CONTRIBUTION OF VENTRAL PALLIDAL CHOLINERGIC NEURONS TO BEHAVIORAL DESPAIR AND FEAR LEARNING

SAHAR HALIM

BOGAZICI UNIVERSITY

2022

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Thesis submitted to the

Institute for Graduate Studies in Social Sciences

in partial fulfillment of the requirement for the degree of

Master of Arts

in

Cognitive Science

by

Sahar Halim

Boğaziçi University

2022

DECLARATION OF ORIGINALITY

I, Sahar Halim, certify that

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ABSTRACT

Contribution of Ventral Pallidal Cholinergic Neurons

to Behavioral Despair and Fear Learning

Cholinergic neurons of the Ventral Pallidum (VP) densely innervate the Basolateral Complex (BLA) and Central Nucleus of Amygdala (CeA), and to a lesser extent project to the Bed Nucleus of the Stria Terminalis (BNST). Although basal forebrain cholinergic projections have been heavily studied in relation to cognition and explicit memory, there are very few studies that investigate the role of this neuromodulatory pathway in affective processes. The purpose of this thesis is to investigate the contribution of ventral pallidal cholinergic neurons to behavioral despair, anxiety, and fear learning. 192 IgG saporin, an immunotoxin selective to cells that contain p75 neurotrophin receptor, was bilaterally injected into the VP of eight adult male Wistar rats to eliminate cholinergic neurons. Lesioned animals were tested in the forced swim test (FST), open field test (OFT), elevated plus maze (EPM), Morris water maze (MWM) and Pavlovian fear conditioning. The results reveal that the elimination of VP cholinergic projections exhibit a reduction of behavioral despair, increased escape latency in MWM, and diminished freezing response in fear conditioning. These results indicate that elimination of VP cholinergic neurons have an antidepressant effect, while suppressing conditioned fear memory. VP cholinergic lesions also lead to deficits in hippocampus-dependent spatial learning. These results suggest that VP cholinergic neurons can be therapeutic targets in clinical depression and fear-related disorders.

ÖZET

Ventral Pallidal Kolinerjik Nöronların Davranışsal Çaresizlik ve Korku Öğrenmeye Katkısı

Ventral Pallidum'un (VP) kolinerjik nöronları yoğun bir şekilde Bazolateral Kompleksini (BLA) ve Amigdala Merkez Çekirdeği'ni (CeA) innerve eder ve bu daha az ölçüde Stria Terminalis'in Yatak Çekirdeğine (BNST) yansır. Bazal ön beyin kolinerjik projeksiyonları, biliş ve açık hafıza ile ilgili olarak yoğun bir şekilde çalışılmış olmasına rağmen, bu nöromodülatör yolun efektif süreçlerdeki rolünü araştıran çok az çalışma vardır. Bu tezin amacı ventral pallidal kolinerjik nöronların davranışsal çaresizlik, kaygı ve korku öğrenmeye katkısını araştırmaktır. p75 nörotrofin reseptörü içeren hücrelere seçici bir immünotoksin olan 192 IgG saporin, kolinerjik nöronları ortadan kaldırmak için sekiz yetişkin erkek Wistar sıçanının VP'sine iki taraflı olarak enjekte edildi. Lezyonlu hayvanlar, zorunlu yüzme testi (FST), açık alan testi (OFT), yükseltilmiş artı labirenti (EPM), Morris su testi (MWM) ve Pavlovian korku koşullanmasında test edildi. Sonuçlar, VP kolinerjik projeksiyonların ortadan kaldırılmasının davranışsal çaresizlikte bir azalma, MWM'de kaçış süresi yükseldi ve korku koşullandırmasında donma tepkisinde azalma gösterdiğini ortaya koymaktadır. Bu sonuçlar, VP kolinerjik nöronların ortadan kaldırılmasının, koşullu korku hafızasını bastırırken antidepresan bir etkiye sahip olduğunu göstermektedir. VP kolinerjik lezyonlar ayrıca hipokampüse bağlı uzaysal öğrenmede eksikliklere neden olur. Bu sonuçlar, VP kolinerjik nöronların klinik depresyon ve korkuyla ilişkili rahatsızlıklarda terapötik hedefler olabileceğini düşündürmektedir.

ACKNOWLEDGEMENTS

I would firstly like to extend my gratitude towards Dr. Güneş Ünal, Dr. Daniela Schulz, the Behavioral Neuroscience Laboratory members, and my jury members. Dr. Ünal, thank you for giving me a chance in accepting me into this program as well as supporting my experiments. Although it was a challenging program, this opportunity gave me the chance to realize what exactly I am capable of and confidently reassured me to pursue a different path. Without this laboratory, I would not have been able to uncover my unknown strengths and capabilities.

I would also extend my sincerest gratitude to Cemal Akmeşe and Cem Sevinç. Cemal, thank you for the great amount of help you extended to me, participating in my experiments, and teaching me everything I needed to know. You were the only lab member I encountered from the first day who stuck by my side through thick and thin. I wish you nothing but the best at UF (go gators!). Cem, thank you for helping take part in my experiments and being there during the hardest days in the laboratory.

My lovely family, thank you to each one of you for providing support to me in each of your unique ways. Momz, thank you for always encouraging me during my many days of doubt regarding my program, your constant encouragement gave me the strength to refrain from giving up. Bobz, your aphasia did not stop you from discussing complex neuroscience topics and I am so beyond grateful to see the joy in your eyes whenever I discussed my work with you. Dogz, thank you for always being there for me and listening to my rants about my program. Rashwan, I cannot thank you enough for always encouraging me and hyping up the skills that I have. You have always believed in me throughout my whole life, and I am incredibly grateful to have you as my uncle.

I would like to extend an enormous thanks to Berkan. Berkan, you are the only one who truly witnessed all the beauty and challenge this program has brought into my life. Whatever the circumstances were, whether it be a bad day at the lab, crying over my courses and experiments, or my inflictions of self-doubt, you were always there. You always believed in me and reassured me that I always achieve the impossible. Although I had many mental breakdowns along the way, you were always there cheering me on and putting a smile on my face. Thank you for always being there, believing in me, and supporting my dreams.

Finally, I would like to thank myself. Sahar, whenever you look back at this time in your life, just remember that you did this. Let this thesis be a reminder of all the hard work you exerted into this program. You came into this program as a foreigner, not knowing a single soul in the university, and navigating through a completely different world without knowing what you got yourself into. Despite the many obstacles thrown directly at your face along the way, somehow you survived and were able to finish this and move on with your life. Although there were days where it felt like you were stuck inside this deep hole and could not get out, you managed to climb out of it. Wherever you are, I hope that you look back at this thesis and let it be a reminder to you that you can achieve anything you put your mind to. Continue achieving the impossible for as long as you live.

This thesis is dedicated to myself.

Let this be a reminder that you can do anything,

even what seems to be the absolute impossible.

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ABBREVIATIONS

AcbC	Central accumbal core
AcbSh	Peripheral accumbal shell
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's Disease
AF64A	Ethylcholine aziridinium ion
BF	Basal Forebrain
BLA	Basolateral complex
BNST	Bed nucleus of the stria terminalis
CeA	Central nucleus of amygdala
CeA_L	Lateral central nucleus of amygdala
CeA_M	Medial central Nucleus of amygdala
ChAT	Acetyltransferase
CS	Conditioned stimulus
DB	Diagonal Band
DG	Dentate Gyrus
DLC	Deep Lab Cut
EPM	Elevated Plus Maze
FST	Forced Swim Test
hDB	Horizontal limb of the diagonal band of Broca
i.c.v.	Intracerebroventricular
i.p.	Intraperitoneal
IHC	Immunohistochemistry

ITI	Intertrial time interval
LA	Lateral Amygdala
MS	Medial Septum
MWM	Morris Water Maze
NBM	Nucleus Basalis Magnocellularis
NGFr	Nerve growth factor receptor
OFT	Open Field Test
PB	Phosphate buffer
PBS	Phosphate-buffered saline
PBS-Tx	Phosphate-buffered saline Triton X-100
PFA	Paraformaldehyde
PV	Parvalbumin
SI	Substantia Innominata
US	Unconditioned stimulus
VP	Ventral Pallidum
VS	Ventral Striatum

CHAPTER 1

INTRODUCTION

The basal forebrain (BF) is a subcortical brain region that consists of a heterogenous neuronal population responsible for limbic system coordination and cognitive function. The cell types of this brain region consist of cholinergic, glutamatergic, and GABAergic and peptidergic neurons that are intermingled among the various BF nuclei. These nuclei include the ventral pallidum (VP), medial septum (MS), diagonal band (DB), and substantia innominata (SI). The BF cell types have been shown to project to various regions such as the neocortex, hippocampus, and basolateral complex of the amygdala (BLA). Several neurons of this system are implicated in different forms of neural plasticity as well as the mediation of affective processes and memory (Knox, 2016). This is seen in the coordination of cholinergic and GABAergic projections of the MS to the hippocampus. The projections of the MS to the hippocampus are responsible for generating hippocampal theta oscillations which are correlated to spatial navigation and learning and memory (Unal, Joshi, Viney, Kis, & Somogyi, 2015; Unal, Crump, Viney, Éltes, Katona, Klausberger & Somogyu, 2018). A loss of cholinergic cell types yields cognitive impairments given their critical role in cognitive function (Do, Xu, Lee, Chang, Zhang, Chung, Yung, Jiang, Kazunari, Luo, & Dan, 2016). This can be seen in cholinergic lesion studies of the BF, such as that of Nilsson et al. (1992) in which the elimination of BF cholinergic neurons using 192 IgG saporin caused impairments in spatial learning during the Morris Water Maze task. It is important to note that cholinergic projections extend through numerous basal forebrain nuclei including the VP, SI, and the DB (Carlsen, Záborszky, & Heimer, 1985; McDonald, Muller, & Mascagni,

2011). The basal forebrain's densely populated cholinergic cell group innervates the cortex and two major subcortical limbic system structures, the hippocampus and amygdala (Mesulam, Mufson, Wainer, & Levey, 1983; Carlsen et al., 1985; Ottersen, 1980). Although the BF consists of several nuclei contributing to cholinergic innervations to these structures, the focus of this thesis will focus on another key nucleus: the ventral pallidum.

