micromolar range. Abbreviations: CHO: Chinese hamster ovary; Eq: equine; Ee: electric eel.

2.4 Study design

Pregnant mice (TO and C57) were i.p. injected with either VPA (500 mg/kg) or saline on embryonic day 12.5 (E12.5) (Moy, Nadler, Perez et al., 2004) and returned to their home cages. VPA was dissolved in isotonic 0.9% NaCl solution (saline). After the injection of VPA few pregnant mice died, and some gave still birth or underwent desorption. From the successfully delivered pups, male offspring only from both VPA-exposed mothers and from mothers that received saline were divided into 2 groups (Group 1: prenatally VPA-exposed offspring; Group 2: prenatally saline-exposed offspring). Then the mice in each group were further divided into subgroups and i.p. treated as shown in the schematic experimental design (Figure 9). Group 1 was

subdivided into eight subgroups of VPA-exposed mice and Group 2 was subdivided into three subgroups of saline-exposed mice as follows:

Group 1: prenatally VPA-exposed offspring (5-7 mice/group)

Group I: VPA-exposed mice injected with saline

Group II: VPA-exposed mice injected with DL77 (5 mg/kg, i.p.)

Group III: VPA-exposed mice injected with DL77 (10 mg/kg, i.p.)

Group IV: VPA-exposed mice injected with DL77 (15 mg/kg, i.p.)

Group V: VPA-exposed mice injected with DOZ (1 mg/kg, i.p.)

Group VI: DL77 (10 mg/kg, i.p.) was co-administered with RAM (10 mg/kg, i.p.)

Group VII: DL77 (10 mg/kg, i.p.) was co-administered with PYR (10 mg/kg, i.p.)

Group VIII: DL77 (10 mg/kg, i.p.) was co-administered with ZOL (10 mg/kg, i.p.)

Group 2**:** prenatally saline-exposed offspring (5-7 mice/group)

Group I: saline-exposed mice injected with saline

Group II: saline-exposed mice injected with DL77 (10 mg/kg, i.p.)

Group III: saline-exposed mice injected with DOZ (1 mg/kg, i.p.)

All treatments and vehicle (saline) were injected once daily for 21 days from P44 onwards. After one week of treatment, the battery of behavioral tests was started in a sequence shown in Figure 9. Treatments or saline were administered 30–45 min before each behavioral test. To reduce the number of animals used, the biochemical

analysis, Western blotting and immunofluorescence staining were performed in the same groups of animals that were subjected to behavioral tests. The behavioral experiments of the study were conducted between 9:00 am and 3:00 pm. Before the behavioral tests animals were habituated in the study place for at least 1 h. All procedures were approved by the Institutional Animal Ethics Committee of College of Medicine and Health Sciences/United Arab Emirates University (approval no. ERA-2017-5603). All methods were carried out in accordance with relevant guidelines and regulations.

The same study design (Figure 9) was followed using DL77 and E100 as treatments on C57 mice prenatally exposed to VPA, and again the offspring were divided as follows: (Group 1: prenatally VPA-exposed offspring; Group 2: prenatally saline-exposed offspring). Group 1 was subdivided into fifteen subgroups of VPA-exposed mice and Group 2 was subdivided into four subgroups of saline-exposed mice as follows:

<u>Group 1: prenatally VPA-exposed offspring (8-12 mice/group)</u>

Group I: VPA-exposed mice injected with 1% aqueous solution of Tween 80

Group II: VPA-exposed mice injected with DL77 (5 mg/kg, i.p.)

Group III: VPA-exposed mice injected with DL77 (10 mg/kg, i.p.)

Group IV: VPA-exposed mice injected with DL77 (15 mg/kg, i.p.)

Group V: VPA-exposed mice injected with E100 (5 mg/kg, i.p.)

Group VI: VPA-exposed mice injected with E100 (10 mg/kg, i.p.)

Group VII: VPA-exposed mice injected with E100 (15 mg/kg, i.p.)

Group VIII: VPA-exposed mice injected with DOZ (1 mg/kg, i.p.)

Group IX: DL77 (15 mg/kg, i.p.) was co-administered with RAM (10 mg/kg, i.p.)

Group X: DL77 (15 mg/kg, i.p.) was co-administered with PYR (10 mg/kg, i.p.)

Group XI: DL77 (15 mg/kg, i.p.) was co-administered with ZOL (10 mg/kg, i.p.)

Group XII: E100 (10 mg/kg, i.p.) was co-administered with RAM (10 mg/kg, i.p.)

Group XIII: E100 (10 mg/kg, i.p.) was co-administered with PYR (10 mg/kg, i.p.)

Group XIV: E100 (10 mg/kg, i.p.) was co-administered with ZOL (10 mg/kg, i.p.)

Group XV: E100 (10 mg/kg, i.p.) was co-administered with SCO (0.3 mg/kg, i.p.)

Group 2: prenatally saline-exposed offspring (8-12 mice/group)

Group I: saline-exposed mice injected with 1% aqueous solution of Tween 80

Group II: saline-exposed mice injected with DL77 (15 mg/kg, i.p.),

Group III: saline-exposed mice injected with E100 (10 mg/kg, i.p.),

Group IV: saline-exposed mice injected with DOZ (1 mg/kg, i.p.)

For further elaboration and authentication, effects of systematic pre-treatment of E100 was investigated in male BTBR mice following the same sequence of behavioral tests (Figure 9) and the groups were divided as follows (6-8 mice/group):

Group I: C57 mice injected with 1% aqueous solution of Tween 80

Group II: BTBR mice injected with 1% aqueous solution of Tween 80

Group III: BTBR mice injected with E100 (5 mg/kg, i.p.)

Group IV: BTBR mice injected with E100 (10 mg/kg, i.p.)

Group V: BTBR mice injected with E100 (15 mg/kg, i.p.)

Group VI: BTBR mice injected with DOZ (1 mg/kg, i.p.)

Group VII: E100 (5 mg/kg, i.p.) was co-administered with RAM (10 mg/kg, i.p.)

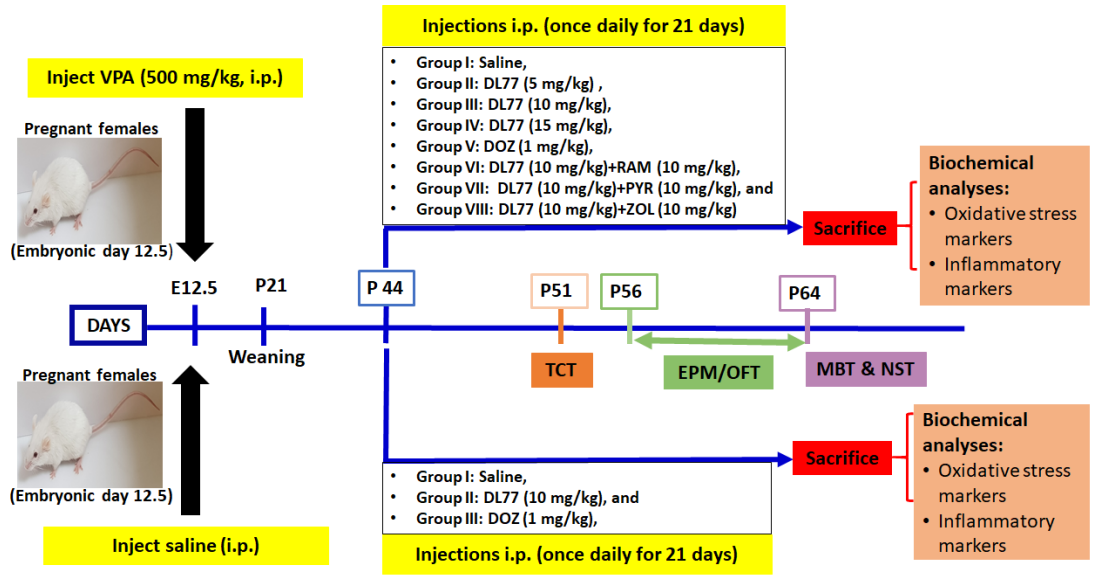


Figure 9: Schematic diagram of drug treatments, behavioral studies, and biochemical assessments with VPA mice.

Pregnant mice were injected with VPA (500 mg/kg, i.p.) at embryonic day 12.5 (E12.5). Treatments started from postnatal day (P44). Injections (once daily) continued for 21 days until P64. Behavioral studies was started from P51. All mice were then sacrificed at P64 for biochemical analyses. Abbreviations: VPA: Valproic acid; i.p.: intraperitoneal; DOZ: Donepezil; RAM: R-alpha methyl histamine; PYR: Pyrilamine; ZOL: Zolantidine; TCT: Three-chamber test; EPM: Elevated plus-maze; OFT: Open field test; MBT: Marble Burying test; NST: Nestlet shredding test.

2.5 Behavioral tests

2.5.1 Three-chamber test (TCT)

TCT assesses cognition in the form of general sociability and social novelty preference in rodents, identifying rodents with deficits in sociability and/or social novelty. The TCT of social interaction and social novelty recognition was performed as described previously (Silverman, Yang, Lord et al., 2010), but with a slight modification in the apparatus, with one center chamber (40 cm × 20 cm × 22 cm) and two side chambers (40 cm × 20 cm × 22 cm) separated by two sliding doors under a light intensity of ~120 lux (Figure 10). The test was composed of four 10-min sessions. In the first session, a test mouse was habituated to the center chamber with the two doors closed for 5 min. In the second session, the doors were opened, and a test mouse was allowed to explore all three chambers for 5 min. Before starting the third session, a stranger mouse (same age and sex with no previous contact with the test mouse), referred to as a novel mouse 1, was placed in a small plastic cage in either the left or right chamber, selected randomly to avoid side preference, while the other cage in the opposite chamber was kept empty and was referred to as a novel object. In the third session, the test mouse was allowed to explore all three chambers and cages for 10 min. The time spent exploring (direct contact) the novel mouse 1 was compared with the time spent exploring the novel object. As previously described, the sociability index (SI) and social novelty preference index (SNI) were calculated by applying a mathematical equation to allow the direct comparison of social behavior of the treated groups (Bambini-Junior, Zanatta, Della Flora Nunes et al., 2014). As the score for SI became more positive and closer to 1, the more social was the tested animal. The SI was calculated using the following equation:

$$SI = \frac{\text{Time exploring novel mouse 1} - \text{Time exploring novel object}}{\text{Time exploring novel mouse 1} + \text{Time exploring novel object}}$$

After the end of the third session a new stranger mouse was added to the empty cage after which the test mouse was allowed to explore the environment for 10 min, which made up the fourth session. The new stranger mouse was referred to as novel mouse 2, while the novel mouse 1 from the previous session was then referred to as the familiar mouse. The exploratory time was measured as in the previous session to evaluate the preference of the test mouse to novel mouse over familiar mouse in the cage. Similarly, SNI was calculated where a value closer to 1 indicated that an animal was more interested in social novelty. The SNI was calculated as follows:

$$SNI = \frac{\text{Time exploring novel mouse 2} - \text{Time exploring familiar mouse}}{\text{Time exploring novel mouse 2} + \text{Time exploring familiar mouse}}$$

The reason for the first session is that it might increase mouse exploration of side chambers relative to the center chamber in sessions 3 and 4. Another objective of habituation in the center chamber before the whole-apparatus habituation was to make the center chamber a familiar “home base” of the test mouse (Crawley, 2004), as reported in the original papers (Moy et al., 2004; Nadler, Moy, Dold et al., 2004). In addition, the time of habituations could be flexible (5–30 min) (Crawley, 2004). Stranger mice (8–16 weeks old) were habituated to the plastic cage in the three-chamber apparatus for 30 minutes 24 h before the test, as described previously (Moy et al., 2004). The trace movements and sniffing during the experiment were automatically recorded using EthoVision® Software (Noldus, Information Technology Netherlands).

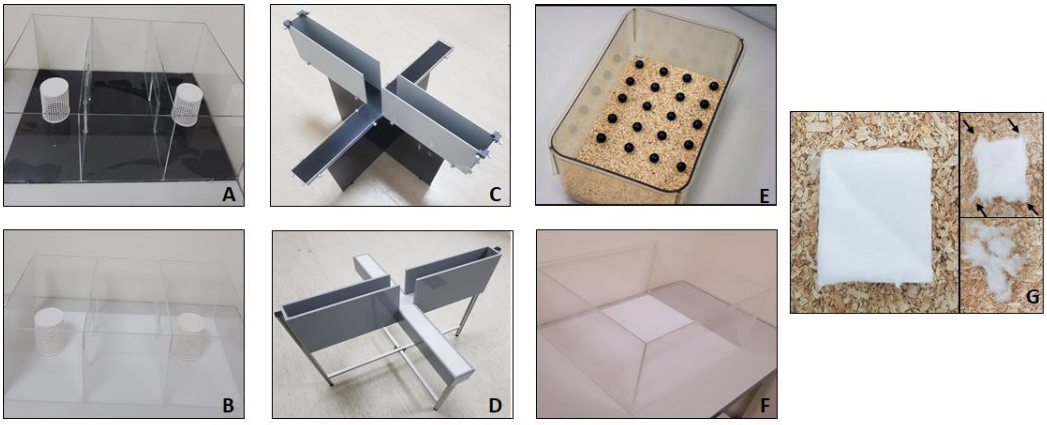


Figure 10: Apparatus used for behavior tests.
Three chamber compartment used for three chamber test **(**A and B), Elevated Plus-Maze used for anxiety and locomotor activity chamber test measures (C and D), cage with equidistant 4 × 5 arrangement of black marbles used for marble burying test (E), Open field used for open-field test (E), and nestlets with specific dimensions used for nestlet shredding test (F). Black and white floorings are selected to contrast with the colour of mouse used, for accurate detection by the software.

2.5.2 Marble burying test (MBT)

MBT behavior is an accurate reflection of repetitive digging behavior (Angoa-Perez, Kane, Briggs et al., 2013; Thomas, Burant, Bui et al., 2009). The test was performed as previously reported with slight modifications (A. Bahi, J. S. Schwed, M. Walter et al., 2014; Theoharides et al., 2016; Thomas et al., 2009). Briefly, cages (26 cm × 48 cm × 20 cm) were filled with fresh, unscented mouse bedding material to a depth of 5 cm, and the bedding surface was leveled by placing another cage of the same size onto the surface of the bedding. Each mouse was housed individually for habituation. After 10-min habituation, the mouse was removed, and 20 glass marbles (15 mm diameter) were carefully overlaid equidistantly in a 4 × 5 arrangement inside the cage. Subsequently, each mouse was returned to its designated test cage and allowed to explore for 30 min. The number of marbles buried (>50% marbles covered by the bedding) was recorded (Angoa-Perez et al., 2013; Kim, Seung, Kwon et al., 2014).

2.5.3 Nestlet shredding test (NST)

NST is a powerful behavioral assay to assess repetitive and obsessive/compulsive-like behavior. It was performed by placing commercially available cotton fiber (nestlet) (5 cm × 5 cm, 5 mm thick, ~2.5 g each) after weighing it on an analytical balance, into a cage (19 cm × 29 cm × 13 cm) filled with fresh, unscented mouse bedding material to a depth of 0.5 cm (Figure 10). The bedding was packed by placing another cage of the same size onto the surface of the bedding. Each nestlet was placed on top of the bedding in one test cage, and the filter-top cover was placed on the cage. No food and water were provided during the test. One mouse was added to each cage and left undisturbed with the nestlet for 30 min. The mouse was then removed and returned to its home cage after test completion. The remaining intact nestlet was removed from the cage using forceps and allowed to dry overnight. The remaining un-shredded nestlet was weighed, and the weight difference was divided by the starting weight to calculate the percentage of nestlet shredded. The remaining shredded nestlet material and bedding was discarded (Angoa-Perez et al., 2013).

2.5.4 Elevated plus-maze (EPM) test

The EPM test was performed as previously described (Bahi, 2013a, 2013b; Bahi & Dreyer, 2012, 2014) Briefly, a four-armed apparatus was elevated 40 cm above the ground. It consisted of two opposite open arms (30 cm × 6 cm) and two closed arms of the same size with 15 cm high walls. The arms were connected by a central square (6 cm × 6 cm) (Figure 10). An animal was placed in the center of the maze facing an open arm. The maze was lighted approximately one meter above the maze. The amounts of time spent with the head and forepaws in the open arms and closed

arms of the maze as well as the number of entries into each arm were measured over 5 min using EthoVision® Sofware. The maze was thoroughly cleaned with a tissue dampened with 70% (volume/volume; v/v) alcohol to remove any odor after each mouse was tested. The total number of entries into the closed arms is used as an index of locomotor activity in the test (Bahi, 2013a, 2013b; Bahi et al., 2012; Bahi & Dreyer, 2014).

2.5.5 Open-field test (OFT)

OFT involves exploratory activity performed in a novel environment, assessed in an open field box (45 cm × 45 cm × 30 cm) (Lucchina et al., 2014) (Figure 10). This test systematically assesses novel environment exploration, general locomotor activity, and anxiety-related behavior in rodents. The center was defined as the central 23 cm × 23 cm area. Mice were introduced into the center area of the arena and allowed 5 min for habituation before actual behavioral recording. The total distance moved in the whole arena, and time spent at the center and periphery were recorded for 10 min using charged-coupled device camera-assisted motion tracking apparatus and EthoVision software. After each trial, the apparatus was cleaned using 70% ethanol and allowed to dry (Kim et al., 2014; Prut & Belzung, 2003). When evaluating the results, a longer time spent in the center indicated lower levels of anxiety-like behaviors and the total distance travelled reflected locomotor activity.

2.6 Brain collection and tissue preparation for biochemical studies

After the 21 days of treatment as described above, the animals were injected with LPS (25 µg/kg, i.p.) two hours before the sacrifice according to previously published protocols (Lucchina et al., 2014).The animals were deeply anesthetized with

pentobarbital (40 mg/kg, i.p.), and cardiac perfusion was performed using 1× PBS (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride) at pH 7.4 to wash out the blood. The brains were quickly removed and placed on an ice plate. The cerebellum and the hippocampus were excised from the brain and snap-frozen in liquid nitrogen for further use in biochemical tests (Ojha, Javed, Azimullah et al., 2015). On the day of assay, the tissues were homogenized on ice in the extraction buffer recommended by the manufacturer, RIPA buffer (50 mM Tris HCl, pH 7.4, 140 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 and 0.5% sodium deoxycholate) and phosphatase and protease inhibitors. The homogenates were sonicated and centrifuged for 30 min at 14000 rpm at 4°C to remove tissue debris, and the resulting supernatant was used for the proinflammatory cytokine assessment, oxidative stress and Western blot analysis. After being transcardially perfused with PBS four animals from respective groups were further perfused by 4% paraformaldehyde (cold) in 1× PBS. After removing the brains from the skulls, they were post-fixed in the same fixative (4% paraformaldehyde) for 48 h at 4°C. The brains were then immersed in 10% sucrose solution for three consecutive days at 4°C. Finally, the brains were stored in -80°C for cryostat sectioning and immunofluorescence staining analyses (Javed, Azimullah, Abul Khair et al., 2016; Ojha et al., 2015).

2.6.1 Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was performed to quantify the levels of proinflammatory cytokines, IL-1β, IL-6, TNF-α and TGF-β in the cerebellum. The levels of IL-1β, IL-6, TNF-α and TGF-β were estimated as recommended by the manufacturer's instructions. For proinflammatory cytokine analysis, the protein concentrations were determined using the Pierce® BCA Protein Assay Reagent Kit. Briefly, 96-well plates were coated with

the diluted capture antibody (100 µl) overnight at room temperature. Each well was aspirated and washed with wash buffer (0.05% Tween 20 in PBS 0.01 M, pH 7.4). The plate was blocked by adding reagent diluent (1% bovine serum albumin in PBS [300 µl]) for 1 hour, except for TFG-β the blocking buffer was 5% Tween 20 in PBS, and the plate was then washed with wash buffer. Samples or standards (100 µl) were added to the well and incubated for 2 hours, then washed with wash buffer. Each well received detection antibody (100 µl), was incubated for 2 hours at room temperature, and was washed with wash buffer. The well then received working solution (1:40) of streptavidin horseradish peroxidase (100 µl) and was further incubated for 30–40 minutes at room temperature and then washed with wash buffer. The wells received substrate solution (100 µl) and incubated for 20 minutes (avoid direct light). Finally, stop solution (2 N H_2SO_4 [50 µl]) was added and mixed by gentle plate tapping. Immediately, the optical density of each well was determined at 450nm using a microplate absorbance reader (Molecular devices Versamax ®, USA). The results are expressed as pg/mg protein (Ojha et al., 2015; Tyrtyshnaia, Lysenko, Madamba et al., 2016).

2.6.2 Oxidative stress marker estimations

Lipid peroxidation product: malondialdehyde (MDA): An MDA detection kit was used to determine the amount of lipid peroxidation using the manufacturer's instructions. Briefly, samples or calibrators (250 µl) were added to acid reagent (1 M Phosphoric acid) (250 µl) and 2-thiobarbituric acid (250 µl). Subsequently, butylated hydroxytoluene in ethanol (10 µl) was added and vortexed vigorously. Then, the samples were incubated for 60 min at 60°C and centrifuged at 10,000 × *g* for 2–3 min.

The reaction mixture was transferred to a cuvette and its spectra at 532 nm recorded. The results are expressed as μM MDA/mg protein.

Glutathione (GSH): For the estimation of GSH, a commercially available GSH kit was used following the manufacturer's instructions. Briefly, the samples were first deproteinized with 5% 5-sulfosalicylic acid solution and centrifuged to remove the precipitated protein. The supernatant was used to measure the GSH levels. Samples or standards (10 μl) were incubated at room temperature for 5 minutes with 150 μl of working mixture (assay buffer + 5,5′-Dithiobis (2-nitrobenzoic acid) + GSH reductase) in 96-well plates. Diluted NADPH solution (50 μl) was added to each well and mixed properly. The absorbance of the samples was measured at 412 nm, along with the kinetics for 5 min by using the microplate reader. The results are expressed as μM GSH/mg protein.

Superoxide dismutase and Catalase (SOD and CAT): For estimation of the activity of antioxidant enzymes SOD and CAT, manufacturer's instructions for the commercially available kits were followed. Briefly, CAT was estimated by mixing the samples or different standard concentrations (20 μl) with assay buffer (100 μl) and methanol (30 μl) in a 96-well plate. To initiate the reaction, H_2O_2 (20 μl) was added to each well and incubated on a shaker for 20 min at room temperature. To terminate the reaction, Potassium hydroxide (30 μl) was added and subsequently followed by the addition of CAT purpald (30 μl). The plate was covered and incubated for 10 minutes at room temperature on a shaker. Then CAT potassium periodate (10 μl) was added and the plate was incubated again for 5 min at room temperature on a shaker and absorbance was determined using a microplate reader at 540 nm. CAT activity was expressed as nmol/min/mg protein. For SOD estimation, samples or standard (10 μl)

were added to diluted radical detector (tetrazolium salt solution) (200 µl) in a 96-well plate. Xanthine oxidase (20 µl) was added to all wells to initiate the reaction. The plate was shaken carefully for a few seconds, covered with plate cover and incubated for 30 min at room temperature. Absorbance was determined at 450 nm using a microplate reader. SOD activity was expressed as units/mg protein (Ojha, Javed, Azimullah et al., 2016).

2.6.3 Western blot analysis

Western blot analysis was conducted to measure the levels of expression of COX-2, iNOS, and NF-κB p65 in the cerebellum and hippocampus of different animal groups, as described previously (Javed, Azimullah, Abul Khair, et al., 2016; Ojha et al., 2015). As mentioned earlier the prepared cell lysates containing the extracted protein from the mouse brain tissues were used. The protein content in the sample was estimated using the Pierce® BCA Protein Assay Reagent Kit. The protein samples containing equal amounts of protein (30 µg) adjusted with RIPA buffer and 4 × Laemmli sample buffer, were loaded and separated in Mini-Protean TGX® precast electrophoresis gels. The proteins were then transferred onto polyvinylidene difluoride membranes that were first activated by soaking in 100% methanol. The transfer was carried in a Trans-blot Turbo semi-dry-transfer system (BIO-RAD, Bio-Rad laboratories, Inc, USA,) using a transfer buffer (25 mM Tris base, 192 mM glycine, 20% v/v methanol and adjust volume to 1000 ml distilled water, pH 8.3). Subsequently, the membranes were incubated for 1 h with the blocking buffer (non-fat dry milk) and then washed to avoid non-specific binding. The membranes were then incubated overnight at 4°C with the specific primary antibody (Table 2). The second day the membranes were washed and incubated for 1 h with the horseradish

peroxidase conjugated secondary anti-rabbit antibody, at room temperature. The bands of proteins detected by the antibodies were visualized using an enhanced chemiluminescence Pico kit as substrate. Subsequently, the blots were stripped and re-probed for actin (used as loading control) using horseradish peroxidase anti-mouse secondary antibody (Table 2). The intensity of the band was measured using densitometry and quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA)

Table 2: Primary and secondary antibodies used in Western blot and their dilutions.

Primary Ab	Dilution	Secondary Ab	Dilution
Rabbit anti-COX-2	1:1000	HRP anti Rabbit	1:20000
Rabbit anti-iNOS	1:5000	HRP anti Rabbit	1:20000
Rabbit anti-NF-κB p65	1:50000	HRP anti Rabbit	1:10000
Mouse anti-actin	1:7000	HRP anti Mouse	1:20000

2.7 Immunofluorescence staining of Iba-1

Brains were collected, post fixed and stored in -80°C as mentioned earlier. On the day of sectioning, the brains were sliced using a cryostat (Leica, Germany) into 20 µm sections. Immunofluorescence staining was performed with the coronal sections of the cerebellum to examine the Iba-1 positive microglia (activated). In 24-well plate brain sections were washed twice with PBS (500 µl/well) and incubated with blocking reagent (10% normal goat serum in PBS 0.3% Triton-X 100) (250 µl/well) for 1 h at

room temperature. The sections were then washed and incubated with the primary polyclonal rabbit antibody against Iba-1 (1:700) (250 µl/well) overnight at 4°C. On the second day, the sections were washed twice with PBS (500 µl/well) and incubated with fluorescent anti-rabbit secondary antibody (Alexa Fluor488) (1:1000) (250 µl/well) for 1 h at room temperature. Subsequently, the sections were washed again and mounted using Vectashield® mounting media. The images were then captured using the fluorescent microscope (Nikon Eclipse Ni). A minimum of three sections per brain from four animals were used (three sections per brain) to analyse microglia activation. From each section the activated microglia were evaluated by measuring the integrated density of the Iba-1 signal from three different randomly selected fields of equal areas, using the Image J software. The expression levels of Iba-1 were detected by the green fluorescence emitted by Alexa Fluor488. The total corrected cellular fluorescence (TCCF) was then calculated using the following equation: (TCCF) = integrated density – (area of selected cell × mean fluorescence of the background) (McCloy, Rogers, Caldon et al., 2014). This TCCF was calculated and normalized against the mean of the control, with results presented as percentage fold increase from the control.

2.8. Determination of acetylcholinesterase (AChE) activity in BTBR mice cerebellum

The acetylcholinesterase assay kit was used, and the procedure followed was according to the manufacturer. The assay relates the hydrolysis of ACh to choline by AChE enzyme. Briefly, 5 µl of supernatant of homogenate (cerebellum tissue) was placed into the plate. Then, 45 µl of working reagent that consists of AChE assay buffer, and 50 µl reaction mix were added into each well. After incubation for 20-30

min at 37ºC, absorbance was read in a kinetic mode, and choosing two time points in a linear range to calculate the AChE activity of the sample, using a microplate reader at 570 nm. AChE activity was expressed as nmol/min/mg protein.

2.9 Statistical analysis

For the SI and SNI, and results of the MBT, NST, OFT and EPM test, statistical significance was assessed with a group (Control, VPA, BTBR) × drug (SAL, DL77, E100) analysis of variance. The data were first analyzed for normality by assessing the sample distribution or skewness (−1.5 to +1.5 considered normally distributed). The source of the detected significances was determined by Bonferroni's multiple comparison post hoc test. *P*-values less than 0.05 were considered statistically significant. The number of mice per group is indicated in the figures. Distinct analyses were performed to enable the calculation of SI and SNI, for the interaction time. Respective group and chamber were considered independent variables, and their influence over the dependent variable time was determined. Statistical significance for biochemical assessments, Western blot, and immunofluorescence analyses was calculated using one-way analysis of variance followed by post hoc Tukey's multiple comparison test. For statistical comparisons, the software package SPSS 25.0 (IBM Middle East, Dubai, UAE) was used. The results are expressed as the means and standard errors of the means (SEM). The *P*-values less than 0.05 were considered statistically significant.

