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MASTER THESIS

ÖZGE BAŞER

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ADVISOR

PROF. DR. BAYRAM YILMAZ

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LIST OF SYMBOLS AND ABBREVIATIONS

AChAcetylcholine

AChEAcetylcholinesterase

ACTH Adrenocorticotropic Hormone

AGRPAgouti-Related Peptide

AMYAmygdala

ARCArcuate Nucleus

AVPArginine Vasopressin

BFBasal Forebrain

BFCThe Basal Forebrain Complex

BLABasolateral Amygdala

cAMPCyclic Adenosine Monophosphate

CART Cocaineand Amphetamine-Regulated Transcript

CCCingulate Cortex

ChATCholine Acetyltransferase

CHTCholine Uptake Transporter

CNOClozapine-N-Oxide

CortCorticosterone

CRECre Recombinase

CRFCorticotropin-Releasing Factor

DMHDorsomedial Hypothalamus

DNADeoxyribonucleic Acid

GABAGamma-Aminobutyric Acid

GPCRsG Protein Coupled Receptors

HDB The Horizontal Diagonal Band Nucleus

HPA Hypothalamic-Pituitary-Adrenal

IC The Islands of Calleja

IN Intermediate nucleus

IPN The Interpeduncular Nucleus

iPVN Intraparaventricular Nuclear

LDT Laterodorsal Tegmental Nuclei

mAChRs Muscarinic Receptors

MCPO The Magnocellular Preoptic Area

MHb The Medial Habenula

mRNA Messenger Ribonucleic acid

MSThe Medial Septal Nucleus

NAc Nucleus Accumbens

nAChRs Nicotinic Receptors

NE Norepinephrine

NPY Neuropeptide Y

NRM Nucleus Raphe Magnus

OT The Olfactory Tubercle

PCR Polymerase Chain Reaction

PFC Prefrontal Cortex

POMC Pro-opiomelanocortin

PPT Pedunculopontine Tegmental Nuclei

PVN Paraventricular Nucleus

REM Rapid-Eye Movement

SPECT Single Photon Emission Computed Tomography

SI Substantia Innominata

NBM The Nucleus Basalis

VDB Vertical Diagonal Band Nucleus

VTAVentral Tegmental Area

αMSH α- melanocyte stimulating hormone

ABSTRACT

THE ROLE OF CHAT IN ARCUATE NUCLEUS ON ANXIETY BEHAVIOUR

Anxiety and stress are extremely crucial for the consistency of survival under the conditions of wild nature. For human beings, the level of stress becomes deeper and could be observed in every aspect of our daily lives. Impairments of the mechanism which controls this system leads to anxiety disorder and depression that affect the lives of more than 300 million people worldwide. The stress suppressive effect of nicotine by causing an addiction commonly in everyday life became a focus of interest for researchers. The pathways that nicotine stimulates are physiologically stimulated by the neurotransmitter acetylcholine. The mechanism of anxiety, insomnia, muscle tension, careful reduction, and forgetfulness, which are came along with anxiety disorder and depression, are associated with acetylcholine. The development of effective treatment for these conditions is possible with a correct understanding of the connected systems. Chemogenetic activation technique allows us to target cholinergic neurons of ChAT-IRES-Cre transgenic mice.

The aim of this study is chemogenetic activation of cholinergic neurons in the arcuate nucleus in order to understand the role of it in the anxiety behavior. Elevated plus test, open field test, and light-dark box tests were performed. The determined stress factor for those tests are; the height, the open and unprotected area, and the over-lightened area. Spent time at the areas related with stress factors was associated with anxiolytic effects and it was shown that the cholinergic neuron group in the arcuate nucleus has an effect of tolerance enhancement of the stress; however, does not show any effect on locomotor activity.

ÖZET

Arkuat Çekirdek Kolin Asetiltransferaz Nöronlarının Anksiyete ile İlişkisi

Anksiyete ve stres, canlılığın devamı için doğada karşılaşılan koşullarda hayati önem taşır. İnsanlarda ise stresin boyutu biraz daha değişerek hayatın her alanında karşımıza çıkmaktadır. Bu sistemi kontrol eden mekanizma üzerindeki bozulmalar dünya çapında 300 milyondan fazla kişinin yaşamını etkileyen yaygın anksiyete bozukluğu ve depresyona yol açmaktadır. Nikotinin stres baskılayıcı etkisi günlük yaşam içerisinde sıklıkla başvurulan bir bağımlılığa sebep olmasıyla araştırmacıların dikkatini çekmiştir. Nikotinin uyarıcı etki gösterdiği yolaklar, fizyolojik olarak asetilkolin nörotransmitteri tarafından uyarılır. Anksiyete ve depresyon durumlarında hissedilen kaygı artışı, uykusuzluk, kas gerginliği, dikkatte azalma, unutkanlık gibi durumlardan sorumlu sistemlerin asetilkolin ile ilişkili olduğu bilinmektedir. Bu rahatsızlıklara etkin tedavinin geliştirilmesi, bağlantılı olan sistemlerin doğru anlaşılması ile mümkündür. Yapılan tez çalışmasında kemogenetik aktivasyon tekniği, ChAT-IRES-Cre transgenik farelerde kolinerjik nöronların hedeflenmesine olanak sağlamaktadır. Çalışmada arkuat nükleustaki kolinerjik nöronlar kemogenetik olarak aktive edilmiş. Yükseltilmiş artı testi, açık alan testi ve aydınlık-karanlık kutu testleri yapılmıştır. Yapılan testlerde kontrol grubuna kıyasla fareler için stres faktörü olan yükseklik, açık ve korunmasız alanda bulunma ve aşırı ışık ile aydınlatılmış alan parametreleri kullanılmıştır. Grupların stres faktörleri ile ilişkili bölgelerde bulunma sürelerinin artması anksiyolitik etki ile ilişkilendirilmiş ve arkuat nükleusta bulunan kolinerjik nöron grubunun, strese olan toleransı arttırıcı bir etkisi olduğu ve lokomotor aktiviteye herhangi bir etkisi olmadığı gösterilmiştir.

1. INTRODUCTION and PURPOSE

Acetylcholine (ACh), is a rapid-acting neurotransmitter on neuromuscular and autonomic ganglia. The Cholinergic system, affects relieasing of neurotransmitters act as neuromodulators[1]. Acetylcholine is recognized by two subtype receptors, namely muscarinic (mAChRs) and nicotinic (nAChRs) receptors [2,3]. mAChRs affect many signal pathways by activating the intracellular second messenger system through G proteins. Nicotinic receptors, which are non-selective cation channels, have a regulatory effect on metabotropic signaling pathways [3,4].

Recent studies have investigated the association and pathophysiology of acetylcholine with anxiety and depression-like behaviors. Clinical and preclinical studies have shown antidepressant-like property of acetylcholine receptor antagonists [5,6]. In a human brain study, using single photon emission computed tomography (SPECT), the number of nAChRs decreased significantly in depressed individuals[7]. Stimulation of nAChRs result in relief effect on anxiety through decreasing activity of beta 2 subtypes on the basolateral amygdala (BLA) [8]. Suppression of mAChR (M1 Subtype) and nAChRs on dorsal hippocampus has an anxiety enhancing effect[9]. While the association of the acetylcholine system with anxiety was shown in the studies performed, Jeong et al in 2016 demonstrated the existence of a new group of cholinergic neurons in the arcuate nucleus (ARC) [10]. The new finding occurs a new question about physiological functions of ARC choline acetyltransferase (ChAT) neurons. This study aimed to reveal the role of the hypothalamus and ARC^{ChAT} anxiety neurons in the relationship.

2. LITERATURE REVIEW

2.1 Cholinergic System Overview

Acetylcholine is a neurotransmitter, usually a stimulatory, that acts for a few milliseconds before break down with acetylcholinesterase (AChE) enzymes to cholines and acetates. Neurons, which secrete the ChAT, are commonly regarded as cholinergic[11]. The cholinergic system modulates neural activity with AChRs and modulates various aspects of brain function including sensory processing[12] attention[13], sleep, arousal[14], cognition[15] and memory[15]. As well as nerve cells directly affected by ACh, there are also regulatory, synaptic plasticity enhancing effects on modulator and excitability of neurotransmitter release from the presynaptic cell[16-19].

