THE ROLE OF VENTRAL PALLIDAL GABAERGIC NEURONS IN AFFECTIVE AND COGNITIVE PROCESSES

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THE ROLE OF VENTRAL PALLIDAL GABAERGIC NEURONS IN AFFECTIVE AND COGNITIVE PROCESSES

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DECLARATION OF ORIGINALITY

I, Cemal Akmeşe, certify that

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ABSTRACT

The Role of Ventral Pallidal Gabaergic Neurons in Affective and Cognitive Processes

GABAergic projections from the ventral pallidum (VP) and the associated *substantia innominata* (SI) are relayed to two limbic structures respectively associated with acute and chronic forms of fear: the amygdala and the bed nucleus of the stria terminalis (BNST). This implicates a key role for VP GABAergic neurons in affective processing. I carried out selective lesion experiments to identify the role of VP GABAergic neurons in several implicit and explicit processes. To reveal the functional role of these neurons, bilateral injections of GAT1-Saporin or vehicle (saline) injections were made into the VP of adult male Wistar rats (*n = 16*). The animals were then assessed in the forced swim test (FST), open field test (OFT), elevated plus maze (EPM), Morris water maze (MWM) and Pavlovian fear conditioning. I found that VP GAT1-Saporin lesions reduced behavioral despair while not altering general locomotor activity. The experimental animals also exhibited a reduced freezing response and increased darting behavior through the fear conditioning acquisition trials. This indicates that selectively inactivating the GABAergic neurons in the VP have an antidepressant effect while active coping mechanisms are promoted. Despair and fear memory-related differences observed in the GAT1-Saporin group may be related to the local inhibition in the basal forebrain as well as long-range GABAergic projections to the amygdala and the extended amygdala. Silencing these long-range VP GABAergic neurons may prove useful in the treatment of depressive and fear-related disorders.

ÖZET

Ventral Pallidal Gabaerjik Hücrelerin Afektif ve Bilişsel Süreçlerdeki Rolü

Basal ön beyin yapılarından olan ventral pallidum (VP) ve substantia innominatadaki (SI) GABAerjik hücreler akut ve kronik korkuyla ilişkili olan Amigdala ve Stria terminalis yatağı bölgelerine girdi sağlar. Bu çalışma VP ve SI beyin bölgelerindeki bu GABAerjik hücrelerin farklı davranışsal ve bilişsel süreçlerdeki rolünü ortaya koymak adına yürütülmüştür. Bu hücrelerin spesifik rollerini ortaya çıkarmak için, GAT1-Saporin toksinini ve %.9 derişime sahip salini yetişkin Wistar sıçanlarında stereotaksik ameliyat ile enjekte ettim. Ameliyat sonrası iyileşme için gerekli süreden sonra bu hayvanların zorunlu yüzme testi, açık alan testi, yükseltilmiş artı labirenti, Morris su testi ve Pavlovian korku koşullanması deneylerinde sergiledikleri davranışları gruplar arasında karşılaştırdım. VP ve SI GABAerjik hücreleri kalıcı olarak elimine edilen hayvanların davranışsal çaresizlik belirtisi olarak kabul edilen hareketsiz kalma sürelerinde azalma tespit ettik. Ayrıca bu azalma genel motor aktivitede bir azalma olmaksızın ortaya çıktı. Benzer şekilde, GAT1-Saporin grubundaki hayvanların korku koşullaması edinim gününde ve koşulamadan farklı bir bağlamda test edildiklerinde daha az donma tepkisi verdiklerini gözlemledim. VP ve SI bölgelerindeki GABAerjik hücrelerin susturulmasının anti-depresan etkisi olduğunu ve aktif baş etme stratejilerinin de kontrol grubuna kıyasla daha fazla olduğunu buldum. Bu anlamlı fark VP ve SI'daki amygdala'ya projeksiyon yapan kolinerjik hücreler üzerinde GABAerjik hücrelerin etkisiyle ortaya çıkıyor olabilir. Bu GABAerjik hücreler, klinik depresyon ve korku bozukluklarında yeni tip terapötik ilaçların geliştirilmesinde önemli bir rol oynayabilir.

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ABBREVIATIONS

ac	Anterior commissure
BF	Basal forebrain
BLA	Basolateral amygdaloid nucleus, anterior part
BMA	Basomedial amygdaloid nucleus, anterior part
BNST	Bed nucleus of the stria terminalis
CB	Calbindin
CeA	Central amygdaloid nuclei
CeL	Central amygdaloid nucleus, lateral part
CeM	Central amygdaloid nucleus, medial part
ChAT	Choline acetyltransferase
CR	Calretinin
CS	Conditioned Stimulus
EA	Extended amygdala
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
hDB	Nucleus of the horizontal limb of the diagonal band
ITC	Intercalated nuclei of the amygdala
i.p.	Intraperitoneal
LA	Lateral amygdaloid nucleus
MeA	Medial amygdaloid nuclei
MS	Medial septum
NGS	Normal goat serum

NHS	Normal horse serum
PB	Phosphate buffer
PBS	Phosphate-buffered saline
PBS-TX	Phosphate-buffered saline-Triton X-100
PFA	Paraformaldehyde
PV	Parvalbumin
s.c.	Subcutaneous
SI	Substantia innominata
SIB	Substantia innominata, basal part
st	Stria terminalis
US	Unconditioned stimulus
VP	Ventral pallidum

CHAPTER 1

INTRODUCTION

1.1 Connection of Amygdala and BNST (extended amygdala)

In his seminal book, the *Expression of the Emotions in Man and Animals*, Charles Darwin emphasized two properties of all emotional expressions: they are the product of the evolutionary process, and they must strengthen the ability to adapt. He presented a wide range of emotional expressions in that they are universal in humans and animals (Darwin, 1872). After a long debate regarding the properties of the emotional expressions by him, a group of scientists, led by Paul Ekman, came up with convincing evidence that validates the propositions of Darwin (Ekman et al., 1987). A constituent of the six to seven basic emotions asserted by Ekman (Ekman, 1992), fear helps to form associations between aversive experiences and sensory input from the environment and increases the chance of our survival (Maren, 2001). It is an appraisal of and reactions to immediate danger, which starts quickly on facing physically or psychologically dangerous circumstances and terminates once perceived situations disappear. The rapid onset of reactions to fearful situations constitutes the adaptive benefit of emotions (fear) and mobilizes us unconsciously through our autonomous nervous system. The other adaptive property of the reactions to emotions (fear-related reactions) gives a chance to modify the adaptive response easily since these reactions do not continue for a long time (Ekman, 1999). On the other hand, sustained fear, or anxiety, is induced by

psychologically or physically more distant or nonexistent threats. Since these threats are not proximate and not predictable due to their anonymous sources, reactions to anxiogenic situations lead to a state of alert and agitation in the organism. In their comprehensive review, Davis and Walker explained the difference of fear and anxiety definitions in terms of proximity to threat, duration of symptoms, and mediating brain regions (Michael Davis, Walker, Miles, & Grillon, 2009).

Revealing neuroanatomical regions or circuits encoding anxiety and fear memory help to understand etiology and provide information to develop selective clinical treatments without side effects. The amygdala and the bed nucleus of the stria terminalis (BNST) respectively mediate in the mammalian brain fear memory and anxiety (Michael Davis et al., 2009). In the early 1920s, Johnston put forward the existence of a link between these two structures constituted by the bed nuclei stria terminalis. His way of identifying the "bed of the stria terminalis" was an uninterrupted forebrain formation that also contains the centromedial segments of the amygdala (Johnston, 1923). Continuous cell formation and fibers in the caudal substantia innominata, which ties the centromedial amygdala to the bed nucleus of stria terminalis, were referred to as the ‘extended amygdala’ (Alheid & Heimer, 1988). These two forebrain structures, amygdala and the BNST, are at the core of the conception of the limbic system proposed by Paul McLean following Papez's suggestion about the involvement of the medial temporal lobe structures in emotion processing (Maclean, 1949; Papez, 1937). Nevertheless, the importance of the amygdala in emotional expression arose from the large medial temporal lobe lesioning study in monkeys. Lesions of large medial temporal lobe structures comprising the hippocampus, amygdala, and encompassing areas caused ‘psychic blindness’ such as loss of fear, hypersexuality, hyperorality, and

losing the concept alpha-dominance in monkeys, were defined as Klüver-Bucy syndrome (H Klüver & Bucy, 1937; Heinrich Klüver & Bucy, 1939). Later on, Weiskrantz demonstrated that limited lesions in the amygdala stimulate Klüver-Bucy syndrome on its own, which manifests the emotion-processing function of the amygdala (Weiskrantz, 1956). Although lesion studies have always attracted the attention of neuroscientists studying the amygdala, it became one of the most studied regions after the introduction of the fear conditioning paradigm. A neutral stimulus, such as light or sound, is associated with an aversive stimulus, in general a mild-foot shock. A couple of repetitions is sufficient to elicit fear reactions, such as freezing, caused by aversive stimuli to the initial neutral stimuli. This association between the neutral and aversive stimuli is highly resistant to extinction, and the nature of this paired reaction is acknowledged as a good model for a wide variety of disorders such as simple phobias and anxiety (M. Davis & Whalen, 2001; M Davis, 1992; Rosen & Schulkin, 1998; Sah, Faber, Lopez de Aementia, & Power, 2003).

1.2 Amygdala

The amygdaloid complex consists of more than ten nuclei. These nuclei and their subareas are characterized based on their afferent and efferent connections, cellular composition, and histology (Krettek & Price, 1978). Although the considerable effort put in to enlighten the structure and function of the amygdaloid nuclei, there is no consensus on its nomenclature (J. LeDoux, 2007). A well-known idea, which argues that

the amygdala comprises rudimentary and evolutionarily recently-developed brain regions, divides nuclei of the amygdaloid complex into three parts: basolateral nuclei, cortical nuclei, and centromedial nuclei. The lateral nucleus, the basal nucleus (also known as the BLA), and the accessory basal nucleus constitute the basolateral nuclei/complex (BLC). Close to the surface of the brain, ventrally located to the basolateral complex, the collection of nuclei is named cortical nuclei. The central amygdala (CeA), medial amygdala (MeA), and the parts of the BNST that proximate the amygdaloid complex form the so called centromedial nuclei (Johnston, 1923; A J McDonald, 1982; Alexander J McDonald, 1998; Sah et al., 2003). On the other hand, it is proposed that we cannot classify the amygdala neither structurally nor functionally as a separate region. It stresses that while the lateral and basal amygdala nuclei are part of the cortex phylogenetically, the medial and central amygdaloid nuclei are extensions of the ventral striatum (J. LeDoux, 2007; Swanson & Petrovich, 1998). The present study is based on the traditionally-delineated nuclei and approach the amygdala as a separate region. There are reciprocal connections among the BNST, substantia innominata and the amygdala. Thus we should think of them as distinct but interrelated components of the amygdaloid complex (Alheid & Heimer, 1988).

Afferent and efferent connections of the amygdala are revealed by tract-tracing studies. In addition to the sensory stimuli from all modalities (visual, auditory, somatosensory, gustatory/visceral, etc.), the amygdala also receives polymodal inputs frWhile the first set of inputs originated from cortical and thalamic regions and plays a role in feeding the amygdala with sensory information and memory-related information, the second set of inputs that come from the hypothalamus and brainstem participate in starting behavioral actions and autonomic response. The neocortex provides a majority

part of the sensory inputs that arrive in the amygdala (Alexander J McDonald, 1998; Sah et al., 2003). To be precise, the lateral nucleus of the basolateral amygdala is the hub that gathers sensory information from sensory systems (J. LeDoux, 2007). There is convincing evidence about the role of the lateral amygdala nucleus in the acquisition of fear memories and it was shown that inactivation of the lateral nuclei interfere with acquiring fear conditioning (Amorapanth, LeDoux, & Nader, 2000; Goosens & Maren, 2001).