Chapter 3: Results

3.1 Effects of DL77 and DOZ on sociability deficits assessed in VPA-exposed TO mice in TCT

The effect of subchronic systemic injection of DL77 at three different doses (5, 10, and 15 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.) on ASD-like sociability deficits in the TCT task in VPA-exposed mice are shown in Figure 11A. The results of statistical analyses revealed that subchronic treatment with DL77 (10 mg/kg, i.p.) prior to TCT significantly enhanced sociability of VPA-exposed mice by increasing SI when compared to saline-treated VPA-exposed mice with [$F_{(1,10)}$ = 7.63; P < 0.05]. However, DL77 (5 and 15 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.) failed to enhance sociability of VPA-exposed mice with p=0.92, p=0.16, and p=0.16, respectively (Figure 11A). Moreover, no significant difference in the SI was observed for saline-exposed mice pretreated with 10 mg/kg of DL77 (10 mg/kg) with p=0.43, indicating that VPA-exposed mice exhibited enhanced sociability performance when pretreated with DL77 (10 mg/kg) (Figure 11A). Furthermore, DOZ (1 mg/kg, i.p.) failed to alter saline-exposed with p=0.19 as compared to saline-treated Saline exposed mice (Figure 11A). Additionally, the observed results for SI values showed that the DL77 (10 mg)-provided enhancement in sociability performance was completely abrogated when co-administered with the CNS-penetrant H3R agonist RAM (10 mg/kg, i.p., P<0.05) or the CNS-penetrant H2R antagonist ZOL (10 mg/kg, i.p.), with [$F_{(1,10)}$ = 30.72; P < 0.001] as compared to DL77 (10 mg)-pretreated VPA-exposed mice (Figure 11B). However, the CNS-penetrant H1R antagonist PYR (10 mg/kg, i.p.) failed to reverse the observed effects of DL77 (10 mg/kg) with p=0.71 for the comparison of DL77 (10

mg)-treated VPA-exposed mice with DL77 (10 mg) + PYR (10 mg)-treated VPA-exposed mice (Figure 11B).

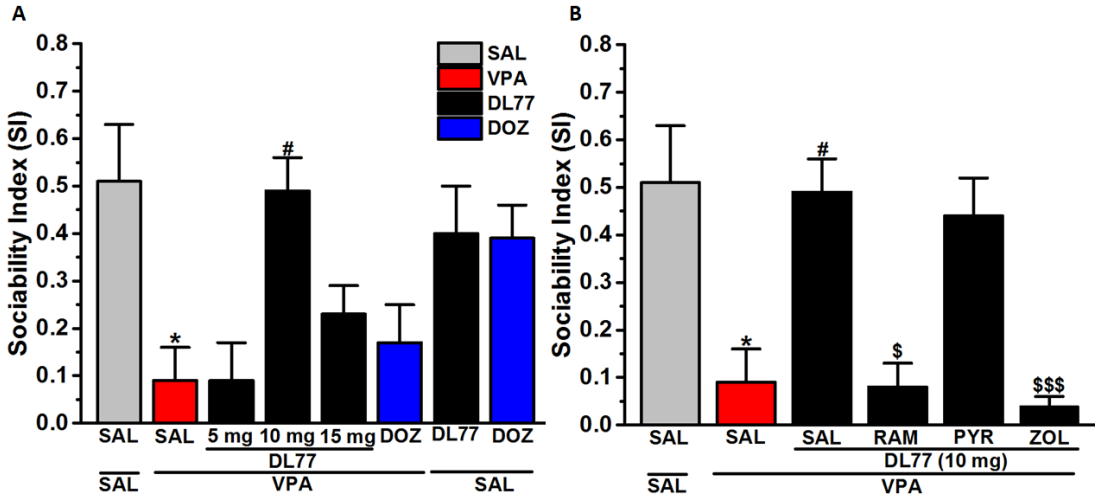


Figure 11: Effects of DL77 and DOZ on sociability deficits assessed in VPA-exposed TO mice in TCT.

After 10 minutes of acclimatization, male subjects were allowed to explore all chambers for 10 min. The results obtained were Sociability index (SI) **A)** Abrogation of DL77 (10 mg)-provided effect on SI (B). Saline-exposed mice were injected with saline in SAL group (gray), DL77 (5, 10, and 15 mg/kg, i.p. in black), or DOZ (1 mg/kg, i.p. in blue) was administered subchronically for 21 days. Abrogative studies of subchronic (21 days) systemic co-injection of RAM (10 mg/kg, i.p. for RAM group), PYR (10 mg/kg, i.p. PYR group), or ZOL (10 mg/kg, i.p., for ZOL group) on the DL77 (10 mg)-provided improvement of sociability. Saline exposed mice were injected with saline, DL77 (10 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.). Figures show mean ± SEM (n=6-7). $^{*}P<0.05$ vs.SI of saline-treated saline-exposed mice. $^{\#}P<0.05$ vs. SI of saline-treated VPA-exposed mice. $^{\$}P<0.05$, $^{\$\$\$}P<0.001$ vs. SI of DL77 (10 mg)-treated VPA-exposed mice.

3.2 Effects of DL77 and DOZ on social novelty deficits assessed in VPA-exposed TO mice in TCT

Figure 12 shows the effects of subchronic administration of three different doses of DL77 (5, 10, and 15 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.) on social novelty deficits of VPA-exposed mice. The results of post hoc analysis indicated that saline-exposed mice exhibited significantly higher SNI when compared to VPA-exposed mice, with [$F_{(1,10)} = 5.87$; $P < 0.05$] (Figure 12A). VPA-exposed mice pretreated with DL77 (10 or 15 mg/kg, i.p.) showed significant improvement in SNI when compared

to saline-treated VPA-exposed mice with [$F_{(1,10)}$ = 5.73; P < 0.05] and [$F_{(1,10)}$ = 5.76; P < 0.05], respectively (Figure 12A). However, DL77 (5 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.) failed to improve the social novelty of VPA-exposed mice, with p=0.93 and p=0.60, respectively (Figure 12A). Furthermore, no significant difference between saline-treated saline-exposed mice and DL77 (10 mg)-treated saline-exposed mice was found (p=0.51) (Figure 12B). As depicted in Figure 12B and observed in the post hoc analysis, the results for SNI values showed that the DL77 (10 mg)-provided enhancement in social novelty performance was completely counteracted when the centrally active H3R agonist RAM (10 mg/kg, i.p.) or the CNS-penetrant H2R antagonist ZOL (10 mg/kg, i.p.) was co-administered (P < 0.05 for both comparisons) (Figure 12B). However, the CNS-penetrant H1R antagonist PYR (10 mg/kg, i.p., P>0.05) failed to reverse the enhancement of DL77-provided social novelty performance (Figure 12B).

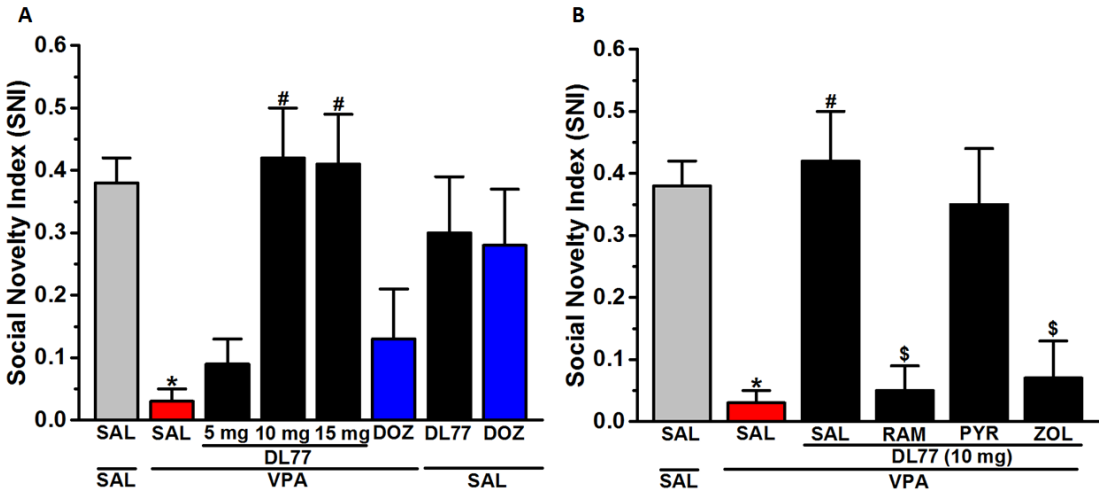


Figure 12: Effects of DL77 and DOZ on social novelty deficits assessed in VPA-exposed TO mice in TCT.

After 10 minutes of exploration, male subjects were allowed to explore all chambers for another 10 min. The results obtained were Social novelty index (SNI) **(A)** Abrogation of DL77 (10 mg)-provided effect on SNI (B). Saline-exposed mice were injected with saline in SAL group (gray), DL77 (5, 10, and 15 mg/kg, i.p. in black), or DOZ (1 mg/kg, i.p. in blue) was administered subchronically for 21 days. Abrogative studies of subchronic (21 days) systemic co-injection of RAM (10 mg/kg, i.p. for RAM group), PYR (10 mg/kg, i.p. PYR group), or ZOL (10 mg/kg, i.p., for ZOL group) on the DL77 (10 mg)-provided improvement of social novelty. Saline exposed mice were injected with saline, DL77 10 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.). Figures show mean ± SEM (n=5-7). $^{*}P<0.05$ vs. SNI of saline-treated saline-exposed mice. $^{\#}P<0.05$ vs. SNI of saline-treated VPA-exposed mice. $^{\$}P<0.05$ vs. SNI of DL77 (10 mg)-treated VPA-exposed mice.

3.3 Effects of DL77 and DOZ on elevated stereotyped repetitive behavior in VPA-exposed TO mice in MBT

The effect of subchronic systemic injection of DL77 (5, 10, or 15 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.) on the increase in repetitive behavior of VPA-exposed mice in MBT is shown in Figure 13A and B. The results of statistical analyses showed that subchronic treatment with either 10 or 15 mg/kg DL77 or 1 mg/kg DOZ prior to MBT significantly decreased the increased percentage of marbles buried by VPA-exposed mice, with [$F_{(7,48)} = 6.65$; $P<0.001$] (Figure 13A). As observed in the post hoc analysis, VPA-exposed mice buried significantly more marbles compared to the saline-exposed animals, with [$F_{(1,12)} = 8.03$; $P<0.05$]. However, VPA-exposed mice pretreated with

DL77 at 10 mg/kg, or 15 mg/kg exhibited significantly decreased percentages of buried marbles compared to saline-treated VPA-exposed mice, with [$F_{(1,12)}$ = 24.14; P < 0.001] and [$F_{(1,10)}$ = 27.33; P< 0.001], respectively (Figure 13A). In contrast, DL77 (5 mg/kg, i.p.) failed to significantly decrease the percentage of buried marbles in VPA-exposed mice, with [$F_{(1,12)}$ = 3.45; p = 0.09] (Figure 13A). Notably, no significant difference in the DL77-provided effect on percentage of buried marbles was observed between DL77 10 mg/kg and 15 mg/kg, with p = 0.59. Importantly, subchronic systemic administration of DOZ (1 mg/kg, i.p.) significantly decreased the percentage of marbles buried by VPA-exposed mice, with [$F_{(1,12)}$ = 8.62; P < 0.05]. Furthermore, no significant difference in the percentage of buried marbles was observed in the comparison of saline + saline group vs. saline + DOZ (1 mg) group (p = 0.88) (Figure 13A). However, there was a significant decrease in the percentage of buried marbles in saline-exposed mice subchronically treated with DL77 (10 mg/kg, i.p.) vs. saline + saline group, with [$F_{(1,12)}$ = 16.178; P < 0.05] (Figure 13A). In another experiment, the DL77 (10 mg)-provided decrease in the percentage of buried marbles was reversed by RAM, with [$F_{(1,12)}$ = 1.43; p = 0.26] for the comparison of VPA-exposed mice vs. VPA-exposed mice + DL77 (10 mg) + RAM (10 mg). However, PYR and ZOL failed to reverse this DL77 (10 mg)-provided decrease in the percentage of buried marbles, as they had no significant effect compared to VPA-exposed mice treated with DL77 (10 mg/kg, i.p.), with [$F_{(1,12)}$ = 2.73; p = 0.12] and [$F_{(1,12)}$ = 0.00; p = 0.98], respectively (Figure 13B).

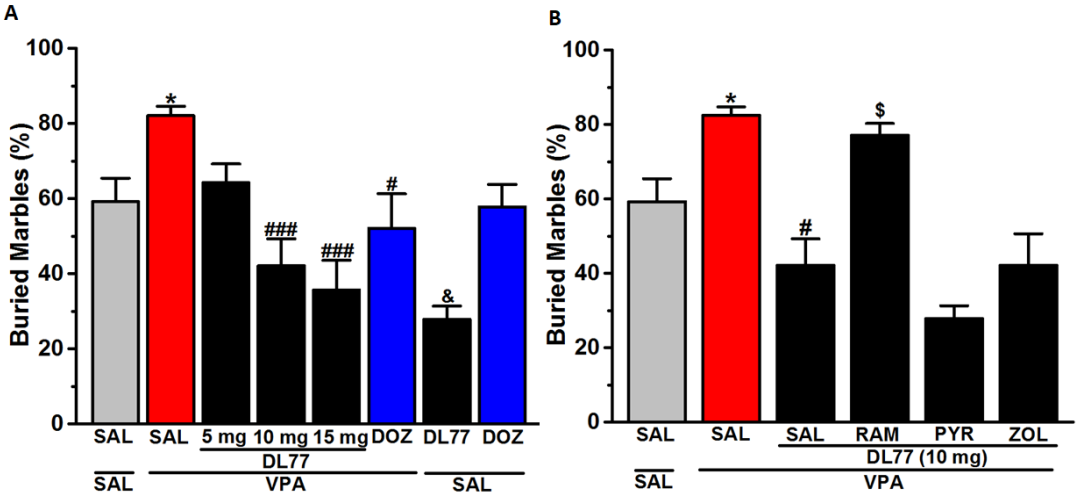


Figure 13: Effects of DL77 and DOZ on elevated stereotyped repetitive behavior in VPA-exposed TO mice in MBT.

A) Repetitive marble-burying behavior was measured after a 30-minute testing session. VPA-exposed mice (red) demonstrated elevated stereotyped, repetitive behaviors that were significantly increased compared to saline-exposed mice (gray). DL77 (at a dose of 5, 10, or 15 mg/kg, i.p. (black)) or DOZ (1 mg/kg, i.p. in blue) was administered subchronically for 21 days. B) Effects of sub-chronic (21 days) systemic co-administration of RAM (10 mg/kg, i.p.), PYR (10 mg/kg, i.p.), or ZOL (10 mg/kg, i.p.) on the DL77 (10 mg)-provided attenuation of stereotyped repetitive behavior of VPA-exposed mice in MBT. Saline-exposed mice were injected with saline in group, DL77 (10 mg/kg, i.p.), or DOZ (1 mg/kg, i.p.). Figures show mean ± SEM (n = 7). $^{*}P < 0.05$ vs. saline-treated saline-exposed mice. $^{\#}P < 0.05$ vs. saline-treated VPA-exposed mice. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. saline-treated VPA-exposed mice. $^{\&}P < 0.001$ vs. saline-treated saline-exposed mice. $^{\$}P<0.05$ vs. DL77 (10 mg)-treated VPA-exposed mice.

3.4 Effects of DL77 and DOZ on elevated stereotyped repetitive behavior in VPA-exposed TO mice in NST

The effect of subchronic systemic injection of DL77 (5, 10, or 15 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.) on the increase in the percentage of shredded nestlet was assessed in NST (Figure 14A and B). ANOVA indicated that VPA-exposed mice shredded significantly more nestlet compared to the saline-exposed mice, with [$F_{(1,12)} = 20.90$; $P < 0.05$]. VPA-exposed mice treated with DL77 (10 mg/kg or 15 mg/kg) exhibited a significantly lower percentage of shredded nestlet compared to VPA-exposed mice treated with saline, with [$F_{(1,12)} = 7.34$; $P < 0.05$] and [$F_{(1,12)} = 9.63$; $P < 0.05$], respectively (Figure 14A). However, DL77 (5 mg/kg) failed to modulate the

percentage of shredded nestlet in VPA-exposed mice, with [$F_{(1,12)}$ = 0.39; p = 0.54]. Notably, no significant difference in the DL77-provided effect on percentage of shredded nestlet was observed between the two doses of 10 and 15 mg/kg, with p = 0.84. Moreover, subchronic systemic administration of DOZ (1 mg/kg, i.p.) significantly lowered the percentage of shredded nestlet in VPA-exposed mice, with [$F_{(1,12)}$ = 10.99; $P < 0.05$]. Furthermore, neither saline + saline group vs. saline + DL77 (10 mg) group nor saline + saline group vs. saline + DOZ (1 mg) group showed significant differences in shredded nestlet (p = 0.56 and p = 0.53, respectively) (Figure 14A). As depicted in Figure 14B and observed in the post hoc analysis, the DL77 (10 mg)-provided decrease in the percentage of shredded nestlet was reversed following RAM, with [$F_{(1,12)}$ = 0.002; p = 0.98] compared with saline-treated VPA-exposed mice (Figure 14B). However, PYR and ZOL failed to reverse this DL77-provided decrease in the percentage of shredded nestlet, as they had no significant effect compared to VPA-exposed mice treated with DL77 (10 mg/kg, i.p.), with [$F_{(1,12)}$ = 2.73; p = 0.12] and [$F_{(1,12)}$ = 0; p = 0.98], respectively (Figure 14B).

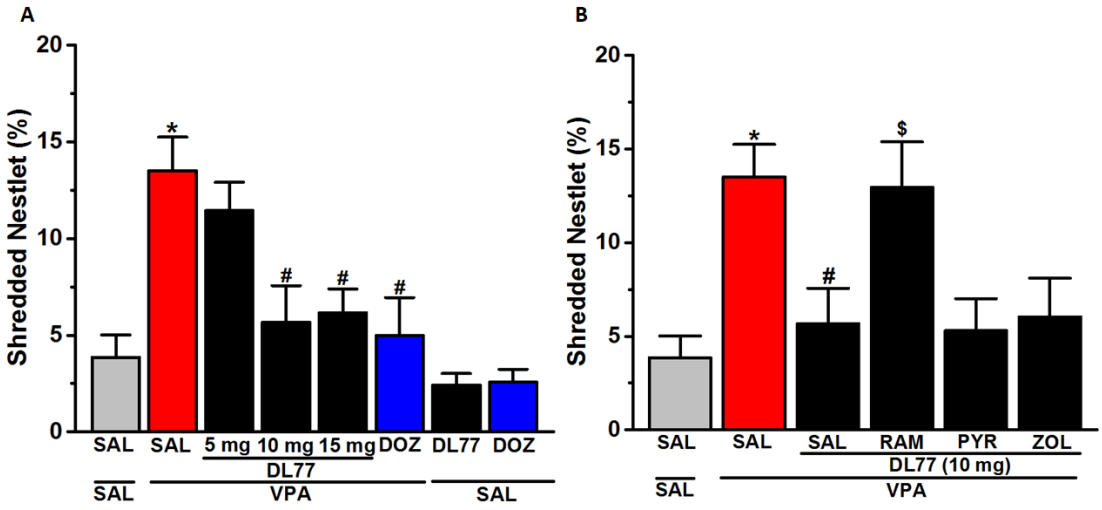


Figure 14: Effects of DL77 and DOZ on elevated stereotyped repetitive behavior in VPA-exposed TO mice in NST.

(A) Repetitive shredding behavior was measured after a 30-minute testing session. VPA-exposed mice (red) demonstrated elevated stereotyped, repetitive behaviors that were significantly increased compared to saline-exposed mice (gray). DL77 (at a dose of 5, 10, or 15 mg/kg, i.p. (black)) or DOZ (1 mg/kg, i.p. in blue) was administered subchronically for 21 days. (B) Effects of sub-chronic (21 days) systemic co-administration of RAM (10 mg/kg, i.p.), PYR (10 mg/kg, i.p.), or ZOL (10 mg/kg, i.p.) on the DL77 (10 mg)-provided attenuation of stereotyped repetitive behavior of VPA-exposed mice in NST. Saline-exposed mice were injected with saline in group, DL77 (10 mg/kg, i.p.), or DOZ (1 mg/kg, i.p.). Figures show mean ± SEM (n=7). $^{*}P < 0.05$ vs. saline-treated saline-exposed mice. $^{\#}P < 0.05$ vs. saline-treated VPA-exposed mice. $^{\$}P<0.05$ vs. DL77 (10 mg)-treated VPA-exposed mice.

3.5 Effects of DL77 and DOZ pretreatment on exploratory behavior in VPA-exposed TO mice in EPM test

Figure 15 shows the observed effects of subchronic systemic injection of saline or the H3R antagonist DL77 (5, 10, or 15 mg/kg, i.p.) on the anxiety parameters of VPA-exposed mice tested in the EPM, namely, the percentage of time spent in open arms (Figure 15A), the number of entries into open arms (Figure 15B), and locomotor activity expressed as the number of entries into closed arms (Figure 15C). Subsequent post hoc analyses showed that DL77 administered at 5, 10, or 15 mg/kg i.p. did not alter the percentage of time spent exploring the open arms of the maze during a 5 min session compared to saline-treated VPA-exposed mice, with [$F_{(1,9)} = 0.30$; p = 0.59], [$F_{(1,9)} = 0.18$; p = 0.68], or [$F_{(1,9)} = 0.56$; p = 0.47], respectively (Figure 15A). However,

VPA-exposed mice pretreated with DOZ (1 mg) spent a significantly lesser percentage of time exploring the open arms compared to saline-treated VPA-exposed mice, with [$F_{(1,7)} = 11.84$; $P < 0.05$] (Figure 15A). Further analyses of data describing the number of entries and percentage of time in the open arms of the maze yielded for DL77 (5, 10, and 15 mg/kg i.p.) and DOZ (1 mg/kg, i.p.) practically the same results, with [$F_{(1,9)} = 1.75$; p = 0.22], [$F_{(1,9)} = 1.20$; p = 0.30], [$F_{(1,9)} = 1.21$; p = 0.30], and [$F_{(1,7)} = 7.22$; $P < 0.05$], respectively (Figure 15B). Interestingly, the number of closed arm entries following subchronic systemic injection of DL77 (5, 10, or 15 mg/kg) was not significantly different, with [$F_{(1,10)} = 0.99$; p = 0.34], [$F_{(1,10)} = 0.29$; p = 0.60], and [$F_{(1,10)} = 0.06$; p = 0.81], respectively. However, DOZ (1 mg)-treated VPA-exposed mice showed fewer closed arm entries compared to saline-treated VPA-exposed mice, with [$F_{(1,10)} = 6.60$; $P < 0.05$] (Figure 15C).

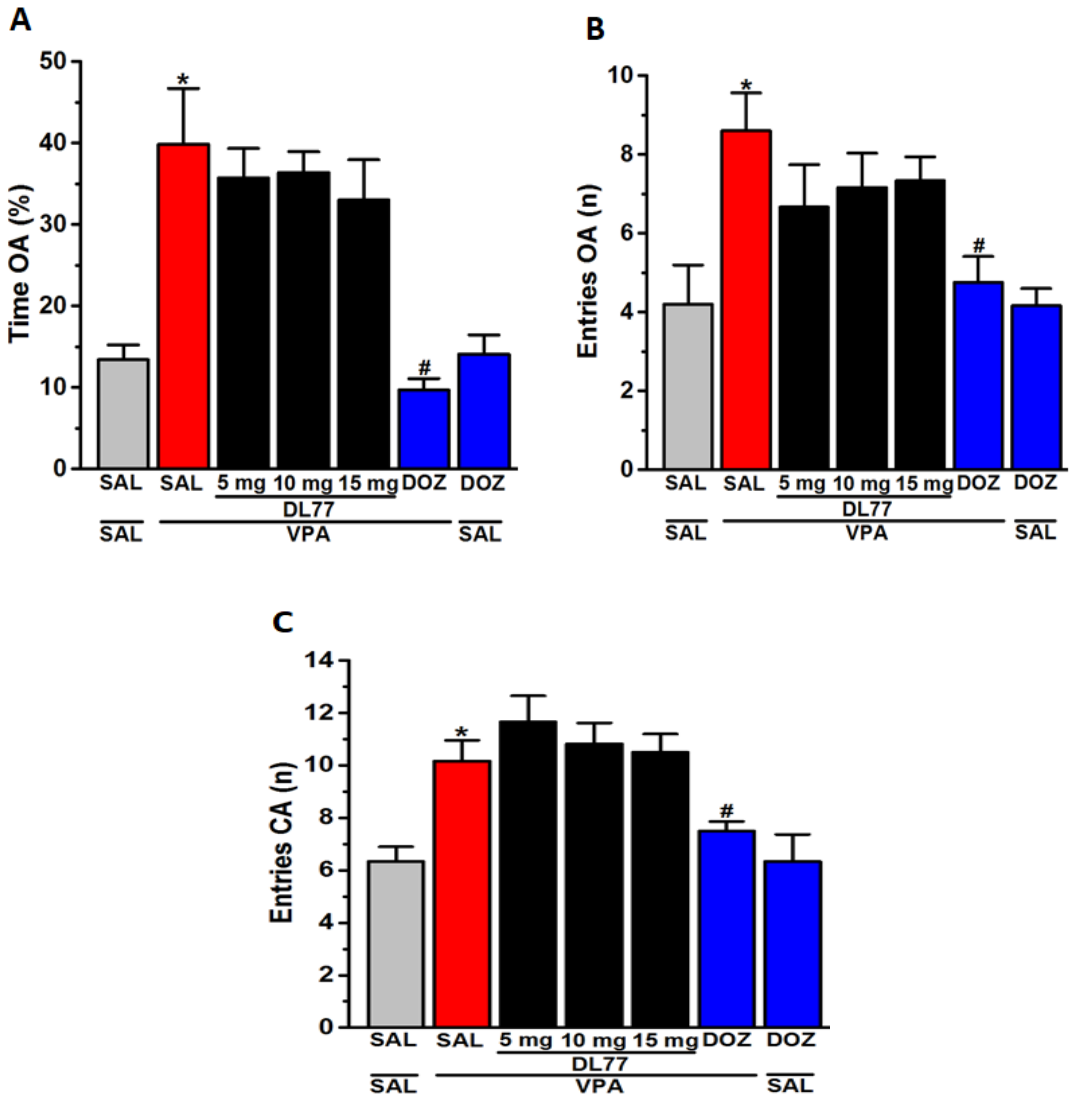


Figure 15: Effects of DL77 and DOZ pretreatment on exploratory behavior in VPA-exposed TO mice in EPM test.

VPA-exposed mice (red) demonstrated elevated impulsive attitude and deficits in cognition behaviors that were significantly increased compared to saline-exposed mice (gray). DL77 (5, 10, or 15 mg/kg, i.p. in black) or DOZ (1 mg/kg, i.p., in blue) was administered subchronically for 21 days. DOZ (1 mg/kg, i.p.) attenuated the increased percentage of time spent on the open arms of the EPM (**A**) the increased number of entries into the open arms (B) and the increased number of entries into the closed arms (C) in VPA mice. However, pretreatment with the H3R antagonist DL77 (5, 10, or 15 mg/kg, i.p.) did not affect any of the three parameters (A-C). Saline-exposed mice were injected with saline or DOZ (1 mg/kg, i.p.) (A-C). Data are expressed as the mean ± SEM (n=5-7). $^{*}P < 0.05$ vs. saline-treated saline-exposed mice. $^{\#}P < 0.05$ vs. saline-treated VPA-exposed mice.

3.6 Effects of DL77 and DOZ pretreatment on levels of malondialdehyde (MDA) and glutathione (GSH) in the cerebellum of VPA-exposed TO mice

The ability of DL77 to reduce oxidative stress in VPA-exposed mice was evaluated. Two parameters were measured, the MDA and GSH levels in VPA-exposed mouse cerebellum, which responded in an exacerbated way to the inflammatory stimulus LPS. The results in Figure 16A and B show that MDA was significantly elevated ($P<0.05$), and GSH was significantly decreased ($P<0.05$) in the brain tissues of VPA-exposed mice compared to saline-exposed mice (Figure 16A and B). However, brain tissues of VPA-exposed mice subchronically pretreated with DL77 (10 or 15 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.) showed a significant reduction of MDA (from 214.88 ± 12.58 μM to 112.77 ± 19.56, 102.24 ± 16.60, and 97.32 ± 16.55 μM for DL77 (10 and 15 mg/kg) and DOZ (1 mg/kg), respectively) (all $P<0.05$) (Figure 16A). In addition, subchronic administration of DL77 (10 or 15 mg/kg) or DOZ (1 mg/kg) significantly increased GSH compared to the saline-treated VPA-exposed animals (all $P<0.05$) (Figure 16B). Meanwhile, the VPA-exposed mice subchronically pretreated with 5 mg/kg did not show any significant difference from the saline-treated VPA-exposed group (Figure 16A and B). Moreover, subchronic systemic co-administration of RAM (10 mg/kg, i.p.) partially abrogated ($P< 0.05$) the protective effects of DL77 (10 mg/kg, i.p.) against the VPA-induced decreased level of MDA (Figure 16A), and it completely reversed the DL77 (10 mg)-provided increase in GSH in VPA-exposed mice (Figure 16B).