Acetylcholine (ACh), a widespread receptor in the brain's neuromodulatory system, has proven to be significant in various cognitive functions, such as memory and synaptic plasticity, as seen in localized infusion studies of cholinergic receptors (Hasselmo, 2006). There are two key sites within the brain in which ACh is primarily released: the basal forebrain and the brainstem. The cholinergic system of the basal forebrain projects to various brain regions such as the cerebral cortex, limbic structures including the hippocampus and amygdala, and the olfactory bulb while the cholinergic brainstem projects to the basal forebrain, thalamus, and basal ganglia structures (Fig. 1) (Mesulam, 1988; Wrenn & Wiley, 1998; Avery & Krichmar, 2017). Acetylcholine is firstly synthesized via the enzyme choline acetyltransferase (ChAT) from two key compounds: choline and Acetyl-CoA (Fig. 2) (Coyle, Price, & DeLong, 1983). ACh is then stored into vesicles along the axon terminal in which it is released into the synaptic cleft when released. ACh diffusion then activates either muscarinic or nicotinic receptors. The enzyme acetylcholinesterase (AChE), located on the neuron's surface, terminates the action of ACh within the synapse through breaking down ACh into acetate and choline. Choline is then transported back into the neuron to continue the process of acetylcholine synthesis. The biochemical processes seen in acetylcholine synthesis, storage, and inactivation are key markers in studying the cholinergic effects on brain

function (Coyle et al., 1983). Such markers have been commonly used to identify the impact of Alzheimer's disease, a degenerative disorder displaying cholinergic hypofunction (Coyle et al., 1983; Rossner, Härtig, Schliebs, Brückner, Brauer, Perez-Polo, Wiley, & Bigl, 1995).

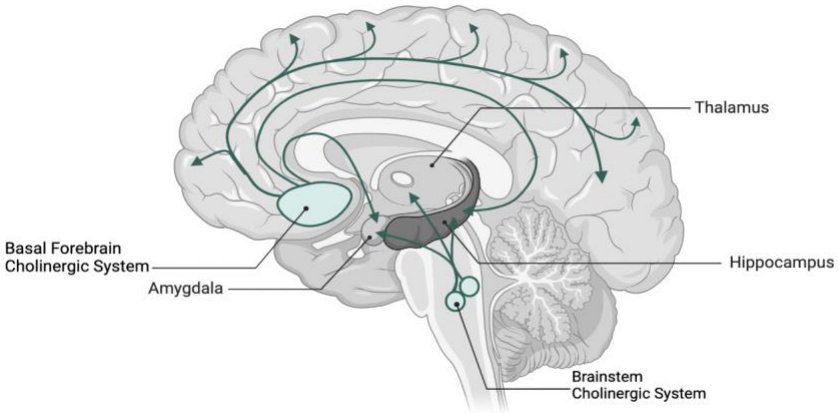

Fig. 1 Illustration of cholinergic basal forebrain and brainstem projections
Note: The basal forebrain cholinergic projections target the neocortex and limbic structures such as the hippocampus and amygdala. The brainstem cholinergic projections target the basal forebrain, thalamus, and basal ganglia structures. Adapted from Coyle et al., 1983.

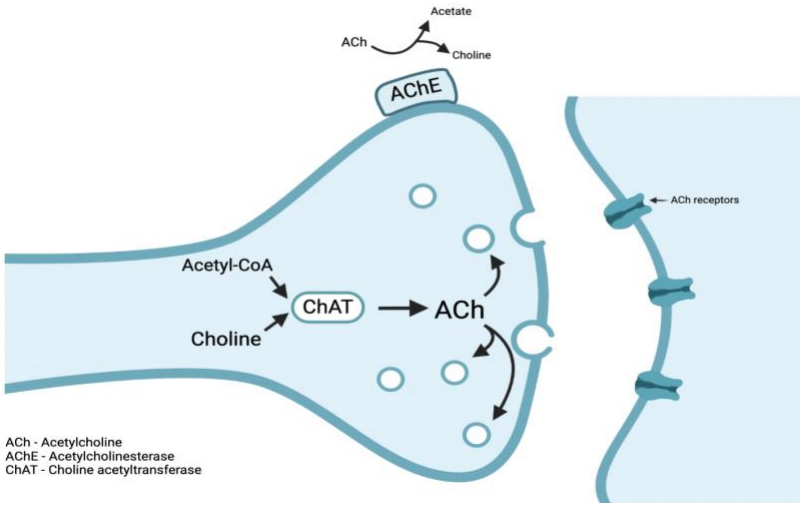

Fig. 2 Simple schematic representation of a cholinergic synapse
Note: Acetyl-CoA and choline synthesize acetylcholine (ACh) via the choline acetyltransferase enzyme (ChAT). ACh is then stored into vesicles within the cell to be released across the cleft to muscarinic or nicotinic receptors. This process is then terminated by acetylcholinesterase which breaks down ACh into acetate and choline. Adapted from Coyle et al., 1983.

Alzheimer's disease (AD) is characterized by a widespread loss of cholinergic neurons, primarily localized within the basal forebrain, suggesting that these cholinergic systems are implicated in learning and memory. A reduction in biological markers including ChAT and AChE are key characteristics typically displayed in Alzheimer's patients. The cholinergic innervations from the BF to the BLA are reduced by 40% in AD (McDonald, 2020). This loss of cholinergic innervation to the amygdala yields impairments in emotional and event-related memory. Furthermore, impairments of fear conditioning are also displayed among AD patients given that fear conditioning is an amygdala-dependent form of memory (Hamann, Monarch, & Goldstein, 2002). Inactivity of cholinergic receptors in the BLA also display increased fear and anxiety among AD patients (Pidoplichko, Prager, Aroniadou-Anderjaska, & Brega, 2013). Furthermore, when compared to normal control individuals, the brains of Alzheimer's patients after death displayed a 60% to 90% significant decrease in ChAT throughout the cerebral cortex and hippocampus, related to impairments in declarative memory retrieval (Coyle et al., 1983, Dunnett, Everitt, & Robbins, 1991).

The investigation of clearly identifying neurochemical abnormalities in AD led to the development of the cholinergic hypothesis. The cholinergic hypothesis states that the decline in cognitive function is due to BF cholinergic dysfunction resulting in the loss of ACh transmission in the cortex and other brain regions (Terry & Buccafusco, 2003). Specifically, the degeneration of cholinergic projections originating from the nucleus basalis magnocellularis (NBM) to the cerebral cortex as indicated by a decrease of ChAT is correlated to dementia (Dunnett et al., 1991). This pathway in specific has been demonstrated to be the earliest projections severely affected in AD (Schliebs, Rossner, & Bigl, 1996). It was hypothesized that

lesions of the NBM would serve as the ideal animal model of AD given its implications in memory when its cholinergic cells are degenerated. However, NBM cholinergic lesions did not prove to be a simple animal model to explain the cognitive deficits brought on by AD (Dunnett et al., 1991). Furthermore, the reduction of markers, such as ChAT and AChE, and their interpretations within AD are problematic in that the location of these cholinergic cell bodies and their innervations remain uncertain (Coyle et al., 1993). Overall, the alteration or blockade of cholinergic systems does not provide clear evidence as to how learning, memory, and fear conditioning are specifically affected (Ennaceaur, 1998).

Given the ambiguity in elucidating a singular role of the cholinergic BF projections in learning, memory, and fear systems, it is essential to anatomically localize cholinergic cell groups to further define how they affect cognitive functions and behavioral effects. Although many studies have used ablation techniques of basal forebrain cholinergic neurons, there exists a gap in research regarding cholinergic signaling implicated in fear learning (Jiang, Kundu, Lederman, López-Hernández, Ballinger, Wang, Talmage, & Role, 2016). Furthermore, several animal studies which have investigated the role of BF cholinergic neurons through lesion studies in learning and memory mainly targeted the MS and NBM (Ennaceaur, 1998). The VP, commonly known for its role in reward, motivation, and learning and memory, has implications in limbic functions. The cholinergic system of the VP may have involvements in fear and threat responses given its involvement with the amygdala, a region notorious for its involvement in fear and anxiety (Moaddab, Ray, & Mcdannald, 2021).

The ventral pallidum receives a major input from the ventral striatum (VS) via striatopallidal projections in which these afferent projections can be further categorized from two VS structures, the central accumbal core (AcbC) and peripheral accumbal shell (AcbSh). Specifically, the dorsolateral VP receives afferents from AcbC while the ventromedial VP receives its afferents from AcbSh (Tripathi, Prensa, & Mengual 2013). The ventral striatopallidal system, a basal ganglia extension through the basal forebrain, is the main system in which the VS and accumbens projects to the VP and plays a role in motivational behaviors (Zahm, 2009). It is important to note that the VP receives cholinergic projections from the nucleus accumbens and the amygdala (Alheid & Heimer, 1988; Bengston & Osborne, 2000). VP cholinergic efferents densely innervate the basolateral complex (BLA) and central nucleus of the amygdala (CeA), and to a lesser extent the bed nucleus of the stria terminalis (BNST) as well as the prefrontal cortex (Ottersen, 1980; Mascagni & McDonald, 2009; Muller, Mascangi, & McDonald, 2011; Záborszky, van den Pol, & Gyengesi 2012; Root, Melendez, Záborszky, & Napier, 2015; McDonald 2020; Prasad & McNally, 2020). The cholinergic afferents to the BLA, CeA, and BNST play a neuromodulatory role in that they serve as primary mediators in a variety of cognitive functions. Such functions include memory consolidation as well as implications in fear conditioning and extinction (Muller et al., 2011; Knox, 2016; McDonald, 2020).