Cholinergic motor neurons of somatic and autonomic nervous systems innervate somatic, smooth, and cardiac muscles, and mediates glandular secretions[20]. Most of the preganglionic neurons and postganglionic parasympathetic nerve endings in the autonomic nervous system and a few of the postganglionic sympathetic nerve endings (sweat glands and very few blood vessels) are cholinergic.

The capacity of the high affinity choline uptake transporter (CHT) from the extracellular domain to the choline uptake towards presynaptic terminals is an important value for normal acetylcholine synthesis and therefore cholinergic transmission. Changes in CHT capacity can be vital to fulfilling the tasks of attention processes and capacities[21,22].

Several cholinergic neurons are in the basal forebrain (BF). The BF cholinergic projections are upon the entire allocortex and isocortex[11]. Cholinergic neurons in the BF[23,24] are associated with memory and arousal. The medial septal nucleus (MS) and vertical diagonal band nucleus (VDB) are in rostral part of the BF. The caudal parts are the nucleus basalis, substantia innominata, and nucleus ansa lenticularis. Intermediate nucleus are the horizontal diagonal band nucleus (HDB) and the magnocellular preoptic area (MCPO).

Pontomesencephalic cholinergic neurons whereas cells in the nucleus raphe magnus (NRM) project to the ventral horn of the spinal cord[25] have a role in sleep-wakefullness, locomotor behavior, and memory. The pontomesencephalic cholinergic cells project to the basal telencephalon, the diencephalon, the brainstem, the deep cerebellar nuclei. But the cerebellar cortex and the spinal cord are not innervated by them[26,27].

There are cholinergic interneurons in the striatal structures, such as the islands of Calleja (IC) , the olfactory tubercle (OT) , nucleus accumbens (NAc) and caudate putamen complex[20]. Cholinergic interneurons are tonic-active ACh neurons in the striatum and nucleus accumbens[23,28]. The projection from the medial habenula (MHb) [29] to the interpeduncular nucleus (IPN) pathway [30,31] is to serve as an major link to associated with sleep, stress, pain and nicotine addiction[32-37].

The cholinergic neuron group in the brain stem (laterodorsal tegmental/pedunculopontine tegmental nuclei [LDT/PPT]), which are active in wakefulness and the rapid-eye movement (REM) sleep period.

The key element for homeostatic regulation, the hypothalamus, also has many cholinergic neuron groups and plays a major role in the regulation of energy metabolism. It has recently been found that ChAT neurons in the dorsomedial hypothalamus (DMH) arrange food intake and body weight.

Muscarinic receptors on gamma-aminobutyric acid (GABA) released axon terminals inhibits ARC pro-opiomelanocortin neurons (POMC)[29]. Thus, enhancing inhibitory tone to ARC^{POMC} suppresses the appetite through paraventricular nucleus (PVN). In addition, the physiological role of ARC ChAT neurons is currently unknown[10].

2.2 Acetylcholine and Stress Relation

The studies in the literature suggests that stress change the functioning of the cholinergic system[38,39]. Stress response affects the release of acetylcholine in the forebrain and activate the septohippocampal pathway[40]. The basal forebrain complex (BFC) and the MS and nucleus basalis send a very rich of ACh input to the hippocampus (Hipp.), amygdala (AMY) and prefrontal cortex (PFC)[41].

The hippocampus inhibits the hypothalamic-pituitary-adrenal (HPA) axis[42]. Additionally, the activation of the hippocampus is strongly regulated through both nAChRs and mAChRs. The MS and the diagonal band of Broca send projections to the hippocampus glutamatergic and GABAergic[43]. In a study done with restrained rats, it has been shown that enhancement of cholinergic activity in response to stress in two acetylcholine projection system (in the dorsal hippocampus and PFC), while relief from stress increases the ACh levels in NAc and AMY[44]. In a study conducted under 1h or 24 h of cold stress (5°C), the concentration of ACh in the hypothalamus and dorsal hippocampus decreased in rats[45].

While acute stressors increase acetylcholine release in the hippocampus[44,46], the effects of chronic stress are not yet fully understood, but may involve a gradual decline in cholinergic function[38,47]. The activity of AChE in the hippocampus of animals decreases under chronic stress[48,49]. Cholinergic dysfunctions have been reported in rats subjected to chronic stress or corticosterone (Cort) administration. A decrease in the number of positive AChE-stained neurons was observed in the MS[50,51]. Dysfunction of the stress-induced cholinergic system may impair learning and memory processes[38,39].

Nicotine has a performance-enhancing effect on short-term attention-related tasks and work memory, but is addictive on the long-term after the brain stimulates reinforcement and reward circuits. Nicotine activates a broad array of nAChRs subtypes that can be targeted to pre- as well as peri- and post-synaptic locations. Nicotine can change synaptic properties and modulates sypnaptic plasticity. Thus, stimulation of nicotinic receptors affects a wide range of brain regions including PFC and cingulate cortex (CC), hippocampus, AMY, ventral tegmental area (VTA) and the NAc[52] and ARC. Decreasing nAChR signaling in the AMY occurs anxiolytic and antidepressant-like effects in mice and reverse some of the anxiety- and depression-like symptoms induced by AChE blockade[53].

The acetylcholine effect is mediated by physiological and emotional responses on the HPA axis through activation of nAChRs. Additionally, nicotine directly can stimulate the HPA axis. Studies using a nAChRs antagonist, mecamylamine, indicate that receptor stimulation alleviates activation of the HPA axis and exhibits anxiolytic behavioral effects. The results of this study suggest that mecamylamine has anxiolytic properties that appear to be influenced by basal cholinergic tone and has an attenuating effect on the stress evoked release of Cort[54].

The AMY also receives cholinergic inputs from the BFC[41]. In rodents, neuronal activity in the BLA effects through nAChRs[6]. It is believed that the cholinergic inputs through AMY strengthen the link between environmental stimuli and stressful events. The noradrenergic and cholinergic systems, converge on the BLA and contribute to its modulation. It was shown that fear training could be more persistent when ACh release occurred during the conditioned fear training. With this information, it is thought that ACh affects the acquisition of fear-related behaviors in amygdala-based learning and memory[55].

The cholinergic–adrenergic theory of depression hypothesizes that over-active cholinergic signaling on noradrenergic tone can lead to depression[56]. It is shown that ACh

and norepinephrine (NE) have an opposing effect on behaviors related to anxiety and depression[8].

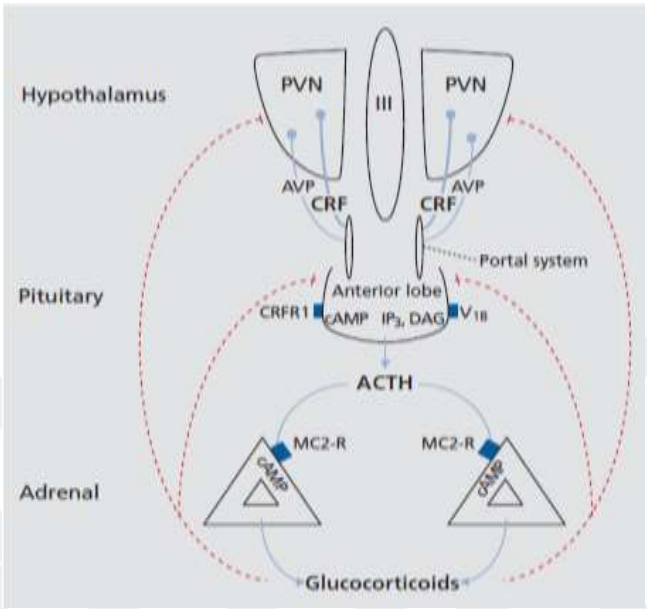

Fig.1 Schematic representation of the hypothalamic-pituitary-adrenal (HPA) axis.

2.3 Arcuate Nucleus and Effects

The hypothalamic centers regulate energy homeostasis by directly innervate PVN neurons. Neurons in the arcuate nucleus has receptors of glucose, insulin, and leptin. These cells also synthesize neuropeptide Y (NPY), agouti-related peptide (AGRP), α-melanocyte stimulating hormone (αMSH), and cocaineand amphetamine-regulated transcript (CART) which play critical roles in the regulation of feeding behaviors[57-59]. POMC and AGRP have the same receptor on PVN that is MC4-R. While POMC neurons are active, energy consumption increases while nutrient uptake decreases, resulting in satiety. In contrast, activation of AGRP neurons is in function of the MCR-4 antagonist on the PVN[60].