Following cortical and subcortical afferent of the amygdala, efferent projections target the same cortical and subcortical regions. The basolateral complex innervates parts of the medial temporal lobe related to the memory systems. For the traditionally accepted view, sensory inputs in different modalities enter from the basolateral complex, which includes the lateral amygdala, and are then transmitted to the output center of the amygdala, the central nuclei (CeA), in which they leave the amygdaloid complex (Sah et al., 2003). The expression of fear responses is induced by the central nuclei projections that terminate in the hypothalamic and brainstem systems, which are necessary to modulate the autonomic and endocrine changes in the brain (J. E. LeDoux, Iwata, Cicchetti, & Reis, 1988). The second output center of the amygdala, the BNST, also sends inputs to the hypothalamus and brainstem modulatory systems. These modulatory systems, targeted by both the CeA and the BNST, contribute to memory-related processes in the temporal lobe, and show their effects slowly and in a long-lasting manner compared to neurotransmitter activation (M. Davis & Whalen, 2001; Dong, Petrovich, & Swanson, 2001).

Furthermore, it was shown that topographical projections exist between the central nuclei of the amygdala (CeA) and the BNST (Dong et al., 2001). Sensory inputs

entering from the basolateral amygdala (BLA) are processed among nuclei of the amygdala and then transmitted separately to the medial (CeA_M) and lateral (CeA_L) division of the central amygdala to produce appropriate responses for threating or anxiogenic stimuli. Inputs from BLA to the CeA_M mediates fear and short-term responses. In contrast, inputs from BLA to the CeA_L which provide dense CRF-containing inputs to the lateral division of the BNST ($BNST_L$), mediates anxiety-related (long duration) reactions. CRF antagonist injections to the CeA and the BNST indicated that the CRF is involved in the reactions to sustained fear (anxiety), but not the phasic fear (simple phobias) reactions (Michael Davis et al., 2009; Walker, Miles, & Davis, 2009). Bilateral lesions of the BNST in rats, a previous study from our lab, increased immobility during uncontrollable and inescapable stress conditions created by the forced swimming test (Schulz & Canbeyli, 2000). The increased immobility observed in the forced swimming test, which induce the prolonged anxiogenic state, would be more meaningful if considered as part of the freeze response, which is an important measure for evaluating depressive behavior in rodent models (Unal & Canbeyli, 2019).

1.3 The Bed Nuclei of Stria Terminalis (BNST)

Similar to the organization of the amygdala, the BNST consists of several nuclei, and these nuclei are mainly classified as anterior and posterior. At least twenty separate cell groups are identified in anterior-posterior axis. The anterior division nuclei are also further sectioned into anterolateral, anteromedial and anteroventral. The idea of dividing

the BNST into anterior and posterior divisions is supported by functional experiments (C. C. Crestani et al., 2013). While posterior BNST nuclei are studied for sexually dimorphic mating behavior (Liu, Salamone, & Sachs, 1997), the anterior division is densely investigated for anxiety due to receiving inputs from the central amygdala (Gungor & Paré, 2016). Not only taking extensive inputs from the central and medial amygdala, the BNST gets inputs from the hippocampus, another key limbic structure, and receives limited inputs from the medial prefrontal cortex. Apart from its reciprocal connections with the limbic system structures and medial prefrontal cortex, as mentioned above, the BNST provides inputs to the regions such as the hypothalamus and the brainstem, involved in the modulation of the autonomic, hormonal, and behavioral responses to the stressful conditions. In control of hypothalamic pituitary adrenal (HPA) axis activations to stressful situations, the BNST is critical in the stress response. Certain BNST nuclei provide monosynaptic inputs to the corticotropin releasing hormone (CRH) releasing cells of the hypothalamic PVN, which then modulates the output of the HPA axis. By considering its reciprocal connection with the cortical and subcortical structures, it was speculated that the BNST is a relay station, through which different cell groups activate, between the limbic system structures and stress/anxiety response mediating brain regions (C. Crestani et al., 2013; Herman et al., 2003).

However Davis et al. (2009) proposed that the BNST is essential only for expressing anxiety (chronic stress) but not for acute fear, memory or imminent threat (acute stress) reactions (Michael Davis et al., 2009). Although widely accepted in rodent and human literature, Gungor and Pare (2016) criticized this limited role of the BNST in a proposed model on elicited stress reactions. Recent findings which indicate that the

BNST modulates responses to the discrete threat through its projections to the central amygdala contradict the propositions of Davis's model. It was shown that different segments of the BNST contain various cell types, which perform different and sometimes opposite functions. Therefore, inference of the lesion studies targeting the BNST studies should be carefully interpreted since a mixture of postulated cell groups might be included. More importantly, Gungor and Pare state that the amygdala and the BNST are not in dissociation in creating responses to stimuli that arouse fear and anxiety. They expressed that they both structures act in cooperation in the handling of the processing of stimuli that trigger fearful and anxiogenic situations. These complementary roles of both the amygdala and the BNST in the expression of fear and anxiety necessitate precise network activity (Michael Davis et al., 2009; Gungor & Paré, 2016). Coordination of these regions belong to two limbic systems might be carried out by the basal forebrain, as it is reported in the hippocampus (Tamás F Freund & Antal, 1988). Let me explain the role of the basal forebrain projections in the hippocampus and the other subcortical and cortical structures before turning to their role in the amygdala and the BNST.

1.4 Hippocampus

The hippocampus, a relatively large part of the limbic system, has a central role in acquiring new declarative memories and managing spatial navigation (John O'Keefe & Recce, 1993; Scoville & Milner, 1957). Declarative memory, requiring conscious

awareness, is originally divided into two distinct types: episodic (events) and semantic (facts) (Tulving, 1972). Electrophysiological recordings from freely moving animals showed that a group of hippocampal pyramidal cells, known as place cells, encode specific locations within an environment (J O'Keefe & Dostrovsky, 1971). Even though the hippocampus is an archicortex buried beneath the neocortical mantle, it has access to the sensory information about the environment (Unal, 2019). Around the same time with the discovery of the spatial encoding properties of the hippocampal cells, the candidate for the cellular model of the memory was revealed, which is the long-term potentiation (LTP) (Bliss & Lomo, 1973). LTP, proposed as a synaptic plasticity mechanism for encoding memories, is also admitted as experimental support for Hebbian learning that relies on associative activity among pre-and post-synaptic cells (Kandel, Dudai, & Mayford, 2014). As well as the hippocampus, later reports showed that LTP is detected in other regions comprising neocortical areas, the amygdala, and the reward-related midbrain regions (Caporale & Dan, 2008; Clugnet & LeDoux, 1990).

The coordinated activity of different cell groups, such as pyramidal cells and interneurons, were recorded in various behavioral states. It was shown that powerful theta oscillations (4-10 Hz) are present in all studied mammalian hippocampus except for bats (Gyorgy, 2002; Yartsev, Witter, & Ulanovsky, 2011). Theta-range oscillations, associated with memory acquisition, exploratory behaviors, and REM sleep, are also present in the other limbic system structures, such as the amygdala and the entorhinal cortex (Buzsáki & Moser, 2013; Gyorgy, 2002; Paré & Collins, 2000). The theta oscillatory activity is vital for regular hippocampal activity. Interrupting this rhythm leads to severe learning difficulties, as observed in behavioral studies with rats that underwent hippocampal lesions (Mitchell, Rawlins, Steward, & Olton, 1982; Winson,

1978). As the phase of the theta oscillation is critical in the emergence and direction of the synaptic plasticity (Gyorgy, 2002; Hölscher, Anwyl, & Rowan, 1997; Huerta & Lisman, 1993), scientists aimed to discover a rhythm generator for the hippocampal theta oscillations. The medial septum and diagonal band of Broca, two neighboring basal forebrain regions, were initially designated as “pacemakers” as their lesions abolish the theta activity in the hippocampus (Mitchell et al., 1982; Rawlins, Feldon, & Gray, 1979; Unal, Joshi, Viney, Kis, & Somogyi, 2015).

1.5 Basal Forebrain

The basal forebrain (BF) is comprised of several subnuclei, including the medial septum (MS), ventral pallidum (VP), diagonal band of Broca (DBB), and substantia innominata (SI). In addition to modulating cortical activity, these complicated basal forebrain regions are involved in attention, memory, motivation, and some neurological diseases such as Alzheimer's disease (I Gritti et al., 2006; Zaborszky, van den Pol, & Gyengesi, 2012). The basal forebrain nuclei provide dense cholinergic and GABAergic inputs, as well as some glutamatergic and peptidergic projections, to the cerebral cortex and various limbic system structures, including as the hippocampus and the amygdaloid complex (Carlsen, Záborszky, & Heimer, 1985; McKinney, Coyle, & Hedreen, 1983; M-M. Mesulam, Mufson, Wainer, & Levey, 1983; Ottersen, 1980; Zaborszky, Pang, Somogyi, Nadasdy, & Kallo, 1999). As a well-studied animal model for Alzheimer's disease (AD), the role of basal forebrain cholinergic neurons in memory has been a

major focus of research for two decades. It was shown that more than half of the ChAT-containing cells in the temporal cortex and hippocampus were reduced in AD patients compared to healthy people. The relatively large cholinergic neurons of the nucleus basalis magnocellularis (Meynert) were studied in detail for their role in Alzheimer's Disease. Degeneration of cholinergic projections that originate from the basal forebrain and target the limbic system -including the hippocampus and amygdala- were identified to be responsible for cognitive decline in AD patients (Dunnett, Everitt, & Robbins, 1991; M.-Marsel Mesulam, 2013). However, loss of cholinergic nucleus basalis magnocellularis neurons do not form a comprehensive model to explain all the symptoms of the AD (Dunnett et al., 1991). The BF cholinergic neuron projections alone were thought to be of great importance in the modulation of cortical activity, which affects memory and motivation. However, lesion studies that selectively target the cholinergic projections of the BF showed that interruptions of these pathways were different from lesioning the GABAergic and cholinergic BF neurons altogether (Gerashchenko, Salin-Pascual, & Shiromani, 2001). It was shown that cholinergic neurons make up only 50% of the entire cellular population of the BF. In contrast, GABAergic neurons account for 35% of the whole cell population in the BF. Since then, the importance of the GABAergic neurons in the modulation of the basal forebrain mediated modulation of the upstream network activity has been understood, and studies involving the manipulation of GABAergic cells have begun to take more space in the literature (I Gritti et al., 2006).

CHAPTER 2

LITERATURE REVIEW

GABAergic BF neurons send long-range projections to the neocortex (Fisher, Buchwald, Hull, & Levine, 1988) and the hippocampus (Köhler, Chan-Palay, & Wu, 1984). The BF GABAergic neurons mainly target the interneurons in the hippocampus and neocortex (T. F. Freund & Buzsáki, 1996; T. F. Freund & Meskenaite, 1992; Henny & Jones, 2008). The interneurons of the hippocampus are inhibited by the GABAergic medial septum neurons, which give rise to disinhibition of the pyramidal neurons, in other words, indirect activation of hippocampal pyramidal neurons. These BF GABAergic medial septum projections, together with the BF cholinergic projections, mediate in generating hippocampal theta oscillations (4-10 Hz), which correlate with spatial navigation and other hippocampal learning and memory forms (Borhegyi, Varga, Szilágyi, Fabo, & Freund, 2004; Tamás F Freund & Antal, 1988; Pascual, Pérez-Sust, & Soriano, 2004; Petsche, Stumpf, & Gogolak, 1962; Unal et al., 2018, 2015). Early studies show that not only the GABAergic inputs (Köhler et al., 1984) but also cholinergic inputs (Shute & Lewis, 1967) of the medial septum target the hippocampus and neocortex. However, the target selectivity and function of these GABAergic and cholinergic projections seems to be distinct. Cholinergic BF projections target both pyramidal neurons and interneurons, while BF GABAergic projections only target interneurons in the hippocampus. This selectivity might be the underlying reason enabling them to fulfill their unique functions (Unal et al., 2018, 2015). The cholinergic inputs have been mainly subject to long-term synaptic plasticity studies. Infusion studies

indicate that cholinergic inputs are responsible for the encoding of new declarative memories (Hasselmo, 2006) whereas the basal forebrain GABAergic inputs usually target GABAergic interneurons in cortical and subcortical structures and involve in the modulation of network oscillations, such as the theta rhythm (Dragoi, Carpi, Recce, Csicsvari, & Buzsáki, 1999; Somogyi, Katona, Klausberger, Lasztóczi, & Viney, 2014).