Threat learning and its related behaviors are involved in a large neural network involving cholinergic projections of the BLA, CeA and BNST along with the VP. Specifically, the BLA and CeA are essential brain regions related to fear in that they are necessary in using and signaling threat information (Moaddab et al., 2021). The BLA has implications in fear conditioning, fear extinction, and anxiety

(Knox, 2016; McDonald, 2020). In a study done by Amorapanth, LeDoux, and Nader (2000), lesions to the BLA, specifically the Lateral Amygdala (LA) of the BLA, revealed its role in the acquisition of fear memory in that when eliminated, fear conditioning acquisition was blocked indicated by the animals' freeze response. This is also in line with Goosens and Maren (2001) who observed impaired freezing responses to an auditory conditioned stimulus (CS) when lesioning the BLA. Jiang et al. (2016) revealed that cholinergic projections to the BLA in specific play a role in conditioned fear learning as indicated by reduced freeze response due to cholinergic inhibition via optogenetic techniques. The VP's cholinergic efferents to the BLA have also proven to be an important circuit involved in memory consolidation. The BLA is able to enhance memory consolidation through interacting with the hippocampus and neocortical regions that serve as memory storage areas (Muller et al., 2011). This interaction allows for the generation of theta oscillations within these regions which further facilitates synaptic plasticity (Muller et al., 2011). Additionally, the BLA serves as the amygdala's primary input center of sensory information provided by sensory modalities (LeDoux, 2007). Projections entering the BLA are then efferently projected onto the amygdala's primary output center, the CeA and BNST (Sah, Faber, Lopez de Aementia, & Power 2003; LeDoux, 2007).

The central amygdala and BNST play a role in processing information retrieved from the BLA in order to formulate an appropriate response to threat stimuli. Given that fear conditioning involves information regarding the CS to be relayed to the BLA, followed by the CeA, lesions of the CeA also demonstrate blocked fear conditioning acquisition (Amorapanth et al., 2000). The CeA can be further divided into medial (CeA_M) and lateral (CeA_L) divisions which process BLA afferents regarding sensory information to produce threat responses. BLA

projections to the CeA_M are implicated in short term fear responses which dissipate quickly, known as phasic fear. BLA projections to the CeA_L on the other hand mediate long terms of apprehension (anxiety), known as sustained fear. Furthermore, the CeA_L releases a corticotropin releasing factor to the BNST which can result in prolonged periods of sustained fear, highlighting the BNST's key role in anxiety (Davis, Walker, Miles, & Grillon, 2009).

In attempts to identify an adequate animal model which reveals the abnormalities caused by reduced BF cholinergic projections, such as that in AD, several paradigms exist which involve the lesioning of BF cholinergic populations. Such paradigms have investigated methods involving mechanical lesions, excitotoxins, ethylcholine aziridinium ion (AF64A), and 192 IgG saporin. In a study by Schliebs et al. (1996), they compared the effectiveness of the previously listed methods in targeting cholinergic neurons in the BF, specifically the NBM. They found serious limitations in the use of mechanical lesions, excitotoxins (quinolinic, ibotenic, and quisqualic acid), and AF64A. Mechanical lesions, produced through radiofrequency or electrolysis, damages the cholinergic system but also impacts fiber bundles which pass through the region of interest, yielding it to be nonspecific for cholinergic populations. Similarly, excitotoxins led to the destruction of non-cholinergic cells. Although AF64A does terminate cholinergic cell population specifically to the BF, its selectivity depends on the dosage and injection site. Contrarily, 192 IgG saporin proved to induce specific cholinergic lesions of the BF.

The selective cholinergic neurotoxin, 192 IgG saporin, consists of a ribosome-inactivating protein paired with a monoclonal antibody that targets cells expressing low affinity p75 nerve growth factor (NGFr) (Wiley, Oeltmann, & Lappi, 1991; Pappas, Davidson, Nallathamby, Park, & Wiley, 1996; Pang & Nocera, 1999).

This neurotoxin has been proven to be successful in selectively eliminating cholinergic cells within the basal forebrain given that only this particular neuronal population within the BF expresses low affinity p75 NGFr. Once injected intracerebrally, 192 IgG saporin binds to the surface of neurons expressing p75 NGFr and enters the cell via endocytosis. Once internalized into the cell's cytoplasm, 192 IgG saporin stops protein synthesis through inactivating the ribosomal subunit which leads to cell death (Perry, Hodges, & Gray, 2001; Wrenn & Wiley, 1998). The selectivity of 192 IgG saporin in its elimination of cholinergic BF neurons can be seen through various injection methods: intracerebroventricularly (i.c.v.) and intracerebrally (Wrenn & Wiley, 1998).

Intracerebroventricular 192 IgG saporin injections lesion two key regions, the cholinergic BF system and cerebellar Purkinje cells consisting of p75 NGFr as indicated by decreased ChAT and AChE (Wrenn & Wiley, 1998). Heckers et al. (1994) demonstrated a complete loss of NGFr positive cells and cholinergic BF neurons in the cerebral ventricle, MS, and DB when injected intracerebroventricularly but not in the NBM-SI complex. They also found a complete loss of cholinergic fibers in the hippocampus and neocortex, with a minimal loss found in the amygdala. This is also in line with Nilsson et al. (1992) in which they found a complete loss of cholinergic hippocampal and neocortical fibers. Furthermore, Nilsson et al. (1992) found that their i.c.v. saporin injections degenerate NGFr positive cholinergic neurons found in the DB and NBM complex. When 192 IgG saporin is injected intracerebroventricularly, several deficits in learning and memory paradigms are displayed. Such deficits are seen in Morris Water Maze tasks in which adult animals demonstrate impaired learning acquisition (Wrenn & Wiley, 1998). This is seen in studies by Nilsson et al. (1992) and Wiley et al. (1995) in

which their i.c.v. saporin injections reduced learning and memory in MWM tasks. Furthermore, i.c.v. 192 IgG saporin injection studies have shown that the cholinergic cells of the ventral pallidum are not affected (Heckers, Ohtake, Wiley, Lappi, Geula, & Mesulam, 1994; Rossner et al., 1995). This suggests that the nature or origin of the degeneration of cholinergic VP cells and their immunotoxin response remains unknown (Rossner et al., 1995). Given that i.c.v. injections have shown to eliminate p75 containing Purkinje cells, this is deemed as a possible contribution to such behavioral deficits seen in MWM tasks and requires further investigation (Wrenn & Wiley, 1998).

Intracerebral 192 IgG saporin injections directly eliminate BF cholinergic neurons of the injection site. Unlike the behavioral deficits observed via i.c.v. injections, intracerebral injection studies have not explicitly demonstrated deficits in learning and memory (Wrenn & Wiley, 1998). In a study by Wenk et al. (1994), NBM injections did not provide sufficient evidence in learning and memory impairments indicated by small ChAT reductions in the hippocampus and cortex. Similarly, Torres et al. (1994), Pang and Nocera (1999), and Perry et al. (2001) found similar results in water maze tasks when injecting the NMB, MS, and DB. Pang and Nocera (1999) however found that the cholinergic population in the ventral pallidum was spared while Heckers et al. (1994) found damaged cholinergic neurons when injecting the SI yielding reduced hippocampal and cortical projections.

Overall, previous studies utilizing 192 IgG saporin, via i.c.v. or intracerebral injections, have not investigated the role of the VP through injecting it directly. Furthermore, the previously mentioned studies primarily focused on the role of BF cholinergic systems in learning and memory paradigms in attempts to find an animal model for AD. Although learning and memory deficits are seen in AD, this disease

also exhibits symptoms of anxiety and depression indicated by reduced cholinergic projections to the BLA. Therefore, in addition to learning and memory, the BF cholinergic population and its role in behavioral despair and anxiety should also be considered.

The ambiguity of the aforementioned results indicate that the cholinergic BF population requires more investigation in that other BF nuclei should be studied. Given what is known about cholinergic cells in memory, as revealed by Alzheimer's disease and prior lesioning studies, it is hypothesized that VP cholinergic lesions will yield impairments in fear learning indicated as a reduced freezing response in the Pavlovian Fear Conditioning task. Furthermore, previous literature primarily focuses on injecting 192 IgG saporin intracerebroventricularly or via intracerebral injections of the substantia innominata, medial septum, diagonal band, and nucleus basalis magnocellularis (Perry et al., 2001). There remains a gap in the literature regarding the implications of VP cholinergic population and its involvement with the BLA, CeA, and BNST in regard to behavioral despair, anxiety, and fear learning remain elusive. Therefore, the purpose of this thesis is to use 192 IgG saporin to selectively inactivate VP cholinergic neurons to investigate their contribution in behavioral despair, anxiety, and fear learning.

CHAPTER 2

MATERIAL AND METHODS

2.1 Animals

This experiment was conducted using eight adult male Wistar rats (n = 8) weighing approximately 240-320 grams. All animals were housed in groups of four in a vivarium in a controlled environment (21 ± 1 °C; 50% humidity; 12:12 day/night cycle with lights on at 8:00) and had *ad libitum* access to food and water. After the surgical procedure, animals were transferred to their own cages throughout the experiment. All procedures used were approved by Boğaziçi University Ethics Committee for the Experimental Use of Animals in Scientific Research (see Appendix).

This thesis compared eight control animals injected with 0.9% saline (NaCl) from a previous study by Cemal Akmeşe carried out in the Behavioral Neuroscience Laboratory. The control animals from the previous study were injected at the same site as the 192 IgG saporin group. The results of this study compared the effects of 192 IgG saporin to the sham injection group obtained from that study.