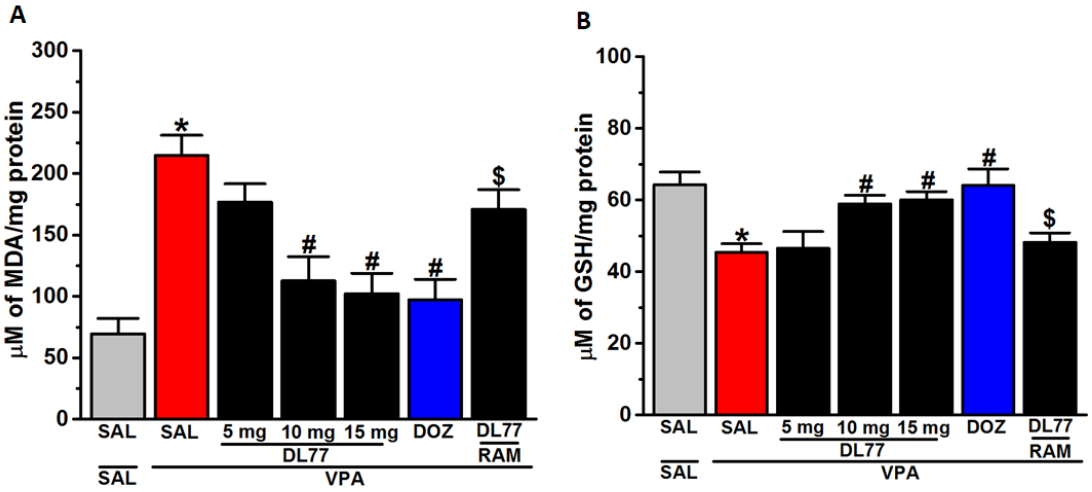


Figure 16: Effects of DL77 and DOZ pretreatment on levels of malondialdehyde (MDA) and glutathione (GSH) in the cerebellum of VPA-exposed TO mice.

VPA-exposed mice (red) showed a significant increase in MDA (A) and significant decrease in GSH (B) compared to saline-exposed mice (gray). DL77 (5, 10, or 15 mg/kg, i.p. in black) or DOZ (1 mg/kg, i.p. in blue) was administered subchronically for 21 days. DL77 (10 or 15 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.) significantly decreased MDA and significantly increased GSH. RAM (10 mg/kg, i.p.) when co-administered abrogated the modulating effects provided with DL77 (10 mg/kg, i.p.) on oxidative stress levels (A, B). Saline-exposed mice were injected with saline. Data are expressed as the mean ± SEM (n=5-7). $^{*}P < 0.05$ vs. saline-treated saline-exposed mice. $^{\#}P < 0.05$ vs. saline-treated VPA-exposed mice. $^{\$}P < 0.05$ vs. DL77 (10 mg)-treated VPA-exposed mice.

3.7 Effects of DL77 and DOZ pretreatment on levels of proinflammatory cytokines IL-1β, IL-6 and TNF-α in the cerebellum of VPA-exposed TO mice

The effects of DL77 on the levels of proinflammatory cytokines IL-1β, IL-6 and TNF-α in brain tissue of VPA-exposed mice that were exacerbated with LPS challenge were also assessed (Figure 17A-C). The induction of ASD-like behaviors by prenatally administered VPA significantly increased IL-1β (Figure 17A), IL-6 (Figure 17B), and TNF-α (Figure 17C) compared to the saline-exposed mice (all $P < 0.001$), while subchronic systemic administration of DL77 (5, 10 or 15 mg/kg, i.p.) or DOZ (1 mg/kg) significantly ($P < 0.001$) attenuated the rise of these proinflammatory cytokines in VPA-exposed mice (Figure 17A-C). Notably, subchronic systemic co-administration of RAM (10 mg/kg, i.p.) partially abrogated ($P < 0.001$) the protective

effects of DL77 (10 mg/kg, i.p.) against VPA-induced elevation of proinflammatory cytokines (Figure 17A-C).

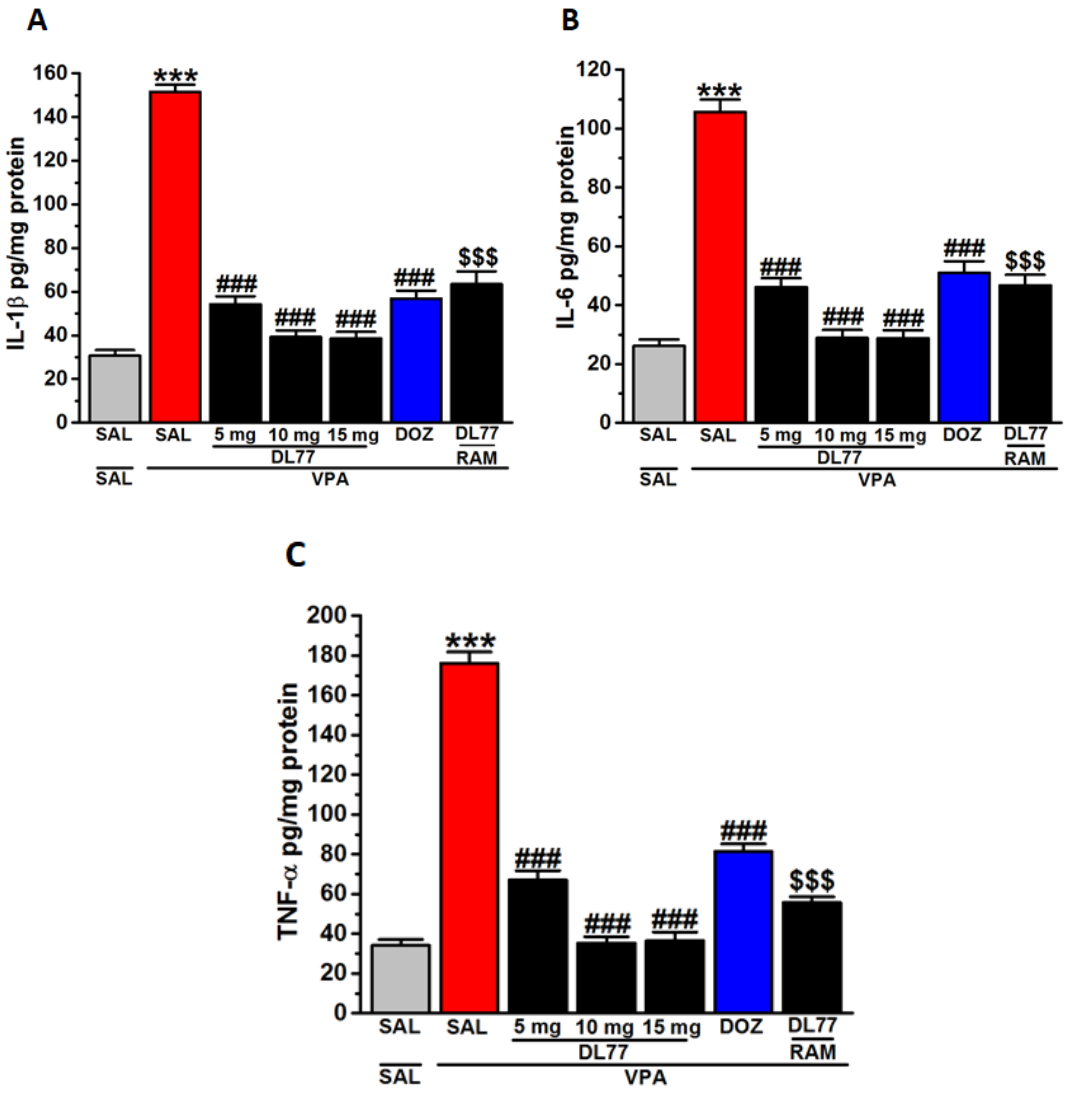


Figure 17: Effects of DL77 and DOZ pretreatment on levels of proinflammatory cytokines IL-1β, IL-6 and TNF-α in the cerebellum of VPA-exposed TO mice.

VPA-exposed mice (red) showed significantly increased levels of IL-1β (A) IL-6 (B) and TNF-α (C) compared to saline-exposed mice (gray) exacerbated by LPS challenge. DL77 (5, 10, or 15 mg/kg, i.p. in black) or DOZ (1 mg/kg, i.p. in blue) was administered subchronically for 21 days. DL77 (5, 10, or 15 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.) significantly decreased IL-1β (A), IL-6 (B) and TNF-α (**C**). RAM (10 mg/kg, i.p.) when co-administered reversed the modulating DL77 (10 mg)-provided effects on proinflammatory cytokines (A-C). Saline-exposed mice were injected with saline (A-C). Data are expressed as the mean ± SEM (n = 5-6). $^{***}P < 0.001$ vs. saline-exposed mice. $^{###}P < 0.001$ vs. VPA-exposed mice. $^{\$\$\$}P < 0.001$ vs. DL77 (10 mg)-treated VPA-exposed mice.

3.8 Effects of DL77 and E100 on sociability deficits assessed in VPA-exposed C57BL/6J mice in TCT

The effect of subchronic systemic administration with DL77 (5, 10, or 15 mg/kg, i.p.), E100 (5, 10, or 15 mg/kg, i.p.), and DOZ (1 mg/kg, i.p.) on ASD-like sociability impairments in the TCT task in VPA-exposed mice are shown in Figure 18A and B. As observed in the post hoc analysis, VPA-exposed mice exhibited significantly lower sociability expressed in form of SI value when compared to saline-exposed animals, with [$F_{(1,12)}$ = 23.38; P<0.05] (Figure 18A). However, DL77 (10 and 15 mg/kg) significantly increased SI of VPA-exposed mice when compared to Saline treated VPA -exposed group, with [$F_{(1,12)}$ = 9.92; P<0.05] and [$F_{(1,12)}$ = 32.82; P<0.05], respectively (Figure 18A). Moreover, the results revealed that the enhancement in SI observed with DL77 (15 mg/kg) was comparable to that shown with DOZ (1 mg/kg), with [$F_{(1,12)}$ = 0.05; p=0.83], and was significantly higher than the DL77 (5 mg)- and DL77 (10 mg)-provided effects, with [$F_{(1,12)}$ = 7.22; P<0.05] and [$F_{(1,12)}$ = 5.74; P<0.05], respectively (Figure 18A). Interestingly, the DL77-provided improvement of sociability was counteracted following subchronic systemic co-administration with centrally acting H3R agonist RAM (10 mg/kg, i.p.) with [$F_{(1,12)}$ = 6.42; P<0.05] compared with DL77 (15 mg)-treated VPA-exposed mice (Figure 18B). However, CNS-penetrant H1R antagonist PYR (10 mg/kg, i.p.) and H2R antagonist ZOL (10 mg/kg, i.p.) failed to reverse the DL77 (15 mg)-provided sociability enhancement, with [$F_{(1,12)}$ = 3.64; p=0.08] and [$F_{(1,12)}$ = 1.32; p=0.27], respectively (Figure 18B). Similarly, the effect of subchronic systemic injection of E100 at three different doses (5, 10, and 15 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.) on ASD-like sociability impairments in the TCT task in VPA-exposed mice are shown in Figure 18A. The results of statistical analyses revealed that subchronic pretreatment with E100 (5, 10,

and 15 mg/kg, i.p.) and DOZ (1 mg/kg) prior to TCT significantly enhanced sociability by increasing SI, with [$F_{(8,54)}$ = 4.51; P<0.001] (Figure 18A). As observed in the post hoc analysis, VPA-exposed mice exhibited significantly lower sociability expressed in form of SI value when compared to saline-exposed animals, with [$F_{(1,12)}$ = 13.62; P<0.05] (Figure 18A). However, E100 (5, 10, and 15 mg/kg) significantly increased SI of VPA-exposed mice when compared to saline-treated VPA-exposed group, with [$F_{(1,12)}$ = 21.22; P<0.05], [$F_{(1,12)}$ = 23.83; P<0.05], and [$F_{(1,12)}$ = 14.24; P<0.05], respectively (Figure 18A). Moreover, the results revealed that the enhancement in SI observed with E100 (10 mg/kg) was comparable to that shown with DOZ (1 mg/kg), with [$F_{(1,12)}$ = 0.08; p=0.78] as well as with DL77 (15 mg/kg, i.p.) with [$F_{(1,12)}$ = 0.44; p=0.52], and was significantly higher than the E100 (5 mg)-provided effects, with [$F_{(1,12)}$ = 5.17; P<0.05] (Figure 18A). Interestingly, the E100-provided improvement of sociability was counteracted following subchronic systemic co-administration with centrally acting H3R agonist RAM (10 mg/kg, i.p.), CNS-penetrant H2R antagonist ZOL (10 mg/kg, i.p.), or muscarinic antagonist SCO (0.3 mg/kg, i.p.), with [$F_{(1,12)}$ = 7.78; P<0.05], [$F_{(1,12)}$ = 8.38; P<0.05], or [$F_{(1,10)}$ = 6.48; P<0.05] respectively, as compared to the E100 (10 mg)-treated VPA-exposed mice. (Figure 18B). However, CNS-penetrant H1R antagonist PYR (10 mg/kg, i.p.) failed to reverse the E100 (10 mg)-provided sociability enhancement, with [$F_{(1,12)}$ = 0.06; p=0.82] for SI of E100 (10 mg)-treated VPA-exposed animals vs. PYR (10 mg) + E100 (10 mg)-treated group (Figure 18B). Notably, neither DL77 (15 mg) nor E100 (10 mg) or DOZ (1 mg) altered SI of Saline pretreated saline-exposed mice, with [$F_{(1,12)}$ = 0.09; p=0.77], [$F_{(1,12)}$ = 0.12; p=0.73], and [$F_{(1,12)}$ = 1.23; p=0.29], respectively (Figure 18A).

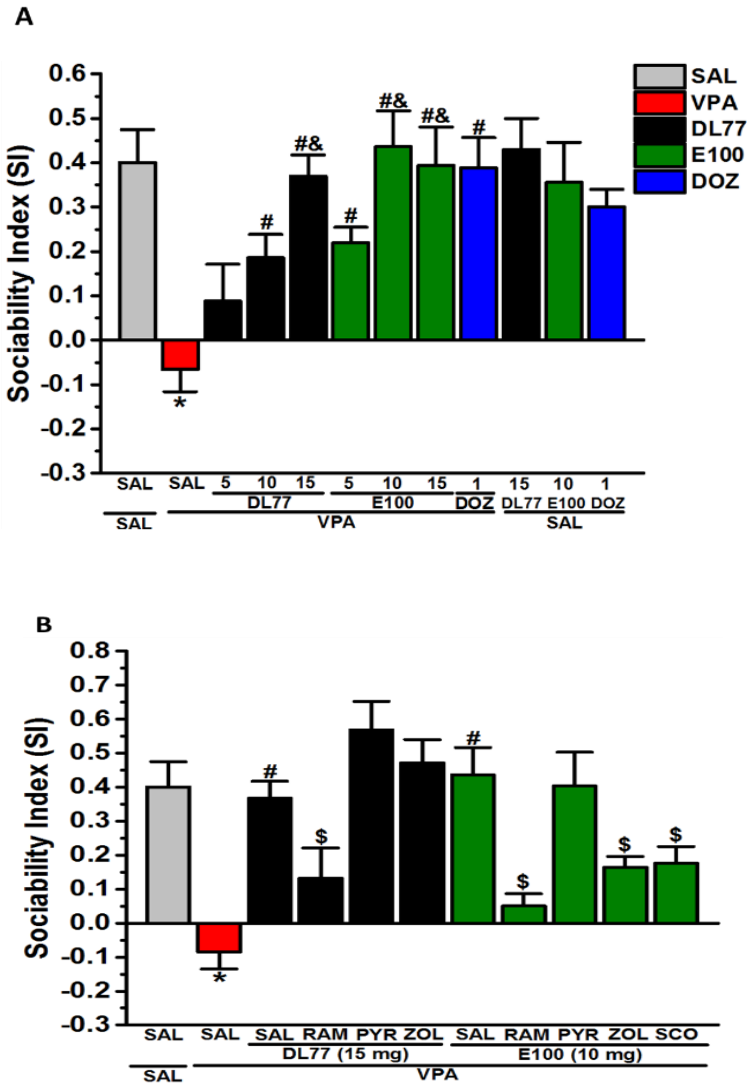


Figure 18: Effects of DL77 and E100 on sociability deficits assessed in VPA-exposed C57BL/6J mice in TCT.

After 10 minutes of acclimatization, male subjects were allowed to explore all chambers for 10 min. The results obtained were Sociability index (SI) (A) Abrogation of DL77 (15 mg)- and E100 (10 mg)-provided effect on SI (B). Saline-exposed mice were injected with saline in SAL group (gray), DL77 (5, 10, and 15 mg/kg, i.p. in black), E100 (5, 10, and 15 mg/kg, i.p. in green), or DOZ (1 mg/kg, i.p. in blue) was administered subchronically for 21 days. Abrogative studies of subchronic (21 days) systemic co-injection of RAM (10 mg/kg, i.p.), PYR (10 mg/kg, i.p.), or ZOL (10 mg/kg, i.p.) on the DL77 (15 mg) or E100 (10 mg)-provided improvement of sociability, additionally SCO (0. 3 mg/kg, i.p.) on E100 (10 mg)-provided improvement in SI. Saline-exposed mice were injected with saline in group, DL77 (15 mg/kg, i.p.), E100 (10 mg/kg,i.p.) or DOZ (1 mg/kg, i.p.). Figures show mean ± SEM (n = 7). $^{*}P<0.05$ vs.SI of saline-treated saline-exposed mice. $^{\#}P<0.05$ vs. SI of saline-treated VPA-exposed mice. $^{\&}P<0.05$ vs. SI of DL77 (5 mg)- or E100 (5 mg)-treated VPA exposed mice. $^{\$}P<0.05$ vs. SI of DL77 (15 mg)- or E100 (10 mg)-treated VPA-exposed mice.

3.9 Effects of DL77 and E100 on social novelty deficits assessed in VPA-exposed C57BL/6J mice in TCT

The effect of subchronic systemic injection of DL77 (5, 10, and 15 mg/kg, i.p.), E100 (5, 10, or 15 mg/kg, i.p.), and DOZ (1 mg/kg, i.p.) on ASD-like social novelty impairments in the TCT task in VPA-exposed mice are shown in Figure 19A and B. As observed in the post hoc analysis, VPA-exposed mice exhibited significantly lower social novelty expressed in form of SNI value when compared to saline-exposed animals, with [$F_{(1,12)}$ = 24.64; P<0.05] (Figure 19A). However, DL77 (10 and 15 mg/kg) significantly increased SNI of VPA-exposed mice when compared to Saline treated VPA-exposed group, with [$F_{(1,12)}$ = 6.36; P<0.05] and [$F_{(1,12)}$ = 17.43; P<0.05], respectively (Figure 19A). Moreover, the results revealed that the enhancement in SNI observed with DL77 (15 mg/kg) was comparable to that shown with DL77 (10 mg/kg) with [$F_{(1,12)}$ = 3.61; p=0.08] and DOZ (1 mg/kg) with [$F_{(1,12)}$ = 0.04; p=0.85], and was significantly higher than the DL77 (5 mg)-provided effects, with [$F_{(1,12)}$ = 27.43; P<0.05] (Figure 19A). Interestingly, the DL77 (15 mg)-provided improvement of social novelty was counteracted following subchronic systemic co-administration with centrally acting H3R agonist RAM (10 mg/kg, i.p.) and the CNS-penetrant H2R antagonist ZOL (10 mg/kg, i.p.) with [$F_{(1,12)}$ = 9.09; P<0.05] and [$F_{(1,10)}$ = 18.71; P<0.05], respectively, and as compared to DL77 (15 mg)-treated VPA-exposed mice (Figure 19B). However, CNS-penetrant H1R antagonist PYR (10 mg/kg, i.p.) failed to reverse the DL77 (15 mg)-provided social novelty enhancement, with [$F_{(1,12)}$ = 0.58; p=0.46] for SNI of DL77 (15 mg)-treated VPA-exposed animals vs. PYR (10 mg) + DL77 (15 mg)-treated group (Figure 19B). Similarly, improvement in social novelty preference was achieved following subchronic systemic pretreatment of VPA-exposed mice with E100 (10 and 15 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.), with [$F_{(1,12)}$ = 15.93;

$P<0.05$], [$F_{(1,12)} = 15.84$; $P<0.05$], and [$F_{(1,12)} = 14.60$; $P<0.05$], respectively (Figure 19A). As depicted in Figure 19B and observed in the post hoc analyses, the E100 (10 mg)-provided improvement of social novelty assessed by SNI was reversed by systemic co-administration of RAM (10 mg/kg, i.p.), ZOL (10 mg/kg, i.p.), or SCO (0.3 mg/kg, i.p.), with [$F_{(1,12)} = 6.77$; $P<0.05$], [$F_{(1,12)} = 14.75$; $P<0.05$], or [$F_{(1,10)} = 25.29$; $P<0.05$], respectively, and as compared to the E100 (10 mg)-treated animals (Figure 19B). However, PYR failed to counteract the improved social novelty provided with E100 (10 mg/kg, i.p.) in treated VPA-exposed mice, since SNI remained not significant following subchronic co-administration of PYR (10 mg/kg, i.p.) and E100 (10 mg/kg, i.p.) with [$F_{(1,12)} = 0.39$; p=0.55] for SNI of E100 (10 mg)-treated VPA-exposed animals vs. PYR (10 mg) + E100 (10 mg)-treated group (Figure 19B). Interestingly, the E100 (10 mg)-provided improvement of social novelty assessed by SNI was comparable to the effects provided by DOZ (1 mg/kg) and DL77 (15 mg/kg), with [$F_{(1,12)} = 0.52$; p=0.49] and [$F_{(1,12)} = 0.04$; p=0.85], respectively (Figure 19A). Notably, neither DL77 (15 mg) nor E100 (10 mg) or DOZ (1 mg) altered SNI of saline pretreated saline-exposed mice, with [$F_{(1,12)} = 0.19$; p=0.67], [$F_{(1,12)} = 0.60$; p=0.46], and [$F_{(1,12)} = 0.86$; p=0.37], respectively (Figure 19A).

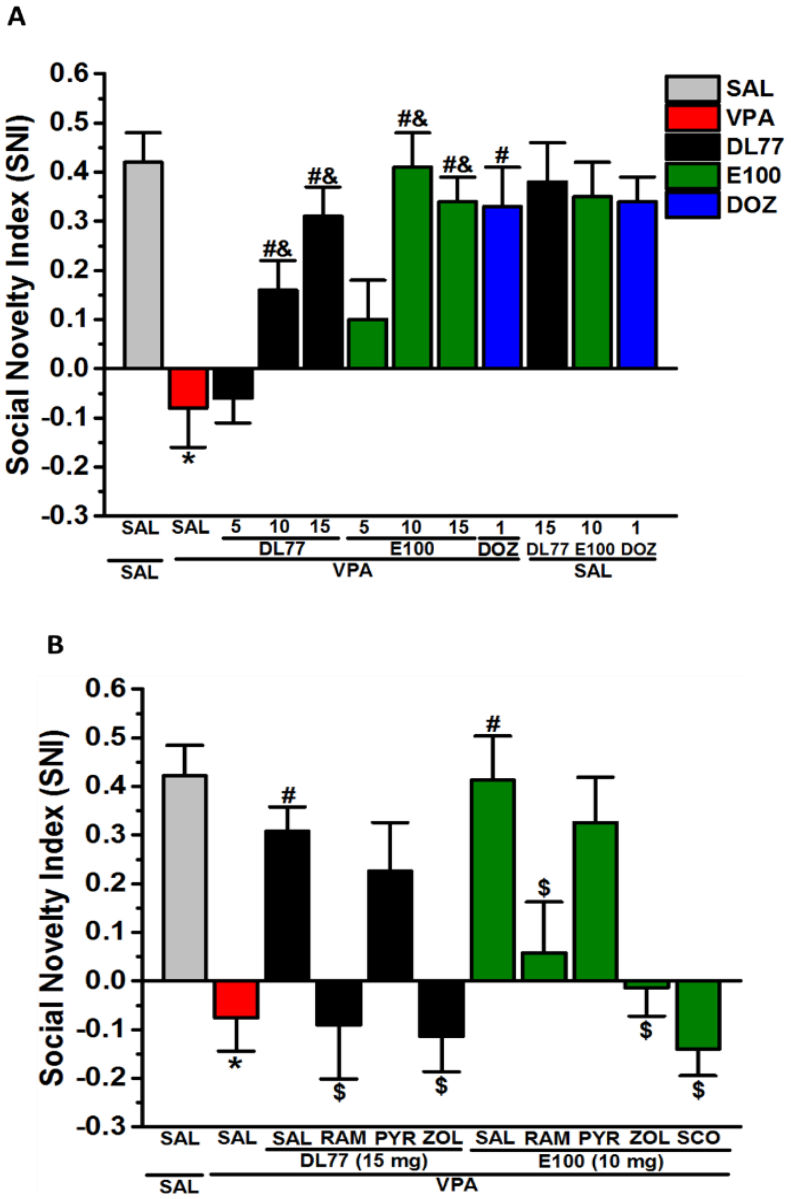


Figure 19: Effects of DL77 and E100 on social novelty deficits assessed in VPA-exposed C57BL/6J mice in TCT.

After 10 minutes of exploration, male subjects were allowed to explore all chambers for another 10 min. The results obtained were Social novelty index (SNI) (A) Abrogation of DL77 (15 mg)- and E100 (10 mg)-provided effect on SNI (B). Saline-exposed mice were injected with saline in SAL group (gray), DL77 (5, 10, and 15 mg/kg, i.p. in black), E100 (5, 10, and 15 mg/kg, i.p. in green), or DOZ (1 mg/kg, i.p. in blue) was administered subchronically for 21 days. Abrogative studies of subchronic (21 days) systemic co-injection of RAM (10 mg/kg, i.p.), PYR (10 mg/kg, i.p.), or ZOL (10 mg/kg, i.p.) on the DL77 (15 mg) or E100 (10 mg)-provided improvement of social novelty, additionally SCO (0.3 mg/kg, i.p.) on E100 (10 mg)-provided improvement in SNI. Saline-exposed mice were injected with saline in group, DL77 (15 mg/kg, i.p.), E100 (10 mg/kg,i.p.) or DOZ (1 mg/kg, i.p.). Figures show mean ± SEM (n = 7). $^{*}P<0.05$ vs. SNI of saline-treated saline-exposed mice. $^{\#}P<0.05$ vs. SNI of saline-treated VPA-exposed mice. $^{\&}P<0.05$ vs. SI of DL77 (5 mg)- or E100 (5 mg)-treated VPA exposed mice. $^{\$}P<0.05$ vs. SNI of DL77 (15 mg)- or E100 (10 mg)-treated VPA-exposed mice.