Following early studies showing that BF also provides inputs to the amygdala, it was revealed that BF neurons primarily target the basolateral nucleus of the basolateral amygdaloid complex (BLC). One group of projecting cells was identified as cholinergic neurons (Mascagni & McDonald, 2009; Woolf, Eckenstein, & Butcher, 1983). While about 75% of the BF neurons projecting to the basolateral amygdala are ChAT-positive, 25% of such cells are non-cholinergic (Carlsen et al., 1985; Záborszky, Heimer, Eckenstein, & Leranth, 1986). The GABAergic cells are estimated to constitute 10-15% of all amigdalopetal basal forebrain projecting neurons (Mascagni & McDonald, 2009). GABAergic interneurons in the amygdala are the target of 80-90% of the GABAergic BF projections (McDonald, Muller, & Mascagni, 2011). Labelling these long range BF GABAergic projection neurons for calcium-binding proteins revealed that all parvalbumin (PV) positive cells are GABAergic and they are mostly found in the SI/VP (Ivana Gritti, Manns, Mainville, & Jones, 2003). Previous studies indicated that stimulating the SI and VP regions of the brain suppressed putative inhibitory neurons in the basolateral amygdaloid complex (Mello, Tan, & Finch, 1992).

Moreover, similar to inputs from the BF GABAergic neurons to the two main types of cells of the basolateral amygdala, there are also inputs from BF cholinergic neurons (Muller, Mascagni, & McDonald, 2011). These BF cholinergic projection cells may discharge at theta frequency ranges (Maan Gee Lee, Hassani, Alonso, & Jones,

2005). It is suggested that, similar to the hippocampus (M G Lee, Chrobak, Sik, Wiley, & Buzsáki, 1994), both cholinergic and GABAergic BF inputs might lead to the emergence of theta rhythms in the basolateral amygdaloid complex (McDonald, Muller, & Mascagni, 2011). There is compelling evidence in the early literature that the amygdala participates in the consolidation of emotionally arousing event memories. While expecting a noxious stimulus or intense arousal periods, the theta oscillations prominently exist in the amygdala. It is worth noting that these amygdaloid theta oscillations appear during either negatively or positively valanced circumstances (Paré & Collins, 2000). Based on the consistency seen between hippocampal and amygdaloid theta activity, it was suggested that amygdaloid theta oscillations link temporal lobe and declarative memory-associated cortical storage areas by providing interactions between these regions (Paré, Collins, & Pelletier, 2002).

BF GABAergic neurons provide dense projections that target mainly PV+ GABAergic basolateral amygdaloid interneurons and, to a less(er) extent, to the principal neurons in the basolateral amygdaloid complex (BLC). As in the hippocampus, inhibition of the interneurons in the basolateral amygdala may cause indirect activation (disinhibition) of principal neurons in the BLC (Mcdonald et al., 2011; Tóth, Freund, & Miles, 1997). The BF GABAergic projection neurons fire at theta frequency, and inputs from these neurons to the hippocampus (Borhegyi et al., 2004) and neocortex (Lin, Gervasoni, & Nicolelis, 2006) generate theta and gamma frequency oscillations. Accordingly, related inputs to the basolateral amygdala are thought to play a part in inducing similar oscillations that might affect emotional arousal and the formation of emotional memory akin to the role of those oscillations in the lateral nucleus (McDonald et al., 2011; Pape, Narayanan, Smid, Stork, & Seidenbecher, 2005; Paré & Collins,

2000). In fact, the importance of the local GABAergic interneurons to control the long-term potentiation (LTP) in the lateral nucleus of the basolateral amygdaloid complex's projection neurons (LA), which is vital for the acquisition of emotional memories, was revealed (Bissieére, Humeau, & Luüthi, 2003; M. Davis & Whalen, 2001; X. F. Li, Armony, & LeDoux, 1996).

These two disinhibitory patterns that are seen in subcortical limbic system structures, including hippocampus and amygdala, are not shown in the BNST, the other limbic system structure. A retrograde tracing study combined with fluorescent immunohistochemistry from our laboratory was carried out to test whether the same BF GABAergic disinhibitory mechanism pattern occurs in the BNST. The results show that from the BF GABAergic, particularly the calcium-binding protein calbindin-expressing neurons, and to a lesser extent parvalbumin-immunopositive neurons, send projections to the BNST. These long-range BF GABAergic neurons targeting the basolateral amygdala and the BNST particularly emerge from the ventral pallidum and the substantia innominata (Tuna & Ünal, 2021).

2.1 Ventral Pallidum

The ventral pallidum (VP), located ventrally to the anterior commissure and covers the sub commissural section of the substantia innominata, was first identified as a distinct region that plays a critical role as an output center of the ventral striatum (nucleus accumbens) by Heimer and Wilson (1975). The VP was proposed as a hub point

receiving the hedonic, motivational signaling from limbic system, and thus final point for movement to reward. However, it does not mean that the VP on its own mediates all reward processing but rather it could be expressed that for the processing of the signals related to the reward and motivation the VP is one of the critical regions in the brain (Haber & Knutson, 2010; K. S. Smith, Tindell, Aldridge, & Berridge, 2009). In addition to this, the VP is involved in processing the emotional inputs (Haber & Knutson, 2010). There are two main parts of the VP, the dorsolateral and the ventromedial sections, characterized according to the inputs they receive and send. A principal constituent of the ventral striatum, two sections of the nucleus accumbens (Acb) send their inputs to distinct parts of the VP. The dorsolateral VP (VPl) receives inputs from the nucleus accumbens core (AcbC), whereas the ventromedial VPm gets inputs by the nucleus accumbens shell (AcbSh) (Tripathi, Prensa, & Mengual, 2013). Due to relaying these dense projections from the nucleus accumbens to the brainstem, it was asserted that the VP is the convergent region where limbic motivational indications were converted into the motor output (Mogenson, Jones, & Yim, 1980). However, that only constitutes one part of the ventral pallidum's role. The VP also receives reward-related inputs from the amygdala, prefrontal, orbitofrontal, and infralimbic cortex, lateral hypothalamus, ventral tegmental area, and from the other regions. With these connections to the limbic system and higher cortical areas, it can be expressed that the ventral pallidum not only mediates transforming motivational signals into movement but mediates reward and motivation related at various levels in the brain (K. S. Smith et al., 2009). Unfortunately, the cell-type diversity of VP makes it difficult to comprehend its exact role in the aforementioned complex cognitive processes. In addition to dominance of the GAD65 and GAD67 expressing GABAergic neurons in each subcomponent, calcium-binding

protein cells, including calbindin, calretinin, parvalbumin, and some peptidergic cells groups such as neuropeptide Y and somatostatin, are also expressed in the VP (Root, Melendez, Zaborszky, & Napier, 2015; Zaborszky et al., 2012).

As discussed above, neuronal tracing studies show that the ventral pallidum sends projections to the basolateral amygdala. While cholinergic neurons make up 75% of these projections (Carlsen et al., 1985), GABAergic neurons constitute 10-15% of these projections (Mascagni & McDonald, 2009). Even though the role of the ubiquitous GABAergic neurons in the ventral pallidum is associated and studied with several cognitive tasks processing including food intake, locomotion, digging (K. S. Smith & Berridge, 2005), rearings, water consumption, aversive taste reactivity (Shimura, Imaoka, & Yamamoto, 2006), maternal behaviour (Numan et al., 2005), and alcohol seeking behavior (Harvey et al., 2002), the role of GABAergic neurons in the anxiety, fear memory and depression were not fully explored. The aim of the present selective inactivation study is to reveal whether BF GABAergic projections arising from the ventral pallidum and targeting the amygdaloid complex have a role in creating pathological fear and anxiety reactions observed in disorders including depression, anxiety, and post-traumatic stress disorders. Another contribution of this study to the literature is the investigation of the Ventral Pallidum GABAergic neurons in both explicit (i.e. Morris Water Maze) and implicit memory (i.e. Pavlovian Fear Conditioning) tasks within the same design.

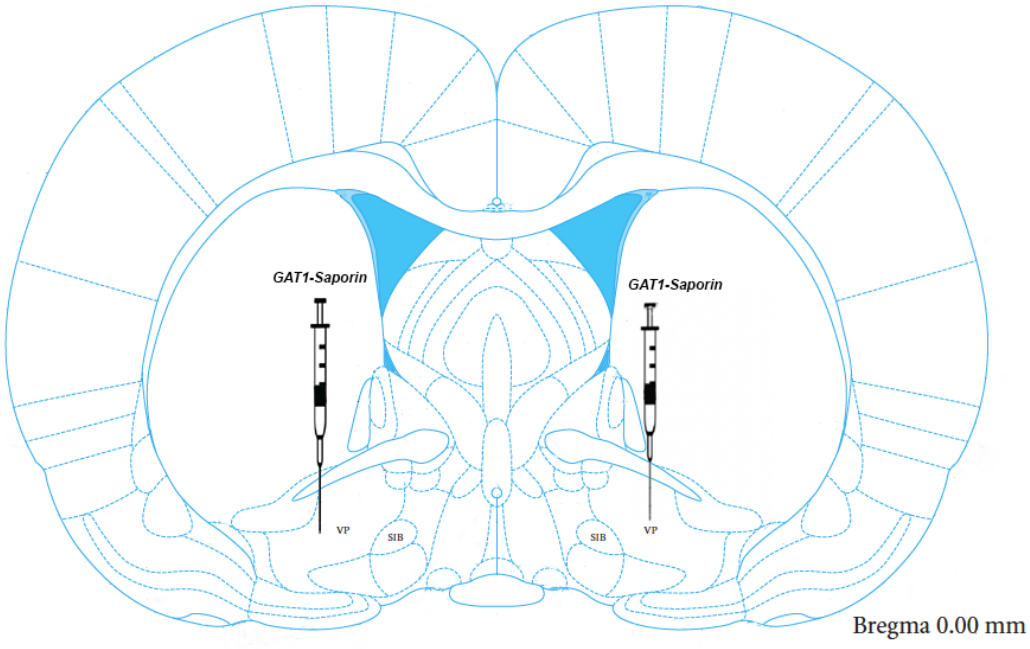


Figure 1. GAT1-Saporin targeting the ventral pallidum and substantia innominate using the Paxinos and Watson's coronal atlas (2007), Check abbreviations.

CHAPTER 3

MATERIAL AND METHODS

3.1 Animals

Adult male Wistar rats weighing 240-320 g (n = 16) housed in the Behavioral Neuroscience Laboratory at Boğaziçi University were used. All animals were housed as groups of four (21 ± 1 °C; ~ 50% humidity; 12:12 day/night cycle with lights on at 8:00) with *ad libitum* access to food (rat chaw) and tap water. All procedures were approved by Boğaziçi University Ethics Committee for the Experimental Use of Animals in Scientific Research.

3.2 Surgical Procedures

Animals were anesthetized using intraperitoneal injection (i.p.) of a mixture of ketamine (80 mg/kg) and xylazine (10.0 mg/kg). After the loss of reflexes, heads were shaved, and the animals were placed to a stereotaxic frame. Throughout the surgeries, muscle reflexes checked regularly, and if necessary, additional anesthetic given. A heating pad and infra-red lamp kept the body temperature at approximately 36 °C. An eye cream was used to avoid drying of the eyes. Before the skull was exposed, a local anesthetic spray

(Vemcaine, 10% lidocaine) and antiseptic (Batticon) were administered on the surface of scalp. Following the absorption, starting from the back of the eyes, the skull was exposed through a vertical incision by using a lancet. The exposed skull was cleaned using saline and the Bregma point determined with a custom-made surgical microscope. The injection coordinates were calculated from a rat brain atlas with reference to the Bregma point (VP: AP = 0.00 , ML = ±2.20, DV = −7.60) (Paxinos & Watson, 2007). Bilateral craniotomies performed above the injection sites (Right VP and Left VP) according to their coordinates (Paxinos & Watson, 2007). Injections were made to the previously defined location reference to the dura mater in the dorsal-ventral axis by a Hamilton injection syringe. With the help of a precise injector pump made according to the size of the Hamilton syringe, agents were slowly injected in desired volume and speed. The syringes were retracted after waiting ten minutes to prohibit dorsal diffusion. Gat1-Saporin (325 ng/ µl, 0.5 µl and at 0.1 µl/min ratios) injections given in each hemisphere in twelve animals (n = 8). In the sham (control or vehicle) condition for the Gat1 saporin administered group, 0.9 % saline (NaCI) was administered in the exact coordinates to the eight animals (n = 8) with the same procedure used in saporin administration. After injection administration into the region of interests, the skull was cleaned, and the incision was firmly sutured. Local anesthetics (Anestol pomade, 5% lidocaine and Jetokain, 5 mg/kg, s.c.) and an antiseptic (Batticon) were applied to the stitch to prevent future infections. To avoid dehydration during the surgery and speed up the recovery phase, animals were given 0.9 % saline (2 ml, i.p.). They took the post-operative care in which they placed under the infrared lamp and regularly watched until they wake up. Their general clinical symptoms checked and scored during the following days, the physical condition of the surgery place, and behavioral indications of pain

using a post-operative evaluation checklist (see Appendix). Additionally, the food and water intake were recorded daily. When animals showed mild indication of pain, they were given an additional dose of Jetokain (5 mg/kg, s.c.). For complete recovery and the completion of Saporin-based elimination of specific cells, there was a waiting time of 7-14 days. Subsequently, animals transcardially perfused with 0.9% saline and 4% paraformaldehyde (PFA) (Check for methods, Tuna 2021).