POMC is a prohormone, which is necessary for sythesis of the adrenocorticotropic hormone (ACTH), β-endorphin, β-lipotropic hormone, and the melanocortins and synthesized from the hypothalamus and the pituitary[61-63].

It has known that ARC has activatory effect on HPA axis. Central injection of the orexigenic factor NPY, which is synthesized with Agrp results in HPA axis activation [61,62]. Intraparaventricular nuclear (iPVN) injection of Agrp significantly also increases corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) release and the plasma level of ACTH[63]. The anorectic peptides αMSH and CART increase circulating levels of ACTH and corticosterone[63-65], induce cylic adenosine monophosphate (cAMP) binding protein phosphorylationin CRF neurons[66], and stimulate CRF release from hypothalamic neurons[63,67]. These studies suggest that the HPA axis is activated in response to positive and negative states of energy balance.

2.4 Spesific cell targeting by using Cre-LoxP System

The regions present in the brain structure do not consist of homogeneous cell groups. There are different types of neurons and glia cells which the physiological function is to be investigated. The electrical stimulation or lesion studies to investigate a type of neurons in a particular region does not give the correct result as it directly affects the whole nerve tissue in the microcircuit unless it is specific to a single cell group. The Cre-lox system is a system implemented to manage gene expression. The Cre-LoxP system allows gene activation only in the target neuron group in the brain, and through this genomic-based change, the corresponding neuron activation or inhibition can be made. Transgenic mice are generated only by insertion of the Cre recombinase (Cre) enzyme gene in the gene region specific to a given group of neurons. Protein Cre binds to 34 bases loxP sites and differs in how the genetic material is arranged using the orientation and position of loxP sites. If loxP sites is on the same deoxyribonucleic acid (DNA) strand and are in opposite orientation, the DNA between the loxP sites in inverted and recombination results in an inversion. If the LoxP regions are looking in the same direction, the sequence is removed as a circular DNA fragment so that the gene is deleted. LoxP sites is on separate DNA molecules, a translocation event occurs. In the study, the Cre-LoxP system provided the applicability of the chemogenetic technique to investigate the anxiety-like behavior of ARC^{ChAT} neurons in ChAT-ires-Cre mice.

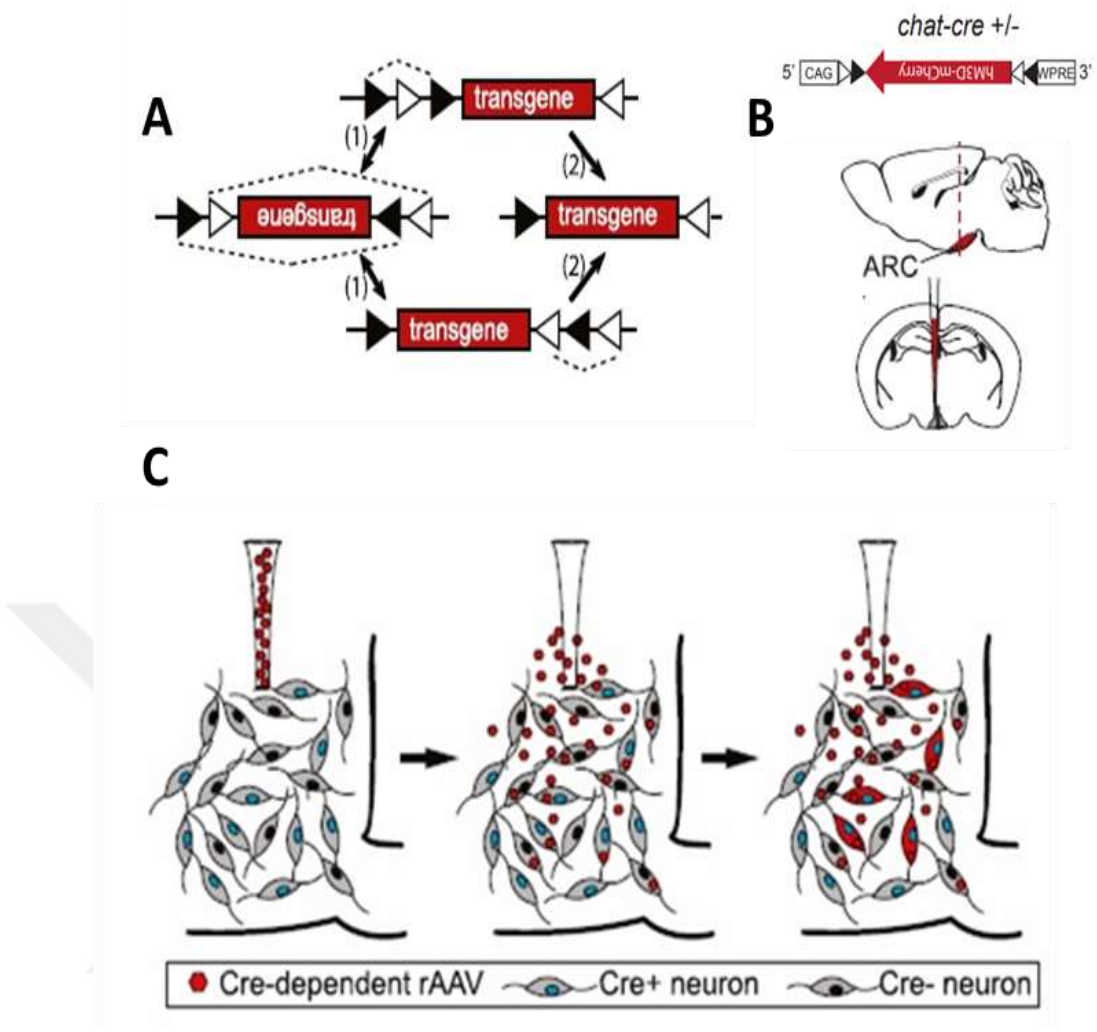

Fig.2: Spesific cell targeting by using Cre-LoxP System.

A. Schematic representation of the flip-excision (FLEX) switch used in Cre-linked viruses and recombination event used to provide stable inversion. **B.** Representation of intracranial injection into the ARC of ChATCre mice. **C.** The selective Cre-dependent virus is expressed only in ChAT-Cre neurons.

2.5 DREADDs- designer receptors exclusively activated by designer drugs

In order to understand a behavioural task that occur in a result of complex brain activity, the relation in between function and behavior of the cell groups must be established. In the central nervous system studies that run with animals, brain lesions and electrical stimulation can be seen as the primitive methods compared with today's techniques. Today, genetically targetable techniques allow rapid and reversible control of neuronal activity. These tools vary widely in terms of ease of use, time and invasiveness, allowing for extensive study of various physiological questions[68]

The method is targeted only to a family of G protein coupled receptors (GPCRs) that can be activated by a pharmacologically inert drug-like compound (clozapine-N-oxide-CNO). Then, it has continued to last in human mAChRs and took its last form[69]. This receptor has been converted to an artificial receptor that responds only to the designer drug (CNO) and is insensitive to the natural hormone (ACh)[70].

In this thesis, pAAV-EF1a-DIO-hM3D (Gq)-mCherry was used for acute activation of ARC^{ChAT} neurons. The control group in chemogenetical experimental design was done with changing the virus type. The control group of our experimental design was intracranially injected and pAAV-CAG-flex-rev-tdTomato virus on the ARC.

3. MATERIALS AND METHODS

3.1 Animals

In the experiments, two months (ChAT-CRE) transgenic male mice were used. The ChAT-Cre line (Code: 006410) was commercially obtained from Jackson Laboratories. Mice were housed 5/cage in a vivairum under standart laboratory conditions (temp 21 ± 4°C, 12:12 light-dark cycle, lights on at 7:00 a.m.). Food and water were available ad libitum. A tissue sample of 30 days old pups was taken. DNA was isolated from tissue samples. Polymerase chain reaction (PCR) was carried out to determine the Cre-gene locus.