3.10 Effects of DL77 and E100 on elevated stereotyped repetitive behavior in VPA-exposed C57BL/6J mice in MBT

The effect of subchronic systemic administration with DL77 (5, 10, or 15 mg/kg, i.p.), E100 (5, 10, or 15 mg/kg, i.p.), and DOZ (1 mg/kg, i.p.) on the elevated repetitive behavior of VPA-exposed mice in MBT is shown in Figure 20A and B. The results of statistical analyses showed that VPA-exposed mice buried significantly more marbles compared to the saline-exposed animals, with [$F_{(1,8)}$ = 18.62; P<0.05]. However, DL77 (10 or 15 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.) significantly obliterated the increased percentage of marbles buried by VPA-exposed mice pretreated when compared to saline-treated VPA-exposed mice, with [$F_{(1,9)}$ = 10.56; P<0.05], [$F_{(1,10)}$ = 14.30; P<0.05], and [$F_{(1,8)}$ = 8.32; P<0.05], respectively (Figure 20A). Noticeably, subchronic systemic administration of DL77 (5 mg/kg, i.p.) failed to significantly modify the percentage of buried marbles in VPA-exposed mice, with [$F_{(1,8)}$ = 2.60; p=0.14] (Figure 20A). Moreover, the DL77 (15 mg)-provided decrease in the percentage of buried marbles was entirely abrogated by RAM and ZOL, with [$F_{(1,11)}$ = 10.18; P<0.05] and [$F_{(1,12)}$ = 4.80; P<0.05], respectively, and as compared with DL77 (15 mg)-treated VPA-exposed mice (Figure 20B). However, PYR failed to counteract this DL77 (15 mg)-provided decrease in the percentage of buried marbles, as PYR had no significant effect compared to VPA-exposed mice treated with DL77 (15 mg/kg, i.p.), with [$F_{(1,11)}$ = 0.14; p= 0.71] (Figure 20B). Similarly, the effect of subchronic systemic administration with E100 (5, 10, or 15 mg/kg, i.p.) on the elevated repetitive behavior of VPA-exposed mice in MBT is shown in Figure 20A. The results of statistical analyses showed that E100 (10 or 15 mg/kg, i.p.) significantly obliterated the increased percentage of marbles buried by VPA-exposed mice pretreated when compared to saline-treated VPA-exposed mice, with [$F_{(1,8)}$ = 17.32; P<0.05] and [$F_{(1,8)}$

= 22.35; $P<0.05$], respectively (Figure 20A). Noticeably, subchronic systemic administration of E100 (5 mg/kg, i.p.) failed to significantly modify the percentage of buried marbles in VPA-exposed mice, with [$F_{(1,8)}$ = 0.52; p=0.49] (Figure 20A). Moreover, the E100 (10 mg)-provided decrease in the percentage of buried marbles was entirely abrogated by RAM, ZOL, and SCO, with [$F_{(1,8)}$ = 17.71; $P<0.05$], [$F_{(1,8)}$ = 5.98; $P<0.05$], and [$F_{(1,8)}$ = 5.92; $P<0.05$], respectively, and as compared with E100 (10 mg)-treated VPA-exposed mice (Figure 20B). However, PYR failed to counteract this E100 (10 mg)-provided decrease in the percentage of buried marbles, as PYR had no significant effect compared to VPA-exposed mice treated with E100 (10 mg/kg, i.p.), with [$F_{(1,8)}$ = 0.09; p= 0.77] (Figure 20B). Interestingly, the E100 (10 mg)-provided mitigation of repetitive behavior assessed by percentage of buried marbles was comparable to the effects provided by DOZ (1 mg/kg) and DL77 (15 mg/kg), with [$F_{(1,10)}$ = 0.10; p=0.75] and [$F_{(1,8)}$ = 3.95; p=0.08], respectively (Figure 20A). Notably, neither DL77 (15 mg) nor E100 (10 mg) or DOZ (1 mg) altered percentage of buried marbles of Saline pretreated saline-exposed mice, with [$F_{(1,8)}$ = 0.30; p=0.60], [$F_{(1,8)}$ = 0.11; p=0.75], , and [$F_{(1,8)}$ = 0.02; p=0.89], respectively (Figure 20A).

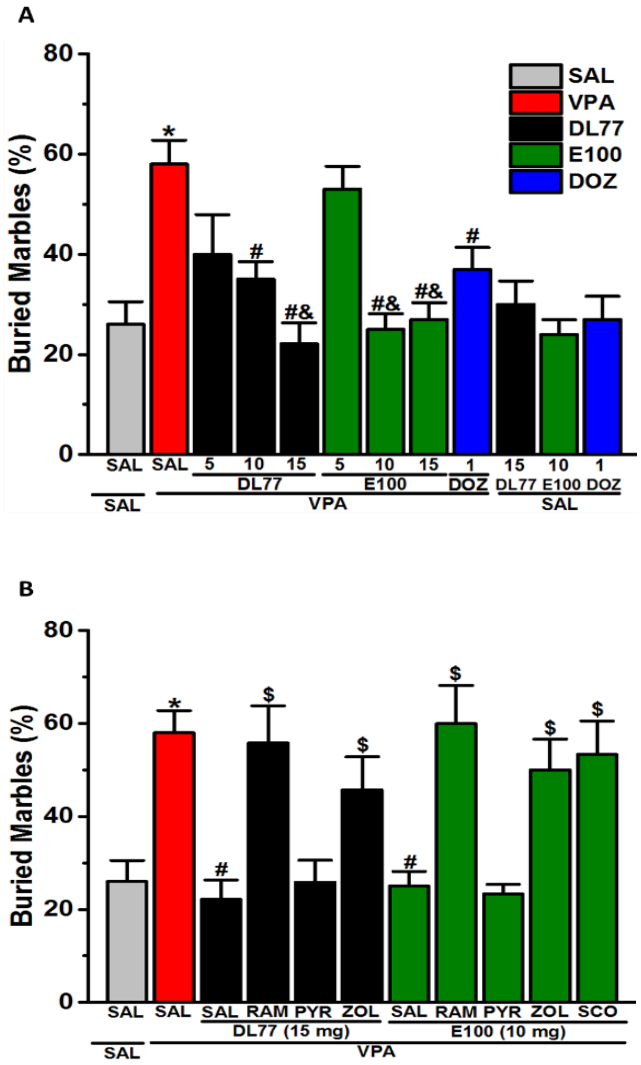


Figure 20: Effects of DL77 and E100 on elevated stereotyped repetitive behavior in VPA-exposed C57BL/6J mice in MBT.

(A) Repetitive marble-burying behavior was measured after a 30-minute testing session. VPA-exposed mice (red) demonstrated elevated stereotyped, repetitive behaviors that were significantly increased compared to saline-exposed mice (gray). DL77 (5, 10, or 15 mg/kg, i.p., in black), E100 (5, 10, or 15 mg/kg, i.p., in green), or DOZ (1 mg/kg, i.p. in blue) was administered subchronically for 21 days. (B) Effects of subchronic (21 days) systemic co-administration of RAM (10 mg/kg, i.p.), PYR (10 mg/kg, i.p.), or ZOL (10 mg/kg, i.p.) on the DL77 (15 mg)- or E100 (10mg)-provided attenuation of stereotyped repetitive behavior and additionally SCO (0.3 mg/kg, i.p.) on E100 (10 mg)-provided improvement of VPA-exposed mice in MBT. Saline-exposed mice were injected with saline, DL77 (15 mg/kg, i.p.), E100 (10 mg/kg, i.p.), or DOZ (1 mg/kg, i.p.). Figures show mean ± SEM (n = 7). $^{*}P < 0.05$ vs. saline-exposed mice. $^{\#}P < 0.05$ vs. VPA-exposed mice. $^{\&}P<0.05$ vs. DL77 (5 mg)- or E100 (5 mg)-treated VPA-exposed mice. $^{\$}P<0.05$ vs. DL77 (15 mg)- or E100 (10 mg)-treated VPA-exposed mice.

3.11 Effects of DL77 and E100 on elevated repetitive and compulsive-like behaviors in VPA-exposed C57BL/6J mice in NST

The effect of subchronic systemic administration with DL77 (5, 10, or 15 mg/kg, i.p.), E100 (5, 10, or 15 mg/kg, i.p.), and DOZ (1 mg/kg, i.p.) on the percentage escalation of shredded nestlet was evaluated in NST (Figure 21A and B). Post hoc analyses showed that VPA-exposed mice shredded significantly more nestlet compared to the saline-exposed mice, with [$F_{(1,8)}$ = 20.31; P<0.05]. However, VPA-exposed mice pretreated with DL77 (10 or 15 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.) exhibited a significantly lower percentage of shredded nestlet compared to VPA-exposed mice treated with saline, with [$F_{(1,8)}$ = 5.65; P<0.05], [$F_{(1,8)}$ = 17.75; P<0.05], and [$F_{(1,8)}$ = 19.42; P<0.05], respectively (Figure 21A). Moreover, subchronic systemic administration of DL77 (5 mg/kg, i.p.) failed to significantly modify the percentage of shredded nestlet in VPA-exposed mice, with [$F_{(1,8)}$ = 0.06; p=0.82] (Figure 21A). Notably, there was a significant difference in the DL77-provided effect on percentage of shredded nestlet detected between the two doses of 10 mg/kg and 15 mg/kg, with [$F_{(1,8)}$ = 13.72; P<0.05] (Figure 21A). As shown in Figure 21B and observed in the post hoc analyses, the DL77 (15 mg)-provided decrease in the percentage of shredded nestlet was countered following subchronic systemic co-administration of RAM (10 mg/kg, i.p.) and ZOL (10 mg/kg, i.p.), with [$F_{(1,8)}$ = 18.45; P<0.05] and [$F_{(1,8)}$ = 6.08; P<0.05], respectively, and compared with DL77 (15 mg)-treated VPA-exposed mice (Figure 21B). However, PYR failed to reverse this DL77-provided decrease in the percentage of shredded nestlet, as it had no significant effect compared to VPA-exposed mice treated with DL77 (15 mg/kg, i.p.), with [$F_{(1,8)}$ = 0.02; p=0.90] (Figure 21B). Similarly, the effect of subchronic systemic administration of E100 (5, 10, or 15 mg/kg, i.p.) on the percentage escalation of shredded nestlet was

evaluated in NSB (Figure 21B). Post hoc analyses showed that VPA-exposed mice pretreated with E100 (5 mg, 10 mg or 15 mg/kg, i.p.) exhibited a significantly lower percentage of shredded nestlet compared to VPA-exposed mice treated with saline, with [$F_{(1,8)}$ = 20.92; P<0.05], [$F_{(1,8)}$ = 33.26; P<0.05], and [$F_{(1,8)}$ = 38.78; P<0.05], respectively (Figure 21A). Moreover, the effects observed with 10 or 15 mg/kg were significantly higher than that witnessed with 5 mg/kg, with [$F_{(1,8)}$ = 6.62; P<0.05] and [$F_{(1,8)}$ = 9.60; P<0.05], respectively. Notably, no significant difference in the E100-provided effect on percentage of shredded nestlet was detected between the two doses of 10 mg/kg and 15 mg/kg, with p=0.93 (Figure 21A). As shown in Figure 21B and observed in the post hoc analyses, the E100 (10 mg)-provided decrease in the percentage of shredded nestlet was countered following subchronic systemic co-administration of RAM (10 mg/kg, i.p.), with [$F_{(1,8)}$ = 11.45; P<0.05] compared with E100 (10 mg)-treated VPA-exposed mice (Figure 21B). However, PYR, ZOL and SCO failed to reverse this E100-provided decrease in the percentage of shredded nestlet, as they had no significant effect compared to VPA-exposed mice treated with E100 (10 mg/kg, i.p.), with [$F_{(1,8)}$ = 0.003; p=0.96], [$F_{(1,8)}$ = 0.74; p= 0.41], and [$F_{(1,8)}$ = 0.11; p=0.75], respectively (Figure 21B). Interestingly, the E100 (10 mg)-provided mitigation of repetitive behavior assessed by percentage of shredded nestlet was comparable to the effects provided by DOZ (1 mg/kg) and DL77 (15 mg/kg), with [$F_{(1,8)}$ = 0.003; p=0.96] and [$F_{(1,8)}$ = 0.009; p=0.93], respectively (Figure 21A). Notably, neither DL77 (15 mg) nor E100 (10 mg) or DOZ (1 mg) altered percentage of shredded nestlet of saline-treated saline-exposed mice, with [$F_{(1,8)}$ = 0.22; p=0.65], [$F_{(1,8)}$ = 1.14; p=0.99], , and [$F_{(1,8)}$ = 0.30; p=0.60], respectively (Figure 21A).

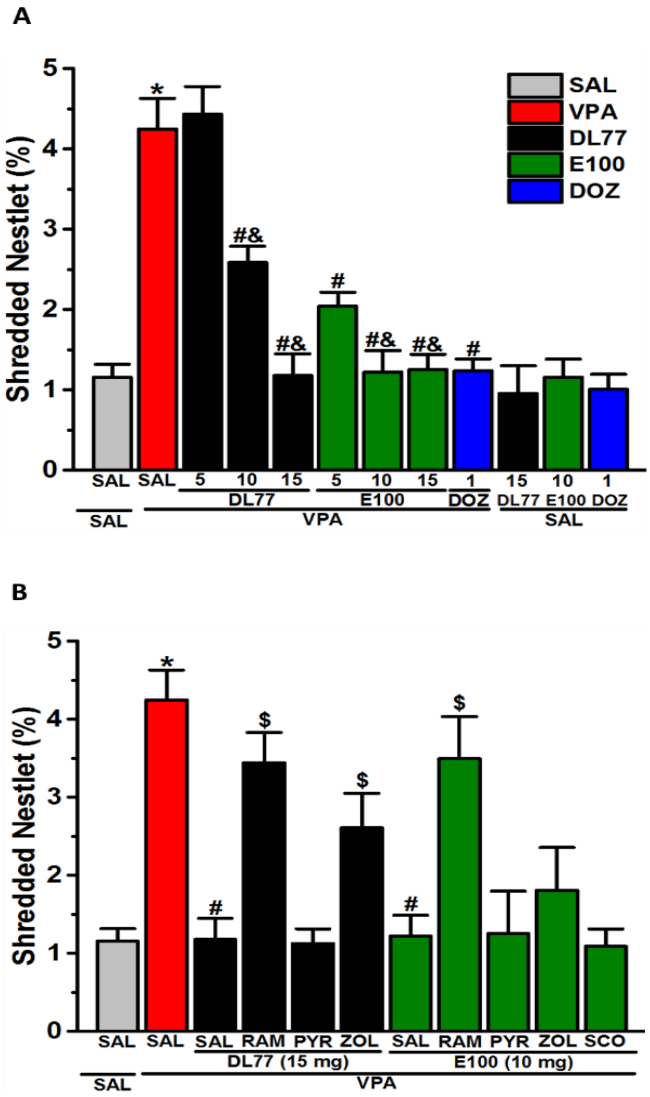


Figure 21: Effects of DL77 and E100 on elevated repetitive and compulsive-like behaviors in VPA-exposed C57BL/6J mice in NST.

(A) Repetitive shredding behavior was measured after a 30-minute testing session. VPA-exposed mice (red) demonstrated elevated stereotyped, repetitive behaviors that were significantly increased compared to saline-exposed mice (gray). DL77 (5, 10, or 15 mg/kg, i.p., in black), E100 (5, 10, or 15 mg/kg, i.p., in olive), or DOZ (1 mg/kg, i.p. in blue) was administered subchronically for 21 days. (B) Effects of subchronic (21 days) systemic co-administration of RAM (10 mg/kg, i.p.), PYR (10 mg/kg, i.p.), or ZOL (10 mg/kg, i.p.) on the DL77 (15 mg)- or E100 (10 mg)-provided attenuation of stereotyped repetitive behavior and additionally SCO (0.3 mg/kg, i.p.) on E100 (10 mg)-provided improvement of VPA-exposed mice in NST. Saline-exposed mice were injected with saline, DL77 (15 mg/kg, i.p.), E100 (10 mg/kg, i.p.), or DOZ (1 mg/kg, i.p.). Figures show mean ± SEM (n = 7). $^{*}P < 0.05$ vs. saline-exposed mice. $^{\#}P < 0.05$ vs. VPA-exposed mice. $^{\&}P<0.05$ vs. DL77 (5 mg)- or E100 (5 mg)-treated VPA-exposed mice. $^{\$}P<0.05$ vs. DL77 (15 mg)- or E100 (10 mg)-treated VPA-exposed mice.

3.12 Effects of DL77 and E100 pretreatment on anxiety responses and locomotor activity in VPA-exposed C57BL/6J mice in EPM test

Figure 22A-C shows the observed effects of subchronic systemic injection of DL77 (5, 10, or 15 mg/kg, i.p.), E100 (5, 10, or 15 mg/kg, i.p.), and DOZ (1 mg/kg, i.p.) on the anxiety parameters of VPA-exposed mice tested in the EPM, namely, the percentage of time spent in open arms (Figure 22A), the number of entries into open arms (Figure 22B), and locomotor activity expressed as the number of entries into closed arms (Figure 22C). The results observed showed that VPA-exposed mice spent significantly less time as well as lower number of entries in open arms when compared to saline-exposed mice, with [$F_{(1,10)}$ = 20.56; P<0.05] and [$F_{(1,10)}$ = 19.84; P<0.05], respectively (Figure 22A and B). However, subsequent post hoc analyses revealed that DL77 when administered at 15 mg/kg, i.p. significantly altered the percentage of time spent exploring the open arms of the maze during a 5 min session compared to saline-treated VPA-exposed mice, with [$F_{(1,10)}$ = 5.37; P<0.05] (Figure 22A). Moreover, DL77 when administered at three different doses (5, 10, and 15 mg/kg, i.p.) failed to significantly increase the number of entries the open arms of the maze during a 5 min session compared to saline-treated VPA-exposed mice, with p = 0.25, p = 0.99, and p = 0.77, respectively (Figure 22B). Interestingly, VPA-exposed mice pretreated with DOZ (1 mg) spent a significantly higher percentage of time exploring the open arms compared to saline-treated VPA-exposed mice, with [$F_{(1,10)}$ = 12.99; P<0.05] (Figure 22A). Further analyses of data describing the number of entries into the open arms of the maze yielded for DOZ (1 mg/kg, i.p.) practically the same results, with [$F_{(1,10)}$ = 9.97; P<0.05] (Figure 22B). Notably, the number of closed arm entries following subchronic systemic injection of DL77 (5, 10, or 15 mg/kg) and DOZ (1 mg/kg, i.p.) was not significantly different, with [$F_{(1,10)}$ = 2.14; p=0.17], [$F_{(1,10)}$ = 2.84; p=0.12],

[$F_{(1,10)}$ = 2.08; p=0.18], and [$F_{(1,10)}$ = 4.40; p=0.06], receptively (Figure 22C). Similarly, the effects of subchronic systemic injection of E100 (5, 10, or 15 mg/kg, i.p.) on the anxiety parameters of VPA-exposed mice in the EPM, namely, the percentage of time spent in open arms (Figure 22A), the number of entries into open arms (Figure 22B), and locomotor activity expressed as the number of entries into closed arms were evaluated (Figure 22C). The results observed revealed that E100 when administered at 10 or 15 mg/kg, i.p. significantly altered the percentage of time spent exploring the open arms of the maze during a 5 min session compared to saline-treated VPA-exposed mice, with [$F_{(1,10)}$ = 21.54; P<0.05] and [$F_{(1,10)}$ = 12.64; P<0.05], respectively (Figure 22A). Moreover, post hoc evaluation revealed that, compared with saline-exposed mice, the VPA-exposed mice displayed similar closed arm entries, with [$F_{(1.10)}$ = 3.76; p=0.08] (Figure 22C). However, E100 when administered at 10 or 15 mg/kg, i.p. significantly increased the number of entries the open arms of the maze during a 5 min session compared to saline-treated VPA-exposed mice, with [$F_{(1,10)}$ = 8.29; P<0.05] and [$F_{(1,10)}$ = 13.24; P<0.05], respectively (Figure 22B). Interestingly, VPA-exposed mice pretreated with DOZ (1 mg) spent a significantly higher percentage of time exploring the open arms compared to saline-treated VPA-exposed mice, with [$F_{(1,10)}$ = 13.74; P<0.05] (Figure 22A). Notably, the number of closed arm entries following subchronic systemic injection of E100 (5, 10, or 15 mg/kg) was not significantly different, with [$F_{(1,10)}$ = 0.51; p=0.49], [$F_{(1,10)}$ = 2.14; p=0.17], and [$F_{(1,10)}$ = 0.47; p=0.51], receptively (Figure 22C). Interestingly, the E100 (10 mg)-provided alteration of time spent in open arms or number of entries into open arms was significantly higher than those provided with DL77 (15 mg/kg), with [$F_{(1,10)}$ = 6.59; P<0.05] and [$F_{(1,10)}$ = 6.43; P<0.05], respectively (Figure 22A and B). As shown in

Figure 22D and observed in the post hoc analyses, the DL77 (15 mg)- or E100 (10 mg)-provided increase in the time spent in open arms was abrogated following subchronic systemic co-administration of RAM (10 mg/kg, i.p.), with [$F_{(1,10)}$ = 8.63; P<0.05] and [$F_{(1,8)}$ = 28.93; P<0.001], respectively, and as compared with DL77 (15 mg)- or E100 (10 mg)-treated VPA-exposed mice (Figure 22D). However, PYR and ZOL failed to abrogate the DL77-provided increase in time spent in open arm, as they had no significant effect compared to VPA-exposed mice treated with DL77 (15 mg/kg, i.p.), with [$F_{(1,10)}$ = 0.08; p=0.79] and [$F_{(1,10)}$ = 0.40; p=0.54], respectively (Figure 22D). However, SCO reversed the E100-provided effects on time spent in open arm and as compared to VPA-exposed mice treated with E100 (10 mg/kg, i.p.), with [$F_{(1,10)}$ = 8.16; P<0.05], while ZOL and PYR failed to nullify the E100 (10 mg)-provided effect, with [$F_{(1,11)}$ = 0.32; p=0.59] and [$F_{(1,11)}$ = 0.60; p=0.45], respectively (Figure 22D). Statistical analyses of number of entries into open arms yielded practically the same results for E100 (10 mg)-provide effect (Figure 22B) and its abrogation with RAM (Figure 22E). Notably, neither DL77 (15 mg) nor E100 (10 mg) or DOZ (1 mg) altered percentage of time spent in open arms of saline-treated saline-exposed mice, with [$F_{(1,10)}$ = 2.34; p=0.16], [$F_{(1,10)}$ = 0.70; p=0.42], , and [$F_{(1,10)}$ = 0.03; p=0.87], respectively (Figure 22A). Similarly, neither DL77 (15 mg) nor E100 (10 mg) or DOZ (1 mg) altered number of entries into open arms of Saline pretreated saline-exposed mice (all P>0.05) (Figure 22B).

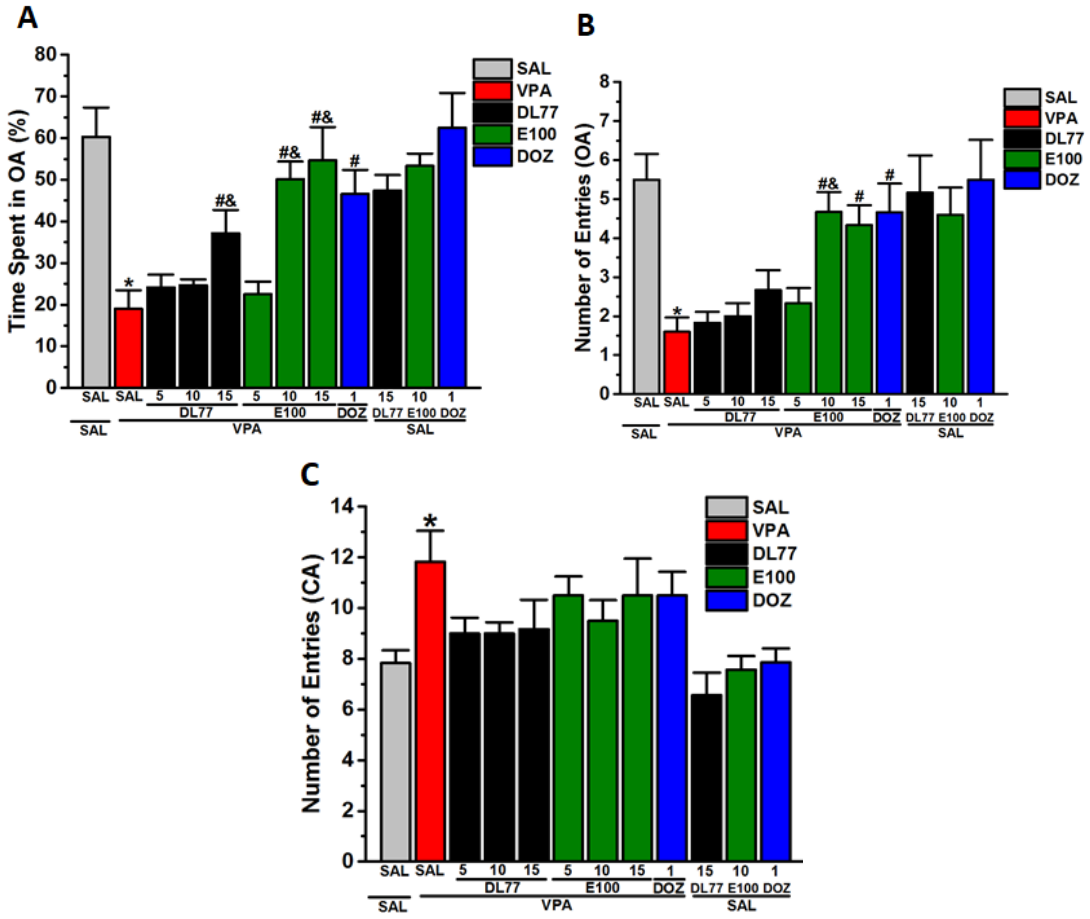


Figure 22: Effects of DL77 and E100 pretreatment on anxiety responses and locomotor activity in VPA-exposed C57BL/6J mice in EPM test.

VPA-exposed mice (red) demonstrated elevated innate anxiety and risk assessment that were significantly increased compared to saline-exposed mice (gray). DL77 (5, 10, or 15 mg/kg, i.p. in black), E100 (5, 10, or 15 mg/kg, i.p., in green), or DOZ (1 mg/kg, i.p., in blue) was administered subchronically for 21 days. DOZ (1 mg/kg, i.p.) attenuated the decreased percentage of time spent on the open arms of the EPM (A) the decreased number of entries into the open arms (B) and the increased number of entries into the closed arms (C) in VPA exposed mice. Abrogation of subchronic (21 days) systemic co-administration of RAM (10 mg/kg, i.p.), PYR (10 mg/kg, i.p.), or ZOL (10 mg/kg, i.p.) on the DL77 (15 mg)- or E100 (10 mg)-provided effects and additionally SCO (0.3 mg/kg, i.p.) on E100 (10 mg)-provided modulation on time spent in the open arms (D) and number of entries into the open arms (E). attenuation of anxiety of VPA-exposed mice in EPM. Saline-exposed mice were injected with saline, DL77 (15 mg/kg, i.p.), E100 (10 mg/kg, i.p.), or DOZ (1 mg/kg, i.p.) (A-C). Data are expressed as the mean ± SEM (n = 6). $^{*}P < 0.05$ vs. saline-exposed mice. $^{\#}P < 0.05$ vs. VPA-exposed mice. $^{\&}P<0.05$ vs. DL77 (5 mg)- or E100 (5 mg)-treated VPA-exposed mice. $^{\$}P<0.05$ vs. DL77 (15 mg)- or E100 (10 mg)-treated VPA-exposed mice.

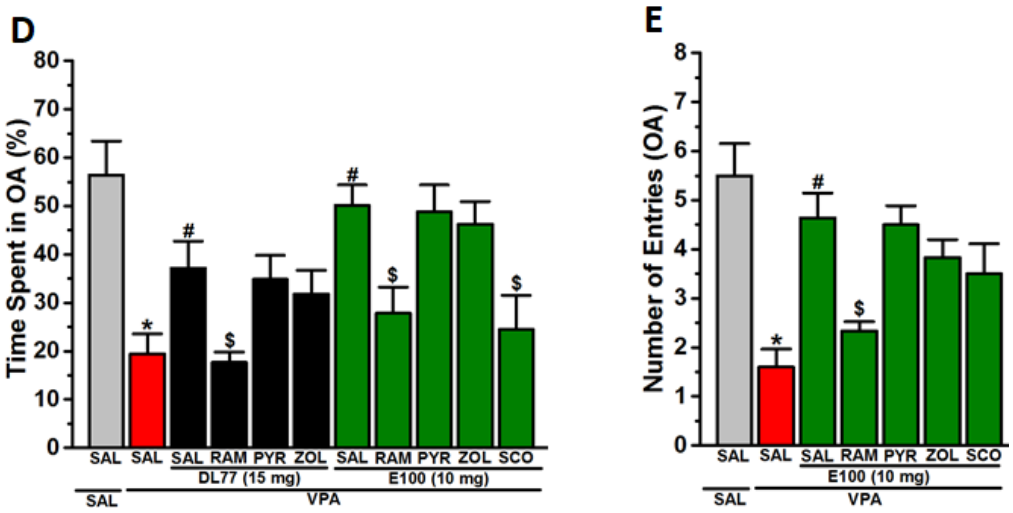


Figure 22: Effects of DL77 and E100 pretreatment on anxiety responses and locomotor activity in VPA-exposed C57BL/6J mice in EPM test (continued).