3.3 Experiment: GAT1-Saporin Injection

To elucidate the possible effect of the GABAergic inputs originating from ventral pallidum targeting the amygdala and the BNST, we used GAT1-Saporin (a ribosome-inactivating toxin that causes cell death) to selectively eliminate the GABAergic cells. Findings from our laboratory, confirming the result of McDonald (McDonald et al., 2011), revealed that VP GABAergic neurons provide dense inputs to the amygdala, and also target neurons in the anterior and central BNST. Based on this data, I tested the hypothesis that eliminating VP GABAergic neurons causes impairment of conditioned fear learning in the amygdala and alerts the anxiety response emerging from the BNST.

3.3.1 Colchicine Injection

The somata of GABAergic neurons, not only the interneurons but also the long-range ones, contain less amount of GAD and GABA due to their fast axonal transportation to the axon terminals after translation. This causes a difficult problem in detecting this major neurotransmitter with immunohistochemistry. Colchicine (100µg diluted in 10.0µl of saline) was injected into the bilateral ventricles of randomly chosen two animals from each group to interrupt microtubules enabling axonal transport. The immunohistochemistry was performed on four colchicine-injected rats. Animals were taken to post-operation care for one day after colchicine injections, and then anaesthetized and perfused with 4% depolymerized paraformaldehyde.

3.4 Behavioral Tests

3.4.1 Forced Swimming Test

In the forced swimming test (FST), rats were placed into a water-filled cylinder, in which they cannot stand on their legs and should swim to avoid drowning (the depth of water is 30 cm, at 25 ± 1°C). Each animal was taken to the test twice with 24 hours break between the pre-test and the test session. While the pre-test takes 15 minutes and is treated as an acclimation period, the main test starts after 24 hours and takes 5 minutes in rats. Animals were assessed for the second day (test) scores. Their behavioral reactions, classified as active behaviours, including swimming, struggling, climbing,

head shaking, and as passive behavior (i.e. immobility) were compared in experimental and control groups (Porsolt, Bertin, & Jalfre, 1978). Immobility signifies behavioral despair, a rodent model of depressed behavior (Unal & Canbeyli, 2019).

3.4.2 Open field test

In the open field test, animals were placed into a box (70x70x45 cm) with a 45 cm high height that prevented their escape. The experiment took 5 minutes for each animal. A lux meter was used to test the light intensity at the box's corners and center to check that the lighting conditions were considerably different. Several dependent variable measurements were recorded, including time spent in the center and peripherals of the maze, time spent on the move, the number of rearings, pace of the movement, and distance travelled in the maze.

3.4.3 Elevated plus maze (EPM)

The EPM consists of two open (transparent acrylic) and two closed (wooden opaque) arms positioned to form a shape like a cross by cutting each other. Animals were placed in the middle of the maze, and their face would be toward an open arm. Experiments continued for 5 minutes, and during which spent time the open and closed arms and the

number of alternations among those arms were recorded with the help of a deep learning-based tracking program and were compared between groups (Mathis et al., 2018; Pellow, Chopin, File, & Briley, 1985; Slattery & Cryan, 2012). A light meter was used to test the light intensity at the box's corners and center to check that the lighting conditions were considerably different. Time spent in the closed arms indicates anxiety-like behavior.

3.4.5 Morris Water Maze (MWM)

The Morris water maze evaluates hippocampus-dependent spatial learning in rodents with the use of distal cues in a large, water-filled tank. The animals were submerged under 30 cm of water into a specialized platform which is 122 cm in diameter. The purpose of this behavioral test is for the animal to find a platform (10 cm in diameter) which is covered by approximately 1-2 cm of water to alleviate them from the unpleasant swimming conditions. The water was kept at around 21 ± 1°C. The animal was placed at random starting points along the tank's perimeter to navigate throughout the maze using proximal/distal cues to help locate the hidden platform. The semi-random set of start locations can be found in Table 1. The cues were attached to the external wall of the tank. Each trial lasted for 2 minutes, with a 15 second intertrial interval time (ITI). If the animals were unable to find the platform within this time frame, they were guided to and placed on the platform for 10-15 seconds. Each day consisted of four trials in which the time spent to find the platform (escape latency), the

movement of the animals, and the time spent in each quarter was encoded with DLC. The experiment also consisted of one probe trial in which animals were taken for only one trial which lasted for one minute without the platform in the tank. During the probe trial, the animals spent time in the target quarter in which the movement of the animals was recorded and analyzed (Vorhees & Williams, 2006).

3.4.6 Fear Conditioning

The fear conditioning chamber was (25)x(40)x(20) cm in dimension with a 16-metal grid floor spaced 2.5 cm apart and was connected to a custom-made shock provider and a custom Arduino setup with a speaker mounted on a back wall. The sound and corresponding shock's onset and duration were controlled via Arduino. A camera was mounted above and in front of the chamber to capture all activity throughout the experiment.

Once the animals were taken from their home cages, they were placed on a counter in the test room for five minutes. They were then placed into the chamber for an additional three-minute acclimation time in the conditioning box. The chamber was cleaned with 70% alcohol before and after each use to eliminate olfactory cues. The test phase then commenced at the end of each acclimation phase. Both testing and acclimation phases were carried out in dim light.

The first experimental day consisted of testing phases which consisted of five delay conditioning trials. A foot shock (2 sec at 1.0 mA) was given directly after a tone

(80 dB, 2kHz, 6 sec) in which the tone lasted for a duration of 60 seconds. After a three-day waiting period, the fourth experimental day consisted of the same acclimation phases followed by the test phases. This experiment consisted of twelve trials in which only the tone was presented. The last experimental day, which took place on the seventh day, animals were again acclimated and placed into the testing environment which was physically and geometrically different than the previous chamber used for the other fear conditioning experiments.

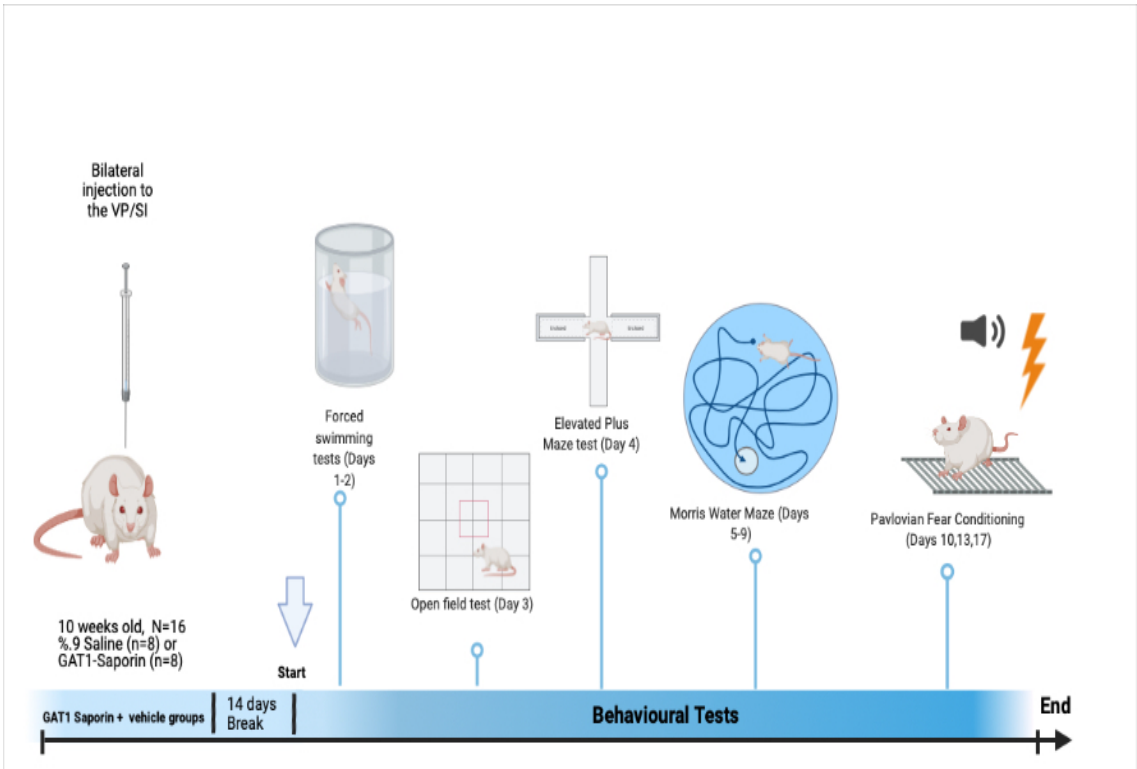


Figure 2. Timeline of behavioral experiments which includes a breakdown of the injections and behavioral tests. Drawn with Biorender.com.

3.5 Histology

After brains were taken out with transcardial perfusion, they were postfixed in 4% PFA for two nights at 4°C. Later, they were rinsed in 0.1 M phosphate buffer (PB) (3x10 min) to purify from PFA. Coronal (frontal) sections of 40-60 μm thickness were taken utilizing a Leica VT1000 S vibratome. Consequently, the brain sections were examined with an Olympus BX43 epifluorescent microscope for whether injection sites (each ventral pallidum) were hit. This preliminary result helped us to decide whether aimed regions were hit.

3.6 Immunohistochemistry (IHC)

Immunohistochemistry (IHC) is a useful technique for diagnosis and experimentation and allows us to make visible biomarkers in the tissues. IHC technique is derived from a collection of information from three scientific fields: immunology, histology, and chemistry. Specific antibodies called primary antibodies, obtained from various animals (e.g., mouse, guinea pig, rabbit, goat) as an immune system reaction to the delivered antigens from outside, is used to bind antigens in the tissues in the first step. And these bindings are visualized in the second step with the help of fluorescence conjugated or other dyes (secondary antibodies), become visible under the epifluorescent or light microscope (Coons, 1958; Coons, Creech, & Jones, 1941; Ramos-Vara, 2005).

Unal et al. (2015) described how to conduct free-floating immunofluorescence labelling. Individual slices were transferred to glass vials and washed (3x10 mins) with 0.3 per cent Triton X-100 in PBS (PBS-TX) at room temperature (Unal et al., 2015). The sections were then submerged in a blocking solution comprising 20% NHS and 80% PBS-TX for 1 hour at room temperature. The slices were then incubated for 48-72 hours at 4 °C in primary antibody solutions in PBS-TX with 1% NHS. In a subset of slices, DAPI (1:2000, code:D3571, ThermoFisher) staining was used to further highlight the borders of distinct basal forebrain nuclei. After the final rinse process, slices were incubated in a DAPI solution in glass vials for 15 minutes. PBS-TX was then used to re-rinse the portions (3x10 mins). Finally, the sections were cover slipped and put on glass slides.

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

3.7 Microscopic observation and anatomical description

All animal brains added to the experiments were observed under microscopy. DAPI (1:2000, code:D3571,ThermoFisher), a blue-fluorescent dye that stains the center of the nucleus, was utilized to delineate the borders of the basal forebrain nuclei. Substance-P

(1:100-500, code: ab216412, Abcam) immunoreactivity was assessed to draw the borders for the ventral pallidum (Bigl, Woolf, & Butcher, 1982; Farrell et al., 2020). This co-staining enabled me to distinguish cortical and subcortical structures.