3.2 Breeding

Transgenic mice were crossed with in-breed females and C57BL / 6 wild type females. Breeding cages had generally 1:2 the ratio of male-female. After the 14th days of pragnancy male mice were taken from the breeding cages. The cleaning of the cages were made before the get birth of pups. During the lactation period, puppies were untouched until 21 days old. After the offspring are 25-30 days old, they became ready for tissue uptake and genotyping. Animals that were determined to be transgenic with the Cre-gene region were injected at 2 months of age.

3.3 Genotyping

Pups were briefly anesthetized under isoflurane anesthesia. A sample of 2 cm in diameter was taken from the ears. Tissues collected at room temperature to PCR tubes. 100µL alcaline buffer (25mM NaOH -Sigma, 306576- and 0.2 mM EDTA) added on the tissue samples and was boiled at 98 ° C for 1 hour in a PCR device (Labnet International, TC9610). After boiling, 100µl of neutralization buffer (40mM Tris HCl, Sigma-T5941) was added and the mixture is centrifuged for 4 minutes at 4000 rpm (SIGMA 1-14 microcentrifuge). The supernatant was taken in a separate tube and stored at -20 ° C. The separated supernatant contains a dirty DNA sample, that was used in the polymerase chain reaction.

3.4 Polimerase Chain Reaction (PCR)

DNA sample and mixture used; Taq polymerase enzyme solution 2X and the primers are taken on ice and run at + 4 ° C. After the prepared mixture was divided into tubes, the DNA sample was finally added into the mixture. The positive and negative controls should be prepared in addition to the sample DNA tubes. The positive control tube contains of DNA from the mother or father known to be ChAT-Cre. The negative control consisted only of the mixture and does not contain DNA.

Primer Designs

5’ primer Primer: GCC AGC TAA ACA TGC TTC ATCTm: 54.5 ºC

3’ primer Primer: ATT GCC CCT GTT TCA CTA TCCTm: 55 ºC

Standard PCR Protocol (12.5 ul per tube)

• 6 µl 2X master mix (New England Biolabs, MO270S)
• 0,125 µl 20µM 5’ primer
• 0,125 µl 20 µM 3’ primer
• 0,5 µl template DNA
• 5,75 µl ddH2O [or It should be calculated as 12.5 µl total volume]

Standard PCR Programm

- 1.95°C, 1 min.
- 2.95°C, 30 sec
- 3.60°C, 20 sec [annealing temperature depends on primer Tm]
- 4.68°C, 45 sec [extension time depends on amplicon size]
- 5.Turn to step 2, 37 repeat
- 6.72°C, 5 min
- 7.4°C, t=0 [∞]

3.5 Agarose Gel Imaging

1X TBE Buffer Solution into 2 g agarose (Bioshop, AGA001.1) was added to a 2% gel mixture was obtained. Removing at short intervals by controlling the mixture in a microwave oven was heated until completely homogeneous. Safeview 5µl per 100ml

boiling in the gel (Applied Biological Materials Inc. Safeview FireRed) was added and shaken to disperse homogeneously. The agar was poured into the electrophoresis cassette.

The following steps in the protocol were followed:

Take care not to leave air bubbles. Place the comb. 20 minutes to freeze. The comb was removed. It was placed with the casette in the electrophoresis tank. 1X TBE buffer was added until the gel will sink completely into the buffer. PCR products were slowly loaded with the help of micropipette by adding 5X gel dye (1 µl, Sigma-G7654 for 1 sample 12µl) to the well. 3 ul HyperLadder (Bioline, H4-215108) is loaded to the first well. DNA samples are run in the gel in the direction of the positive polarity for 15 minutes at 200Volts (VWR, 300V). Gel was displayed in the BioRAD ChemiDoc imaging system. Samples of having 727 base pair are CRE positive.

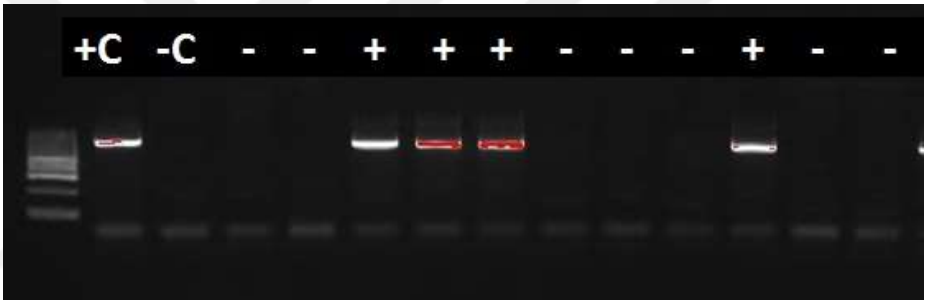

Fig.3: Representative Gel Image for PCR results.

+C: positive control (750bp). -C: negative control. (+) represents as positive results (Cre positive). (-) represents as negative results (Wild Type).

3.6 Adeno-Associated Virus (AAV) Production

Plasmids required for recombinant adeno-associated virus production were obtained from Addgene in the form of Stbl3 E-coli transformed bacterial swabsAfter sterilized-autoclaved LB agar mixed with H2O at the rate of 35 gr / L, 100 µg / mL ampicillin is added for antibiotic resistance. LB agar was poured into 10 cm polystyrene petri dishes at a rate of 2/3. Plasmids were cultivated on solid agar plates and incubated overnight at 37 ° C right after. Resistant bacterial colonies were selected with strips and transferred to 20g / L LB fluid medium for propagation. LB agar were allowed to multiply for 18 hours at 37 ° C, 250 rpm rpm for bacterial growth. The bacteria were centrifuged

at 4 ° C at 6000 rpm for 15 minutes after reaching a sufficient number of bacteria. The pellet was frozen at -80 ° C. These operations were repeated for the main vector, the auxiliary vector 2/1 and the auxiliary vector helper.

3.7 DNA Isolation

Pellets containing vectors were sown. The pellet content was DNA isolated using the Qiagen Endofree Plasmid Kit (Qiagen, 12362). The SpektraMax instrument was used to determine the concentration of DNA obtained. These operations were repeated for the main vector, the auxiliary vector 2/1 and the auxiliary vector helper.

3.8 293AAV Cell transfection

Cells of the 293AAV cell line (Cell BioLabs, AAV-100) were grown in an incubator under conditions of 37 °C, 5% CO_2, 20% O_2, in 10 × 10 cm petri dishes. When the cell density reaches 70-80 %, it is multiplied by passaging and when sufficient amount is reached cells are prepared for transfection. 24 μg helper vector helper, 20 μg helper vector 2/1, 12 μg master vector Opti-MEM transfection medium and polyethyleneimine transfection agent were added to the cells by mixing them in a sterile tube. The cells were allowed to incubate in the incubator at 37°C, 5% CO_2, 20% O_2 for 72 hours. The transfected cells were collected and centrifuged at 1100 rpm for 4 minutes at 4 °C. The pellet containing cells was frozen at -80°C.

3.9 Purification

The pellet resulting from the transfection was added to 9 mL of sterile lysis solution (150 mM NaCl, 20 mM Tris pH = 8,0). Cells were placed in 3 sets of liquid nitrogen (-150 ° C) and 37°C water bath until completely frozen and completely dissolved. Following cold-warm application, the sonication was performed on ice for 2 minutes on ice with 50% frequency. A total of 2 mins of viruses were isolated from the cells in the form of 15 sec active 15 sec wait. 1 mM $MgCl_2$ and 250 U / mL Benzonase (Sigma E8263-25K) for purification were centrifuged at 4000 rpm at 4 ° C for 20 minutes. 17%, 25%, 40% and 60% solutes were prepared for the phase gradient used to isolate viruses. The solution contents are indicated on the table 1. Gradient is generated by slowly dropping in 3 mL, 5 mL, 6 mL, 6 mL volumes with the aid of syringe to OptiSeal ultracentrifuge tubes (Beckman 36625), respectively. The cell lysis was added at the top. It is centrifuged at 60000 rpm for 90 minutes at 18ºC. Lysate was separated into viruses

at 40% solution layer and cell debris at 60% layer. Amicon 100K filtered columns (Millipore, UFC910024) were soaked in 1x storage buffer solution (10% 10x PBS, 50 gr D-Sorbitol, 42,4 mL 5% NaCl). The layer containing the 40% solution was added to the columns and filled to 15 mL with 1x storage buffer solution. And centrifuged for 30 minutes at 3500 rpm at 4 ° C. This procedure was repeated two more times with the addition of 1x storage buffer solution. Viruses remaining on the filter was collected and frozen at -80° C.