2.2 Stereotaxic surgery procedure

The animals were prepared for stereotactic surgery by firstly carrying out intraperitoneal injections (i.p.) using a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) to anesthetize the animal. Once the animal presented a complete loss of all reflexes, the animals' head was shaved and placed into the stereotaxic frame. The animals were then head-fixed to a stereotaxic frame using ear bars. A heating pad was placed underneath the animal and an infra-red lamp was used to keep the body

temperature at a constant 36º C. Eye cream was then placed directly on the eyes to prevent drying. A local anesthetic spray (Vemcaine, 10% lidocaine) and antiseptic (Batticon) were administered to the scalp's surface. Once these were absorbed into the skin, a vertical incision was made and exposed the skull. Once fully exposed, the skull's surface was cleaned with saline to allow for a clear view of the Bregma point using a microscope. Injection sites were previously determined using a rat brain atlas and calculated using a reference to the Bregma point (VP: AP = 0.00, ML = ± 2.20, DV = −7.60) (Paxinos & Watson, 2007). Bilateral craniotomies were then carried out above the injection sites based on their coordinates to the left and right VP. 192 IgG Saporin (Advanced Targeting Systems, 500 ng/µl in PBS, 0.5 µl volume, 0.1 µl/min) was injected to the previously defined locations in the dorsal-ventral axis using a Hamilton syringe. The contents of the Hamilton syringe were injected slowly using a microinjector pump at a desired volume and speed. After a ten-minute period to prevent dorsal diffusion, the syringes were retracted. The exposed skull was cleaned, and the incision was sutured. Once the incision was stitched, local anesthetics (Anestol pomade, 5% lidocaine and Jetokain, 5 mg/kg, s.c.) and antiseptic (Batticon) were applied. The animals were given 0.9% saline (2 ml, i.p.) during the recovery process to speed up their recovery and to prevent dehydration. They were then placed in post-operative care in which they were placed underneath an infrared lamp, and they were constantly monitored until the anesthetic effects wore off. Animals were given additional dosage of analgesics if exhibiting any symptoms or indications of pain. The animals' weight, food and water intake, and symptoms were monitored in the following days after surgery. There was a waiting period of 7-14 days for complete recovery and for targeted cells to be eliminated. Animals were decapitated after being transcardially perfused using

0.9% saline and 4% depolymerized paraformaldehyde (PFA). The behavioral timeline in which all the procedures were performed can be seen in Fig. 3.

2.3 Behavioral Tests

The behavioral tests were carried out over a span of seventeen days. After the injection of 192 IgG saporin to the VP, animals had a two-week recovery period before the start of the battery of behavioral tests. After the behavioral tests were complete, animals had another two-week recovery period before being transcardially perfused. The timeline of the behavioral tests can be seen in Fig. 3.

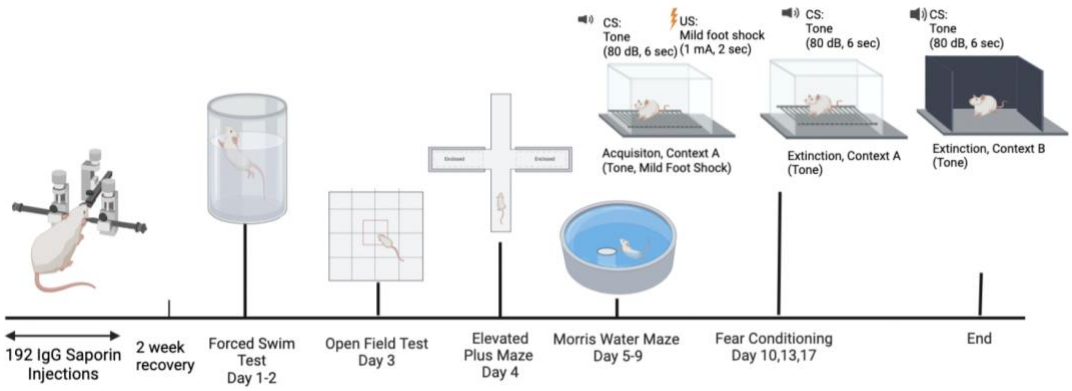

Fig. 3 Simple schematic diagram of the experimental timeline

2.3.1 Forced Swim Test (FST)

In this test, rats were placed into a water-filled cylinder (depth 30 cm, temperature 25 ± 1 °C). The test had a span of two days in which a 24-hour break was given between the first and second test. Before the start of each trial, the animal was brought into the testing environment in which they would acclimate to for a span of five minutes. The first test, which was the pre-test (FST-1), lasted for fifteen minutes after the animal was placed into the water-filled cylinder. The second test (FST-2), occurring 24 hours later, was five minutes long after the animal was placed into the water-filled cylinder. After each trial, animals were taken out and placed into their

home cage and placed underneath an infrared lamp to dry off for thirty minutes. The behavioral responses of the animals were assessed based on the FST-2 scores which were further categorized into three active behaviors: immobility, swimming, and climbing. Immobility behaviors were defined as a complete lack of motion among the animals, climbing was defined as a motion in which the animals' paws were placed above the surface of the water, and swimming was defined as motor movements in which the animal was positioned to be parallel to the water.

2.3.2 Open Field Test (OFT)

The open field test consisted of a black opaque box (70 cm x 70 cm x 45 cm) in which the animals were placed directly into the center at the start of each trial which lasted five minutes. Before each trial, animals were acclimated for five minutes to the testing environment. Additionally, the light intensity of each corner and the center was measured at the start of the behavioral experiment to ensure that each lighting condition was different. In between each trial, 70% ethanol was used to clean the open field test maze. Several variables were measured throughout this test including the time spent moving, time spent along the periphery and in the center, velocity, and distance traveled. A custom adaptation of Mathis et al. (2018) DeepLabCut (DLC) was used to analyze behaviors involving the animals' position and directional movement.

2.3.3 Elevated Plus Maze (EPM)

The elevated plus maze consisted of a cross-like structure built with two transparent acrylic arms (open) and two closed wooden arms (closed) with each arm measuring 50 cm by 10 cm. The arms were placed to be 50 cm above the ground. A lux meter

was used to measure the light intensity at each of the arms to ensure each light condition was significantly different. The animals were acclimated to the testing environment for five minutes. Animals were placed at the center during each trial with their head facing the direction towards one of the open arms. Each trial lasted five minutes and the maze was cleaned with 70% ethanol in between each trial. The time spent in the closed and open arms and the number of alternations between these arms was measured using DLC (Mathis et al., 2018; Pellow, Chopin, File, & Briley, 1985).

2.3.4 Morris Water Maze (MWM)

The Morris water maze consisted of a circular water maze (diameter = 120 cm) with a hidden platform (diameter = 10 cm) submerged underneath 1-2 centimeters of water. This task spanned over a five-day period, the first four days being training trials and the fifth day being a probe trial. The water in the circular maze was constantly monitored and kept at a temperature of 21 ± 1°C. The platform was kept in the same position throughout all of the trial days, except for the probe trial in which it was removed from the maze. There were four cues attached across the perimeter of the maze's wall, spanned 90º from each other. They were attached on the outside of the wall so that the animals were not able to touch or knock down the cues. The four cues consisted of a different shape (star, rectangle, circle, and square) and color (black, green, yellow, and purple). Additionally, the four cues were approximately the size of a standard piece of paper. Throughout the training trials, animals began each trial at random start locations. The training trials consisted of four training sub-trials. Each sub-trial allowed for a maximum of two minutes for the animal to find the hidden platform. If the animal was unable to find the platform

during this two-minute period, they were led to the hidden platform and remained on it for fifteen seconds. If animals found the platform, they stayed on the platform for fifteen seconds before being removed from the maze. At the end of each training sub-trial, the animals were placed into their cage for fifteen seconds before being introduced to a new starting position. The time to find the platform (escape latency), the animals' movement, and the duration spent in each quadrant during the training trials was analyzed using DLC.

The probe trials consisted of one sub-trial for each animal in which the platform was removed from the water maze. Each probe trial had a 60 second duration. The time spent in each target quadrant that the platform was previously in, the total movement, velocity, and the time spent along the periphery and in the center was analyzed using DLC.

2.3.5 Fear conditioning

The fear conditioning task consisted of an apparatus (Context A) with a chamber (20 cm x 40 cm x 20 cm) that had a floor made up of 16 metal grids which were spaced 1cm apart. The chamber was connected to a shock-provider and Arduino setup. Additionally, two cameras were positioned over and in front of the chamber to record each session. Before the start of the experiment, the animals were brought to the testing environment for a five-minute acclimation period. Once complete, animals were then placed into the chamber. In between trials, the chamber was cleaned using 70% ethanol. The acclimation and experimental trials were carried out under dim lighting.

On the acquisition day, animals were placed into the chamber (Context A) for a three-minute baseline measurement phase. After the completion of this phase,

there were five delay conditioning trials in which a mild foot shock (2 sec, 1.0 mA) was given 60 seconds after the presentation of a tone (80 dB, 2 kHz, 6 sec), with an intertrial interval time (ITI) of 66 seconds.

After a three-day waiting period, animals were tested again using the same apparatus (Context A). The test consisted of a three-minute baseline measurement phase followed by twelve trials. Each trial lasted one minute, and the animals received the same tone (80 dB, 2kHz, 6 sec) they had received during the acquisition.

Six days after the acquisition day, animals were placed in a new context (Context B) which was physically different from Context A. Context B consisted of a solid and flat floor that was square in shape. While the walls of context A were made up of a transparent plexiglass, the walls of Context B and opaque walls. Furthermore, Context B was placed in a different location of the behavioral testing room. The testing using Context B consisted of twelve trials followed by a three-minute baseline measurement. Each trial was separated by one minute and consisted of the same tone previously used (80 dB, 2kHz, 6 s).