CHAPTER 4

RESULTS

4.1 Histology and Immunohistochemistry (IHC)

We analyzed our injection sites for randomly chosen eight animals and found that our injections targeted the ventral pallidum and substania innominata from the anterior-posterior (AP) axis from +0.36 to -0.48 (Fig 3A-B). The IHC analysis for PV and ChAT immunoreactivity showed that GAT1-Saporin injection killed the PV positive neurons, sparing the cholinergic neurons (Fig 3C-D). The PV positive cells in the vehicle group outnumbered the PV positive cells in the GAT1-Saporin group (Fig 3E-M). While there was a significant difference in the PV positive cells, there was no statistical difference in the ChAT positive neurons between groups (Fig 3C-D).

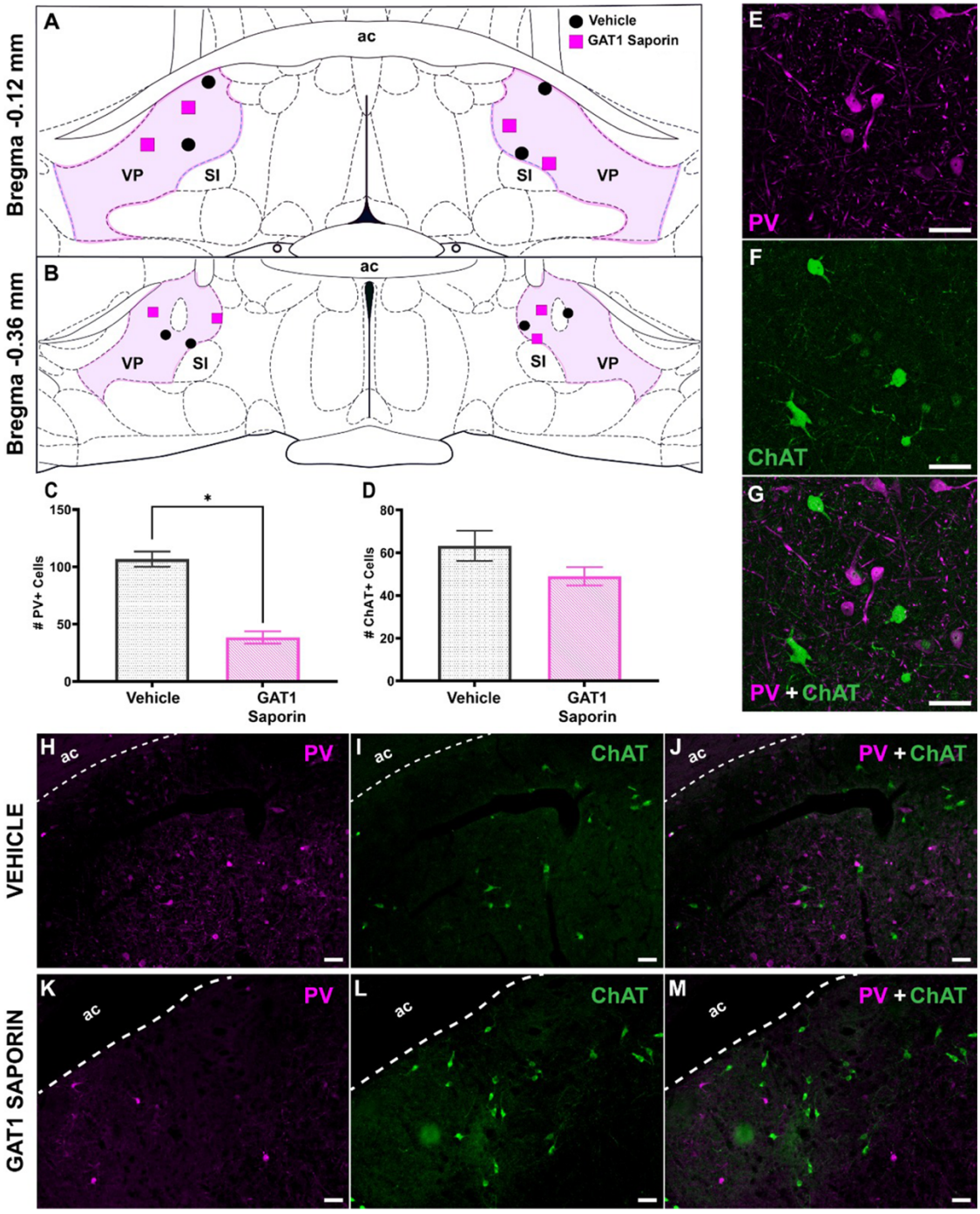


Figure 3. Intraventral pallidal injections of GAT1 saporin reduce the amount of PV+ cells but not ChAT+ cells in the VP. (A-B) Coronal sections displaying the centers of injection sites based on Paxinos and Watson atlas (2007). (C-D) The average number of PV+ and Chat+ cells in each examined region of the VP/SI in Vehicle and GAT1 saporin groups. (E-M) Confocal image showing specific labeling of ChAT+ and PV+ neurons in the VP of each experimental group's animals. SEM is indicated by error bars. All scale bars are 50 µm in length. ac = anterior commissure. * = $p < 0.05$

4.2 Forced Swim Test

The forced swimming test (FST), a reliable/widely used method for measuring psychomotor retardation and anti-depressant effect in the animal depression model, was carried out to see if any ameliorative effect of our (above) manipulation. GAT1-Saporin injection to the VP/SI reduced the immobility (freezing response) in response to depression inducing forced swim test on day 1 ($M_{Vehicle} = 79,43$, $SD_{Vehicle} = 38.04$, $M_{GAT1\text{-}Saporin} = 35.61$, $SD_{GAT1\text{-}Saporin} = 11.13$) (t= 3.127, df= 14, p= 0.0074) (Fig 4A) and day 2 ($M_{Vehicle} = 77,10$, $SD_{Vehicle} = 34.33$, $M_{GAT1\text{-}Saporin} = 48.03$, $SD_{GAT1\text{-}Saporin} = 14.51$) (t= 2.207, df= 14, p= 0.0446) (Fig 4B). Similarly, the observed climbing duration on forced swim test day 2 was more prolonged in GAT1 Saporin group ($M = 205.0$, $SD = 48.05$) than the vehicle group ($M = 142.8$, $SD = 44.49$) (t= 2.683, df= 14, p= 0.0178)(Fig 4C).

However, there was no significant difference between groups in terms of swimming duration ($M_{Vehicle} = 34,69$, $SD_{Vehicle} = 19.84$, $M_{GAT1\text{-}Saporin} = 30.63$, $SD_{GAT1\text{-}Saporin} = 15.45$) (Fig 4D) head shaking ($M_{Vehicle} = 48,13$, $SD_{Vehicle} = 8.127$, $M_{GAT1\text{-}Saporin} = 44.81$, $SD_{GAT1\text{-}Saporin} = 7.792$) (Fig 4E), or the number of diving on FST day 2 (Fig 4F).

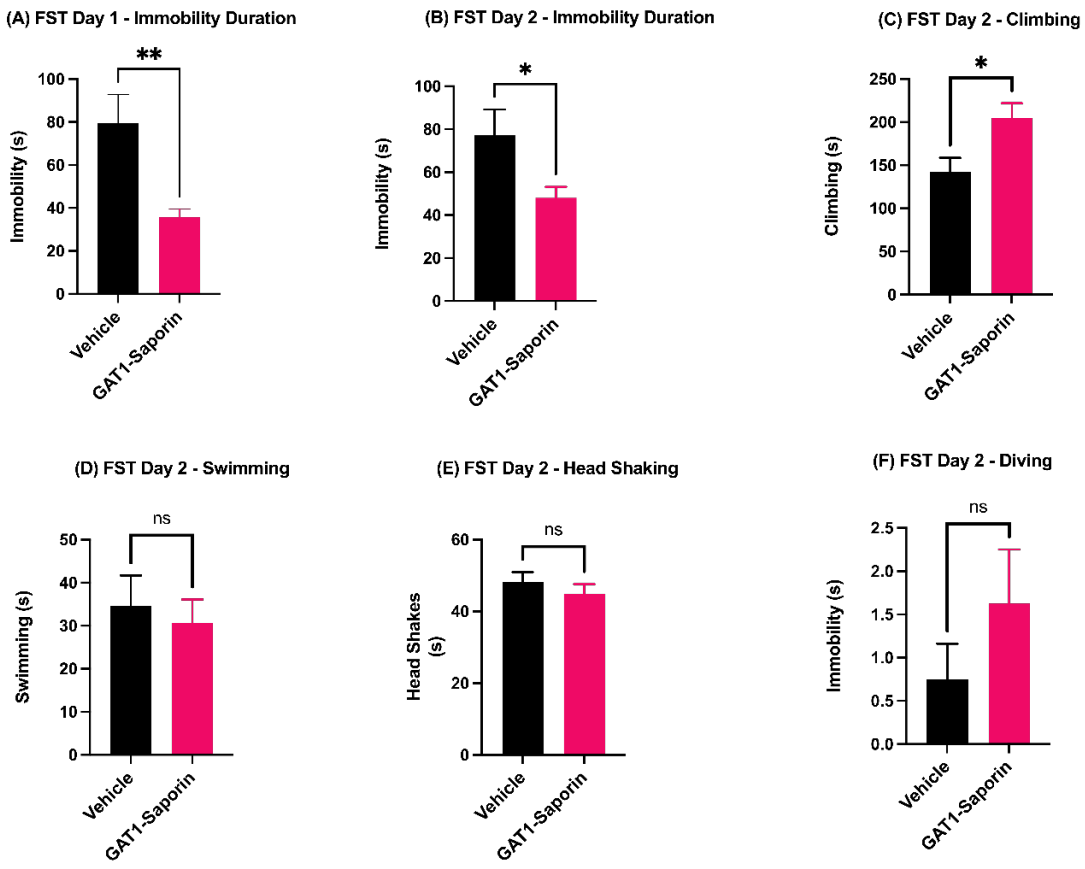


Figure 4. After GAT1-Saporin injections into the ventral pallidum, animals demonstrated less immobility on the second day of FST (A). On the first (A) and second (B) day of the FST, average durations of immobility is exhibited. Additionally, on the second day of the FST climbing (C), swimming (D), head shaking (E), and the number of diving (F) is shown. Error bars show SEM. * = $p < 0.05$

There were no group-level difference in the open field locomotor activity (Fig 5B), indicating that the observed antidepressant-like effects in the FST were independent of treatment-induced changes in general locomotor activity (see below).

4.3 Open Field Test

The open-field test was introduced to measure locomotor activity and the anxiety scores of the animals. These enabled us to see locomotor activity, movement disorders originated from manipulations, and anxiety levels of the animals. Regarding the time spent in the center or periphery of the maze, our tests revealed that GAT1-Saporin infusion to the VP/SI elicited no statistically significant changes between vehicle *(M = 29.01, SD = 15.71*) and saporin-injected rats (*M = 20.35, SD = 10.29*) (t= 1.305 , df =14 , p = 0.2130) (Fig 5A). Addition to that, there were no effect of our manipulation on the locomotory activity of the animals ($M_{Vehicle}$ *= 1301.0,* $SD_{Vehicle}$ *= 204.5,* $M_{GAT1\text{-}Saporin}$ *= 1618.0* $SD_{GAT1\text{-}Saporin}$ *= 747.3*) (t= 1.161, df=14, p= 0.2649) (Fig 5B).