Gradient Table	% 17	% 25	% 40	% 60
10X PBS	5 mL	5 mL	5 mL	-
1M MgCl	0,05 mL	0,05 mL	0,05 mL	0,05 mL
1M KCl	0,125 mL	0,125 mL	0,125 mL	0,125 mL
5M NaCl	10 mL	-	10 mL	-
Iodixanol	12,5 mL	20 mL	33,3	50
% 0,5 Fenol Red	-	0,1 mL	-	0,025 mL
H2O	22,3 mL	24,7 mL	1,5	-
Total Volume	50	50	50	50

Table 1: The phase gradient used to isolate viruses.

3.10 Stereotactic Intracranial Surgery

Mice were anesthetized with isoflurane gas. The mice were placed through the upper teeth of the stereotactic frame. The tongue was taken sideways out of the mouth for opening the breath path. During the operation the mice were inhaled with a mixture of isoflurane and air. The foot was tightened with a forceps to control reflex formation. The mouse skull, were placed in the frame with the help of ear bars. Skin cut vertically with scalpel. The skull was wiped with hydrogen peroxide and bone lines become apparent. The Bregma point is assumed to be zero for the x, y, z coordinates. The glass micropipette was filled with mineral oil to make a phase difference with the virus. The micropipette can be used to determine coordinates in the head of the mice. The x and y coordinates of the skull are straight which is important for the success of injection. The trailing edge of the mouse head will cause entry to a region other than the targeted region. The skull was

drilled with a dental drill. The glass pipette was prepared with a Narshige micromanipulator vertical pipettor (Narishige, PC-10). The glass micropipette filled with mineral oil is filled with 800ul virus. The virus used for the thesis experimental group will be pAAV-EF1a-DIO-hM3D (Gq) -mCherry and pAAV-CAG-flex-rev-tdTomato for the control group. The Z-coordinate was zeroed by touching the cortex with the glass micropipette. The ARC was reached in 2 minutes along the Z coordinate. Injection 80nl / min is carried out at speed. Both sides were given 300 nL and after the injection was complete, 10 minutes was waited to avoid negative pressure before leaving the tissue. After injection, wound lips were brought together and sewed with 4.0 silk rope. The stitched area was wiped with betadine. The anesthetized mouse awake approximately 20 minutes, active within 2 hours. The ARC coordinates are constant as madial/lateral: (-) 0,450/(+)0,350mm; dosal/ventral (-)5,7mm, but anterior/posterior should calculate according the way between lambda-bregma points. The formula if Y coordinate would be ‘’A’’= (A*1,46) /4,2. 1,46 is the coordinate for ARC in Allen Brain Atlas for total brain lenght is 4,2mm Mouse brain.

At the end of the two-week infectious process, the virus would be expressed in the CHAT neurons in the ARC region.

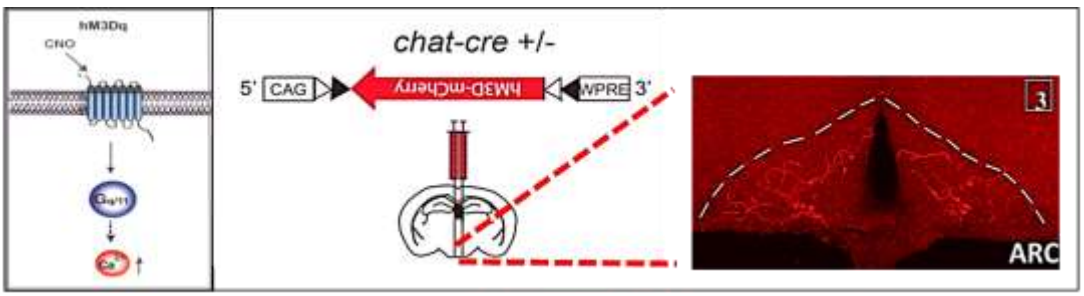

Fig.4: Representative image for intracranial injection of virus containing hM3D channels in ChAT-Cre mice and infected (red) ChAT neurons in ARC.

3.11 Behavioral Tests

Injected hM3D cation channel keep the target neurons active for 15-120 minutes after administration of agonist (CNO) *intraperitoneally* (ip) [71]. The only variable in our control group was the type of virus that is injected. The virus to be given contains tdTomato (red fluorescent protein). The virus-infected control group, which did not contain any channel proteins, becomes inactive after *i.p* administration of CNO. All groups receive CNO *i.p* and are tested 30 min later. There should be one day intervals between all tests to avoid the effects of CNO in the tissues. Tests were performed during the light phase, and are made elevated plus maze test, open field test and light -dark box test, respectively. The animals were brought into the experiment room at least half an hour before the experiment with their own cages to acclimatize. Room temperature was recorded. The room light was adjusted to the type of experiment to be applied.

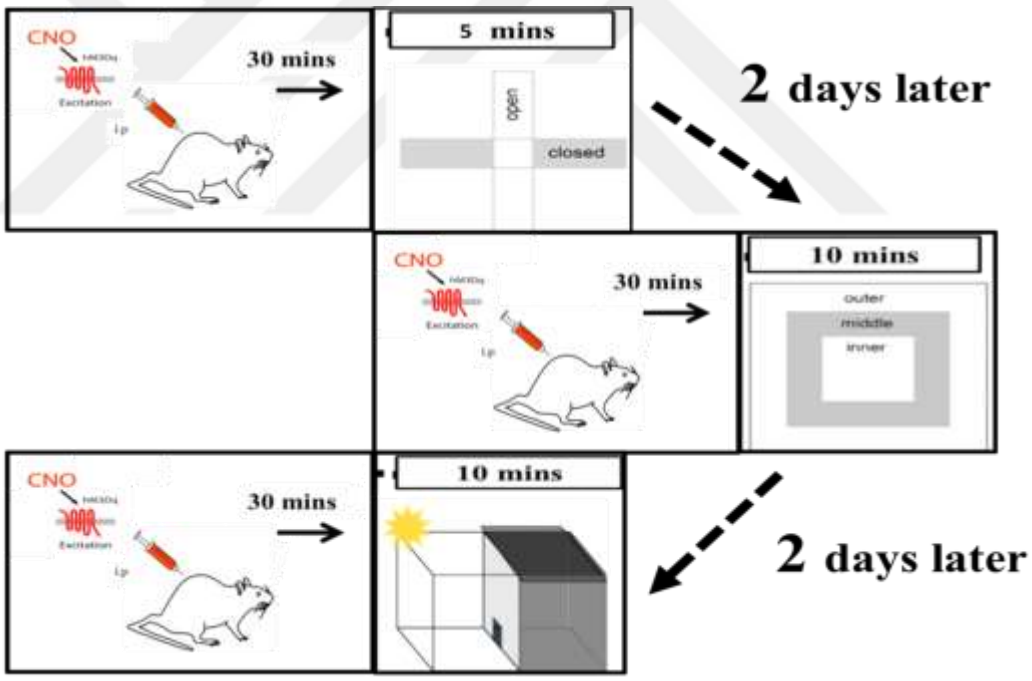

Fig.5: Behavioral tests flowchart

3.11.1 Elevated Plus Maze (EPM) Test

The elevated plus maze, the rodent is a commonly used behavioral tests to measure anxiety behavior. It is easy to apply and it can result in a short 5 minute test period[72].

The parameters are total arm entries, the percentage/ratio of open arm entries relative to total entries, and the percentage / ratio of time spent on the open arms relative to total arm time. Anxiety index[73] = 1 − [([Open arm time/Test duration] + [Open arms entries/Total number of entries])/2] were calculated.

Equipment: The maze consisted of four arms and based on the contrast between two plexiglas platform closed by lateral walls and two perpendicular open arms.