The behaviors in the fear conditioning task were coded based on freezing responses. Additionally, the responses the animals displayed to each foot shock were measured through the quantification of darting behaviors which were classified as a quick movement or escape-like response.

2.4 Histology

Once all behavioral testing was complete, the animals were deeply anesthetized and perfused transcardially using 9% saline followed by 4% depolymerized PFA in phosphate buffered saline (PBS). After the completion of this process, the brains were removed and postfixed in 4% PFA for two nights at 4ºC. The brains were then

rinsed in 0.1M of phosphate buffer (PB) three times for ten minutes each time. Coronal sections were then obtained using the Leica VT1000S vibratome to slice at a thickness of 50-70 µm.

2.5 Immunohistochemistry (IHC)

Free-floating immunofluorescence labeling techniques were used as described by Unal et al. (2015). Individual brain sections were placed into glass vials and washed (3 x 10 min) at room temperature with 0.3% Triton X-100 in PBS (PBS-TX). A blocking solution containing 20% Normal Horse Serum (NHS) and 80% PBS-TX was used to submerge the sections for one hour at room temperature. The sections were then placed to incubate for 48-72 hours at 4ºC in primary antibody solutions in PBS-TX with 1% NHS. Once the primary antibody incubation was complete, sections were washed in PBS-TX (3 x 10 min) and placed to incubate in secondary antibody solutions with 1% NHS for 4 hours at room temperature. DAPI (1:2000, code: D3571, ThermoFisher) staining was used to identify the borders of BF nuclei. Therefore, the sections were incubated for fifteen minutes in DAPI solution in glass vials and were then rinsed in PBS-TX (3 x 10 min). Lastly, the sections were placed onto glass slides and cover slipped.

The primary antibodies used in this process are listed were rabbit anti-Parvalbumin (1:2000, ab11427, Abcam), goat anti-ChAT (1:500, AB144P, Merck-Millipore), goat anti-ChAT (1:350, ab254118, Abcam). The secondary antibodies used were as follows: donkey anti-rabbit Alexa Fluor 488 (1:250, ab150073, Abcam), donkey anti-goat DyLight650 (1:1000, ab96938, Abcam).

2.6 Microscopic observation and anatomical description

All brain sections obtained during this experiment were observed using an Olympus BX43 epifluorescent microscope. A blue fluorescent dye, DAPI (1:2000, code: D3571, ThermoFisher) was used to distinguish the borders of BF nuclei. Additionally, substance-P (1:100-500, code: ab216412, Abcam) immunoreactivity was used to identify and distinguish the borders of the ventral pallidum. The combination of these staining techniques allowed for all the cell nuclei to be distinguishable and visible.

CHAPTER 3

RESULTS

3.1 Histological and Immunohistochemistry (IHC)

Immunohistochemistry and histological analysis were carried out to further verify the effectiveness of the 192 IgG saporin injections. The injection sites from eight randomly selected animals revealed that the ventral pallidum was targeted from the anterior-posterior (AP) axis. The estimated center of the injection sites was observed in both hemispheres to be within the ventral pallidal boundaries (Fig. 3A). The 192 IgG saporin toxin had a significant impact on the alteration of the Parvalbumin positive (PV+) ($M_{Vehicle}$ = 94.50, $SD_{Vehicle}$ = 18.33, $M_{192IgG\text{-}sap}$ = 44.20, $SD_{192IgG\text{-}sap}$ = 17.38) (t = 6.298, df = 18, p = 0.001) (Fig. 3B), which was previously reported in the findings of Torres et al. (1994). The 192 IgG saporin toxin had a significant impact on the number of the ChAT positive cells (ChAT+) ($M_{Vehicle}$ = 50.00, $SD_{Vehicle}$ = 14.73, $M_{192IgG\text{-}sap}$ = 14.30, $SD_{192IgG\text{-}sap}$ = 6.816) (t = 6.954, df = 18, p = 0.001) (Fig. 3B).

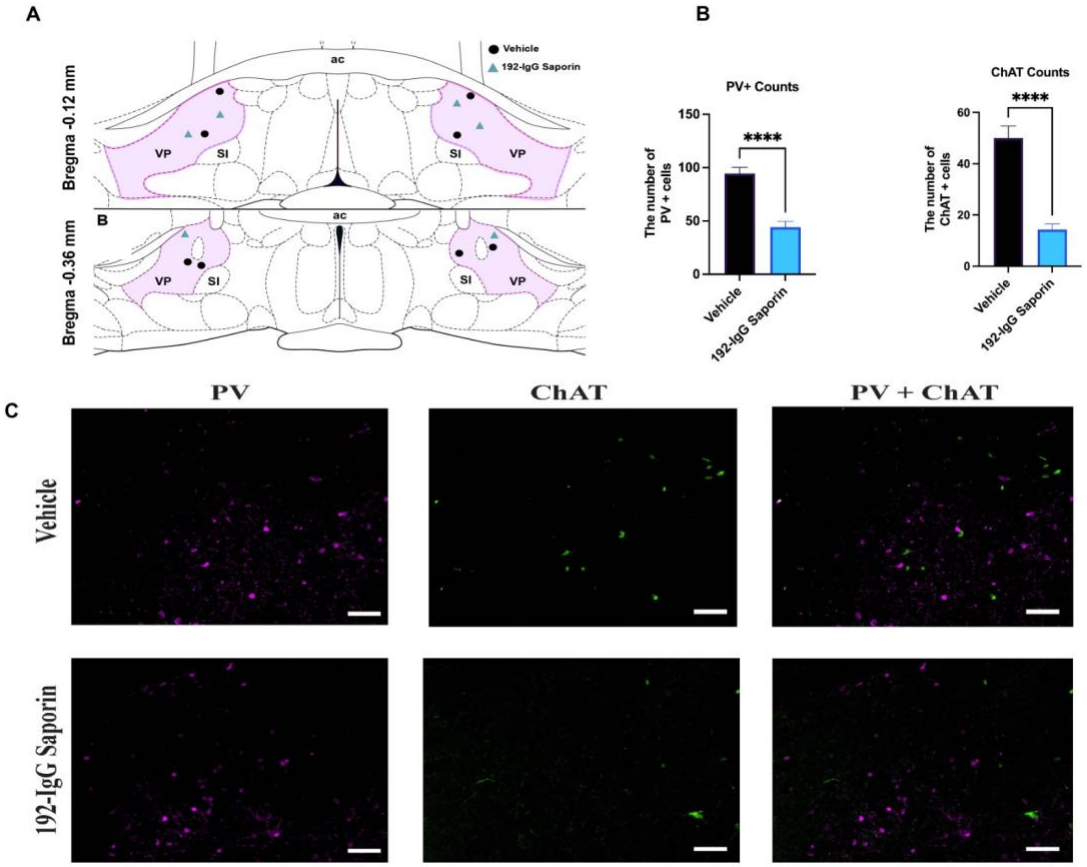

Fig. 4 Histological and Immunohistochemistry analysis of VP 192 IgG saporin injections

Note: (A) Schematic diagrams of coronal sections showing the center of the injection site. (B) The average number of PV+ and ChAT+ cells in the VP. (C) Micrographs depicting the PV and ChAT immunoreactivity of the VP within the vehicle group and 192 IgG saporin group. Error bars depict SEM. Scale bars denote 50μm.

3.2 Forced Swim Test (FST)

FST was carried out to identify behaviors in regard to a depression-induced environment. This was measured through taking the average duration of immobility displayed among animals. The results of this test were taken from the second day of testing to ensure results are obtained from behavioral despair in which they learned during FST day one trials. The results indicate a significant difference between groups in terms of their immobility. 192 IgG saporin injections display a reduced average duration in comparison to the vehicle animals ($M_{Vehicle}$ = 77.10, $SD_{Vehicle}$ = 34.33, $M_{192IgG\text{-}sap}$ = 42.09, $SD_{192IgG\text{-}sap}$ = 24.85) (t = 2.337, df = 14, p = 0.0348) (Fig.

4A). To further identify immobility as a result of depression, climbing was also measured and displayed a statistically significant difference between groups ($M_{Vehicle}$ = 200.9, $SD_{Vehicle}$ = 37.20, $M_{192IgG\text{-}sap}$ = 142.8, $SD_{192IgG\text{-}sap}$ = 44.49). The saporin injected group showed an increased duration in climbing compared to the vehicle group (t = 2.831, df = 14, p = 0.0133) (Fig. 4B). However, there was no significant difference between groups in terms of swimming duration ($M_{Vehicle}$ = 34.69, $SD_{Vehicle}$ = 19.84, $M_{192IgG\text{-}sap}$ = 43.44, $SD_{192IgG\text{-}sap}$ = 30.24) (Fig. 4C) head shaking ($M_{Vehicle}$ = 48.13, $SD_{Vehicle}$ = 8.127, $M_{192IgG\text{-}sap}$ = 46.63, $SD_{192IgG\text{-}sap}$ = 10.31) (Fig. 4D), or the number of diving on FST day 2 (Fig. 4E).