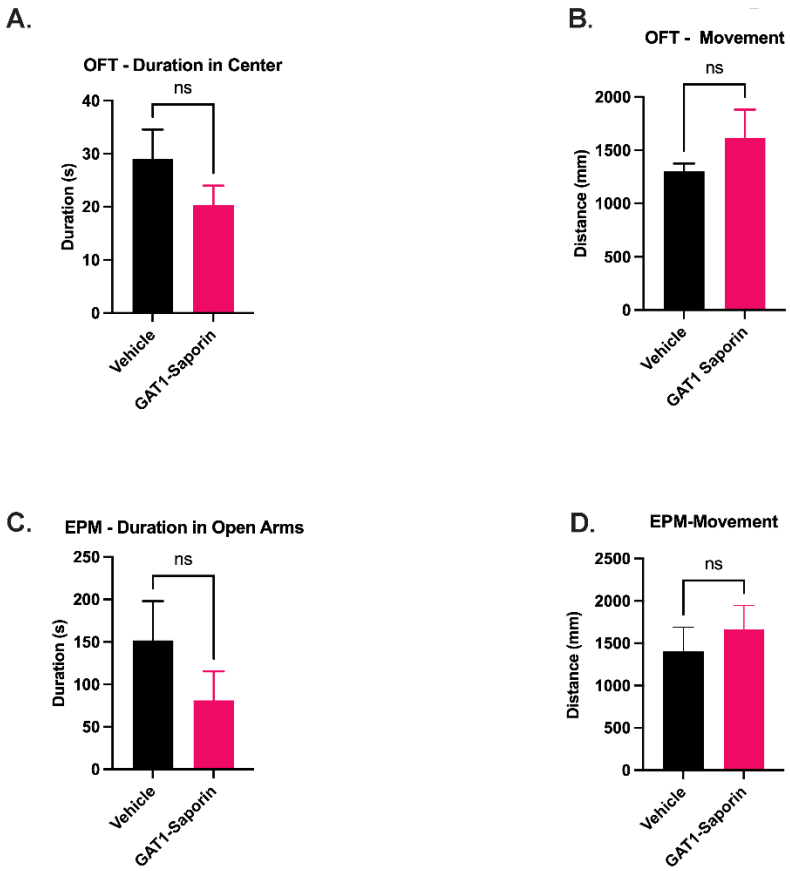


Figure 5. Graphs of behavioral tests indicating that GAT1 Saporin injections into the VP do not have an effect on the overall locomotor activity or anxiety-like behaviors. (A) The average duration spent in the center of the OFT test in seconds. (B) Average movement measured from the total distance (mm) throughout the OFT test. (C) The average time (s) spent in the open arms of EPM (D) General movement during EPM tests.

4.4 Elevated Plus Maze

Elevated plus-maze was originally introduced for its ability to test the efficacy of anxiety-inducing (anxiogenic) or anxiety-relieving (anxiolytic) drugs, but soon became a general anxiety-level screening tool. To test the effect of the saporin based elimination of GABAergic cells, in our experiment the EPM constituted one of the test measures anxiety-related behaviors. There was no statistical difference in the amount of time spent in the open arms between groups ($M_{Vehicle} = 151.8$, $SD_{Vehicle} = 122.7$, $M_{GAT1\text{-}Saporin} = 81.19$, $SD_{GAT1\text{-}Saporin} = 90.28$)(t= 1.226, df=12 p= 0.2438) (Fig 5C-D) Two animals' elevated plus-maze data were removed from the behavioral analysis because they spent less than 3 seconds exploring in the open arms throughout the trial.

4.5 Morris Water Maze

The escape latency of all groups of animals decreased over the four-day training period. The results showed a significant decrease in escape latency after four days of training

sessions (F (3, 42) = 21.52, p = 0.0001) (Fig 6A), indicating that the animals learned the location of the hidden platform.

There was no significant main effect of manipulation on the time it took the rats to find the platform (F (1, 14) = 1,531 p 0.2363) (Fig 6A), indicating that GAT1-Saporin treatment did not impair spatial learning and memory. A probe trial was carried out on the fifth day after the four-day training. The manipulations had no significant effect on the amount of time spent in the target quadrant ($M_{Vehicle} = 21.70$, $SD_{Vehicle} = 15.28$, $M_{GAT1\text{-}Saporin} = 23.28$, $SD_{GAT1\text{-}Saporin} = 12.76$) (t = 0.2245, df= 14, p = 0.8256). (Fig 6B) Similarly, during the probe trial, there was no group difference in time spent in the center zone of the water maze ($M_{Vehicle} = 34.47$, $SD_{Vehicle} = 11.73$, $M_{GAT1\text{-}Saporin} = 37.14$, $SD_{GAT1\text{-}Saporin} = 10.39$) (t = 0.4827, df= 14, p = 0.6368) (Fig 6C). The average distance traveled (t = 0.3412, df=14 ,p= 0.7380) and average speed (t = 1.318, df=14 ,p= 0.2088) of the animals in the probe trial were both unaffected by manipulation (Fig 6D-E).

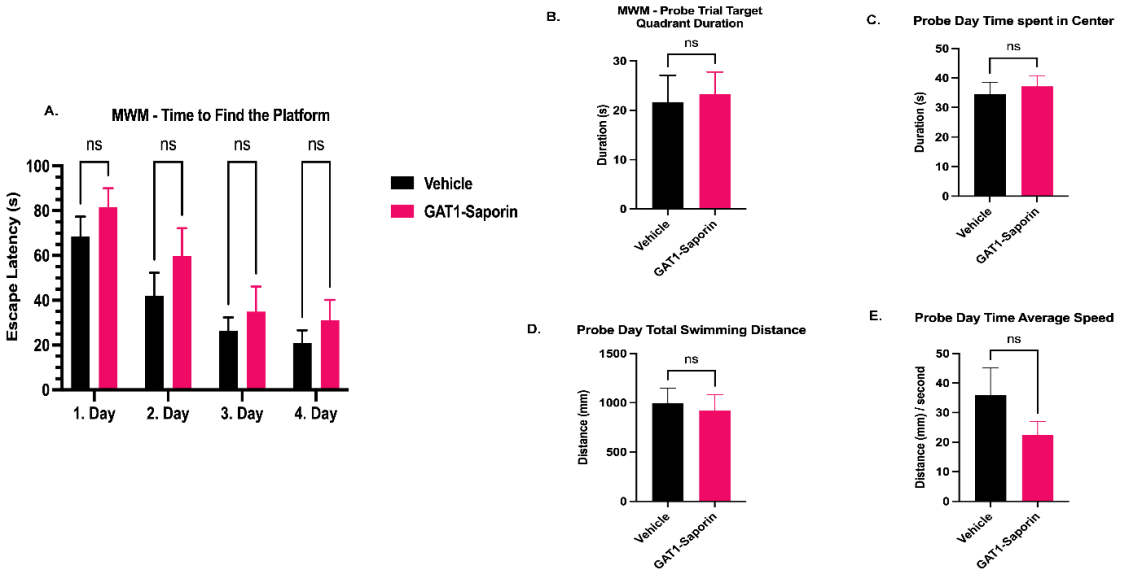


Figure 6**.** Injections of GAT1 Saporin into the VP did not result in MWM deficiencies (A). Average escape latency (s) in the MWM between groups on each training day. (B) The average time (s) spent in the target (platform) quadrant during the probe trial. (C) The average time spent in the MWM's center region during the probe trial. (D-F)

4. 6 Fear Conditioning

Pavlovian fear conditioning is an implicit form of learning, in which specific environmental cues anticipate often negative occurrences. It is the most-common behavioral method to delineate the functional role of fear subcircuits in rodents.

In the first 3 minutes of the day 1 training, none of the animals in either group had a freezing reaction in the baseline period before the acquisition training (Figure 7B). Both groups learned the conditioned freezing response ($F(3, 42) = 4.713$, $p = 0.0063$) (Fig 7B), but there were differences in the conditioned freezing response to shock

between groups ($M_{Vehicle} = 45.12$, $SD_{Vehicle} = 5.293$, $M_{GAT1\text{-}Saporin} = 33.48$, $SD_{GAT1\text{-}Saporin} = 3.691$) ($F(1,14) = 7.601$, $p = 0.154$) (Fig 7B). Except for the first trial, post hoc analyses revealed that animals in the GAT1-saporin groups froze considerably less than rats in the vehicle groups during the acquisition trials. Saporin injection also had a significant effect on active avoidance behaviours. Following saporin injections, occurrences of darting ($U=12$, $p =0.0416$,)(Fig 8A) and jumping ($U=11.50$, $p = 0.0303$, Fig 8B) behaviors in response to mild footshocks were significantly changed. In comparison to the vehicle group (*Mdn=2*), GAT1-Saporin injections *(Mdn=4)* increased the occurrences of darting behavior and the frequency of jumping ($GAT1_{Mdn} = 2$, $Vehicle_{Mdn} = 1$).

Cued fear reactions (freezing to the sound) reduced after the 12-trial extinction trial in both Context A ($F(11, 154) = 4.504$, $p < 0.0001$)(Fig 7C) and Context B ($F(4.800, 67.21) = 6.804$, $p < 0.0001$)(Fig 7D). Although cued fear reactions (freezing to the sound) decreased in both groups of rats after the 12-trial extinction trial, there was no significant difference in the fear reaction to the sound (CS) in the same context (context A) ($F(1,14) = 0.4056$, $p= 0.5345$)(Fig 7C). In GAT1-Saporin animals, however, the cued fear reflex in a different situation (context B) is significantly different ($M_{Vehicle} = 45.04$, $SD_{Vehicle} = 7.690$, $M_{GAT1\text{-}Saporin} = 31.76$, $SD_{GAT1\text{-}Saporin} = 7.154$) ($F(1,14)= 4.735$, $p= 0.0472$)(Fig 7D), they showed less freezing.

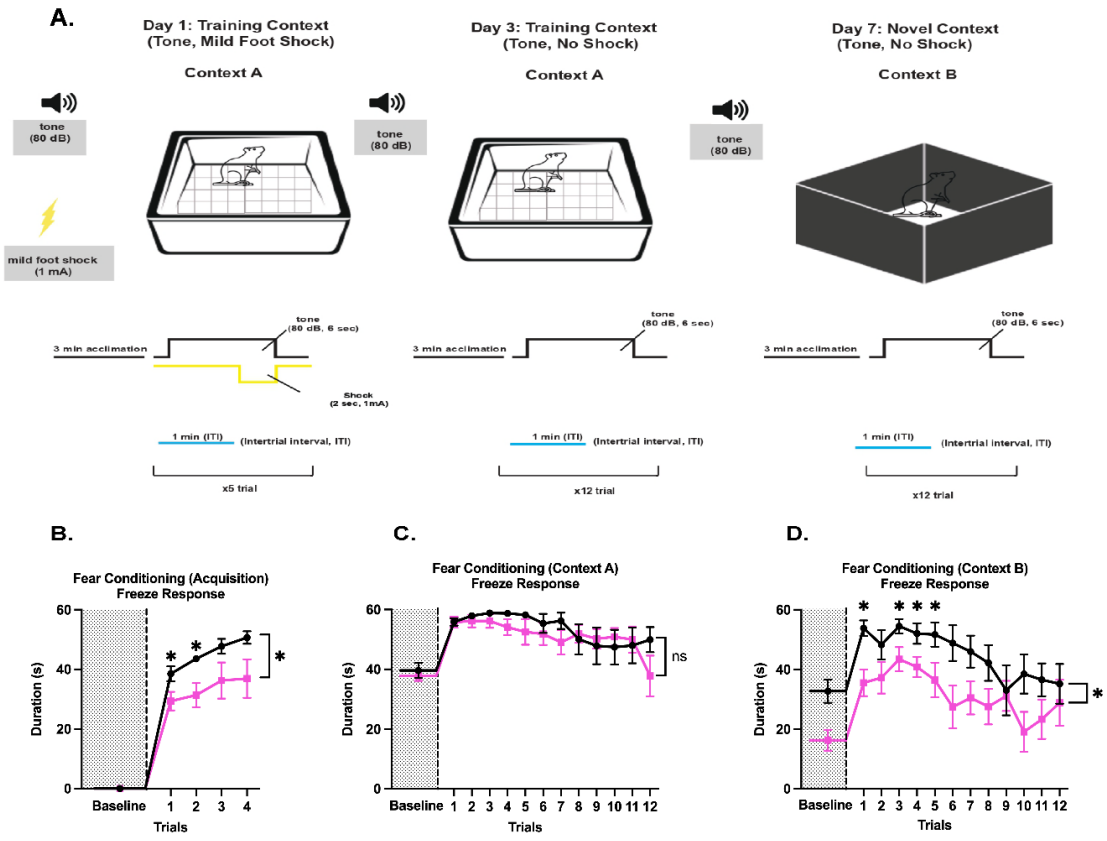


Figure 7. (A) Schematic representation of auditory fear conditioning tests in Context A and Context B for training and novel contexts. (B) The average baseline and duration (s) of freeze responses in the acquisition fear conditioning trials. (C) The average baseline and duration (s) of freeze responses in the Context A fear conditioning trials. (D) The average baseline and duration (s) of freeze responses in the Context B fear conditioning trials. Error bars show SEM. * = $p < 0.05$