Procedure: The light reception of the center and endmost points of the platform must be measured before the test starts. The center is set to receive 100 lux. Room temperature measured at 22 degrees. Animals initially place in the intersection between the arms and exhibit a high level of risk assessment from the central platform toward the open arms. Their faces would be the direction to the open arm, in this study for each animal. Mice are allowed to move freely for 5 min and recorded[74]. After each trial, all chambers are cleaned with Ethanol 70% to inhibit a bias based on olfactory sense.

3.11.2 Open Field (OF) Test

The test is used to systematically assess new environment discovery, general locomotor activity, and detect behavioral changes in anxiety in rodents[75]. The OF is known to have two factors that affect anxiety-like behaviors. The first reason is the social isolation that is exposed when the test is conducted, and the second is the stratigraphy created by the brightly illuminated and unprotected new test environment[75,76].

Equipment: A large square chamber ranging in size from 80X80 cm and 45cm high. Chamber walls and floor are Plexiglas.

Procedure: The light reception of the center and endmost points of the square must be measured before the test starts. The center is set to receive 125 lux. room temperature measured at 22 degrees. Place each mouse in the center of a clean chamber. Mice are allowed to freely explore the chamber for 10 minutes. Mice that spend significantly more time exploring the unprotected center area demonstrate anxiolytic-like baseline behavior[77]. After each trial, all chambers are cleaned with Ethanol 70% to inhibit a bias based on olfactory sense.

3.11.3 Light-Dark Box (LDB) Test

In order to assess anxiety-related behavior in this design, the key criteria are the change in the number of passes between the illuminated and unprotected areas and sections reflected in the amount of time spent in each segment[77].

Equipment: The testing chamber comprised an equal two-compartment box (30X45X30) connected by an opening (7X7 cm), with one covered dark (0-5 lux) compartment and a second light compartment illuminated (200-400 lux).

Procedure: Mice were placed into the light compartment that their faces would be opposite direction to the dark compartment. It allows the mice to move freely between the two boxes with the open door for 10 min[74]. For acquiring knowledge and analyzing the behavioral data is used EthoVision XT 6 - Noldus. Total time spent in the light side during the light-dark box, velocity, number of entries to the light zone and the latency to enter the dark zone recorded by EthoVision XT 6 – Noldus. After trials, the all boxes are cleaned with Ethanol 70% to inhibit a bias based on olfactory sense.

The laboratory where the thesis work is done: Yeditepe University Brain Research Laboratory

Equipment used in thesis study: Stereotaxic frame, elevated plus maze test, open field test, light-dark box test

Chemicals used during the study: Clozapine-N-oxide, paraformaldehyde, 100% ethyl alcohol

Types of viruses used during thesis work: pAAV-EF1a-DIO-hM3D(Gq)- mCherry and pAAV-CAG-flex-rev-tdTomato

3.12 Cardiac Perfusion and Brain Slicing

Mice are anesthetized with isoflurane gas 30 min. after *i.p* administration of CNO. A small incision is made in the right atrium and blood circulation is terminated at this point. Left ventricular circulation was given 0.9% isotonic sodium chloride solution and 4% paraformaldehyde (PFA), respectively. The brain was taken up in 4% PFA solution

and left for 4 hours. Cross sections are taken from the brains to a thickness of 70 μm in the vibrotome.

3.13 C-Fos Immunohistochemistry Assay

The c-FOS protein assay was performed on the receive sections to detect active neurons after CNO administration. The sections are washed 2 times with PBS-Tween 20 for 20 minutes. Tissues are shaking at 216 rpm in a blocking solution containing 3.5% 4% goat serum (Sigma, 69023) and 0.3% Triton-X-100 (Sigma, T9284-12) 1X PBS (Invitrogen, 00-3002). C-Fos antibody (Abcam, ab190289) was prepared in 4: 5% goat serum at a ratio of 1: 5000. the tissues were allowed to shake at 4 ° C overnight. The sections washed with 1XPBS for 2 times for 20 minutes before second antibody administration. The secondary antibody consists of a 1: 1000 ratio Goat anti-Rabbit Alexa Fluor 488 (Invitrogen, A11008) antibody, 1XPBS, 0.1% Triton-X-100. Sections were incubated at room temperature for 2 hours with covered aluminium folio (to avoid the light) in the secondary antibody solution. the tissues were washed 2 times for 20 minutes with 1X PBS, then taken up on slides, dried and covered with coverslip with mounting medium (Sigma, F4680-2ml).

3.14 Imaging

Photos were taken with Zen Confocal microscopy. Red images refered mCherry and tdTomato flourescen in activation group's tissue and control group's tissue respectively. Green flourescent refers c-Fos active neurons result of ımmunostaining GFP tagged c-Fos second antibody. The hM3d infection and c-Fos staining are shown in figure 16.

3.15 Analysis

Statistical analyses were performed using the GraphPad Prism®6. All data are expressed as mean ± SD. Normal distribution of the data was tested using Shapiro-Wilk normality test. For the data passed normality test were analyzed by unpaired Student's t-test or else Mann Whitney U test. P values lower than 0.05 were considered significant.

4. RESULTS

4.1. Elevated Plus Maze (EPM) Test

EPM test results show that there is a significant increase in the ChAT neuron activation group when evaluated on the basis of time spent on open arms (Fig.1). In Figure 2, the results of all subjects are shown together with the mean values of the groups. When calculated according to the anxiety index (AI) wrote in the method of thesis, the release of ACh from the ARC was found to be a significant relief effect on anxiety (Fig.3). According to the velocity (cm/s) results it can say that activation of arcuate ACh has not a significant effect on locomotor activity (Fig.4). It was observed that there was no significant increase in the numbers of active neurons entering the open arm when the number of open arms entry rate to all arms was examined. But it is noteworthy that there is a slight increase (Fig.5). Experimental design is based on detecting the behavioral reflections of the physiological effects of active ARC^{ChAT} neurons. Therefore, when the experiment is terminated, the number of neurons carrying the respective receptor should be illuminated so that the observed behavior can be correlated with the chemogenetic activation performed. hM3D is a cation channel as mentioned above. Neurons carrying hM3D carry a fluorescent protein, that is necessary to be appearance of neurons easily under the microscope as well as this gene locus genetically. It should be additionally noted that this correlation analysis was conducted among all groups of animals in which behavioral data were analyzed and included animals with at least 10 infected neurons analyzed. Animals with fewer than 10 infected neurons were not included in the data analysis (Fig.6).

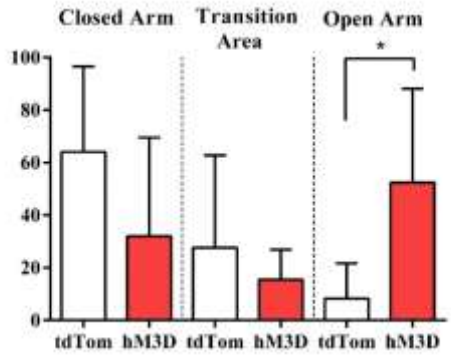

Fig 6. The spent time (%) in elevetad plus maze after 30 min CNO administration

Data are mean ± SD. *p <0.05; n= 9 in activation group(hM3D); n= 7 in control group (tdTom).

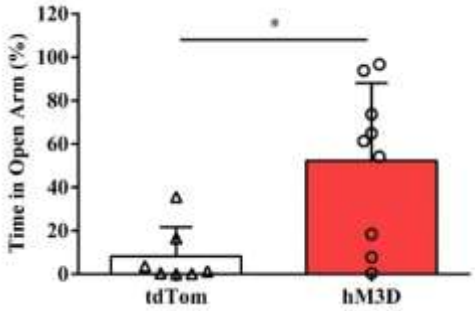

Fig 7. The spent time (%) in open arm with all subjects after 30 min CNO administration

Data are mean ± SD. *p < 0.05; n= 9 in activation group(hM3D); n= 7 in control group (tdTom).

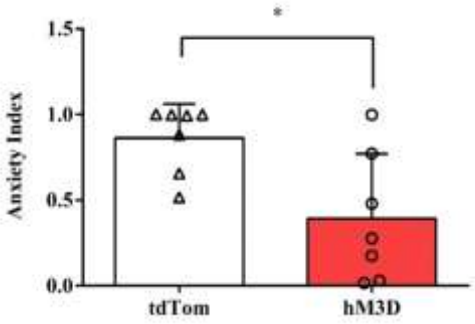

Fig 8. Anxiety Index

The activation group has less anxietic as compared with the controls. Data are mean ± SD. *p < 0.05; n= 7 in each group.