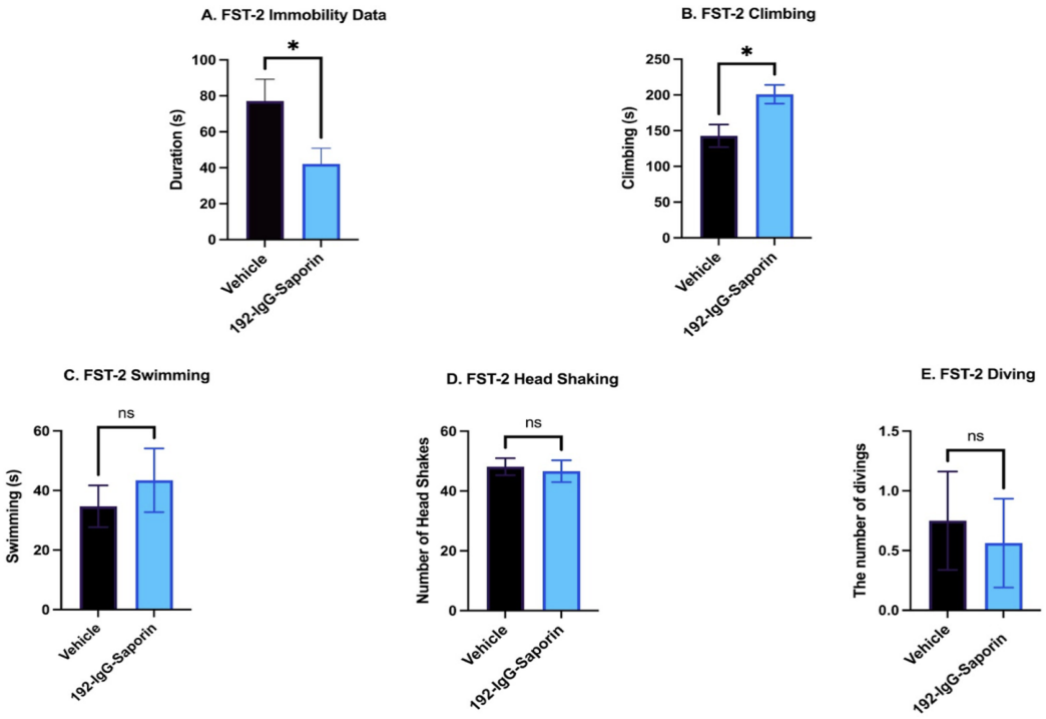

Fig. 5 Graphical representation of behavioral responses during the FST-2
Note: (A) Significant difference in the average time (seconds) exhibiting immobile behaviors between vehicle and 192 IgG saporin group. (B) Significant difference in average climbing time (seconds) between vehicle and 192 IgG saporin group. (C) No significant difference in average swimming time between groups. (D) No significant difference in number of head shakes between groups. (E) No significant difference in the number of dives between groups. Error bars depict SEM.

3.3 Open Field Test (OFT)

OFT was used to measure anxiety through locomotor activity displayed by the animals through measuring the duration spent in the center of the maze as well as the total movement throughout the task. The results indicate that the 192 IgG saporin group did not display a significant difference compared to the vehicle group in terms of the duration spent in the center of the maze as opposed to the periphery ($M_{Vehicle}$ = 29.01, $SD_{Vehicle}$ = 15.71, $M_{192IgG\text{-}sap}$ = 46.19, $SD_{192IgG\text{-}sap}$ = 48.30) (t =0.9563, df = 14, p = 0.3551) (Fig. 5A). Similarly, there was no significant difference between groups in general locomotion as measured by their total movement in the maze throughout the task ($M_{Vehicle}$ = 1301.0, $SD_{Vehicle}$ = 204.5, $M_{192IgG\text{-}sap}$ = 1123.0, $SD_{192IgG\text{-}sap}$ = 491.2) (t = 0.9483, df = 14, p = 0.3591) (Fig. 5B).

3.4 Elevated Plus Maze (EPM)

EPM was carried out to further determine the efficiency of eliminating cholinergic cells in anxiety tasks and behaviors. The results of this task did not reveal a significant difference of the time spent in the open arms between vehicle and 192 IgG saporin groups ($M_{Vehicle}$ = 127.8, $SD_{Vehicle}$ = 113.7, $M_{192IgG\text{-}sap}$ = 127.19, $SD_{192IgG\text{-}sap}$ = 130.4) (t = 0.09, df =10, p = 0.99) (Fig. 5C). The data of three animals during the EPM test were eliminated from behavioral analysis since they spent less than three seconds exploring the open arms throughout the trial. Additionally, there was no significant difference in terms of the total movement between groups ($M_{Vehicle}$ = 127.8, $SD_{Vehicle}$ = 113.7, $M_{192IgG\text{-}sap}$ = 127.19, $SD_{192IgG\text{-}sap}$ = 130.4) (t = 0.66, df = 10, p = 0.5200) (Fig. 5D).

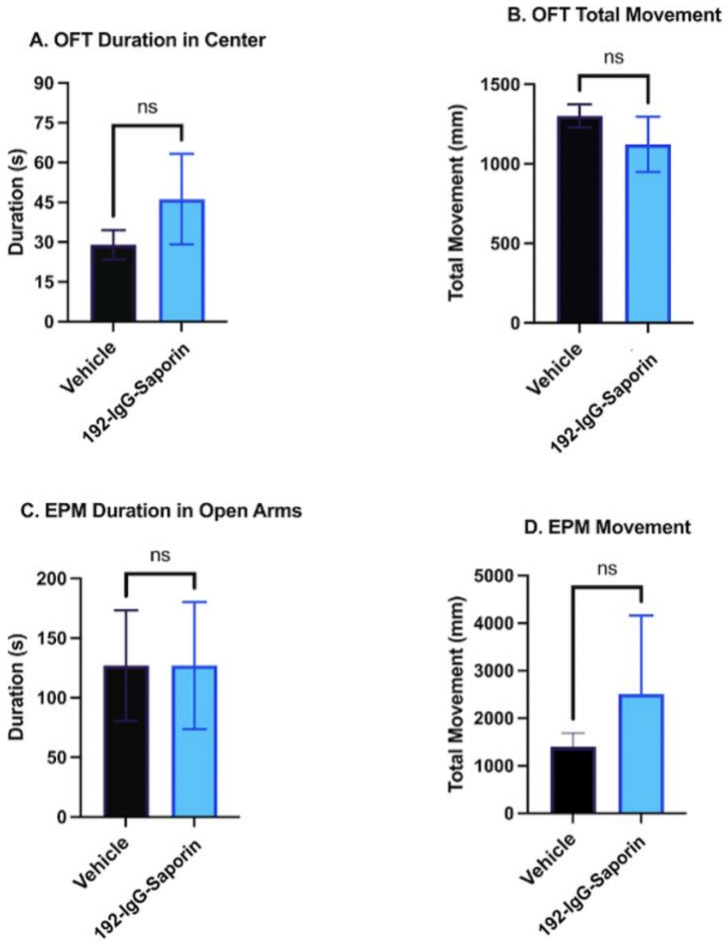

Fig. 6 Graphical representation of behavioral responses during the OFT and EPM Note: (A) No significant difference in the average time (seconds) spent in the center throughout OFT. (B) No significant difference bound in the total movement (mm) throughout OFT. (C) No significant difference in the average time spent in the open arms throughout EPM. (D) No significant difference in the total movement (mm) throughout EPM. Error bars depict SEM.

3.5 Morris Water Maze (MWM)

In the MWM task, spatial learning and memory was tested between groups through measuring the time spent to find the hidden platform (escape latency) in addition to durations spent in target quadrants, the center of the maze, swimming distance and average speed. The results indicate a significant difference in escape latencies between groups throughout four days of trials ($F(1.792, 25.09) = 11.27$, $p = 0.0005$) (Fig. 6A). The escape latencies of the 192 IgG saporin group were significantly

different ($F(1, 14) = 11.00$, $p = 0.0051$) in that they displayed longer durations to find the hidden platform when compared to the control group, specifically on day 2 (Sidak multiple comparisons, $p = 0.0127$ and day 3 ($p = 0.0312$) (Fig. 6A). On the fifth day of the MWM task, there was no significant difference observed in the duration spent in the probe trial's target quadrant ($M_{Vehicle} = 21.70$, $SD_{Vehicle} = 15.28$, $M_{192IgG\text{-}sap} = 16.89$, $SD_{192IgG\text{-}sap} = 9.391$) ($t = 0.7586$, $df = 14$, $p = 0.4607$) (Fig. 6B). However, 192 IgG saporin injections did significantly affect the duration of time spent in the center of the maze in that the saporin injected animals spent less time exploring the center ($M_{Vehicle} = 34.47$, $SD_{Vehicle} = 11.73$, $M_{192IgG\text{-}sap} = 18.96$, $SD_{192IgG\text{-}sap} = 11.65$) ($t = 2.653$, $df = 14$, $p = 0.0189$) (Fig. 6C). The injections nonetheless did not display a significant difference on the total average swimming distance ($t = 0.4235$, $df = 14$, $p = 0.6784$) (Fig. 6D) or the average speed during the probe trials ($t = 0.4543$, $df = 14$, $p = 0.6566$) (Fig. 6E).

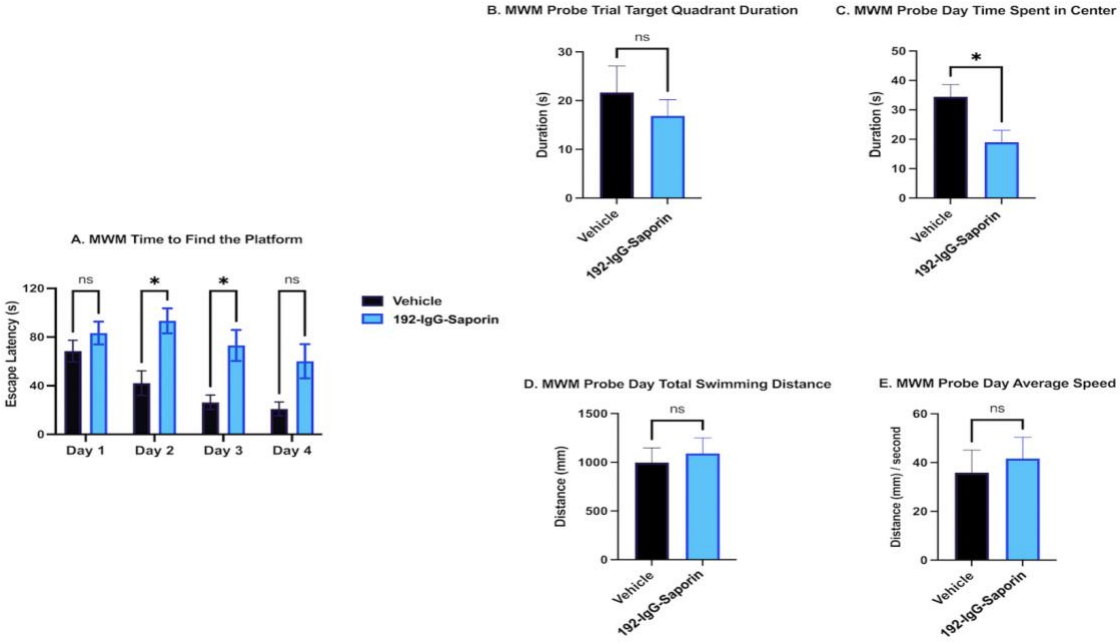

Fig. 7 Graphical representation of the MWM
Note: This indicates 192 IgG saporin animals have impaired spatial learning and are less likely to explore the center of the maze for the platform during probe trials. (A) Significant difference in the average time (seconds) to find the platform on day 2 and day 3 as indicated by increased escape latencies of the 192 IgG saporin group. (B) No significant difference in the average time (seconds) spent in target quadrants. (C) Significant difference in average time (seconds) spent in the center during probe trials. (D) No significant difference in average distance (mm) traveled during probe trials. (E). No significant difference in average speed (mm/s) during probe trials. Error bars indicate SEM.