3.13 Effects of DL77 and E100 pretreatment on exploratory and anxiety-like behaviors in VPA-exposed C57BL/6J mice in OFT

Figure 23 A-D shows the observed effects of subchronic systemic injection of DL77 (5, 10, or 15 mg/kg, i.p.), E100 (5, 10, or 15 mg/kg, i.p.), and DOZ (1 mg/kg, i.p.) on the anxiety parameters of VPA-exposed mice tested in the OFT. As seen in Figure 23 A-D, there were no significant effects of subchronic systemic exposure of VPA-exposed mice to DL77 (5, 10, and 15 mg/kg, i.p.) on time spent in the central arena, time spent in the periphery and total distance travelled (all *P* values >0.05) (Figure 23A-D). In contrast, analysis of variance demonstrated that VPA-exposed mice pretreated with E100 (10 and 15 mg/kg, i.p.) spent a significantly higher percentage of time in the center of the arena, with [$F_{(1,8)}$ = 19.95; P<0.05] and [$F_{(1,8)}$ = 10.31; P<0.05], respectively (Figure 23A). As shown in Figure 23B and observed in the post hoc analyses, the E100 (10 mg)-provided increase in the time spent in the center of arena was abrogated following subchronic systemic co-administration of RAM (10 mg/kg, i.p.), with [$F_{(1,8)}$ = 28.93; P<0.05] compared with E100 (10 mg)-

treated VPA-exposed mice (Figure 23B). However, PYR, ZOL, and SCO failed to abrogate this E100-provided increase in time spent in central arena, as they had no significant effect compared to VPA-exposed mice treated with E100 (10 mg/kg, i.p.), with [$F_{(1,8)}$ = 0.054; p=0.82], [$F_{(1,8)}$ = 0.18; p= 0.69], and [$F_{(1,8)}$ = 0.33; p=0.86], respectively (Figure 23B). In addition, there were no significant effects of subchronic systemic exposure of VPA-exposed mice to E100 (5, 10, and 15 mg/kg, i.p.) on increased total distance travelled in the test arena neither on increased time spent in the periphery (all *P* values >0.05) (Figure 23C and D). Notably, subchronic treatment of saline-exposed mice with E100 (10 mg/kg, i.p.) had no significant influence on time spent in the central arena compared to saline-pretreated saline-exposed mice, with [$F_{(1,8)}$ = 0.01; p=0.92] (Figure 23A).

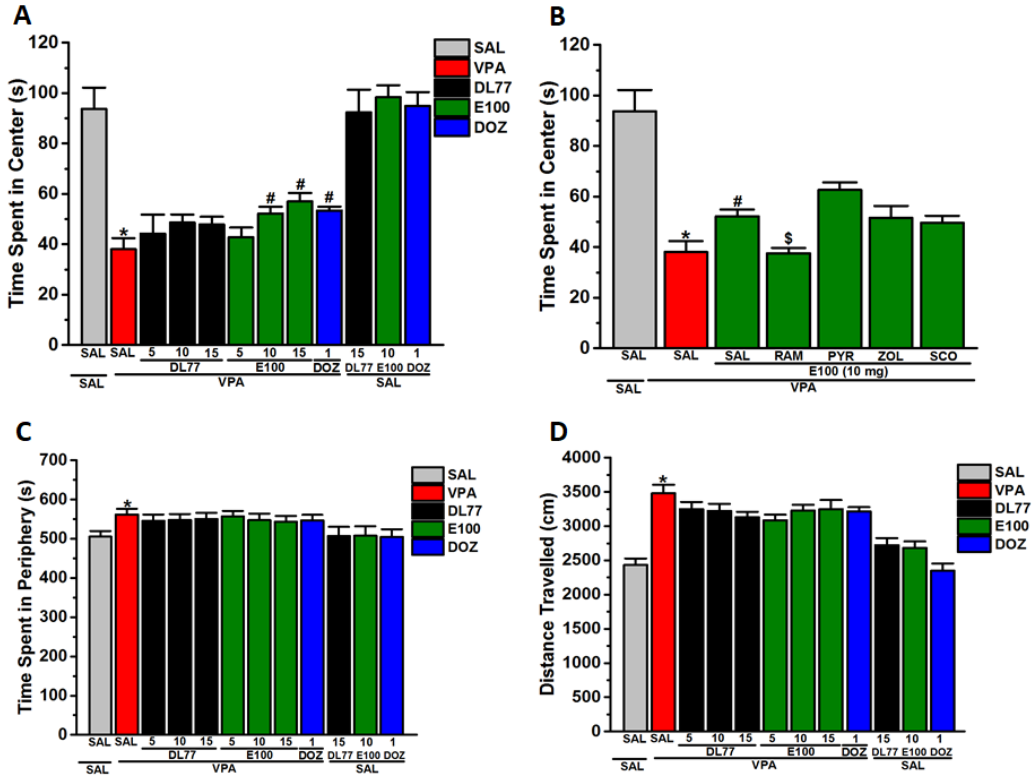


Figure 23: Effects of DL77 and E100 pretreatment on exploratory and anxiety-like behaviors in VPA-exposed C57BL/6J mice in OFT.

VPA-exposed mice (red) demonstrated general locomotor activity and anxiety-related emotional behaviors that were significantly increased compared to saline-exposed mice (gray). DL77 (5, 10, or 15 mg/kg, i.p. in black), E100 (5, 10, or 15 mg/kg, i.p. in green) or DOZ (1 mg/kg, i.p. in blue) was administered subchronically for 21 days. E100 (10 and 15 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.) increased the decreased time spent in the central arena (A) but failed to modulate the increased time spent in the periphery (C) as well as the increased total distance travelled (D) in VPA-exposed mice in the OFT. Effects of subchronic (21 days) systemic co-injection of RAM (10 mg/kg, i.p.), PYR (10 mg/kg, i.p.), ZOL (10 mg/kg, i.p.), or SCO (0.3 mg/kg, i.p.) on the E100 (10 mg)-provided amelioration of time spent in the center of VPA-exposed mice in OFT (B). Data are expressed as the mean ± SEM (n = 5). $^{\#}P<0.05$ vs. saline-exposed mice. $^{*}P<0.05$ vs. saline-treated VPA-exposed mice. $^{\$}P<0.05$ vs. E100 (10 mg)-treated VPA-exposed mice.

3.14 DL77 and E100 restored levels of proinflammatory cytokines in the cerebellum of VPA-exposed C57BL/6J mice

The effects of subchronic systemic treatment of DL77 (5, 10, or 15 mg/kg, i.p.), E100 (5, 10, or 15 mg/kg, i.p.), and DOZ (1 mg/kg, i.p.) on the levels of proinflammatory cytokines IL-1β (Figure 24A), IL-6 (Figure 24C), TNF-α (Figure 24E), and TGF-β (Figure 24G) exacerbated with LPS challenge in cerebellum tissue

of VPA-exposed mice were evaluated. The results observed revealed that systemic prenatal injection of VPA (500 mg/kg, i.p.) induced ASD-like behaviors in VPA-exposed mice and significantly increased IL-1β (Figure 24A), IL-6 (Figure 24C), TNF-α (Figure 24E), and TGF-β (Figure 24G) compared to the saline-exposed mice (all $P<0.05$). However, subchronic systemic administration of DL77 (10 and 15 mg/kg, i.p.) or DOZ (1 mg/kg) significantly (all $P<0.05$) mitigated the rise of these proinflammatory cytokines in VPA-exposed mice (Figures 24A, C, E, and G). Notably, subchronic systemic co-administration of RAM (10 mg/kg, i.p.) abrogated (all $P<0.05$) the protective effects of DL77 (15 mg/kg, i.p.) against VPA-induced elevation of IL-1β (Figure 24B), IL-6 (Figure 24D), TNF-α (Figure 24F), and TGF- β (Figure 24H). Similarly, the effects of E100 (5,10 and 15 mg/kg, i.p.) on the levels of proinflammatory cytokines IL-1β (Figure 24A), IL-6 (Figure 24C), TNF-α (Figure 24E), and TGF-β (Figure 24G) in the cerebellum tissue of VPA-exposed mice were evaluated. The results observed revealed that subchronic systemic administration of E100 (5, 10, and 15 mg/kg, i.p.) significantly (all $P<0.05$) alleviated the rise of these proinflammatory cytokines in VPA-exposed mice. Also, subchronic systemic co-administration of RAM (10 mg/kg, i.p.) nullified (all $P<0.05$) the protective effects of E100 (10 mg/kg, i.p.) against VPA-induced elevation of IL-1β (Figure 24B), IL-6 (Figure 24D), TNF-α (Figure 24F), and TGF- β (Figure 24H).

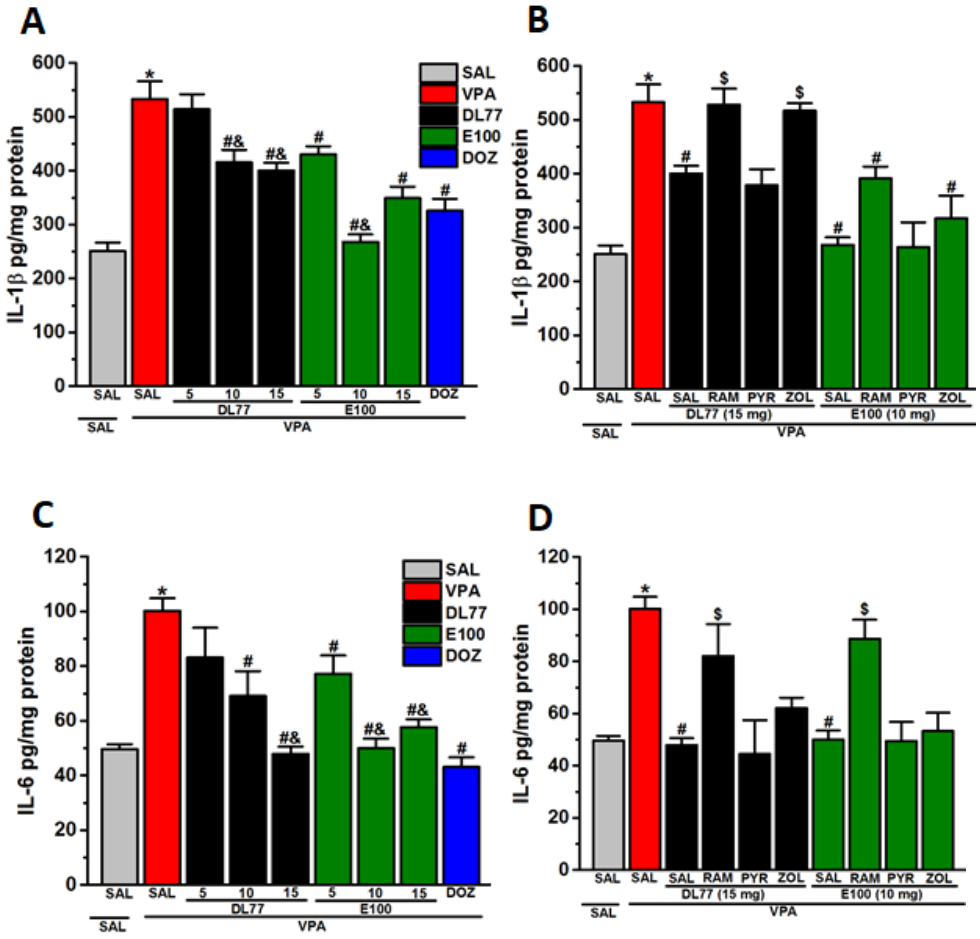


Figure 24: DL77 and E100 restored levels of proinflammatory cytokines in the cerebellum of VPA-exposed C57BL/6J mice.

VPA-exposed mice (red) showed a significant increase in IL-1β (A), IL-6 (C), TNF-α (E), and TGF-β (G) compared to saline-exposed mice (gray). DL77 (5, 10, or 15 mg/kg, i.p. in black), E100 (5, 10, or 15 mg/kg, i.p. in green), or DOZ (1 mg/kg, i.p. in blue) was administered subchronically for 21 days. DL77 (10 or 15 mg/kg, i.p.), E100 (5, 10, or 15 mg/kg, i.p.), or DOZ (1 mg/kg, i.p.) significantly decreased the elevated levels of these cytokines. RAM (10 mg/kg, i.p.), PYR (10 mg/kg, i.p.), and ZOL (10 mg/kg, i.p.) were co-administered for abrogative studies of the modulating DL77 (15 mg)-, or E100 (10 mg)-provided effects on cytokines levels (B, D, F, and H). Data are expressed as the mean ± SEM (n = 5). $^{*}P< 0.05$ vs. saline-exposed mice. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. VPA-exposed mice. $^{\&}P<0.05$ vs. DL77 (5 mg)- or E100 (5 mg)-provided improvement. $^{\$}P<0.05$ vs. DL77 (15 mg)-, or E100 (10 mg)-treated VPA-exposed mice.

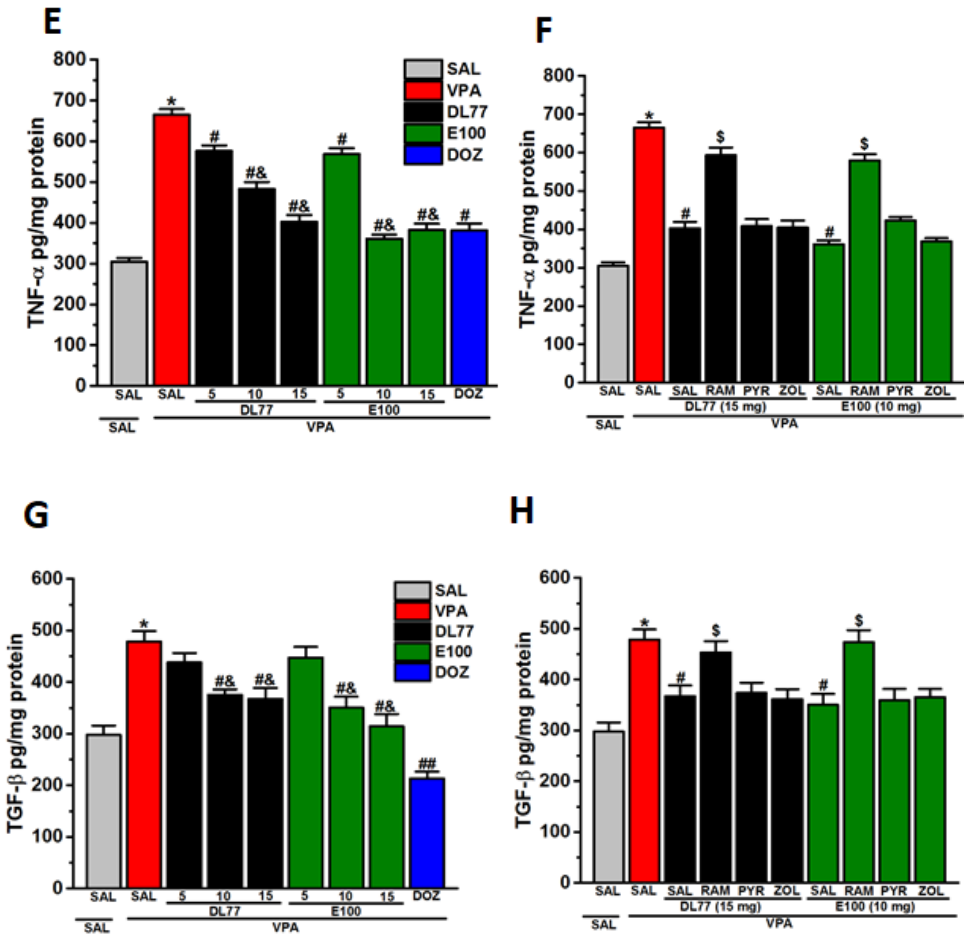


Figure 24: DL77 and E100 restored levels of proinflammatory cytokines in the cerebellum of VPA-exposed C57BL/6J mice (continued).

3.15 DL77 and E100 restored levels of oxidative stress markers in the cerebellum of VPA-exposed C57BL/6J mice

The ability of subchronic systemic administration with DL77 (5, 10, or 15 mg/kg, i.p.), E100 (5, 10, or 25 mg/kg, i.p.), and DOZ (1 mg/kg, i.p.) to decrease oxidative stress in VPA-exposed mice was assessed (Figure 25A-H). Four parameters were measured, namely the levels of MDA, GSH, SOD, and CAT in VPA-exposed mouse cerebellum. The results show that MDA was significantly raised (P<0.05) and GSH, SOD, and CAT were significantly reduced (P<0.05) in the brain tissues of VPA-exposed mice compared to saline-exposed mice (Figure 25A, C, E, and G). However,

brain tissues of VPA-exposed mice subchronically pretreated with DL77 (15 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.) displayed a significant reduction of MDA as well as significant elevation of GSH, SOD, and CAT (P<0.05) (Figure 25A, C, E, and G). Moreover, subchronic systemic co-administration of RAM (10 mg/kg, i.p.) reversed (P<0.05) the protective effects of DL77 (15 mg/kg, i.p.) against the VPA-induced increased level of MDA (Figure 25B), and it also reversed (all P<0.05) the DL77 (15 mg)-provided increase in GSH, SOD, and CAT in VPA-exposed mice (Figure 25D, F and H). Similarly, brain tissues of VPA-exposed mice subchronically pretreated with E100 (, 10, and 15 mg/kg, i.p.) displayed a significant reduction of MDA (Figure 25A) as well as significant elevation of GSH (Figure 25C), SOD (Figure 25E), and CAT (Figure 25G) (all P<0.05). Moreover, subchronic systemic co-administration of RAM (10 mg/kg, i.p.) partially abrogated (P<0.05) the protective effects of E100 (10 mg/kg, i.p.) against the VPA-induced increased level of MDA (Figure 25B), and it also partially reversed (P<0.05) the E100 (10 mg)-provided increase in GSH, SOD, and CAT in VPA-exposed mice (Figure 25D, F and H).

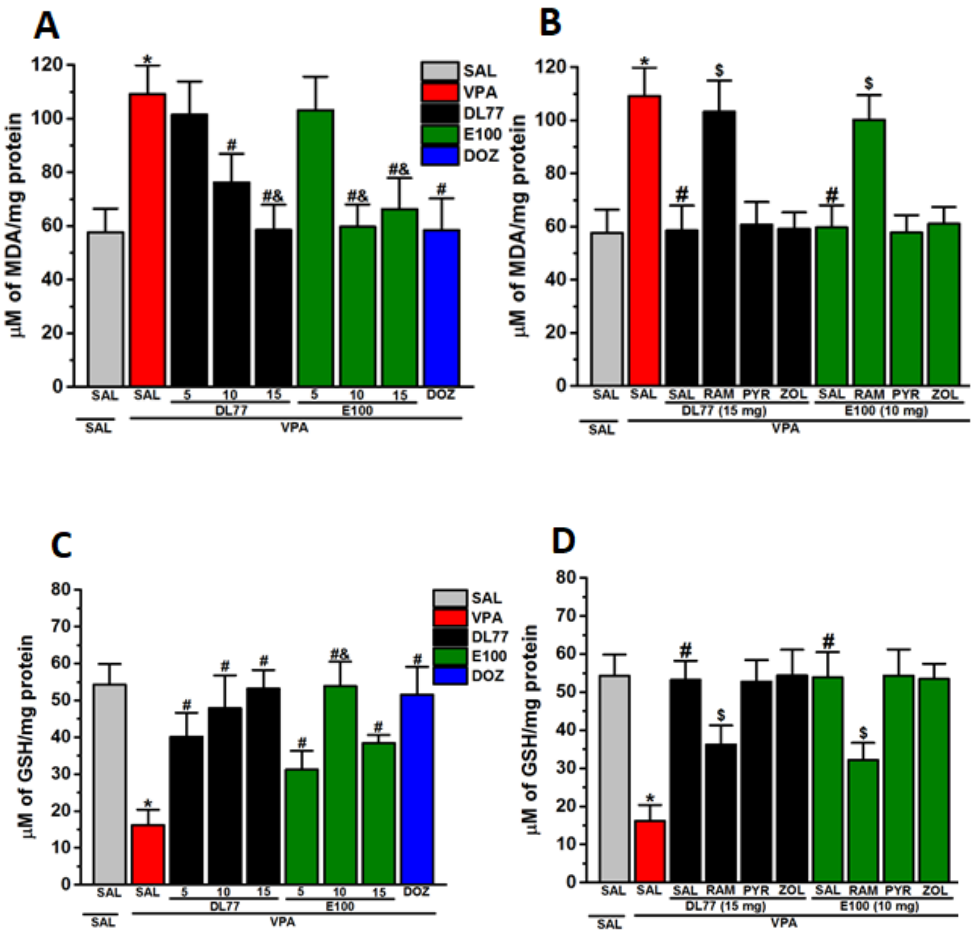


Figure 25: DL77 and E100 restored levels of oxidative stress markers in the cerebellum of VPA-exposed C57BL/6J mice.

VPA-exposed mice (red) showed a significant increase in MDA (A) and decrease in GSH (C), SOD (E), and CAT (G) compared to saline-exposed mice (gray). DL77 (5, 10, or 15 mg/kg, i.p. in black), E100 (5, 10, or 15 mg/kg, i.p. in green), or DOZ (1 mg/kg, i.p. in blue) was administered subchronically for 21 days. DL77 (5, 10, or 15 mg/kg, i.p.), E100 (5, 10, or 15 mg/kg, i.p.), or DOZ (1 mg/kg, i.p.) significantly modulated the levels of these markers. RAM (10 mg/kg, i.p.), PYR (10 mg/kg, i.p.), and ZOL (10 mg/kg, i.p.) were co-administered for abrogative studies of the modulating DL77 (15 mg)-, or E100 (10 mg)-provided effects on oxidative stress levels (B, D, F, and H). Data are expressed as the mean ±SEM (n = 5). $^{*}P< 0.05$ vs. saline-exposed mice. $^{\#}P<0.05$ vs. VPA-exposed mice. $^{\&}P<0.05$ vs. DL77 (5 mg)- or E100 (5 mg)-provided effects. $^{\$}P<0.05$ vs. DL77 (15 mg)-, or E100 (10 mg)-treated VPA-exposed mice.

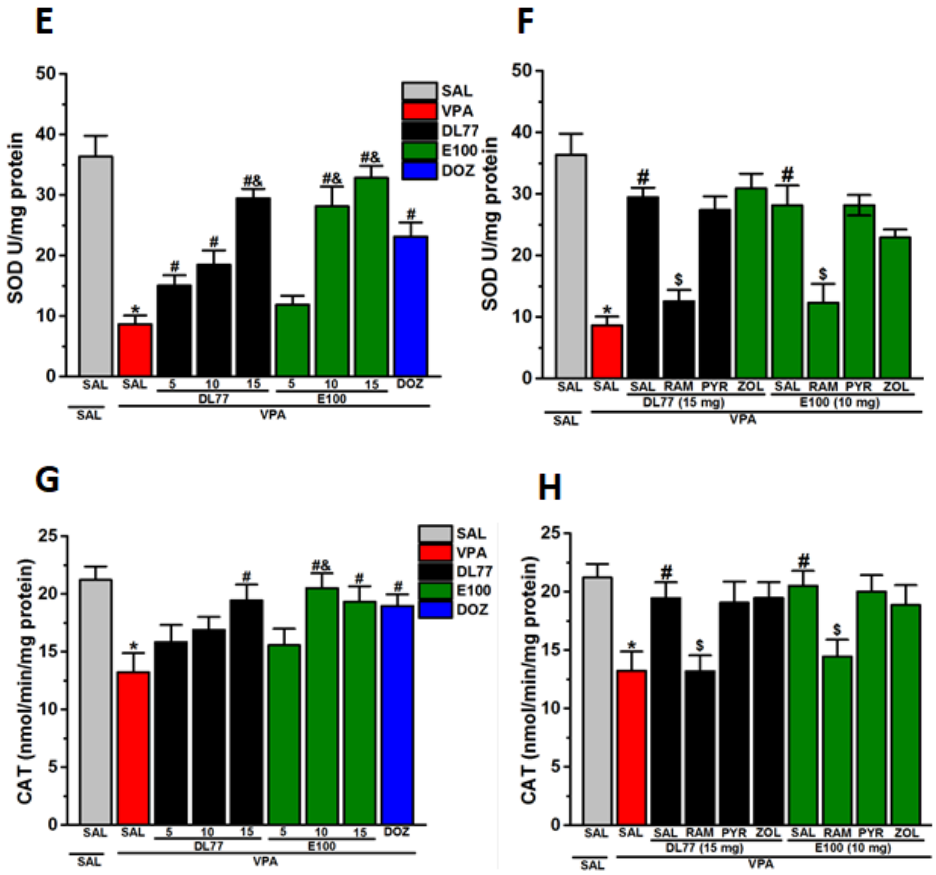


Figure 25: DL77 and E100 restored levels of oxidative stress markers in the cerebellum of VPA-exposed C57BL/6J mice (continued).

3.16 Effects of E100 on expression of NF-κB p65, iNOS & COX-2 in cerebellum and hippocampus of VPA-exposed C57BL/6J mice

The expressions of NF-κBp65, iNOS, and COX-2 were also tested using Western blot in tissue lysates that were isolated from cerebellum (Figure 26A) and hippocampus regions (Figure 26B). A significant increase in expression of NF-κBp65, iNOS, and COX-2 (Figure 26A) was observed in the cerebellum of VPA-exposed mice when compared to the saline-exposed control group of mice (all $P<0.05$). However, following subchronic systemic pretreatment of VPA-exposed mice with E100 (10 mg/kg), a remarkable reduction in the level of NF-κBp65, iNOS, and COX-2 (all $P<0.05$) was observed when compared to the VPA-exposed mice (Figure 26A).

Similarly, hippocampal tissues of VPA-exposed mice also showed a significant increase ($P< 0.05$) in expression of NF-κBp65, iNOS, and COX-2 (Figure 26B) when compared to the saline-exposed mice, however, subchronic systemic pretreatment of VPA-exposed mice with E100 (10 mg/kg) resulted in a remarkable decrease in expression of NF-κBp65, iNOS, and COX-2 (all $P<0.05$) when compared to the VPA-exposed mice (Figure 26B). Interestingly, subchronic systemic co-administration of RAM (10 mg/kg, i.p.) entirely nullified the mitigating effects of E100 (10 mg/kg, i.p.) on expression of NF-κBp65 and COX-2 in cerebellum and hippocampus tissues (all $P<0.05$), and it also partly counteracted the E100 (10 mg)-provided effects on iNOS in VPA-exposed mice (all $P<0.05$) (Figure 26A and B).

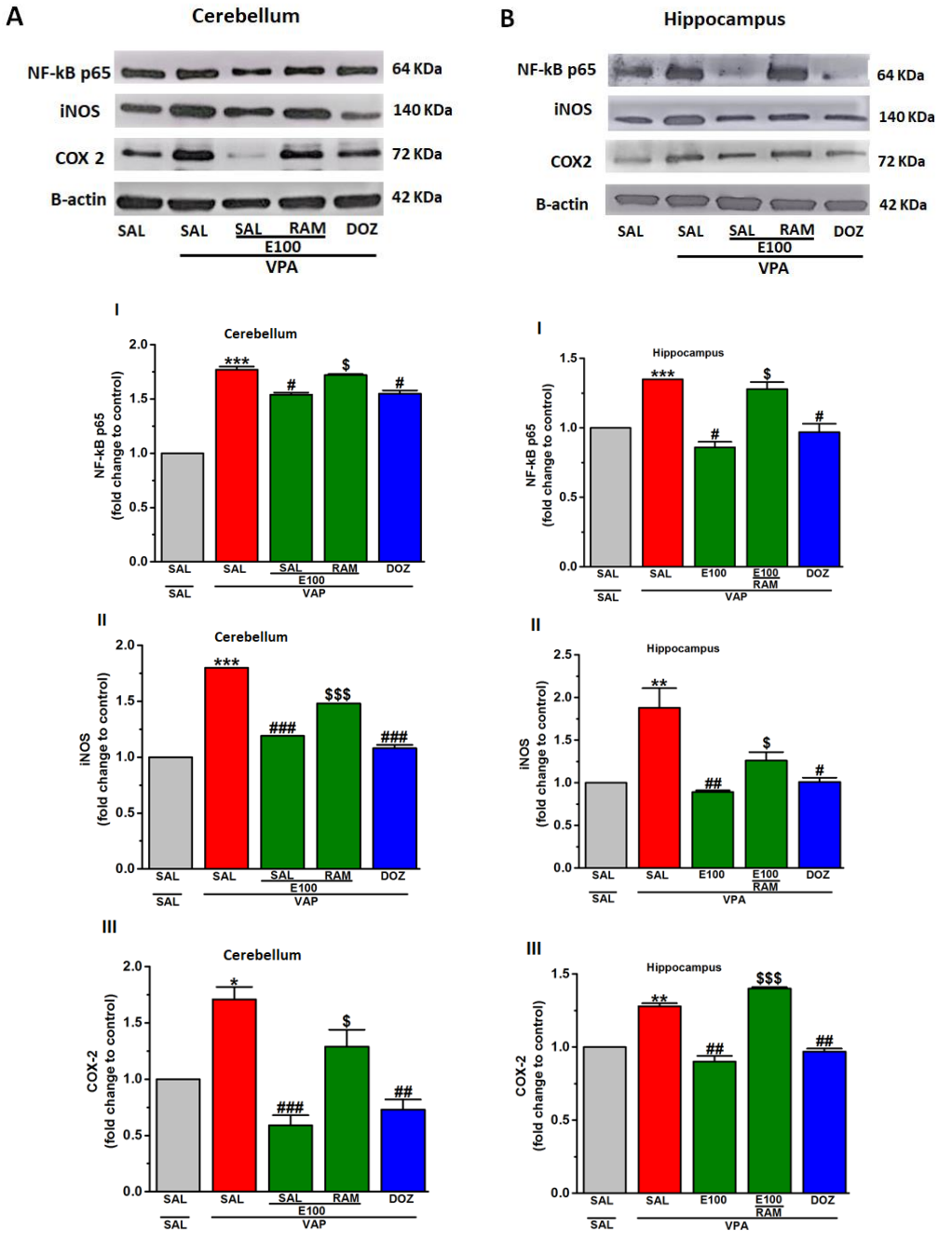


Figure 26: Effects of E100 on expression of NF-κB p65, iNOS & COX-2 in cerebellum and hippocampus of VPA-exposed C57BL/6J mice.