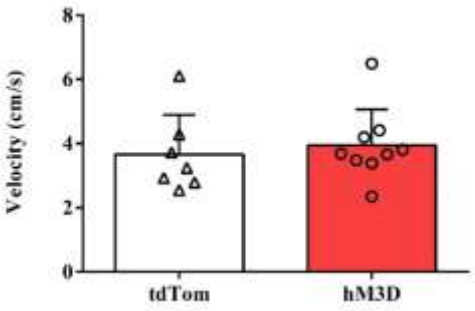

Fig 9. **Velocity**

There is not significant differance between two groups. Data are mean ± SD. n= 9 in activation group(hM3D) ; n= 7 in control group (tdTom).

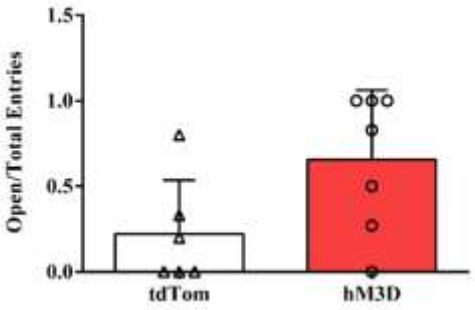

Fig 10. The ratio of entries number of open arm / total number of entries

There is not significant difference between two groups. Data are mean ±SD. n= 9 in activation group (hM3D) ; n= 7 in control group (tdTom).

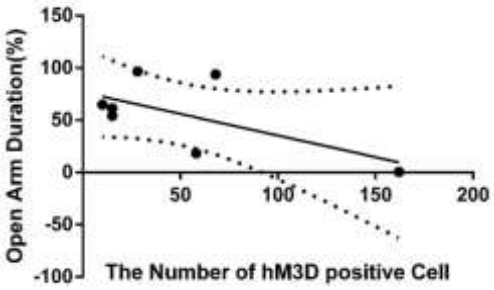

Fig 11.The correlation between the number of active ChAT neurons and spent time in open arm

There is not significant correlation. Data are mean ± SD. n= 7 in each groups.

4.2. Open Field (OF)

In the open field test, time spent in 3 areas was evaluated among the groups. Observed, the time spent in the outer area (area thought to be relatively sheltered relative to animals) of in the chemogenetically activated group decreased significantly compared to the control group (Fig.7). Figure 8 shows the time spent in the outer area together with the results of all subjects together with the mean values in groups. When the ratio of the total distance taken by the mice on the platform to the total arena duration examined activation of ARC ChAT neurons does not appear to have any effect on the locomotor movement of animals (Fig.9). The number of fecal boli can show us a scale that related with the emotional stress of mice. In the OF test, it is said that high defecation behavior is countered by fewer entries in the central part of the arena. In this thesis, the number of fecal boli in OF test does not give a meaningful difference between the groups (Fig.10). When the open field test evaluates data together, it is clear that the role of acetylcholine in reducing stress does not significantly affect physiological defecation.

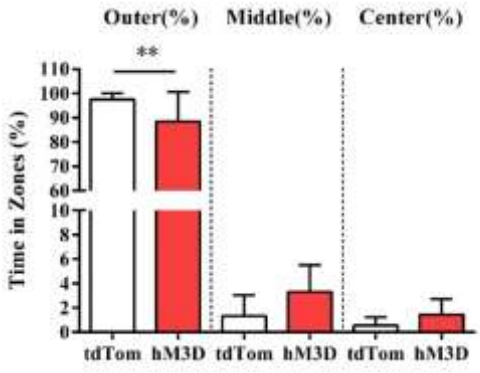

Fig 12. The spent time (%) in zones after 30 min CNO administration

The activation group spent significantly less time in the outer zone. Data are mean ± SD. *$p < 0.05$; n= 9 in activation group(hM3D); n= 7 in control group (tdTom).

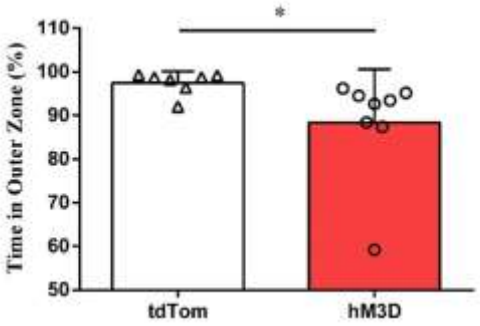

Fig 13. The spent time (%) in outer zone with all subjects after 30 min CNO administration

The activation group spent significantly less time in the outer zone. Data are mean ± SD. *p < 0.05; n= 9 in activation group(hM3D); n= 7 in control group (tdTom).

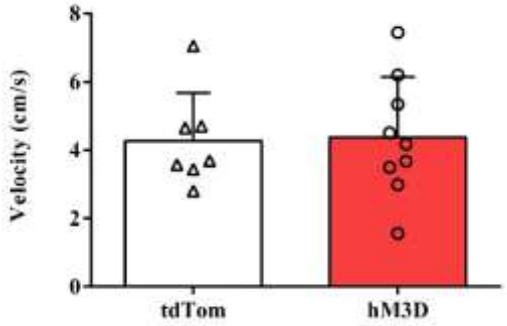

Fig 14. **Velocity**

There is not significant differance between two groups. Data are mean ± SD. n= 9 in activation group(hM3D) ; n= 7 in control group (tdTom).

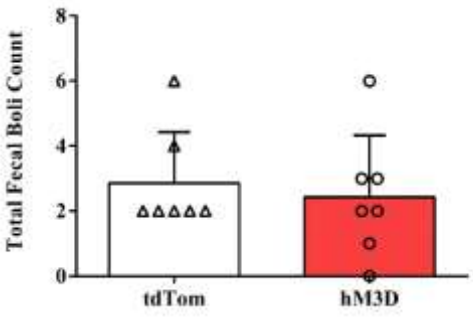

Fig 15. Total Fecal Boli Count

There is not significant differance between two groups. Data are mean ± SD. n= 7 in each group.

4.3. Light-Dark Box (LDB) Test

The light–dark box test is used the nonforced transition behavior from a dark and closed box to a bright and open space. Rodents prefer to stay away from brightly lit areas when they feel comfortable during movement in the dark areas of the surrounding area. The avoidance of movement in the light field is interpreted as an indication of anxiety. The reduction in anxiety is associated with the elongation at the time of the first transition to the dark compartment or to the light compartment. Given the results of the LDB test, it was noted that the activation group has significantly spent more time in light compartment. Additionally, the spent time in the dark compartment of ARC^{ChAT} activation group decreased significantly compared to the control group (Fig.11). There was no significant difference in speed between the two groups (Fig.12). There is a significant difference between the two groups in the number of dark box entries of mice left in the light compartment. Activation group mice exhibited a large number of inputs and outputs, exploring two areas, and greatly and significantly increased the number of entering the dark area (Fig.13). Based on this, it can be said that the behavior of avoiding the anxiety-forming environment also suppresses the idea of discovering the environment. When we look at the number of entries into the dark area while there is no change in the locomotor activity, the control group avoids going out to the light field and the activation group wanders in both areas. As mentioned above, the longer latency of the first time entrance to the dark compartment gives us information about the less anxiety. We leave the animals in light compartment for the first time and record the time to first

transition to the dark compartment. There was no significant difference between the two groups in terms of the delay in the first entry time (Fig.14). The number of fecal boli in LDB test does not give a meaningful difference between the groups (Fig.15).

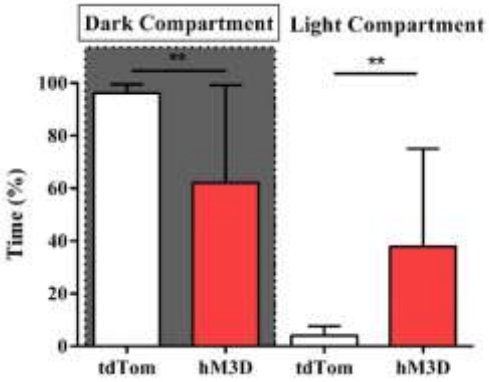

Fig 16. The spent time (%) in all compartments after 30 min CNO administration

The activation group spent significantly more time in the light compartment. Data are mean ± SD. **p < 0.01; n= 7 in each groups.