3.6 Fear Conditioning

The fear conditioning task was used to characterize the functional effect of the elimination of cholinergic cell circuits in fear memory through sound-shock pairings and a change in environment. The experiment consisted of three distinct tests throughout a seven-day period which measured freeze response during acquisition (CS-US pairings) and cued fear reactions (CS) in the same exact conditioned location (Context A), and in a new location (Context B).

In the fear conditioning acquisition trails (day 1), neither group displayed a freeze response during the three-minute baseline period (Fig. 7A). Both groups displayed a learned conditioned freeze response ($F(2.258, 31.61) = 133.1$, $p =$

0.0017) (Fig. 7A), however, there was a statistically significant difference between groups in terms of their conditioned freeze response when shocked ($M_{Vehicle}$ = 45.12, $SD_{Vehicle}$ = 5.293, $M_{192IgG\text{-}sap}$ = 31.84, $SD_{192IgG\text{-}sap}$ = 6.727) ($F(1,14) = 6.430$, $p = 0.238$) (Fig. 7A). Furthermore, the 192 IgG saporin injection displayed a significant main effect only on one type of active avoidance behavior in response to the mild footstock. This can be seen by the increased darting response among saporin injected animals in Fig. 7D ($U = 6$, $p = 0.0039$). The 192 IgG injection group did not exhibit any jumping response differences.

In the cued fear conditioning trials (day 4 and day 7), 192 IgG saporin injections exhibited a significant main effect regarding cued fear responses in both Context A ($F(1,14) = 8.399$, $p < 0.0001$) (Fig. 7C) and Context B ($F(1,14) = 24.22$, $p < 0.0001$). Furthermore, there was a significant interaction between trials and manipulation in both Context A ($F(14,196) = 3.849$, $p < 0.0001$) and Context B ($F(14,196) = 1.843$, $p = 0.0349$) as seen in the overall decreased freeze response (Fig. 7B-C). Overall, there was a significant reduction in cued fear responses.

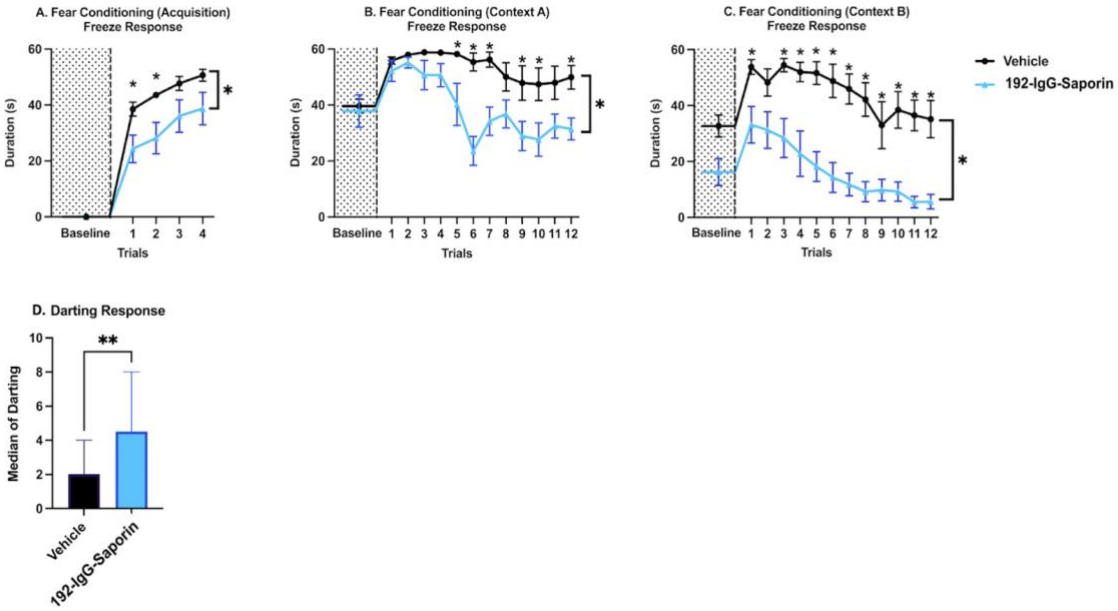

Fig. 8 Graphical representation of fear responses

Note: The fear responses during acquisition and context dependent trials indicates reduced fear response among the 192 IgG saporin group. (A) Significant difference in the average freezing response to mild foot shock during acquisition trials (day 1). (B) Significant difference in the average freezing response in Context A to sound. (C) Significant difference in the average freezing response in Context B to sound. (D) Significant difference in the average darting response during the acquisition trials. Error bars indicate SEM.

CHAPTER 4

DISCUSSION

The ventral pallidum is commonly associated with reward and motivation related behaviors. Furthermore, the VP's neurobiological involvement in psychiatric disorders, such as anxiety and depression, has been overlooked (Root et al., 2015). Therefore, this study aimed to further identify the role of VP cholinergic neurons in various neuromodulatory functions involved in behavioral despair, anxiety, and fear learning. Injections of the neurotoxin 192 IgG saporin eliminated the cholinergic neurons in the VP and the effects of this injection were measured in behavioral tests: FST, OFT, EPM, MWM, and Fear Conditioning. The results of these behavioral tests indicate that the elimination VP cholinergic system clearly exhibited antidepressant effects in FST, increased escape latencies in MWM, and decreased freezing responses in Fear Conditioning. Importantly, histological analysis reveals that 192 IgG saporin effectively eliminates cholinergic neurons in the VP given the reduced number of cholinergic cell types. In prior lesioning studies, it has also been shown that the cerebellar Purkinje neurons, which also contain p75 NGF, are especially impacted when injecting 192 IgG saporin intracerebroventricularly (Wrenn & Wiley, 1998). Although the present study carried out direct injections of the VP, it may be possible that the cerebellar p75 containing Purkinje neurons may also have been affected and should be taken into consideration when analyzing the results, specifically those which primarily focus on analyzing motor movements. Overall, the VP cholinergic neurons can serve as a potential target in therapeutically targeting clinical depression and fear-related disorders.

FST is commonly used to investigate behavioral despair or learned helplessness, a state in which the test animal appears to be completely immobile, a common sign of depression. When exposed to water, animals typically exhibit active motor behavior, such as swimming and climbing, in the attempt to escape from the water. The disengagement from active stress coping mechanisms indicates the animal's state of immobility from the situation. An animal's decrease in immobility typically indicates an antidepressant effect (Ünal & Canbeyli, 2019). In this study, the 192 IgG saporin group demonstrates decreased immobility and increased climbing in attempts to escape from an unpleasant situation. Immobility is characterized by the animal displaying a lack of movement, such as floating in the water and not making any motor movements in the attempt to escape. Additionally, climbing, an active coping response, is characterized by the animal exhibiting an upward movement in which its paws are above the water, as if it is trying to escape. This indicates that 192 IgG saporin injections to the VP cholinergic system yields an antidepressant effect in rodents. However, these findings are not in accordance with that of Chen et al. (2021) in which they investigated the elimination of cholinergic neurons in the horizontal limb of the diagonal band of Broca (hDB). They found that the elimination of hDB cholinergic neurons elicited depressive-like behaviors during forced swimming as indicated by increased immobility. Furthermore, they found that their 192 IgG saporin injections to the hDB resulted in complete neuronal loss of the hippocampus. This indicates that the cholinergic system of the hDB contributes to depressive-like behaviors (Chen, Ke, Ma, Gao, Zhou, Zhu, Liu, Zhang, & Zhou, 2021). Although the present study and that of Chen et al. (2021) carry out similar experimental manipulations, it is interesting that the results are opposite of each other. The present study also found a loss of hippocampal populations in the 192

IgG saporin group indicated by a loss of ChAT positive and Parvalbumin (PV) positive cells. However, it is important to note that Chen et al. (2021) administered FST after an array of behavioral tests, potentially increasing stress exposure to their test groups. Therefore, the potential increase in stress exposure may pose as a factor to the differences found in the present study and that of Chen et al. (2021). Given the significant decrease of immobility found in saporin injected animals, the VP cholinergic system can serve as a model to investigate antidepressants.