NF-κBp65, iNOS and COX-2 are modulated by subchronic treatment with E100 (10 mg/kg, i.p.) in cerebellum and hippocampus of prenatally VPA treated mice (red). Representative immunoblots of cerebellum tissues of VPA-exposed group subjected to immunoreactions with anti-NF-κB p65, anti-iNOS and anti-COX2 (A). Representative immunoblots of hippocampal tissues of VPA-exposed group subjected to immunoreactions with anti-NF-κB p65, anti-iNOS and anti-COX2 (B). Blots were quantified using Image J and corresponding results were represented as fold change of control. Data are expressed as mean ± SEM (n=3). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs saline-exposed mice; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P< 0.001$ vs. VPA-exposed mice and $^{\$}P<0.05$, $^{\$\$}P<0.01$, $^{\$\$\$}P<0.001$ vs. E100 (10 mg)-pretreated group VPA exposed mice.

3.17 Effects of E100 on iba-1 positive microglia in cerebellum tissues of VPA-exposed C57BL/6J mice

Activation of glial cell (microglia) in VPA-exposed mice has been observed and is considered to be an index of the inflammatory responses (Figure 27A and B). The observed results showed that VPA-exposed mice exhibited a significant increase in the expression of Iba-1, which is a marker of activated microglia (P<0.05) (Figure 27A and B). Immunofluorescence staining revealed a significant increase (P<0.05) in the number of activated microglia in VPA-exposed mice compared to the saline-exposed control mice (Figure 27A and B). However, subchronic systemic treatment of VPA-exposed mice with E100 (10 mg/kg, i.p.) significantly decreased the number of activated microglia compared to the saline-exposed control mice (P< 0.05) (Figure 27C). Moreover, subchronic systemic co-administration of RAM (10 mg/kg, i.p.) entirely reversed the effects of E100 (10 mg/kg, i.p.) on the number of activated microglia compared to the E100 (10 mg)-treated VPA-exposed animals (P< 0.05) (Figure 27D).

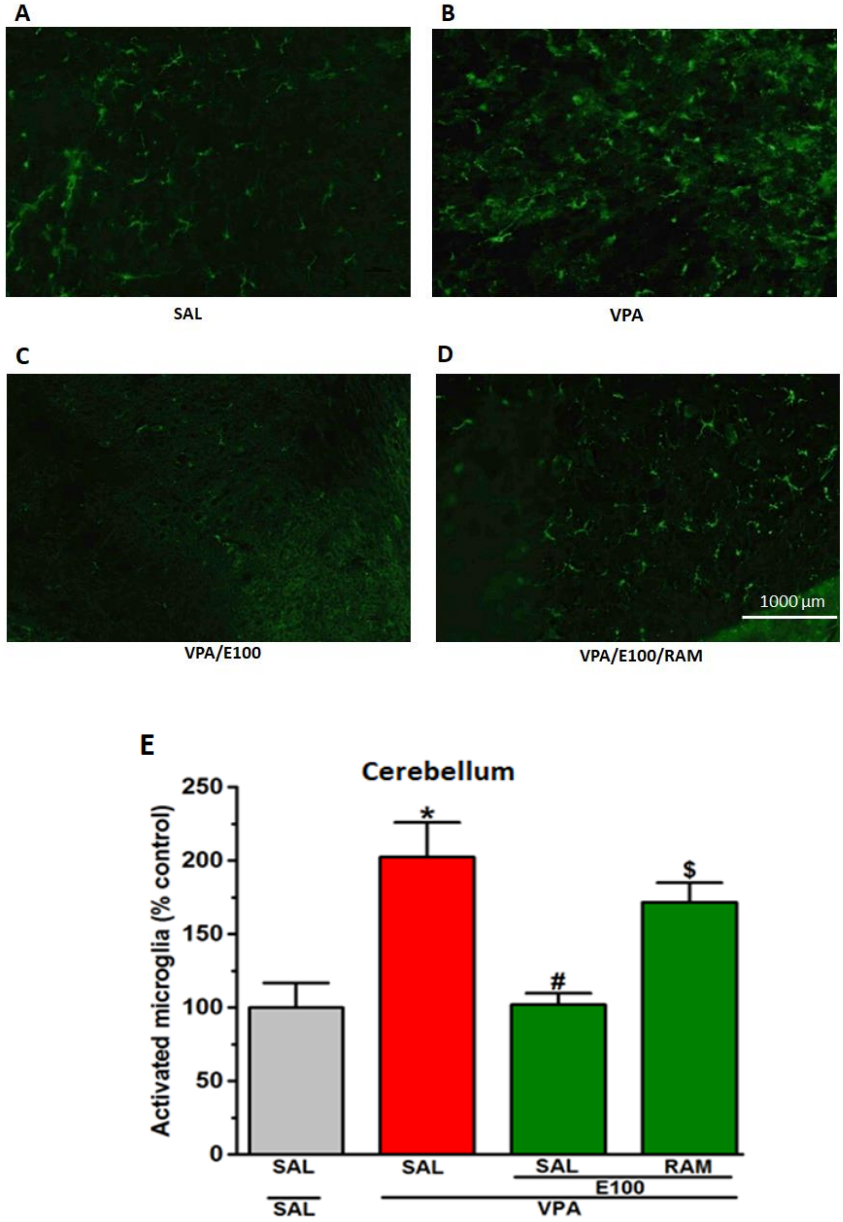


Figure 27: Effects of E100 on iba-1 positive microglia in cerebellum tissues of VPA-exposed C57BL/6J mice.

Profound expression of iba-1-positive microglia was found in the VPA-exposed mice (B) compared to the saline-exposed mice (A). In contrast, subchronic treatment with E100 to the VPA-exposed mice showed significantly lower staining of Iba-1 compared to the VPA-exposed mice (C). Subchronic (21 days) systemic co-injection of RAM (10 mg/kg, i.p) counteracted the E100 (10 mg)-provided amelioration of iba-expression of VPA-exposed mice ($^{\$}P < 0.05$) (D). Quantitative analysis of activated microglia (E) revealed a significant increase ($^{*}P<0.05$) in the number of activated microglia in cerebellum of VPA-exposed mice compared to the saline-exposed mice. However, subchronic treatment with E100 (10 mg/kg, i.p.) to the VPA-exposed mice significantly reduced ($^{\#}P<0.05$) the number of activated microglia in the E100-treated VPA-exposed mice compared to the saline-treated VPA-exposed mice group. Values are expressed as the percent mean ± SEM (n = 3).

3.18 Effects of E100 on sociability and social novelty impairments of BTBR mouse model of ASD

The effect of subchronic systemic injection of E100 at three different doses (5, 10, and 15 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.) on ASD-like sociability and social novelty impairments in the TCT task in BTBR mice are shown in Figure 28 A and B. The results of statistical analyses revealed that subchronic pretreatment with E100 (5, 10, and 15 mg/kg, i.p.) and DOZ (1 mg/kg) prior to TCT significantly enhanced sociability by increasing SI, with [$F_{(5,24)}$ = 5.79; P<0.05] (Figure 28A). As observed in the post hoc analysis, BTBR mice exhibited significantly lower sociability expressed in form of SI value when compared to control animals, with [$F_{(1,8)}$ = 13.66; P<0.05] (Figure 28A). However, E100 (5 and 10 mg/kg) and DOZ (1 mg/kg) significantly increased SI of BTBR mice when compared to saline treated BTBR mice group, with [$F_{(1,8)}$ = 9.36; P<0.05], [$F_{(1,8)}$ = 8.74; P<0.05], and [$F_{(1,8)}$ = 6.21; P<0.05], respectively (Figure 28A). Moreover, the results revealed that the enhancement in SI observed with E100 (5 mg/kg) was comparable to that shown with DOZ (1 mg/kg) and E100 (10 mg/kg), with [$F_{(1,8)}$ = 0.46; p=0.52] and [$F_{(1,8)}$ = 0.26; p=0.63], respectively (Figure 28A). Notably, E100 (15 mg/kg) failed to restore sociability deficits of BTBR mice, with [$F_{(1,8)}$ = 4.21; p=0.07] (Figure 28A). Interestingly, the E100 (5 mg)-provided improvement of sociability was counteracted following subchronic systemic co-administration with centrally acting H3R agonist RAM (10 mg/kg, i.p.), with [$F_{(1,8)}$ = 10.90; P<0.05] (Figure 28A). Similarly, the results of post hoc analyses indicated that BTBR mice demonstrated significantly impaired social novelty preference when compared to the control mice, with [$F_{(1,8)}$ = 6.69; P<0.05] (Figure 28B). However, improvement in social novelty preference was achieved following subchronic systemic pretreatment of BTBR mice with E100 (5 and 10 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.),

with [$F_{(1,8)}$ = 8.65; P<0.05], [$F_{(1,8)}$ = 5.47; P<0.05], and [$F_{(1,8)}$ = 5.37; P<0.05], respectively (Figure 28B). E100 (15 mg/kg) failed to restore social novelty deficits of BTBR mice, with [$F_{(1,8)}$ = 1.83; p=0.21] (Figure 28B). As depicted in Figure 28B and observed in the post hoc analyses, the E100 (5 mg)-provided improvement of social novelty assessed by SNI was nullified by RAM (10 mg/kg, i.p.), with [$F_{(1,8)}$ = 7.08; P<0.05], and as compared to the E100 (5 mg)-treated BTBR mice (Figure 28B). Interestingly, subchronic systemic pretreatment of control mice with the most promising dose of E100 (5 mg/kg, i.p.) did not alter SI or SNI as compared to saline-treated control mice (P>0.05).

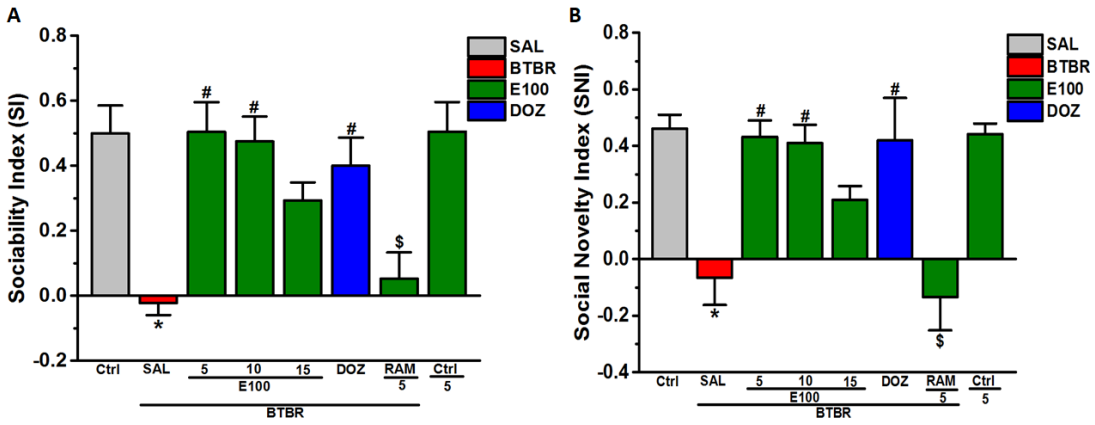


Figure 28: E100 improved sociability and social novelty deficits of BTBR mouse model of ASD.

After 10 minutes of acclimatization, male subjects were allowed to explore all chambers for two 10 min sessions. The results obtained were Sociability index (SI) (A) and Social novelty index (SNI) (B). C57BL/6J (gray) were injected with saline in Ctrl group, E100 (5,10 or 15 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.) were administered subchronically for 21 days. Abrogative study of subchronic (21 days) systemic co-injection of RAM (10 mg/kg, i.p.) on the E100 (5 mg)-provided improvement of sociability (A) and social novelty (B). Figures show mean ± SEM (n = 5). $^{*}P$<0.05 *vs.* SI or SNI of saline-treated Ctrl mice. $^{\#}P$<0.05 *vs.* SI or SNI of saline-treated BTBR mice. $^{\$}P$<0.05 *vs*. E100 (5 mg)-treated BTBR mice.

3.19 Effects of E100 on acetylcholine esterase activity in cerebellum tissues of BTBR mice

Acetylcholine esterase activity in BTBR mice has been assessed (Figure 29). The observed results showed that BTBR mice exhibited a significant increase in the

activity of acetylcholine esterase enzyme in cerebellum tissues of BTBR mice as compared to control mice (P<0.05) (Figure 29). However, subchronic systemic treatment of BTBR mice with E100 (5 mg/kg, i.p.) significantly decreased the acetylcholine activity of BTBR mice when compared with the saline-treated BTBR mice (P< 0.01) (Figure 29). Similarly, subchronic systemic pretreatment with the reference drug DOZ (1 mg/kg, i.p.) significantly decreased the acetylcholine activity of BTBR mice when compared with the saline-treated BTBR mice (P< 0.01) (Figure 29).

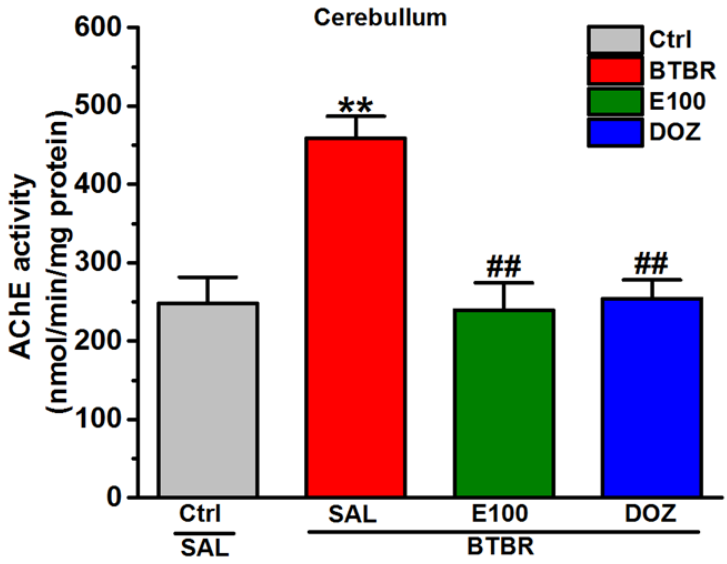


Figure 29: Effects of E100 on acetylcholine esterase activity in cerebellum tissues of BTBR mice.

Inhibitory effects of E100 (5 mg/kg, i.p.) on acetylcholine esterase enzyme in the cerebellum of BTBR mice. Quantitative analysis revealed a significant increase ($^{**}P$<0.01) in the acetylcholine esterase enzyme activity in cerebellum of BTBR mice compared to the control mice. However, subchronic treatment with E100 (5 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.) to the BTBR mice significantly reduced ($^{\#\#}P$<0.01) this activity compared to the saline-treated BTBR mice. Values are expressed as the percent mean ± SEM (n = 3-5).

3.20 Effects E100 on stereotyped repetitive and obsessive-compulsive behaviors of BTBR mouse model of ASD in MBT and NST

The effect of subchronic systemic administration with E100 (5, 10, or 15 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.) on the elevated repetitive behavior of BTBR mice in MBT is shown in Figure 30A. The results of statistical analyses showed that subchronic pretreatment with 5, 10 or 15 mg/kg of E100 or 1 mg/kg DOZ prior to MBT significantly reduced the elevated percentage of marbles buried by BTBR mice, with [$F_{(5,30)}$ = 14.22; P<0.05] (Figure 30A). BTBR mice buried significantly more marbles compared to the control mice, with [$F_{(1,10)}$ = 34.37; P<0.05]. However, pretreatment with E100 (5, 10 or 15 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.) significantly obliterated the increased percentage of marbles buried by BTBR mice when compared to saline-treated BTBR mice, with [$F_{(1,10)}$ = 29.41; P<0.05], [$F_{(1,10)}$ = 19.61; P<0.05], [$F_{(1,10)}$ = 13.24; P<0.05], and [$F_{(1,10)}$ = 22.85; P<0.05], respectively (Figure 30A). Moreover, the E100 (5 mg)-provided decrease in the percentage of buried marbles was comparable to the DOZ-provided effect, with [$F_{(1,10)}$ = 0.12; p=0.73], and was entirely abrogated by RAM, with [$F_{(1,10)}$ = 2.08; p=0.18] and as compared with slaine-treated BTBR mice (Figure 30A). Notably, subchronic systemic pretreatment of control mice with the most promising dose of E100 (5 mg/kg, i.p.) did not alter percentage of buried marbles in MBT as compared to saline-treated control mice (P>0.05). Similarly, the effect of subchronic systemic administration of E100 (5, 10, or 15 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.) on the percentage escalation of shredded nestlet was evaluated in NST (Figure 30B). Post hoc analyses showed that BTBR mice shredded significantly more nestlet compared to the control mice, with [$F_{(1,10)}$ = 27.94; P<0.05]. However, BTBR mice pretreated with E100 (5 or 10 mg/kg, i.p.) exhibited a significantly lower percentage of shredded nestlet compared to BTBR mice treated with saline, with

[$F_{(1,10)}$ = 12.72; P<0.05] and [$F_{(1,10)}$ = 6.63; P<0.05], respectively (Figure 30B). Moreover, the effect observed with 5 mg/kg was comparable to that witnessed with 10 mg/kg, with [$F_{(1,10)}$ = 0.54; p = 0.48], and was significantly higher than that witnessed with 15 mg/kg, with [$F_{(1,10)}$ = 6.11; P<0.05] (Figure 30B). Notably, no significant difference in the E100-provided effect on percentage of shredded nestlet was detected between the two doses of 10 mg/kg and 15 mg/kg, with p=0.17 (Figure 30B). Moreover, subchronic systemic administration of DOZ (1 mg/kg, i.p.) significantly lowered the percentage of shredded nestlet in BTBR mice, with [$F_{(1,10)}$ = 7.79; P<0.05]. As shown in Figure 30B and observed in the post hoc analyses, the E100 (5 mg)-provided decrease in the percentage of shredded nestlet was countered following subchronic systemic co-administration of RAM (10 mg/kg, i.p.), with [$F_{(1,10)}$ = 6.05; P<0.05] and as compared with E100 (5 mg)-treated BTBR mice (Figure 30B). Notably, subchronic systemic pretreatment of control mice with the most promising dose of E100 (5 mg/kg, i.p.) did not alter percentage of shredded nestlets in NST as compared to saline-treated control mice (P>0.05).

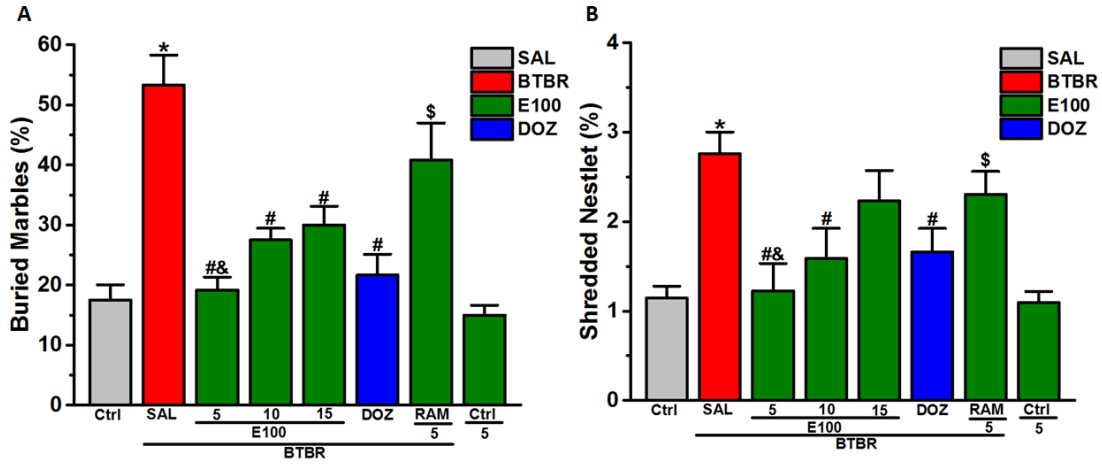


Figure 30: E100 mitigated stereotyped repetitive behavior in MBT and attenuated increased obsessive-compulsive features in NST.

Repetitive marble-burying (A) and obsessive compulsive nestlet shredding (B) behavior were measured after a 30-minute testing session. BTBR mice (red) demonstrated elevated stereotyped, repetitive and compulsive behaviors that were significantly increased compared to Ctrl mice (gray). E100 (at a dose of 5, 10, or 15 mg/kg, i.p in green) or DOZ (1 mg/kg, i.p. in blue) was administered subchronically for 21 days. Effects of subchronic (21 days) systemic co-injection of RAM (10 mg/kg, i.p.) on the E100 (5 mg)-provided attenuation of stereotyped repetitive and compulsive behaviors of BTBR mice were assessed in MBT (A) and NST (B). Figures show mean ± SEM (n = 6). $^{*}P<0.05$ vs. saline-treated Ctrl mice. $^{\#}P<0.05$ vs. saline-treated BTBR mice. $^{\&}P<0.05$ vs. E100 (10 or 15 mg)-treated BTBR mice. $^{\$}P<0.05$ vs. E100 (5 mg)-treated BTBR mice.

3.21 Effects of E100 on anxiety levels and locomotor activity of BTBR model of ASD in EPM

Figure 31A-C shows the observed effects of subchronic systemic injection of saline or E100 (5, 10, or 15 mg/kg, i.p.) on the anxiety parameters of BTBR mice tested in the EPM, namely, the percentage of time spent in open arms (Figure 31A), the number of entries into open arms (Figure 31B), and locomotor activity expressed as the number of entries into closed arms (Figure 31C). The results observed showed that BTBR mice spent significantly less time and lower number of entries in open arms when compared to control mice, with [$F_{(1,10)} = 36.82$; $P<0.05$] and [$F_{(1,10)} = 8.83$; $P<0.05$], respectively (Figure 31A and B). However, subsequent post hoc analyses revealed that E100 when administered at 5, 10, or 15 mg/kg, i.p. significantly altered

the percentage of time spent exploring the open arms of the maze during a 5 min session compared to saline-treated BTBR mice, with [$F_{(1,10)}$ = 23.99; P<0.05], [$F_{(1,10)}$ = 5.33; P<0.05], and [$F_{(1,10)}$ = 17.91; P<0.05], respectively (Figure 31A). Moreover, post hoc evaluation revealed that, compared with control mice, the BTBR mice displayed a lower number of entries into the open arms, with [$F_{(1.10)}$ = 8.83; P<0.05] (Figure 31B). However, E100 when administered at 5, 10 or 15 mg/kg, i.p. significantly increased the number of entries the open arms of the maze during a 5 min session compared to saline-treated BTBR mice, with [$F_{(1,10)}$ = 13.24; P<0.05], [$F_{(1,10)}$ = 12.31; P<0.05], and [$F_{(1,10)}$ = 18.85; P<0.05], respectively (Figure 31B). Interestingly, BTBR mice pretreated with DOZ (1 mg) spent a significantly lower percentage of time exploring the open arms compared to saline-treated BTBR mice, with [$F_{(1,10)}$ = 14.28; P<0.05] (Figure 31A). Further analyses of data describing the number of entries into the open arms of the maze yielded for DOZ (1 mg/kg, i.p.) higher number of entries into open arms when compared to saline-treated BTBR mice, with [$F_{(1,10)}$ = 8.42; P<0.05] (Figure 31B). As shown in Figure 31A and B, the E100 (5 mg)-provided improvement in anxiety levels was abrogated by RAM (10 mg/kg, i.p.) with [$F_{(1,10)}$ = 10.74; P<0.05] and [$F_{(1,10)}$ = 6.17; P<0.05], for time spent in open arms and number of open arm entries, respectively, and as compared to E100 (5 mg)-treated BTBR mice. Notably, the number of closed arm entries following subchronic systemic injection of E100 (5, 10, or 15 mg/kg) and DOZ (1 mg/kg, i.p.) was not significantly different as compared to saline treated BTBR mice, with [$F_{(1,10)}$ = 1.90; p=0.20], [$F_{(1,10)}$ = 0.34; p=0.57], [$F_{(1,10)}$ = 2.46; p=0.15], and [$F_{(1,10)}$ = 2.73; p=0.13], receptively (Figure 31C). Interestingly, subchronic systemic pretreatment of control

mice with the most promising dose of E100 (5 mg/kg, i.p.) did not alter any of the tested parameters in EPM and as compared to saline-treated control mice (all P>0.05).

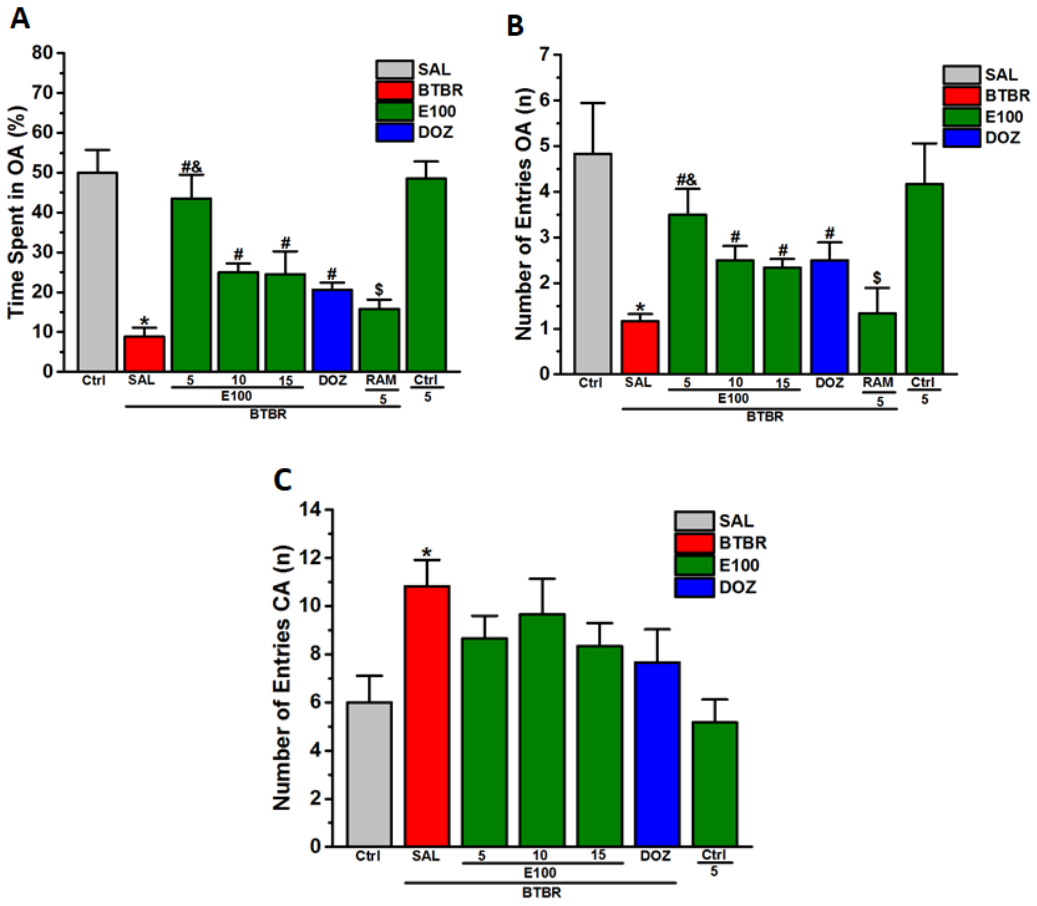


Figure 31: Effects of E100 and DOZ pretreatment on anxiety levels and locomotor activity in BTBR mice in EPM test.