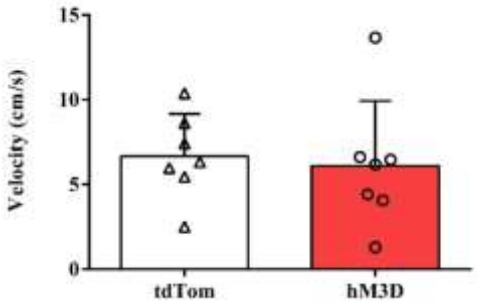

Fig 17.Velocity

There is not significant differance between two groups. Data are mean ± SD. n= 7 in each group.

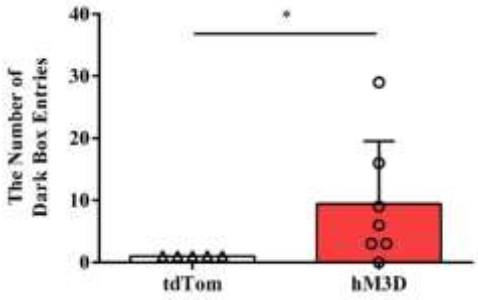

Fig 18. The number of entries to dark compartment

There is a significant increase in the activation group as compare control group. N= 7 in each groups.

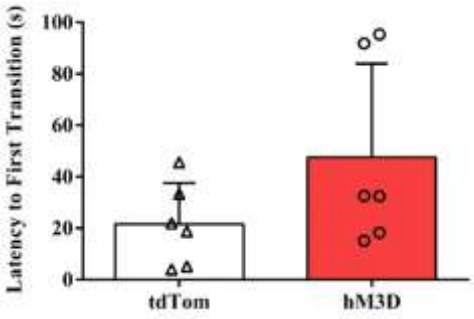

Fig 19.The time of the latency to first entrance into the dark compartment

Total time spent (%) during the light-dark box. There is not significant differance Averages ± SD are shown. n= 7 per groups.

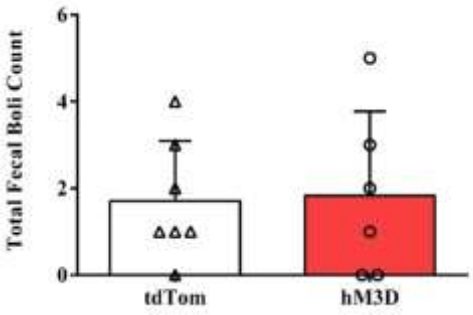

Fig 20. Total Fecal Boli Count

There is not significant differance between physiological two groups. Data are mean ± SD. n = 7 in each group.

4.4. Imaging and Cell Counting

Ip. CNO administration to animals was performed 30 min before cardiac perfusion. It is aimed to display neurons active during the experiment. The cFos stained sections were imaged under a confocal microscope. The number of double-stained cells were counted. Experimental animals with the number of infected cells under 10 neurons were removed from the group. Activation group staining was given with representative image in Figure 16. We have c-Fos negative hM3D infected cells in Image 16. This depends on the success rate of cFos staining.

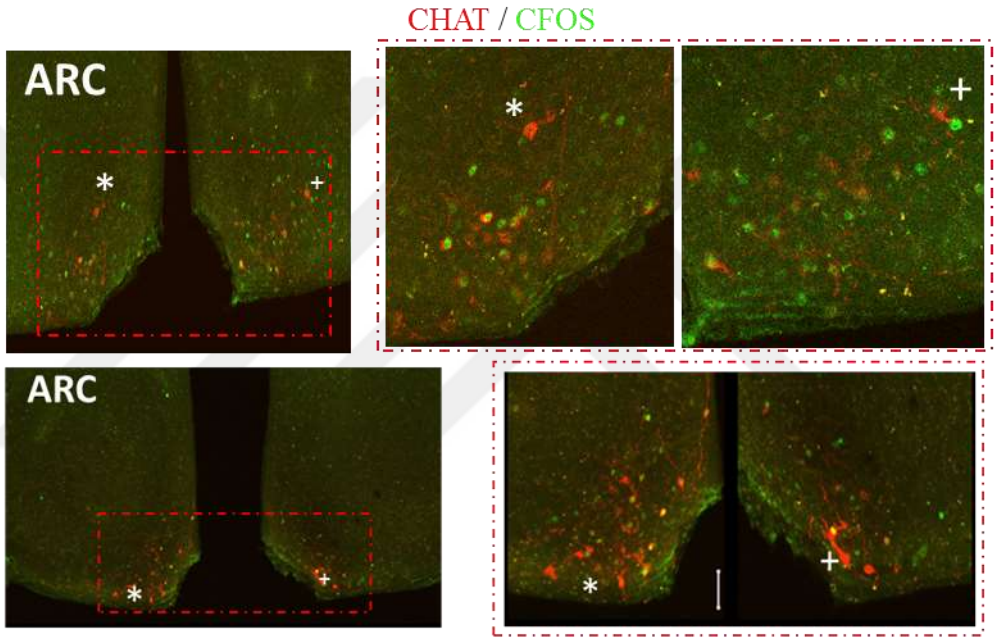

Fig 21. Representative images of C-Fos staining with hM3D-mCherry infected in ARC region of ChAT-Cre mice. Active cells were visualized using cFos staining Green Flourescent Protein (GFP). MCheery (Red) flourescent protein has genetically secreted with hM3D in ChAT neurons. Images were taken by Confocal microscopy. Scale bar, 100μm.

Denek No	Cfos+	Cfos-	Total
1	0	162	162
2	12	3	15
3	0	68	68
4	0	28	28
5	12	3	15
6	0	10	10
7	45	13	58

Table 2. A table of the number of C-Fos positive and negative neurons infected with hM3D-mCherry in the ARC region of ChAT-Cre mice.

5. DISCUSSION and CONCLUSION

Stress has been shown to induce anxiogenic-like behavior in mice. For example, the spent time in open arm decrease after predator odor [78] and unpredictable mild stress[79], in the EPM. Our experimental findings indicate that the level of anxiety is reduced when the acetylcholine system gets activated in the arcuate nucleus. However, there is not a significant change in the locomotor activity. Our results of this study are consistent with anxiety tests on Agrp activated animals [80] which show that the activation of Agrp neurons decreases anxiety levels. The activation of Agrp neurons increased the time in the open arms in the zero- and plus-maze tests, while activation of them did not change in total activity or time that animals explored the center of the arena in a novel open-field exploratory test. If the activations of AgRP and ChAT in ARC are considered to have separately the same, anxiolytic, effects, it may be thought that they are either synaptically linked or co-operated in a common regulatory network where they are effective. Because it is known that both the agouti-related peptide (AgRP) / neuropeptide Y (NPY) neurons and the POMC neurons carry the muscarinic acetylcholine receptor type (Chrm3) mRNA, while the ChAT neurons carry the NPY1R. As a newly discovered feature of Pomc neurons, plays a role in the perception of discomfort by activating the Mc4r receptors in the striatum[81]. It is known that ChAT neurons in ARC have a wide range of peptides, amines, which are enzymes responsible for the synthesis and release of GABA, glutamate, catecholamines, POMC-derived peptides as well as other neuropeptides, and neurotransmitters. Any dysregulation of this neuron group of the hypothalamus can be unpredictable effects on all circuits, as an example aversive perception system as can be seen.

In summary, all of the ARC ChAT neurons, which have molecular heterogenity, are targeted. The Cre-loxP system provided us an opportunity can activate all subgroups of ChAT. The anxiolytic effect we have seen may change when it occurs under physiological conditions. Although stimulation in the experimental environment provides only a certain amount of physiological conditions, neuromodulation effects of the neurons which are responsible for activation of the targeted neurons are neglected. The ARC^{ChAT} neurons activation was demonstrated by three anxiety tests that suppressed anxiety compared to the control group. The target regions involved in this behavior, which arise in further studies, should be identified and compared with known information in the literature.

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