When administering FST, it is important to assess locomotor activity to prevent false-positive or false-negative results to further determine whether the saporin injection alters general activity. Therefore, OFT is used to investigate locomotion and anxiety through measuring animal activity. An animal's path along the periphery is an anxiety-like behavior while animals spending time in the center are deemed less anxious (Kraeter, Guest, & Sarnyai, 2018). Similarly, EPM measures anxiety-like behaviors indicated by the time spent in open arms. In this study, there was no significant difference between the saporin injected group and the vehicle group in both the OFT and EPM. This indicates that the elimination of the VP cholinergic neurons do not play an important role in anxiety like behaviors. In a study by Torres et al. (1994), they found 192 IgG saporin injections to the MS and hDB significantly reduced the time spent in the center of the maze and increased the time spent along the periphery. Therefore, their results demonstrate that the MS and hDB cholinergic systems play a role in anxiety-like behaviors. Chen et al. (2021) also found similar results in that their hDB saporin group spent less time in open arms. This demonstrates that the hDB cholinergic system, along with the MS have a role in anxiety-like behaviors. However, the present study demonstrates that the VP cholinergic system does not explicitly play a role in these types of behaviors.

MWM measures spatial navigation through the use of distal cues used to find a hidden platform. This test has been extensively shown to be a valid measure of spatial learning and navigation, a hippocampal-dependent task (Vorhees & Williams, 2006). The time spent to find the platform, escape latency, is used to measure learning. In this study, 192 IgG saporin injected animals displayed a significant increase in escape latency, indicating impairments in hippocampal-dependent spatial learning. These findings are not in accordance with Perry et al. (2001) in which the NBM and MS were intracerebrally injected using 192 IgG saporin. They found that during the MWM task, the loss of these cholinergic neurons did not significantly impair learning and memory. Similarly, Torres et al. (1994) and Wenk et al. (1994) found no detectable impairments during the MWM task. In Pang and Nocera's study (1999), their intracerebral lesions of the MS and vertical diagonal band indicate that, although there were no significant impairments, these cholinergic systems are involved in spatial learning and memory. Intracerebroventricular 192 IgG saporin injections however produce different results in terms of spatial learning and memory during the MWM. Nilsson et al.'s (1992) i.c.v. injection study found deficits in the MWM task indicating BF cholinergic neurons' role in spatial learning and memory. Wiley et al. (1995) demonstrates similar results in that, after administering i.c.v. injections, they observed a significant decrease in escape latencies during the MWM. Although deficits in spatial learning and memory are commonly seen during i.c.v. administration of 192 IgG saporin, this method does not explicitly address which BF region's cholinergic neurons contribute to this deficit. The present study however provides clarification in the VP cholinergic system's role in spatial learning and memory as indicated by deficits observed in the MWM. This indicates that the VP cholinergic system plays a role in hippocampal processing.

Fear Conditioning is used to further understand the expression of innate fear responses through creating an aversive environment through presenting threatening stimuli. The stimuli in this experiment include a tone, the conditioned stimulus (CS), and pairing it with a foot shock, the unconditioned stimulus (US), to which the defense reaction among rats is a freeze response. In this study, 192 IgG saporin injected animals displayed a significant decrease in freeze response, indicating impairments in fear memory, during the acquisition and context trials. Reduced freeze response during acquisition was also found in Jiang et al.'s (2016) study in which the cholinergic inputs to the BLA were optogenetically regulated. Their study demonstrates cholinergic input to the BLA is essential in fear memory formation and highlights the role of acetylcholine as not only a neuromodulator for fear memories but also for conditioned fear learning (Jiang et al., 2016). This is also in line with Vazdarjanova and McGaugh (1999) in which they found a reduced freeze response when inactivating the BLA using lidocaine. They also found an increased freeze response when administering a muscarinic receptor agonist, oxotremorine, to the BLA. Their results demonstrate that acetylcholine is essential in enhancing memory as indicated by the freeze response during fear conditioning. Chen et al. (2021) found similar results when administering 192 IgG saporin to the hDB in that there was a decrease in the freeze response during contextual fear conditioning. Given that the cholinergic neurons of BF project to the hippocampus, the elimination of these neurons can yield deficits in learning and memory, as seen in Chen et al's study (2021). More specifically, contextual fear conditioning is a hippocampus-dependent form of learning. In the fear conditioning task, the dentate gyrus (DG) and CA1 region of the hippocampus respond to associations made during the fear conditioning task (Chen et al., 2021). Neuronal loss of the hippocampus is also seen in the present

study indicating the elimination of VP cholinergic neurons projecting to the hippocampus yields similar deficits seen in that of Chen et al. (2021). Therefore, reduced freezing response indicates a deficit in contextual memory, potentially due to neuronal loss of these hippocampal regions as well as decreased cholinergic projections to the amygdala. However, it is important to note that the reduced freeze response found in the 192 IgG saporin injected group may be correlated to pain mediation. The nucleus accumbens, which plays a role in addiction, also has implications in the mediation of pain (Harris & Peng, 2020). Furthermore, the nucleus accumbens projects to the ventral pallidum and amygdala which has been shown to play a role in the modulation of pain (Kato, Ide, & Minami, 2016). Therefore, it is a possibility that the elimination of the VP cholinergic cells was involved in the previously mentioned pain circuitry. The results found in the fear conditioning task may therefore have been the result of pain avoidance rather than behavioral despair. Additionally, there was a significant increase in the darting response, which is an active coping mechanism that is characterized by a quick movement or escape-like behavior. It is possible that, although there was a decrease in the overall freeze response, that the pain level of the experimental group was increased. This may indicate that the decreased freeze response may not have been due to deficits in fear learning and memory, but rather to escape from pain. It is also important to consider that the decrease in fear conditioning may also be due to a decrease in sensory perception rather than deficits in fear learning in that the overall threat perception may have been shifted and may be a sign of attentional deficits.

Overall, the central cholinergic pathway is affiliated with various behaviors including motor function, sleep and arousal states, mood, and learning and memory (Mesulam, 1988). More specifically, the BF cholinergic system extensively

innervates structures involved in various cognitive functions, namely learning and memory, stress, cognition, extinction memory, and attentional mechanisms given its major innervations to limbic structures and the cerebral cortex (Coyle et al. 1983; Carlsen et al., 1985; Mesulam, 1990; Page & Sofronview, 1996; Paul, Jeon, Bizon, & Han, 2015; Knox & Keller, 2016). Approximately 19% of the BF cholinergic projections target the prefrontal cortex (Zaborszky et al., 2012). Given the BF cholinergic projections to the cortex, loss of these innervations have implications in memory deficits. Furthermore, an excessive loss of BF cholinergic pathways to both the cortex and limbic structures is characterized by memory impairments, such as that found in Alzheimer's Disease (Mesulam, 1988). Lesions of the BF cholinergic system projecting to the cortex impairs perception, plasticity, and executive function. However, the mechanism of BF cholinergic projections to the cortex and their implication with these functions remains elusive (Gombkoto, Gielow, Varsanyi, Chavez, & Zaborszky, 2021). Additionally, the BF cholinergic system densely innervates the amygdala and has been shown to be involved in memory modulation and consolidation during aversive events (McDonald, 1998; Vazdaranova & McGaugh, 1999; Muller et al., 2011). Approximately 75% of cholinergic projections to the amygdala originate from the ventral pallidum (Root et al., 2015). However, cholinergic signaling from the BF to the amygdala and its implications in fear learning remains a challenge (Jiang et al. 2016). Despite the elusivity involved in understanding the mechanisms and signaling of BF cholinergic neurons to these regions, an intervention of this cholinergic system produces various deficits in cognitive function. This study provides evidence in that such intervention using 192 IgG saporin elicits changes in cognitive functions, such as learning and memory, as well as producing antidepressant-like effects.

In summary, the present thesis revealed the involvement of VP cholinergic neurons in various affective functions such as fear learning and memory and elicited their role in despair/depression-like behavior in the FST. These findings suggest that the BF cholinergic neuronal group can serve an enhancement therapy to treat and target psychiatric disorders in which impaired cholinergic input is present. Future work should focus on pairing antidepressant or anxiolytic related drugs with the cholinergic lesion when carrying out behavioral tasks. In addition, memory enhancement drugs, such as reversible acetylcholinesterase inhibitors, should also be paired with the inhibited cholinergic BF projections to further determine if memory will be enhanced during spatial learning and memory tasks. Overall, this will reveal the effectiveness of various drug therapies in the attempts to target clinical depression and fear-related disorders.

APPENDIX

ETHICS COMMITTEE APROVAL

T.C.
BOĞAZİÇİ ÜNİVERSİTESİ
Kurumsal Hayvan Deneyleri Yerel Etik Kurulu (Bühadyek)

Sayı : 44697215-050.01.04-E.7375 29/04/2020
Konu : 2020-09 Kodlu Başvurunuz
Hakkında

Sayın Dr. Öğr. Üyesi Güneş ÜNAL
Psikoloji Bölüm Başkanlığı - Öğretim Üyesi

Yürütücülüğünü üstlendiğiniz " Neuronal correlates of limbic functions (Limbik fonksiyonların nöronal bağıntıları)" adlı, 25.02.2020 başvuru tarihli ve 2020-09 kodlu başvurunuz, Boğaziçi Üniversitesi Kurumsal Hayvan Deneyleri Yerel Etik Kurulu'nun (BÜHADYEK) 20.02.2020 tarihli toplantısında görüşülmüş ve KABUL edilmiştir..

Bu karar tüm üyelerin toplantıya on-line olarak katılımıyla ve oybirliği ile alınmıştır. COVID-19 önlemleri nedeniyle üyelerden ıslak imza alınamadığından bu onam mektubu tüm üyeler adına Komisyon Başkanı tarafından e-imzalanmıştır

Saygılarımızla bilginize sunarız,

e-imzalıdır
Prof. Dr. Burak GÜÇLÜ
Komisyon Başkanı

Adres:34342 Bebek-İstanbul
Telefon:0212 287 17 53 Faks:0212 265 70 06
Elektronik Ağ:www.boun.edu.tr
Bilgi için: Lale DİLEK

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