BTBR mice (red) demonstrated elevated anxiety-related behavior and hyperactivity that were significantly increased compared to Ctrl mice (gray). E100 (5, 10, or 15 mg/kg, i.p. in green) or DOZ (1 mg/kg, i.p., in blue) was administered subchronically for 21 days. E100 (5, 10, or 15 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.) attenuated the decreased percentage of time spent on the open arms of the EPM (A) the decreased number of entries into the open arms (B) and the increased number of entries into the closed arms (C) in BTBR mice. Effects of subchronic (21 days) systemic co-injection of RAM (10 mg/kg, i.p.) on the E100 (5 mg)-provided elevation of time spent in open arms (A) or number of entries into open arms (B) of BTBR mice were assessed in EPM test. Data are expressed as the mean ± SEM (n = 6). $^{*}P$ < 0.05 vs. Saline treated Ctrl mice. $^{\#}P$ < 0.05 vs. Saline treated BTBR mice. $^{\&}P$<0.05 vs. E100 (10 or 15 mg)-treated BTBR mice. $^{\$}P$<0.05 vs. E100 (5 mg)-treated BTBR mice.

3.22 Effects of E100 on anxiety levels and locomotor activity of BTBR model of ASD in OFT

In addition to EPM, the OFT was used to test anxiety-like behavior and locomotion. As seen in Figure 32A-C, there were no significant effects of subchronic systemic exposure of BTBR mice to E100 (5, 10, and 15 mg/kg, i.p.) on time spent in the periphery (all $P>0.05$) (Figure 32B). In contrast, analysis of variance demonstrated that BTBR mice pretreated with E100 (5, 10, and 15 mg/kg, i.p.) spent a significantly lower percentage of time in the center of the arena, with [$F_{(1,6)} = 6.96$; $P<0.05$], [$F_{(1,6)} = 15.25$; $P<0.05$], and [$F_{(1,6)} = 14.56$; $P<0.05$], respectively (Figure 32A). As shown in Figure 32A and observed in the post hoc analyses, the E100 (5 mg)-provided decrease in the time spent in the center of arena was abrogated following subchronic systemic co-administration of RAM (10 mg/kg, i.p.), with [$F_{(1,6)} = 6.42$; $P<0.05$] compared with E100 (5 mg)-treated BTBR mice (Figure 32A). Moreover, BTBR mice pretreated with E100 (5, 10, and 15 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.) demonstrated significantly lower travelled distance when compared to saline-treated BTBR mice, with [$F_{(1,6)} = 10.60$; $P<0.05$], [$F_{(1,6)} = 10.57$; $P<0.05$], [$F_{(1,6)} = 8.84$; $P<0.05$], and [$F_{(1,6)} = 7.76$; $P<0.05$], respectively (Figure 32C). Notably, subchronic treatment of control mice with E100 (5 mg/kg, i.p.) had no significant influence on time spent in the central arena, time spent in the periphery, or total distance travelled as compared to saline-pretreated control mice (all $P>0.05$) (Figure 32A-C).

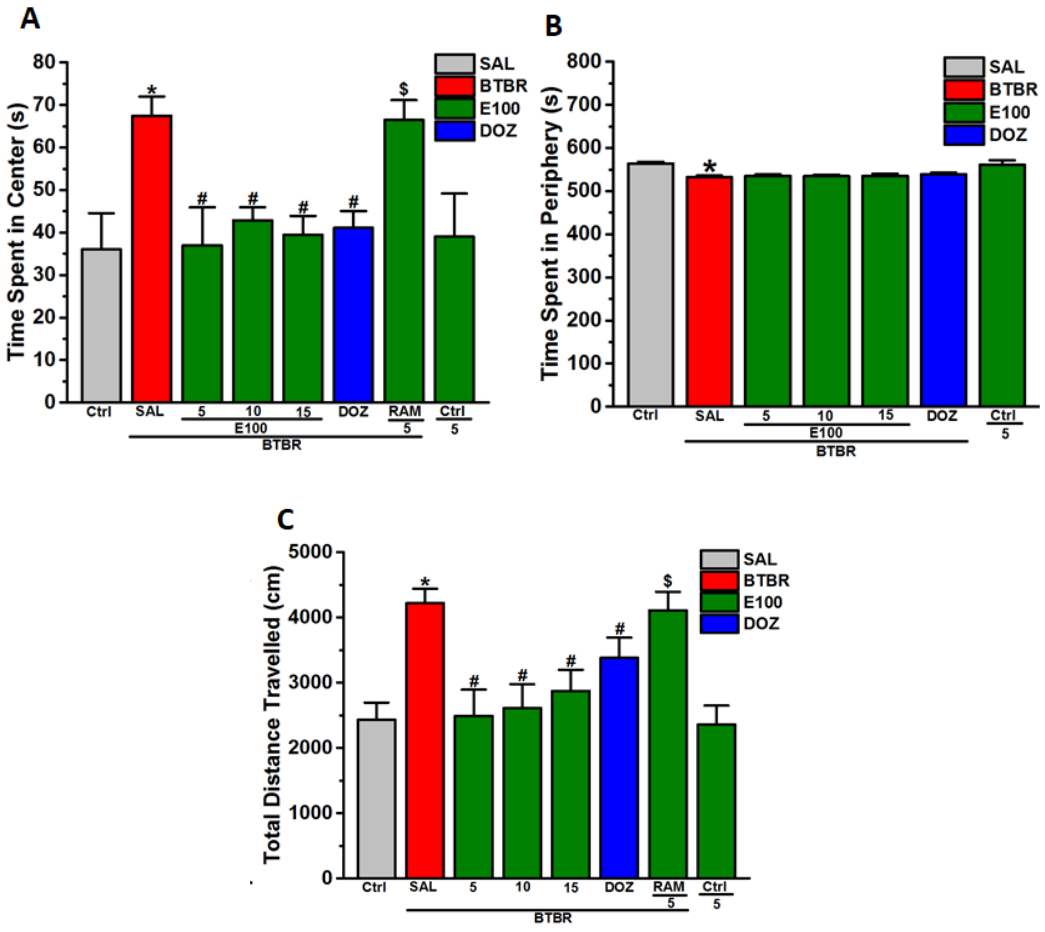


Figure 32: Effects of E100 and DOZ pretreatment on anxiety levels and locomotor activity in BTBR mice in OFT.

BTBR mice (red) demonstrated elevated impulsive attitude and deficits in cognition as well as locomotor activity behaviors that were significantly increased compared to Ctrl mice (gray). E100 (5, 10, or 15 mg/kg, i.p. in green) or DOZ (1 mg/kg, i.p. in blue) was administered subchronically for 21 days. E100 (5 and 15 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.) attenuated the increased time spent in the central arena (A) as well as the increased total distance travelled (C) but failed to modulate the time spent in the periphery (B) in BTBR mice in the OFT. Effects of subchronic (21 days) systemic co-injection of RAM (10 mg/kg, i.p.) on the E100 (5 mg)-provided amelioration of time spent in the center and increased distance travelled (C) of BTBR mice in OFT. Data are expressed as the mean ±SEM (n = 4). $^{*}P<0.05$ vs. Saline treated Ctrl mice. $^{\#}P<0.05$ vs. Saline treated BTBR mice. $^{\$}P<0.05$ vs. E100 (5 mg)-treated BTBR mice.

3.23 Effects of E100 on activated microglia in cerebellum tissues of BTBR mice

Activation of glial cell (microglia) in BTBR mice has been observed (Figure 33). The observed results showed that BTBR mice exhibited a significant increase in the expression of iba-1, which is a marker of activated microglia ($P<0.05$) (Figure 33).

However, subchronic systemic treatment of BTBR mice with E100 (5 mg/kg, i.p.) significantly decreased the number of activated microglia compared to the saline-treated BTBR mice (P<0.05) (Figure 33). Moreover, subchronic systemic co-administration of RAM (10 mg/kg, i.p.) entirely reversed the effects of E100 (5 mg/kg, i.p.) on the number of activated microglia compared to the E100 (5 mg)-treated BTBR animals (P< 0.05) (Figure 33).

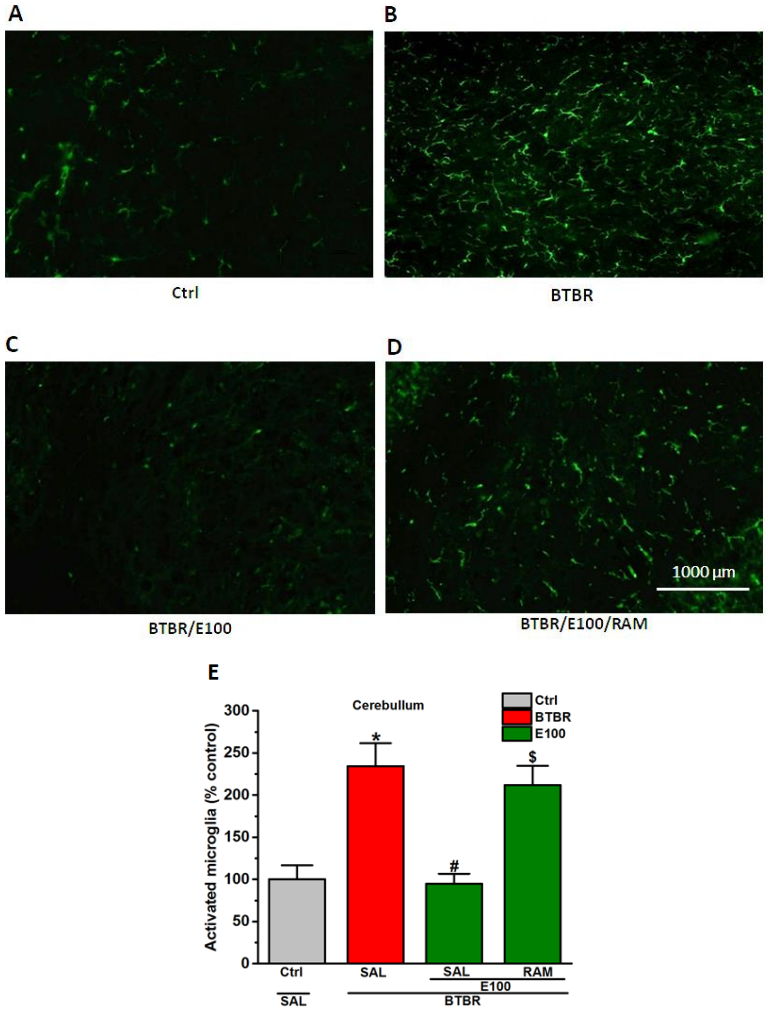


Figure 33: Effects of E100 on iba-1 positive microglia in cerebellum tissues of BTBR mice.

Quantitative analysis of activated microglia revealed a significant increase ($^{*}P$<0.05) in the number of activated microglia in cerebellum of BTBR mice compared to control mice. However, subchronic treatment with E100 (5 mg/kg, i.p.) to the BTBR mice significantly reduced ($^{\#}P$<0.05) the number of activated microglia compared to the saline-treated BTBR mice. Effects of subchronic (21 days) systemic co-injection of RAM (10 mg/kg, i.p.) on the E100 (5 mg)-provided mitigation of activated microglia in BTBR mice $^{\$}P$<0.05. Values are expressed as the percent mean ± SEM (n = 3).

Chapter 4: Discussion

Antagonists targeting H3Rs are considered promising alternative treatments for different brain disorders, such as SCH, AD and narcolepsy (Passani et al., 2011; Sadek et al., 2016c). In the current study we investigated, for the first time, the effects of the H3R antagonist DL77 on a mouse model of ASD-like behaviors induced by prenatal exposure to VPA. DL77 was chosen for the current study because it belongs to the class of non-imidazole H3R antagonists with improved selectivity and safety profile compared to the imidazole-based H3R antagonists, e.g., ciproxifan, which have several limitations as clinical candidates due to the presence of an imidazole heterocycle, which is responsible for numerous possible pharmacokinetic drawbacks, such as CYP450 inhibition, off-target activity, or lack of subtype selectivity, especially over H4R (Passani et al., 2011).

Subchronic systemic administration of DL77 demonstrated ameliorating effects on social interaction deficits and stereotypies in VPA-exposed mice. In the TCT paradigm, sociability or social novelty is the tendency to spend time exploring an unfamiliar animal, compared to time spent exploring an object or a familiar animal, respectively. Systemic subchronic pretreatment with DL77 normalized the impairment in sociability demonstrated by VPA-exposed mice, since these animals, when pretreated with DL77, presented increased sociability index (improved sociability, Figure 11A) and social novelty index (improved social novelty, Figure 12A), to levels similar to the saline-exposed mice. Numerous previous studies have focused on the procognitive effects of several H3R antagonists on social memory (Alachkar, Lazewska, Kiec-Kononowicz et al., 2017; Fox, Esbenshade, Pan et al., 2005; Fox, Pan, Esbenshade, Bennani et al., 2002; Fox, Pan, Esbenshade, Bitner et al., 2002; Fox, Pan,

Lewis et al., 2004; Fox, Pan, Radek et al., 2003; Sadek et al., 2016d), a behavioral feature that is also altered in ASD (Noland, Steven Reznick, Stone et al., 2010), but our study is the first one to assess the effects on of a non-imidazole based H3R antagonist on sociability deficits as ASD-like behaviors in VPA-exposed mice. Importantly, the sociability- and social novelty-enhancing effect observed for DL77 was dose-dependent, since DL77 (5 mg/kg) failed to improve sociability (Figure 11A) or social novelty preference (Figure 12A), while a dose of 10 mg/kg or 15 mg/kg provided significant enhancement in sociability as well as social novelty behavior. An optimum effect was observed when the H3R antagonist DL77 was applied at a dose of 10 mg/kg, and a dose of 15 mg/kg DL77 did not significantly improve upon the DL77 (10 mg)-provided sociability or social novelty enhancement (Figures 11A and 12A). Interestingly, the observations for the dose-dependent effects of DL77 are in line with those previously observed for numerous H3R antagonists in preclinical experiments in different rodents (Benetti and Izquierdo, 2013; Benetti et al., 2013; Sadek et al., 2016c). In addition, the observed results with regard to dose dependency strongly support previous procognitive effects observed for the H3R antagonist DL77 (2.5, 5, or 10 mg/kg, i.p.) on different memory stages, namely, acquisition, consolidation, and retrieval in rats (Sadek et al., 2016c). Notably, the observations of sociability- and social novelty-enhancing effects for DL77 are in agreement with earlier experimental results observed with the imidazole-based H3R antagonist ciproxifan in Swiss mice (Baronio et al., 2015). In that study, ciproxifan (3 mg/kg, i.p.) attenuated sociability deficits in the VPA-induced ASD-like behaviors (Baronio et. al 2015).

Interestingly, the DL77 (10 mg)-provided enhancing effects on sociability and social novelty were completely reversed when mice were co-administered the CNS-

penetrant H3R agonist RAM or with the CNS-penetrant H2R antagonist ZOL, but not with the centrally acting H1R antagonist PYR (Figures 11B and 12B). The latter observations indicate that brain histaminergic neurotransmission appears to be involved in the capacity of the H3R antagonist DL77 to facilitate the release brain histamine in specific brain areas (Brioni, Esbenshade, Garrison et al., 2011; Panula et al., 2015; Sadek et al., 2016c; Sadek & Stark, 2016e). The current results further indicate that histaminergic pathways, through activation of H2Rs, fundamentally contribute to neuronal pathways important for alteration of sociability and social novelty processes in the TCT paradigm in VPA-exposed mice. Notably, the reference drug DOZ failed to improve both social impairment parameters of VPA exposed mice following subchronic systemic treatment (Figure 11A and 12A). These observations could be explained by the results of a previous study in which the integrity of the central histaminergic system was found to be a crucial requirement for the biochemical and behavioral effects elicited by two procognitive compounds, namely, the H3R antagonist ABT-239 and the acetylcholine esterase inhibitor DOZ (Provensi, Costa, Passani et al., 2016). Consequently, further investigations are necessary to assess the functionality of the central histaminergic system in VPA-exposed mice to further explain the behavioral results observed for DOZ in sociability as well as social novelty tests.

Stereotypy and rigidity of behavior are considered core features of ASD (Peter, Oliphant, & Fernandez, 2017; Zhao, Jiang, & Zhang, 2018). Moreover, the involvement of the brain histaminergic neurotransmission in the pathophysiology of Tourette syndrome, a condition commonly comorbid among ASD patients and featured by stereotypies, has been proposed and has been associated with a premature

termination codon (W317X) in the L-histidine decarboxylase (HDC) gene. Consequently, such a premature termination of codon (W317X) impairs brain histaminergic neurotransmission (Frick et al., 2016; Paschou et al., 2013; Rapanelli, 2017a; Rapanelli, Frick, Horn et al., 2016a; Rapanelli & Pittenger, 2016b). In the current study, VPA-exposed mice subchronically pretreated with DL77 (10 or 15 mg/kg, i.p.) or with the reference drug DOZ (1 mg/kg, i.p.) displayed comparable reductions in stereotyped repetitive behavior in MBT (Figure 13A). Moreover, the DL77 (10 mg/kg)-provided effects in MBT were entirely abrogated when mice were administered the CNS penetrant H3R agonist RAM, but not with the CNS-penetrant H1R antagonist PYR or the H2R antagonists ZOL (Figure 13B). The mechanism by which the repetitive behavior is improved is not clear, but it might be explained by the capability of DL77, as a potent H3R antagonist, to mediate the release of different neurotransmitters besides histamine, such as dopamine, serotonin and acetylcholine, in several specific brain areas (Brioni et al., 2011; Sadek et al., 2016c; Sadek & Stark, 2016e; Witkin et al., 2004). Therefore, measuring the levels of different brain neurotransmitters, including histamine, in various brain areas of the VPA-exposed mice with ASD-like behaviors as well as when treated with DL77 would further help us understand which neural circuits could be involved in this observed behavioral improvement. Interestingly, the results observed for DL77 in MBT are in agreement with a previous study in which acute systemic administration of the non-imidazole H3R antagonist ST-1283 significantly decreased the number of buried marbles and significantly shortened the digging duration in adult male C57BL/6 mice, without altering locomotor activity tested in an open field (A. Bahi, J. S. Schwed, et al., 2014).

Similarly, DL77 (10 or 15 mg/kg) attenuated the percentage of shredded nestlet in VPA-exposed mice with ASD-like behaviors in NST, and this reducing effect of DL77 on stereotyped repetitive behavior in NST was reversed with co-administration of the H3R agonist RAM, but not the H1R antagonist PYR or the H2R antagonist ZOL (Figure 14A and B). The results observed for DL77 in NST are consistent withthe current observations in MBT because DL77 facilitates the release of numerous central neurotransmitters besides histamine that are involved in repetitive as well as anxious behaviors in different rodents (Brioni et al., 2011; Sadek et al., 2016c; Sadek & Stark, 2016e; Witkin et al., 2004).

Ligands modulating anxiety levels or locomotion may give rise to a false-positive effect in these behavioral tests (A. Bahi, J. S. Schwed, et al., 2014; Borsini & Meli, 1988). Therefore, the numbers of entries into the closed arms and into open arms in the EPM test were used as indicators of locomotor activity and anxiety levels, respectively. The results showed that DL77 at a dose of 5, 10 or 15 mg/kg did not alter anxiety-like levels nor locomotor activity in VPA-exposed mice as measured by the percentage of time spent or the number of entries into open arms and closed arms, respectively (Figure 15A-C). Thus, the improvements in sociability, social novelty and repetitive behaviors observed for DL77 in TCT, MBT and NST, respectively, appear unlikely to be associated with a modulating effect on anxiety levels or an increase in locomotor activity of the tested mice. In contrast, the reference drug DOZ (1 mg/kg, i.p.) attenuated the percentage of time spent and the number of entries into open arms, indicating its enhancing effects on cognitive functions of VPA-exposed mice to assess risk and evaluate anxiety, as reflected in the decreased number of entries into open arms following subchronic systemic pretreatment VPA-exposed mice with DOZ

(Figure 15A-C). Moreover, the failure of DL77 at all doses to improve this abnormal anxiety and hyperactivity seen in VPA-exposed mice may have been due to the imbalance of several neurotransmitters that are reported to be dysregulated in ASD patients, such as serotonin (Chugani, 2002; Mabunga, Gonzales, Kim et al., 2015), glutamate and GABA (Casanova, Buxhoeveden, & Gomez, 2003; Mabunga et al., 2015; Orekhova, Stroganova, Prokofyev et al., 2008; Rubenstein & Merzenich, 2003).

Several hypotheses have been proposed about the dual role of brain histamine in neurological disorders, and evidence has shown its critical involvement in the modulation of microglia-mediated neuroinflammation (Barata-Antunes, Cristovao, Pires et al., 2017b). To determine a possible function of DL77 in tissue defense, oxidative stress parameters, MDA and GSH were assessed in the cerebellum (Figure 16A and B). The existence of lipid peroxidation is considered a key pathogenic event in brain tissues, resulting from an unbalanced ratio of radical oxygen species generation and the capacity for endogenous cellular antioxidant defense (Javed, Azimullah, Haque et al., 2016). The results showed that VPA-exposed mice with ASD-like behaviors had a significant increase in MDA, with a concomitant decline in GSH in cerebellar tissue. GSH is considered to protect against lipid peroxidation, and it is depleted in oxidative stress (Thomas, Maiorino, Ursini et al., 1990), as demonstrated in the observed results (Figure 16A and B). The results of the current study are in accordance with recent findings that histamine may provide protective effects under an inflammatory context (Barata-Antunes et al., 2017b). Subchronic systemic pretreatment with DL77 (10 or 15 mg/kg, i.p.) inhibited lipid peroxidation, as evidenced by the reduced MDA followed by restoration of GSH (Figure 16A and B). Interestingly, the imidazole-based H3R antagonists, namely, ciproxifan and

clobenpropit, control the elevation of various oxidative stress markers, including MDA and GSH, in amphetamine- or dizocilpine-augmented oxidative stress in an experimental mice model of SCH, indicating that H3R antagonists possess antioxidant activity, which might provide antioxidant actions and at the same time control the symptomatic features of SCH (Mahmood, Khanam, Pillai et al., 2012a; Mahmood et al., 2012b). These results in an SCH experimental model strongly support the current observations regarding DL77 in VPA-exposed mice with ASD-like behaviors, since SCH and ASD are often comorbid and share several symptomatic features (Mahmood et al., 2012a; Sadek et al., 2016c).

The protective role of histamine has also been reported in neurological conditions featured by microglia-induced neuroinflammation and, consequently, the involvement of brain histamine in the pathophysiology of multiple sclerosis and other neurological diseases of several animal models (Barata-Antunes et al., 2017b; Jadidi-Niaragh & Mirshafiey, 2010). The current study demonstrated a significant LPS-induced exacerbated rise in the expression of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) in VPA-exposed mice (Figure 17A-C). However, subchronic systemic pretreatment with DL77 (5, 10, or 15 mg/kg, i.p.) significantly decreased the elevated levels of these proinflammatory cytokines in VPA-exposed mice. Interestingly, the CNS-penetrant H3R agonist, when co-administered with DL77, completely reversed the protective effect of DL77 against the elevated markers of oxidative stress and partially abrogated the DL77-provided protective effects on proinflammatory cytokines (Figure 17A-C), indicating the involvement of central histaminergic neurotransmission in mediating the anti-inflammatory action of DL77 in VPA-exposed mice with ASD-like behaviors under inflammatory conditions.

Taken together the promising results of DL77 in palliating sociability deficits and stereotypies present in the VPA-exposed TO mice model of ASD-like behaviors, as well as its modulation of neuroinflammation and oxidative stress levels under the inflammatory context, encouraged us to expand these initial data. Accordingly, we further carried preclinical experiments using other H3R ligands and on other rodent species to understand the translational potential of H3R antagonists in the therapy of ASD and the neural circuits involved.

Accumulated evidence suggests that a variety of brain neurotransmitters such as ACh, 5-HT, DA, GABA, Glu, and HA are implicated in the onset and progression of ASD, substantiating the significance of this research area in the study of ASD etiology (Bacchelli et al., 2015; Chen et al., 2017; Ellenbroek et al., 2015; Hellings et al., 2017; Hellmer et al., 2017; Naaijen et al., 2017; Nakai et al., 2017; Paval, 2017; Paval et al., 2017; Shah et al., 2006; Wang et al., 2015). Alterations in histaminergic as well as cholinergic neurotransmission are thought to play a crucial role in the phenotypic outcomes of ASD-related behavioral features (Baronio et al., 2015; Karvat et al., 2014; Wright et al., 2017). Previous reports suggested that an impaired cholinergic system causes cognitive problems that may include social problems, which were reversed by donepezil treatments (Karvat et al., 2014; Riedel, Kang, Choi et al., 2009). Numerous lines of evidence from preclinical studies indicated notably that H3R antagonists/inverse agonists have been found to exhibit cognition-enhancing properties (Passani et al., 2011; Sadek et al., 2016c; Sadek & Stark, 2016e; Witkin et al., 2004). In view of that both acetylcholine esterase (AChE) and H3Rs (auto- and heteroreceptors) are suggested to be involved in the modulation of several central neurotransmitters including ACh and HA that are cognition associated. Consistent

with these reports, we assessed for the first time the pharmacological modulation of H3R and AChE by investigating the effects of a novel dual-active AChEI and H3R antagonist E100 on ASD-like behaviors in C57 VPA-exposed mice. Our major findings showed that systemic administration of E100 significantly improved social interaction deficits in VPA-exposed mice in TCT, and stereotypies in MBT and NST paradigm (Figure 18 -21). Importantly, the sociability- and social novelty-enhancing effect observed for E100 was dose-dependent, although E100 (5 mg/kg) significantly increased the SI (Figure 18A), it failed to improve SNI (Figure 19A), while significant enhancement in sociability as well as social novelty behaviors were achieved by a dose of 10 mg/kg or 15 mg/kg. However, E100 (10 mg/kg) effects were similar to the enhancing effects obtained for DOZ and were not significantly further enhanced with the dose of 15 mg/kg E100, (Figure 18A and 19A). On the other hand, DL77 (5 mg/kg) failed to improve both assessed social parameters, while DL77 (10 mg/kg or 15 mg/kg) showed significant improvement dose-dependently, with optimum effect exhibited by DL77 (15 mg/kg). Interestingly, the observations for the dose-dependent effects of E100 and DL77 on social impairments in C57 VPA-exposed mice, are in line with our initial observed results of DL77 on VPA-induced ASD in TO mice. However, the difference in DL77 doses exerting optimum effects (DL77 10 mg/kg in TO mice and DL77 15 mg/kg in C57 mice) on social deficits, may have been due to differences in strains and genetic backgrounds. Notably the observation of sociability and social enhancing-effect of E100 are unlike DL77 and other previous studies in which the effects of H3R antagonists/inverse agonists target H3Rs without inhibitory affinity for AChE (Brabant, Charlier, & Tirelli, 2013; De Almeida & Izquierdo, 1986). As observed, similar enhancing effects were achieved with E100 (10 mg) and a higher

dose of DL77 (15 mg), despite the tests being carried on the same mouse strain (C57), under similar conditions and by the same experimenter. This might be explained by the dual action of E100 with the capability of H3R antagonists to mediate the release of different neurotransmitters other than histamine, such as DA, 5-HT and ACh in specific brain regions (Brioni et al., 2011), together with its AChE inhibitory property that results in modulation of abnormal cholinergic transmission. The latter explanation highlights the significant role of both brain HA and ACh in improving social deficiencies and that not only histaminergic but also cholinergic transmission represents an essential neurophysiological component in cognitive functioning. Interestingly, the results observed for E100 in sociability assessments are in agreement with previous studies in which systemic administration of the dual-acting AChEI and H3R antagonist, namely UW-MD-71, exhibited cognitive enhancing effects that were similar to those obtained for the reference drug DOZ (AChEI) in that study (Sadek & Stark, 2016e).

Furthermore, E100-provided enhancing effects on sociability and social novelty were completely reversed when mice were co-administered the CNS-penetrant H3R agonist RAM, the CNS-penetrant H2R antagonist ZOL, or with muscarinic cholinergic antagonist SCO but not with the centrally acting H1R antagonist PYR (Figures 18B and 19B). This indicated that brain histaminergic and cholinergic neurotransmissions appear to be involved in mediating the capacity of the dual acting E100 to facilitate the release of brain HA and to elevate the levels of ACh in specific brain areas. The results further indicated that histaminergic and cholinergic pathways, through activation of H2Rs and muscarinic ACh receptors, respectively, contribute to

alteration of sociability and social novelty-related processes in VPA-exposed mice as observed in TCT paradigm.