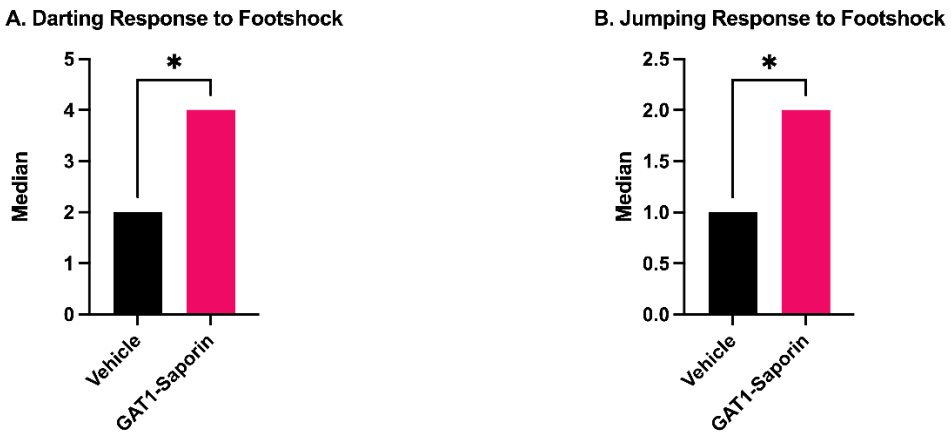
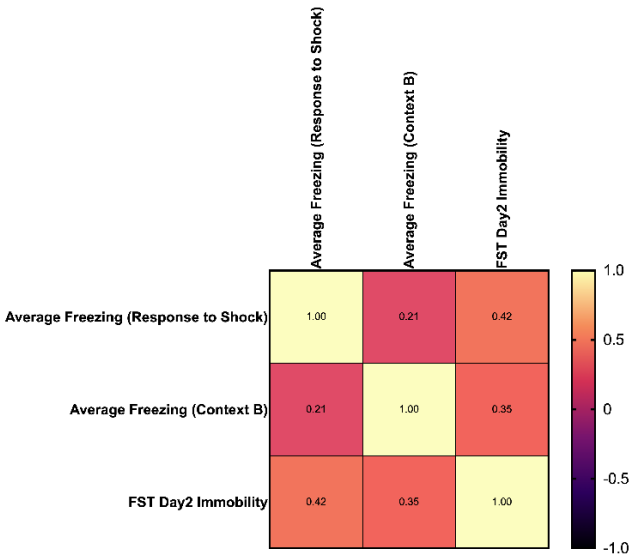
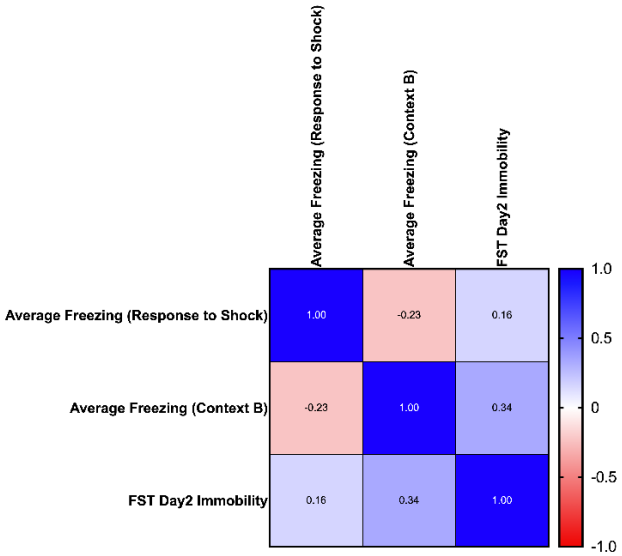


Figure 8. (A) Median darting response to foot shock is significantly different between groups. (B) Median jumping response to foot shock is significantly different between groups.

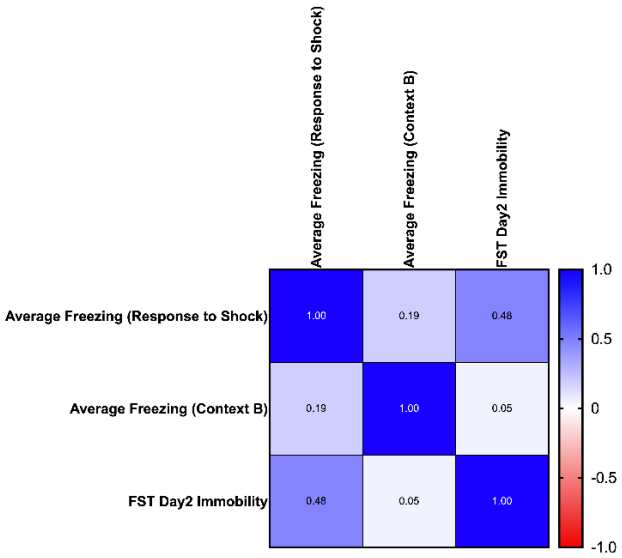
When we calculate the two groups together to check the correlation between different test results, we found a moderate correlation between immobility duration in Forced Swim Test day 2 results and the observed freezing response in the fear acquisition day, $r(16) = .42$, $p = 0.109$, and freezing response to sound in context B, $r(16) = 0.35$, $p = 0.187$ (Fig 9A). Once we consider each group individually, we found a strong correlation between FST Day 2 and average freezing response to shock in the vehicle group , $r(8) = .49$, $p = 0.230$ (Fig 9C), but this is not valid within a GAT1 Saporin group animals reaction $r(8) = .16$, $p = 0.702$ (Figure 9 B).



A. Correlation Matrices of GAT1 + Vehicle Groups



B. Correlation Matrices of GAT1 Saporin Group



C. Correlation Matrices of Vehicle Group

Figure 9. Correlation matrix between fear conditioning (average freezing response to shock and average freezing in context B) and FST (Day2 immobility) depicted for both groups (A), only the GAT1 Saporin group (B), and only the Vehicle group (C).

CHAPTER 5

DISCUSSION

The goal of this work was to explore the idea that the basal forebrain-driven disinhibitory mechanism has a broad modulatory function in the limbic system, extending their role in hippocampal processing to other memory-related structures. This idea originated from our previous neuroanatomical findings, carried out by retrograde tracer injections into different nuclei of the amygdala (including lateral, basal, and central amygdala) and the BNST. Consistent with the previous findings (Do et al., 2016; Mascagni & McDonald, 2009), the study from our laboratory showed that parvalbumin-positive neurons in different basal forebrain nuclei send inputs to the amygdala nuclei. Additionally, we have revealed that calbindin-immunopositive neurons in the basal forebrain nuclei send projections to the basolateral amygdala, centromedial amygdala, and the BNST (Tuna, 2021). These findings pointed to the importance of the non-cholinerigc circuitry between the basal forebrain and the subnuclei of the amygdala and the BNST. To test this hypothesis, ventral palladium/substantia innominate GABAergic neurons were selectively inactivated with GAT1-Saporin. This study is one of the first works investigating the role of the ventral pallidum/substantia innominata GABAergic neurons in wide-ranging affective process including fear memory, anxiety, and behavioral despair.

Three points should be highlighted from the results of this thesis. First, I found a decrease in the immobility during forced swim tests on day 1 and day 2. In other words, selective elimination of VP GABAergic neurons led to an antidepressant effect in

Saporin-injected animals and reduced behavioral despair. Secondly, the groups did not show a statistical difference regarding anxiety. Finally, animals with GAT1-Saporin injections showed increased active avoidance and decreased freezing response.

Similar to our work, Skirzewski et.al (2011) both increased and decreased the GABA level in the ventral pallidum using vigabatrin (an inhibitor of GABA transaminase) and bicuculline (a direct GABAA receptor antagonist) before forced swim tests. While injection of vigabatrin (increasing the extracellular GABA) to the ventral pallidum led to the increase in the immobility, bicuculline injection decreased the immobility reaction in forced swim test (Skirzewski et al., 2011). According to the same study, using micro dialysis and micellar electrokinetic chromatography during FST, it was shown that the forced swim test increased the ratio of extracellular GABA levels in the ventral pallidum. There was a considerable rise in basal GABA levels during swimming time on both days. The second day of FST revealed a significant increase in basal GABA levels compared to day one of FST. These previous results support our findings; however, in a recently published article, there is a finding which indicates that the orexinergic inputs targeting the GABAergic neurons in the VP depolarizes the GABAergic neurons and prevents depressive-like behaviors (immobility). Additionally, blockading the orexinergic receptors which in turn could depolarize the GABAergic neurons in the VP causes increase in immobility (Ji, Zhang, Chen, Wang, & Zhu, 2019).

We observed that the GAT1-Saporin injection to the ventral pallidum had no impact on the animals' total movement (locomotion) compared to the saline-injected vehicle group. This demonstrates that the antidepressant-like effects seen in the FST were irrelevant to treatment-induced changes in general locomotor activity. The orexin study which is attributing different role to the VP GABAergic neurons in forced swim

test than our findings confirms our OFT results (Ji et al., 2019). Nevertheless, these results contrast with the findings of a recently published work, which revealed a significant difference between GABAergic inactivation of ventral pallidum neurons using chemogenetic inactivation and control animals' relation to their locomotion in the OFT (Y.-D. Li et al., 2021). This might be related to the OFT experiment conditions in our laboratory (such as lighting conditions) or the sample size of experimental groups.

Besides its reliability and usefulness for measuring locomotion and anxiety levels, the OFT is used as a control test for the forced swimming test. The observed difference among groups (control/experimental) in terms of the mobility in the FST might be arises from the locomotory activity differences. Thus, to avoid making false positive inferences from the FST results, the OFT is a must and rather than reliable (Gould, Dao, & Kovacsics, 2009; Unal & Canbeyli, 2019).

In accordance with previous research, we discovered that the GAT1-Saporin injection to the ventral pallidum has no effect on the time spent in the open arms or total movement in EPM. EPM is regarded as a reliable way to measure the anxiety levels of animals. We hypothesized that there will be a significant difference between GAT1-Saporin, and vehicle (saline) administered groups of animals if the ventral pallidum GABAergic neurons are involved in the modulation of anxiety process or fear memory. These results may be related to the restricted number of basal forebrain GABAergic neurons projecting to the BNST compared dense projections to the basolateral amygdala (BLA) or central amygdala (CeA). If our manipulations alleviated anxiety levels, GAT1-Saporin group animals would spend much more time on exploratory behavior, such as more rearing and increased time spent in the center of the maze than control groups.

Rats are anxious in novel environments, and because they are nocturnal, the maze's center is much more nervous for them.

I found no significant effect of the manipulation on escape latency during training, and on the time spent in the target quadrant during probe trials of the Morris Water Maze (MWM). It was shown in an earlier study that VP GABAergic neurons are vital for arousal, which mediates motivation. Arousal was reduced by chemogenetic silencing of VP GABAergic neurons (Y. D. Li et al., 2020). This might be the reason for GAT1-Saporin group animals to show relatively increased escape latency without a statistically significant difference. Besides the role of ventral pallidum GABAergic neurons, basal forebrain cholinergic neurons in literature are studied for the possible role of these neurons in spatial learning, especially in the nucleus basalis of Meynert or nucleus basalis magnocellularis (D'Hooge & De Deyn, 2001). As I highlighted above, various subnuclei of the basal forebrain send inputs to both the cerebral cortex and subcortical limbic structures, including the amygdala. This thesis confirms the result of recent work indicating that nucleus basalis magnocellularis GABAergic lesions do not impair long term spatial memory. It is important to note that the toxin-based manipulations targeting the VP/SI GABAergic neurons likely included the GABAergic neurons of the nucleus basalis Meynert (Beselia et al., 2021).

I found that rats that underwent a GABAergic neuron inactivation in VP/SI showed less freezing response during the acquisition day of the Pavlovian fear conditioning and extinction procedure. These GAT1-Saporin injected animals also showed increased activity (more jumping and more darting behaviour) on the acquisition day to the shock. Moreover, observed freezing duration following Pavlovian fear conditioning trials in day 1 and in day 7 at context B modestly correlated with the

immobility time seen in FST day 2. Our findings support the literature indicating that the primary measurement, a decrease in locomotor activity (namely immobility), is similar to the freezing response evaluated in Pavlovian fear conditioning paradigms (Unal & Canbeyli, 2019). Although it is not unquestionably revealed, a slowly growing body of research suggests that the ventral pallidum plays a dynamic function in threat detection. Regarding its afferent inputs, which come from CeA and efferent inputs going to the BLA, it can be said that the ventral pallidum both receive and provide information for threat (Moaddab, Ray, & McDannald, 2021).