Stereotypy, repetitive behavior and restricted interests are considered core features of ASD. Recently, tic disorder has been reported to be associated with histamine dysregulation (Bloch, State, & Pittenger, 2011; M. Rapanelli & Pittenger, 2016b), disorder commonly comorbid with attention difficulties and ASD (Rapanelli, Frick, Pogorelov et al., 2017b). Tics are repetitive and patterned motor actions that are typically associated with preceding uncomfortable sensory experiences (Martino & Hedderly, 2019). Moreover, as alteration in brain's the histamine modulatory system has been identified to be rare genetic cause of tic disorders; histidine decarboxylase knockout (*Hdc*-KO) mouse was generated to represent a promising model of its pathophysiology (Baldan et al., 2014; Ercan-Sencicek, Stillman, Ghosh et al., 2010), implying that diminished histaminergic neurotransmission maybe related to the exhibited repetitive and tic-like stereotypies (Baldan et al., 2014). In addition, Rapanelli et al. have implicated H3R in repetitive behavior-related pathology (Rapanelli et al., 2017b).

VPA-exposed mice subchronically pretreated with E100 (10 or 15 mg/kg, i.p.) or with the reference drug DOZ (1 mg/kg, i.p.) showed comparable reduction in stereotyped repetitive behavior in MBT (Figure 20A). Additionally, the E100 (10 mg/kg)-provided effects observed in MBT were nullified when mice were co-administered the CNS penetrant H3R agonist RAM, the CNS-penetrant H2R antagonist ZOL, or with muscarinic cholinergic antagonist SCO, but not with the CNS-penetrant H1R antagonist PYR (Figure 20B). The mechanism by which the repetitive/compulsive behavior is improved following systemic administration with

E100 may involve its capability as a potent dual-active H3R antagonist and AChE inhibitor, to modulate the release of different neurotransmitters besides HA and ACh, such as DA and 5-HT, in several specific brain areas. Consequently, assessing the levels of different brain neurotransmitters, including HA and ACh, in different brain areas of the VPA-exposed mice with ASD-like behaviors, as well as after pre-treatment with E100 would further help us to understand better which neurotransmitter systems maybe involved in the observed behavioral improvement. However, the results clearly demonstrated that the signaling of H2R and acetylcholine muscarinic receptors postsynaptically are involved. The results observed for E100 in MBT are in agreement with improvement exhibited by DL77, although DL77 (15 mg) showed the optimal improvement comparable to the E100 (10 mg)-provided effect.

Similarly, E100 (5, 10 or 15 mg/kg) reduced the percentage of shredded nestlets in VPA-exposed mice in the NST, and this effect of E100 on stereotyped repetitive and compulsive-like behaviors was counteracted by co-administration of the H3R agonist RAM, but not the H1R antagonist PYR or the H2R antagonist ZOL or with muscarinic cholinergic antagonist SCO (Figure 21B). These results further corroborate the current observations in MBT regarding the capability of E100 to facilitate the release of several brain neurotransmitters, besides HA and ACh that have crucial functions in repetitive/compulsive behaviors in different animal species. Negation of the effect of DL77 (15 mg) on repetitive and compulsive-like behavior in NST with co-administration of ZOL, strongly supports the implication of histamine signaling mainly through H2R. The effects of E100 on locomotor activity as well as anxiety levels were examined to simultaneously rule out possible intrinsic impairment of spontaneous locomotor activity that might mask anxiety parameter. Consequently,

EPM test was carried to study the effects of E100 on locomotor activity as well as anxiety levels. The results showed that E100 at a dose of 10 or 15 mg/kg reduced elevated anxiety levels in VPA-exposed mice, similar to DOZ (1 mg/kg), measured by the percentage of time spent and number of open arms entries (Figure 22A and B). However, systemic pretreatment of VPA-exposed mice with E100 at a dose of 5, 10 or 15 mg/kg or with DOZ (1 mg/kg) did not alter the locomotor activity as measured by number of entries into closed arms (Figure 22C). Similar results were obtained in OFT, confirming the ability of E100 to modulate anxiety-associated fear levels by increasing time spent in the center of the arena, and its failure to reduce hyperactivity, as no effect was exhibited (for all doses), on total distance travelled (Figure 23A-D). Thus, the improvements in sociability, social novelty, and repetitive/compulsive behaviors observed for E100 in TCT, MBT and NST, respectively, appear unlikely to be associated with a modulating effect in locomotor activity of the tested mice. These results are in accordance with previous results that revealed anxiolytic-like effects of a non-imidazole-based H3R antagonist with no differences in spontaneous locomotor activity (Amine Bahi, Johannes Stephan Schwed, Miriam Walter et al., 2014). Moreover, the failure of E100 at all doses and DOZ to alleviate the hyperactivity observed in VPA-exposed mice may have been due to the well-known existing imbalance of excitatory (Glu) and inhibitory (GABA) neurotransmitters, as such imbalance was observed in several clinical trials in patients with ASD (Casanova et al., 2003; Mabunga et al., 2015; Orekhova et al., 2008; Rubenstein et al., 2003). With regard to anxiety, comparing the results of E100 with DL77, it was observed that DL77 (15 mg) only modulated anxiety of VPA-exposed mice by increasing the percentage of time spent in open arms and failed with all doses to increase number of open arm

entries in EPM and time spent in the central arena in OFT. The latter results agreed with our initial data, in which DL77 with all doses failed to restore anxiety levels in VPA- exposed TO mice. These results highlight the role of AChE inhibitory effect of E100 in anxiety modulation. Our findings agreed with previous studies supporting the role of AChE on abnormal behaviors, in which transgenic mice overexpression of synaptic human AChE (hAChE-S) show social recognition impairment, hyperactivity in the novel open field and increased time in open arms of the EPM (Cohen, Erb, Ginzberg et al., 2002; Erb, Troost, Kopf et al., 2001) .

Previous studies have found that several proinflammatory cytokines including TNF-α, IL-1ß, IL-6, and TGF-ß are elevated in the autistic brain (Deckmann, Schwingel, Fontes-Dutra et al., 2018; Depino, 2013; Goines & Ashwood, 2013; Vargas et al., 2005). IL-1ß disruption was reported to have a variety of neurological consequences relevant to ASD. It was also reported earlier that IL-6 overexpression in the mice CNS show alterations in cognition and avoidance behaviors (Heyser, Masliah, Samimi et al., 1997). Additionally, Vargas et al. found that TGF-β was one of the most prevalent cytokines in brain tissues of individuals with ASD and is involved in social behavior. The levels of TGF-β in serum are inversely related to those in the brain of autistic individuals. Notably, increased TGF-β in CNS was reported with activated microglia cells (Ashwood, Krakowiak, Hertz-Picciotto et al., 2011; Ohja, Gozal, Fahnestock et al., 2018; Vargas et al., 2005). In addition, several studies reported that cerebellum of autistic subjects exhibited consistent oxidative stress (Nadeem, Ahmad, Al-Harbi et al., 2019). Consistent with these findings, the results showed that the levels of TNF-α, IL-1ß, IL-6 and TGF-ß were significantly increased in the cerebellum of VPA-exposed C57 mice as compared with age-matched control

mice (Figure 24A, C, E and G). Systemic administration of E100 (10 mg/kg, i.p.) significantly modulated the levels of the proinflammatory cytokines (Figure 24A, C, E and G) and oxidative stress in VPA-exposed mice (Figure 25A, C, E and G). In addition, when co-administered with E100, the CNS-penetrant H3R agonist partially abrogated E100-provided protective effects against increased levels of proinflammatory cytokines (Figure 24B, D, F and H) and entirely nullified the E100-provided effects on oxidative stress levels (Figure 25B, D, F and H).

Similar results were observed with DL77 (15 mg), indicating the involvement of brain histamine in facilitating the neuroprotective action of E100 in VPA-exposed mice with ASD-like features. Interestingly, the current observations are strongly supported by a previous study that demonstrated that JNJ10181457, a histamine H3 receptor inverse agonist suppressed LPS-induced microglial IL-1ß, IL-6 and TNF-α expression, indicating that JNJ inhibited the activation of microglia associated with inflammation (Iida, Yoshikawa, Karpati et al., 2017). In accordance, another study showed that ciproxifan (1 mg/kg, i.p.) reduced the level of pro-inflammatory cytokines IL-1ß and IL-6 in transgenic mouse brain (Mani et al., 2017). These lines of evidence demonstrate the strong impact of histaminergic neurotransmission in modulating microglia-induced neuroinflammation and associated pro-inflammatory cytokine expression. This may suggest that as cytokine imbalances could impact neural activity and mediate behavioral aspects of ASD, H3R antagonists may serve as potential therapeutics for this disorder. In support to our results a previous study provided evidence that fifteen days treatment of ciproxifan prevented cognitive deficits, improved cholinergic transmission, and attenuated neuroinflammation and oxidative stress in APP transgenic mouse model (Mani et al., 2017). The neuroprotective effect

of E100 were further supported by a study that showed the free radical scavenging effect of DOZ associated with memory impairment in mice. The study reported that DOZ (AChEI) may protect neurons against injury in AD through prevention of free radical-mediated neuroinflammation. This suggests that the AChE inhibitory property of E100 may be involved in oxidative stress modulation of VPA exposed mice (Umukoro, Adewole, Eduviere et al., 2014).

The regulation of immune response is mediated by NF-κB via induction of the expression of inflammatory cytokines and chemokines. Activation of NF-κB is significantly increased with ASD (Nadeem, Ahmad, Bakheet et al., 2017; Naik, Gangadharan, Abbagani et al., 2011). Young et. al, also, observed elevated levels of NF-κB in blood and brain of patients with ASD (Young, Campbell, Lynch et al., 2011). Moreover, neuroinflammation is characterized by reactivity of microglia and astrocytes, activation of iNOS signaling and increased expression and release of cytokines and chemokines (Monnet-Tschudi et al., 2011). Evidences indicates that ASD subjects exhibit ongoing neuroinflammatory processes in various regions in the brain involving microglial activation (Rodriguez & Kern, 2011; Vargas et al., 2005), consequently aberrant expression of cytokines and their signaling intermediaries are often observed. Furthermore, recent findings showed that disrupted COX-2 signaling in mice might serve as a novel animal model to assess ASD (Wong, Bestard-Lorigados, & Crawford, 2019). In the current study, the expression of NF-κB, iNOS, and COX-2 were significantly higher in the cerebellum as well as hippocampus of VPA-exposed mice compared with saline-exposed mice (Figure 26). However, systemic administration with E100 (10 mg/kg, i.p.) significantly reduced the increased levels of NF-κB, iNOS, and COX-2, and the CNS-penetrant H3R agonist, when co-

administered with E100, reversed the E100-provided neuroprotection in both brain regions (Figure 26). The latter observations further indicate that brain histamine is strongly involved in facilitating the neuroprotective action of E100 in VPA-exposed mice with ASD-like features. Recent study demonstrated that histamine induce decrease in NF-κB, in inflammatory environment but not in the healthy microglia (Apolloni, Fabbrizio, Amadio et al., 2017)

Microglia, a representative immune cell in the brain, plays an important role in synaptic refinement (Kim, Cho, Shim et al., 2017; Koyama & Ikegaya, 2015), and are also supposed to be associated with the pathogenesis of ASD (Kim et al., 2017; Koyama et al., 2015). Moreover, previous reports have also shown changes in the activation of microglia in patients with ASD (Morgan, Chana, Pardo et al., 2010; Pardo, Vargas, & Zimmerman, 2005). In the current study, the observed results showed that expression of iba-1 positive microglia was significantly increased in the cerebellum of VPA-exposed mice compared to saline-exposed control mice (Figure 27) Microglia activation is also considered to be sign of ROS and inflammatory activation. However, systemic pretreatment of VPA-exposed mice with E100 (10 mg/kg, i.p.) significantly decreased the number of activated microglia comparable to the saline-exposed control mice, and the E100-provided effect on activated microglia was entirely counteracted by co-administration of RAM (Figure 27). The latter results signify the brain HA in facilitating the anti-inflammatory effects of E100 against activated microglia of VPA-exposed mice. These findings agree with the previous study in which the H3R inverse agonist JNJ10181457, exhibited an inhibitory role in vivo on activated microglia and regulated LPS-induced upregulation of microglia pro-inflammatory cytokines in mouse brain (Iida et al., 2017). Therefore, targeting

microglial activation by modulating microglial function and suppressing their deleterious pro-inflammatory neurotoxicity maybe a valid therapeutic strategy for promoting neuroprotection and managing ASD-like behaviors.

Taken together, the novel dual-active H3R antagonist and AChE inhibitor E100 alleviates sociability as well as social novelty deficits and stereotypies (presented as ASD-like behaviors) induced by prenatal exposure of C57 mice to VPA (Figure 34). Moreover, E100 modulates the levels of central HA and ACh in an inflammatory context, mitigating the increase in the levels of proinflammatory cytokines, oxidative stress levels, and expression of NF-κB, iNOS, and COX-2 in the cerebellum as well as the hippocampus (Figure 34). These results demonstrate the palliating effects of E100 in a battery tests conducted in a mouse model of ASD.

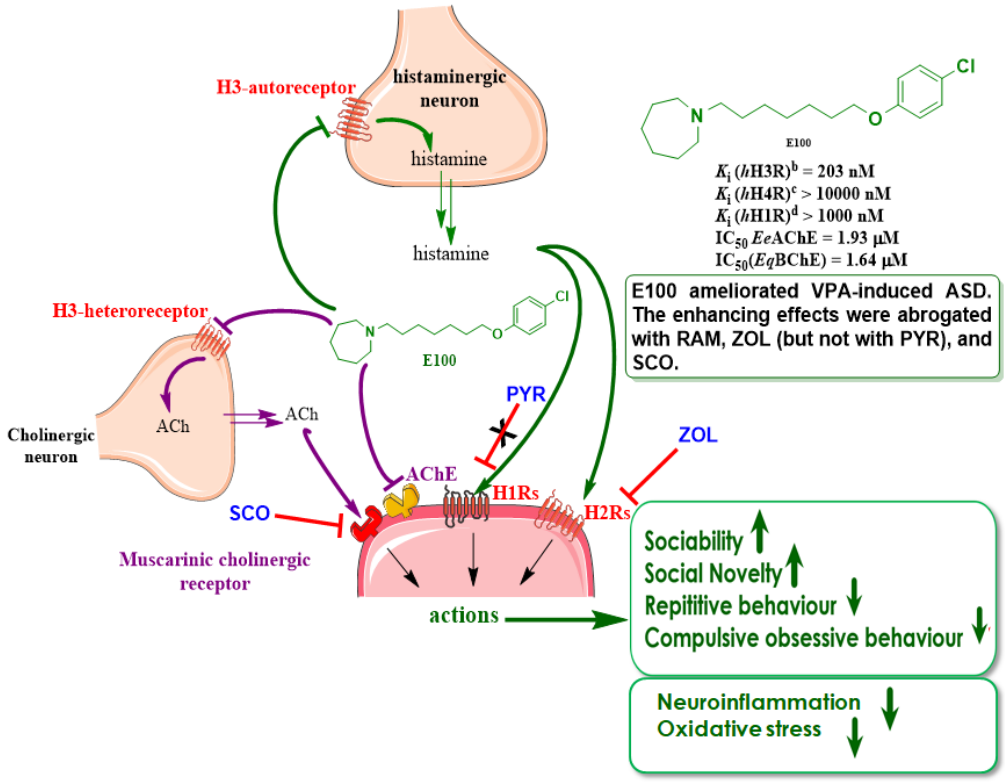


Figure 34: Schematic illustration of possible mechanism of action of E100

Chemical structure of the dual-active hH3R antagonist and AChEI E100, it's in vitro data with regard to HRs, AChE (Ee: electric eel), and BChE (Eq: equine), and its putative mode of action by inhibition of the metabolizing enzyme of ACh and blocking the hH3R acting as auto- and heteroreceptor.

After many years of scientific efforts, the genetic cause of ASD for majority of the cases remains elusive, therefore it was more powerful to include a well-validated model of idiopathic autism in my study to further corroborate our results. This was to authenticate our findings that histaminergic and cholinergic systems dysregulation are implicated in ASD phenotypes and that H3R antagonists maybe a potential therapeutic agent for ASD. BTBR mouse is one of these idiopathic models. The BTBR mouse continues to attract researchers with its complex genetic, molecular and physiological background, a complexity that supports the BTBR mouse as a model for the idiopathic form of autism (Meyza & Blanchard, 2017). It that displays behaviors consistent with the diagnostic categories for ASD, namely impairments in social interaction and increased repetitive behaviors.

Our results determined that E100 significantly improved social deficits exhibited by BTBR mice This was evident by significant enhancement in sociability as well as social novelty behavior provided by dose of 5 mg/kg or 10 mg/kg. Importantly, the sociability- and social novelty-enhancing effect observed for E100 was not dose-dependent, since E100 (15 mg/kg) failed to further improve sociability or social novelty preference (Figure 28A and B). In fact, E100 (5 mg/kg, i.p) was observed to show the optimum effect. The observed results regarding the doses are in agreement with previous results of memory-enhancing effect observed for UW-MD-72, the effect of lower dose (1.25 mg/kg) was significantly higher when compared to the higher doses 2.5 and 5 mg/kg (Sadek, Khan, Darras et al., 2016a). In a further experiment, the E100 (5 mg)-provided enhancing effects on sociability and social novelty were completely reversed when mice were co-administering the CNS-penetrant H3R agonist RAM.

The observed results support the effect of E100 to release several neurotransmitters, such as histamine and acetylcholine, provided by the inhibition of the H3R. These neurotransmitters areimplicated in the regulation of arousal and cognitive processes in several previous studies. Earlier experimental study revealed that antagonism of H3R attenuated impaired social behavior in rodents exposed to phencyclidine (PCP), a finding in line with our result (Griebel et al., 2012). Notably, the reference drug DOZ improved both social impairment parameters of BTBR mice following subchronic systemic treatment (Figure 28A). Recent studies demonstrated cholinergic deficit and low levels of ACh in BTBR mice (McTighe, Neal, Lin et al., 2013). In addition to the well-established positive effect of AChEIs on cognition in a wealth of studies, DOZ treatment was reported by Reidel et al. to relieve social memory deficiency (Riedel et al., 2009). This evidence fits with our findings, as E100 has AChE inhibitory properties which may account for improvements in social deficits observed in E100 (5 mg/kg, i.p.) pretreated BTBR mice. Moreover, the selective H3R antagonist JNJ was reported in a previous study to normalize ACh neurotransmission and positively impact cognition (Galici et al., 2009), supporting our results with E100. These therapeutic effects of E100 may be explained by the modulation of histaminergic and cholinergic systems, hence reduced cognitive rigidity, that consequently increases social attention and elevates sociability levels.

The ability of E100 to enhance cholinergic activity in BTBR mice and exert its potential effect on cognitive deficit associated with sociability impairments was confirmed by measuring AChE activity in the cerebellum. The results revealed that the activity of AChE in E100 (5 mg) -treated mice was significantly reduced compared to BTBR mice and compared to DOZ treated BTBR mice (Figure 29). Considering the

role of this enzyme which is responsible for degrading the ACh, it has been reported in a previous study that AChE inhibition augmented acetylcholine in the synapse, relieve cognitive rigidity and ameliorate social deficiency in BTBR mice (Karvat et al., 2014).

Furthermore, E100 in BTBR mice also exhibited the same trend toward improvement in repetitive and compulsive-like behaviors (Figure 30 A and B). Analysis of MBT and NST results revealed that E100 (5 mg) exhibited the best behavioral improvement that was significant from effects of E100 10 mg and 15 mg. A previous study reported that acute administration of mAChR agonist oxotremorine attenuated the elevated self-grooming and marble-burying behavior in BTBR mice. These findings suggest that activation of mAChR can attenuate certain repetitive behaviors (Amodeo, Yi, Sweeney et al., 2014). This evidence may explain the results observed by E100 on repetitive behavior in MBT, because E100 tend to facilitate the release of ACh through H3 heteroreceptor and increase the level of ACh in synapse by exerting an inhibitory action on AChE. The increased level of ACh may exert mAChR transduction showing the improvement in repetitive behavior observed. Additionally, and as mentioned earlier, several previous studies including our findings reported the positive effects of H3R antagonists on repetitive behaviors of VPA mouse model of ASD in different mouse strains. These findings suggest that the pharmacological manipulation of the histaminergic system by E100 due to its H3R antagonistic effect, may positively impact the elevated repetitive and compulsive-like behaviors in BTBR. On the other hand, BTBR mice have been reported to have elevated oxidative stress with deficient enzymatic anti-oxidant response that is suggested to be associated with the exaggerated repetitive behavior (Nadeem et al., 2019). Based on our finding of the

capacity of E100 to modulate oxidative stress level in VPA exposed mice (Figure 25A, C, E and G), the improvements observed in repetitive/compulsive behavior by E100 in BTBR is strongly supported.

BTBR mice display a variable profile of anxiety-like behaviors in comparison to C57 and other inbred strains. In the current study BTBR mice spent less time and had a reduced number of entries into open arms of the EPM, demonstrating high levels of anxiety (Figure 31A and B). This observation was in agreement with previous studies (Benno, Smirnova, Vera et al., 2009; Pobbe, Defensor, Pearson et al., 2011). Conversely BTBR mice spent more time in the center of the arena of the open field, as compared to C57 in OFT showing low levels of anxiety, reflecting impulsive behavior (Figure 32A and B). This contradictory result is in consistent with several previous studies (Chadman, 2011; Moy et al., 2007; Silverman, Yang, Turner et al., 2010; Yang, Clarke, & Crawley, 2009) and may be explained due the to stress condition exhibited by the EPM over OFT. In fact, previous studies suggested that BTBR mice displayed high risk assessment behaviors (stretched postures and head-outs stretches), as response to high level of stress, reflecting exaggerated responses to stressors which might have influenced responses in EPM. Interestingly, E100 with all doses was able to restore these abnormal levels of anxiety in both tests, hence, our findings suggest interplay between histamine and ACh level dysregulation with abnormal levels of anxiety and impulsive behavior observed in BTBR. This is supported by a recent report, in which lower acetylcholine level in the prefrontal cortex region of BTBR mice showed attention deficit and impulsive behavior (McTighe et al., 2013).

E100 at all doses and DOZ failed to restore the hyperactivity displayed by BTBR mice in EPM (Figure 31C), conversely the hyperactivity was totally restored in

OFT with E100 (5, 10 or 15 mg/kg, i.p.) or DOZ (Figure 32C). As mentioned earlier both OFT and EPM tests are standard tools to analyze activity and profile anxiety in rodents. Open field determines behavior based on combined exploration and aversion against open and bright areas, whereas, in EPM the behavior of rodents is based on openness combined with elevation predominantly related to anxiety (Schmitt & Hiemke, 1998). This explains the sensitivity of each behavioral test in testing the effect of compounds on anxiety and psychomotor activity using the same mouse strain. Although E100 improved increased activity in BTBR mice, it failed to restore hyperactivity in C57 VPA-exposed mice in OFT. This may be due to variability in sensitivity of mouse strains to drugs. This finding agrees with a previous study in which the antidepressant-like effect of fluoxetine was compared in seven inbred mouse strains in the forced swimming test, the results revealed improvement in three strains only (Lucki, Dalvi, & Mayorga, 2001). However, co-administration of the H3R agonist RAM reversed the elevated locomotor activity improvement exhibited by E100 (5 mg) in OFT (Figure 32C), that strongly correlate regulation of both histamine and acetylcholine neurotransmitters with hyperactivity observed in BTBR mice.

Several studies now provide evidence for ongoing neuroinflammatory process in various brain regions involving microglia activation, in individuals with ASD. Microglial activation can then result in a loss of connections or underconnectivity due to the sustained production of mediators longer than usual, contributing to the loss of synaptic connections and neuronal cell death. Several studies reported underconnectivity in ASD individuals (Rodriguez et al., 2011). Emerging evidence implicates compromised inter-hemispherical connectivity in some cases of ASD, where greater callosum reduction was proportional to severity of the disorder.

Moreover, histological analysis showed that the corpus callosum was absent in BTBR mouse brains. Nevertheless, important finding suggested that genes regulating corpus callosum development specifically are not likely to be responsible for the social and repetitive abnormalities in BTBR, evidenced by postnatal surgical disconnection of the corpus callosal fiber tract in C57 mice that exhibited no social deficits or repetitive self-grooming characteristic of BTBR mice (Yang et al., 2009).. Our promising results strongly implicate the histaminergic and cholinergic system dysregulation in the ASD phenotype.

As mentioned earlier, one way to control neuroinflammation is to reduce or inhibit microglial activation. It is plausible that by reducing brain inflammation and microglial activation, the neurodestructive effects of chronic inflammation could be diminished and contribute to improved developmental outcomes. Consistent with previous studies that reported microglia activation in BTBR brains (Heo, Zhang, Gao et al., 2011; Zhang, Gao, Kluetzman et al., 2013) our results demonstrated remarkably a higher expression of Iba-1-positive microglia in BTBR cerebellum compared to C57, reflecting an increase in activated microglia. Subchronic systemic administration of E100 (5 mg) significantly mitigated microglia activation in BTBR, evidenced by attenuation of the expression of Iba-1 (Figure 33). However, co-administration of RAM reversed the suppression exhibited by E100 (5 mg). The data clearly indicate that E100 has the potential to inhibit microglial activation. The current findings complement the earlier observation of E100 (10 mg) on activated microglia of VPA-exposed C57 mice. Since microglia constitutively express all four histamine receptors (H1R, H2R, H3R, and H4R) histamine has a well-established role as neuron-to-glia alarm signal in the brain (Hu & Chen, 2017). It has been reported in a recent study that

histamine counteracts proinflammatory microglia phenotype in the SOD1-G93A mouse model of Amyotrophic Lateral Sclerosis. They demonstrated that histamine exerts its beneficial action only in inflammatory SOD1-G93A microglia, and on the other hand elicits a pro-inflammatory effect in nontransgenic cells (Apolloni et al., 2017). These findings complement an earlier study that demonstrated a dual role for histamine under physiological and inflammatory settings (Barata-Antunes et al., 2017a). Additionally, HA deficiency in *HDC* knock out mice has been reported to make microglial cells more vulnerable to an inflammatory challenge, which further emphasizes the importance of HA in regulating microglial function (Frick et al., 2016).

These reports fit well with our findings that brain HA and Ach are involved in providing an anti-inflammatory action provided by E100 through its action to facilitate the release of brain HA and elevate the levels of ACh in synapse which may be be consequently responsible for the improvement in ASD-like behavioral outcomes in BTBR mice.

Chapter 5: Conclusion

In conclusion, to our knowledge, this is the first study to reveal that DL77, a non-imidazole H3R antagonist palliated core symptoms of ASD, represented as ASD-like behaviors induced by prenatal VPA exposure in two different mouse strains, demonstrating the therapeutic potential of H3R antagonists in the treatment of ASD. In addition, we provided evidences that modulation of central histaminergic neurotransmission by DL77 under an inflammatory context attenuated proinflammatory cytokine release and oxidative stress levels that are considered as predictors of intensified symptoms of ASD.

The promising results of the target drug DL77 and the standard drug DOZ lead to additional investigation into the potential applicability of dual-acting H3R antagonists/AChEIs in the modulation of ASD-like behavior. The observed palliative effects of E100 on behavioral deficits and the mitigating effects on the levels of proinflammatory cytokines as well as oxidative stress provide evidence to suggest that brain histaminergic and cholinergic neurotransmission are potential targets for ASD. The current study is the first in vivo demonstration that a potent dual-acting H3R antagonist and AChE inhibitor is effective in improving ASD-like features induced by prenatal exposure to VPA and in the BTBR mouse model of ASD (environmental and genetic models).

The latter result adds further support to the therapeutic potential of dual-acting H3R antagonists and AChEI in the treatment of CNS disorders, especially those characterized with chronic neuroinflammation such as ASD.

DL77 and E100 showed similar results, with a higher dose of DL77 exhibiting optimal effects, hence we propose histaminergic signalling as a novel mechanism for understanding ASD, and the H3R antagonists as a potential target for therapy.

Future direction

Future studies are essential to investigate which signalling pathways and HRs are involved in this histamine-provided neuroprotective role. Altogether our results opened a novel therapeutic window for managing core symptoms of ASD. However, the examination of neural circuits implicated with the histaminergic and cholinergic neurotransmission systems is an important goal for future work to decipher the pathophysiological mechanisms underlying the observed effects.

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Eissa, N., Jayaprakash, P., Azimullah, S., Ojha, S.K., Al-Houqani, M., Jalal, F.Y., Lazewska, D., Kiec-Kononowicz, K., & Sadek, B. (2018). The histamine H3R antagonist DL77 attenuates autistic behaviors in a prenatal valproic acid-induced mouse model of autism. *Scientific Reports*, *8*, 1-15.

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