Recently, it has been revealed that the role of the cholinergic basal forebrain neurons is not only restricted to arousal, attention, learning and memory but also crucial for fear expression. In detail, it is asserted that the inputs coming from CeA to various basal forebrain nuclei, which, in turn, regulate the activity of the cholinergic basal forebrain neurons, is vital in the transition from passive fear to active fear responses (Gozzi et al., 2010). These might be related to increase darting and jumping behavior in GAT1-Saporin group as well. In this research, it is highlighted that the CeA neuron projections target the GABAergic interneurons, which we probably made a manipulation in our work. Likewise, it was found that the inactivation of the basal forebrain neurons with optogenetics reduced the freezing response during fear memory acquisition, retention, and extinction (Carli & Farabollini, 2022). These results support our findings that GABAergic neurons, which we killed with saporin, could not inhibit the cholinergic neurons in the basal forebrain causing a decrease in freezing response with increased cholinergic neuron activity.

Results of this work gives rise to an important question regarding the theoretical relationship between fear and behavioral despair: does fear with behavioral despair lead

to freezing, or are they different phenomena? The literature indicates that the freezing response observed in Pavlovian fear conditioning and behavioral despair (immobility) in forced swim tests both evaluate a decrease in motor activity. However, the neurobiological underpinnings of the two behavioral systems are different (Unal & Canbeyli, 2019).

It is also important to mention the role of ventral pallidum in pain mediation and analgesia when attributing this structure a role in antidepressant activity and active coping. My findings may partially originate from the pain perception differences between GAT1 Saporin and vehicle animals. However, the ventral pallidum GABAergic neurons are not studied for their role in pain and analgesia. With its high density of opioid receptors and the dense connections with the Ventral pallidum, the Nucleus Accumbens (NAc) responds strongly to painful stimuli (Harris & Peng, 2020).

From beginning to end, this study highlighted the role of the subnuclei of the amygdala in the context of fear memory, anxiety, and behavioural despair. However, functions assigned to the amygdala are not restricted to these a couple of behaviors. In the last decade, cell-type-specific manipulations became possible with the introduction of optogenetics (Deisseroth, 2011). Optogenetics allows neuroscientists to stimulate or inactivate artificially light-sensitive cells with light. Additionally, there is also a chance in the neuroscientists' hands to make cell-selective temporary pharmacological (chemogenetic, such as DREADD) activation or inhibition (Urban & Roth, 2015). These transformations in the lesion tools with new behavioral experiments settings and recently started query in the amygdala literature show us that the role of the amygdala play is not restricted to the encoding of fearful memories has been started to think about. This view is narrow in that there is strong evidence which supports that the role of the amygdala is

beyond fear conditioning. Such roles for this region include other aversive states such as anxiety and reward in addition for being a hub for controlling homeostatic and social behaviours. Giving the amygdala various characteristics, it poses a challenge in determining how neuronal activity contributes to each distinct process. Additionally, the basal amygdala also displays some uncertainties in that its widespread connections to other brain areas remains unknown (Gründemann et al., 2019; Janak & Tye, 2015).

5.1 Contribution of Philosophy Scholars to the Neuroscience

In 1995, a seminal article written by Tim Van Gelder called "What Might Cognition Be, If Not Computation?" highlighted that a dynamic account of cognition may be a better fit for the enquiry for explaining the conception of cognition (Van Gelder, 1995). The embodiment hypothesis, which shares common bases with dynamic systems approach, states that intelligence is the result of the interaction between an organism and its environment via sensory-motor activity. The constant pairing of cognition and the adaptations of the body's external world to cognition allows for developmental change. Being linked to the body and the body's outside world allows cognition to be observable and effective. The physical world consists of its own dynamics in that it can change whenever it wants. Intelligence can be found in the continuous coupling to the physical world. Intelligence does not have to be confined to a single cognitive system but rather can be spread throughout the brain, body, and world. Dynamic systems view any potential for change as a result of the complexity of systems consisting of heterogeneous

parts which self-organize in terms of context and time, which do not consist of a concept's unitary, timeless, and context-free entities. So, cognition is defined as an event in real-time which is the product of heterogeneous systems being bound to each other and the world (L. B. Smith, 2005). Like this embodiment and generally the dynamic system account, Buzsáki (2019) asserted that the brain can be defined as a device in which actions and the examination of the consequences of an action are the basis in which it interacts with the environment. The brain is first filled with nonsensical patterns until it is filled with meaningful action-based interactions. When matching these nonsense patterns to a defined outcome due to a particular action, meaning can be established. The brain can then formulate "what if" situations through reflecting on its own computations otherwise known as cognition through detaching its sensors and actuators. He further explains that the brain is not an absorbent coding machine but rather an explorer who always controls the body to see and test what is out there. The brain, therefore, is not a processor but rather a creator of information (Buzsáki, 2019). I believe that the account of the Buzsaki and the dynamic account of cognition share similarities. These two accounts to highlight the importance of the dynamically changing role of the brain is similar. I tried to address that the amygdala's attributed function has changed from fear expression solely to various cognitive, homeostatic, and internal processes. This change in the attitude of the neuroscientists became possible once they observed the brain is dynamic, and that functional specialization is not in brain regions but in thousands of circuits.

5.2 Limitations

In addition to the control of the parvalbumin, which is calcium-binding proteins used as a marker for GABAergic neurons, carrying out GAD65/GAD67 immunoreactivity presence with other subpopulations (calretinin, calbindin and secretagogin) of the calcium-binding proteins would be meaningful to see any colocalization of these subgroups of neurons with GABAergic marker.

The presence of possible double labelling of GAD65/GAD67 and different calcium binding proteins in sham group would indicate the importance of the calcium-binding proteins in significantly differentiated behavioral tests in the experimental group.

5.3 Implications and Future Directions

GAT1-Saporin experiments related to the animal models of the mentioned affective and cognitive process show us that there is a significant effect of our manipulation in some experiments. To be precise and flexible in our manipulations, DREADD experiments allowing suppression or activation of the cell markers could be planned as a second phase of the study to identify the modulatory role of the GABAergic cell groups in excessive fear or anxiety circuits. Identified cell groups, $VP^{Calbindin/Parvalbumin}$, in retrograde tracing which provide projections to the amygdala and the BNST, could be inhibited with the help of a new state-of-art technique, Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) in rats, and thus their possible role could be investigated both with activation and suppression. To selective inactivation of the

marker (cell type) specific molecules of the ventral pallidum, genetically modified Calbindin/Parvalbumin:cre animals can be used. By injecting the pAAV-hSyn-DIO-hM4D(Gi)-mCherry vector virus into each $VP^{Calbindin/Parvalbumin}$:cre animals, they could be ready to manipulate with clozapine-N-Oxide (CNO), which is a biologically inert agent (Urban & Roth, 2015).

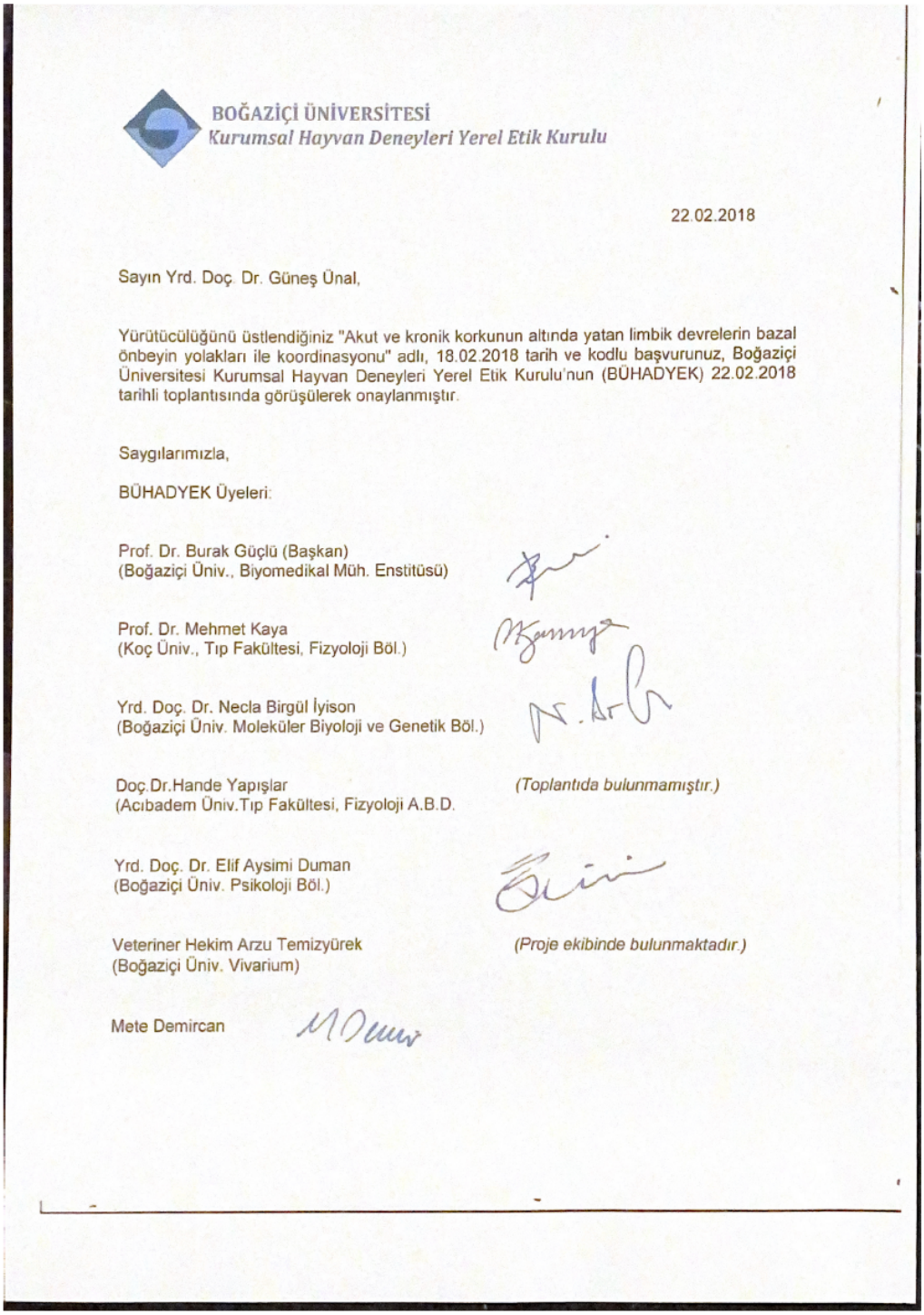
Revealing selective circuits underlying fear memory and anxiety might pave the way for developing effective treatments that target specific symptoms of mood and anxiety disorders. Infusion studies show that reconsolidation of fear memory is sensitive to the protein synthesis blocking manipulations for up to 45 days (Debiec, LeDoux, & Nader, 2002; Kandel et al., 2014). Determining circuits involved in the fear memory and developing suitable types of chemogenetic-like, non invasive tools for humans would be highly promising to relieve or eliminate the symptoms of post-traumatic stress disorder, depression, and anxiety-disorders in the near future.

Acquisition				
Day	**Trial 1**	**Trial 2**	**Trial 3**	**Trial 4**
1	S	E	NE	SW
2	NE	S	SW	E
3	SW	NE	E	S
4	E	SW	S	NE
5 (Probe)	SE			

Table 1: Start Positions of the Morris Water Maze. Table shows the spatial direction of the of animals taken to the Morris water. E, east, N, north S, south and, W, west.

APPENDIX A

ETHICS COMMITTEE APPROVAL



BOĞAZİÇİ ÜNİVERSİTESİ
Kurumsal Hayvan Deneyleri Yerel Etik Kurulu

22.02.2018

Sayın Yrd. Doç. Dr. Güneş Ünal,

Yürütücülüğünü üstlendiğiniz "Akut ve kronik korkunun altında yatan limbik devrelerin bazal önbeyin yolakları ile koordinasyonu" adlı, 18.02.2018 tarih ve kodlu başvurunuz, Boğaziçi Üniversitesi Kurumsal Hayvan Deneyleri Yerel Etik Kurulu'nun (BÜHADYEK) 22.02.2018 tarihli toplantısında görüşülerek onaylanmıştır.

Saygılarımızla,

BÜHADYEK Üyeleri:

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(Boğaziçi Üniv., Biyomedikal Müh. Enstitüsü)

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Mete Demircan

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