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INVESTIGATING EFFECTS OF AGMATINE IN CHRONIC SOCIAL ISOLATION MODEL

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MASTER OF SCIENCE THESIS

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STATEMENT

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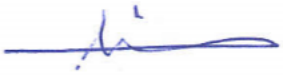

LIST of ABBREVIATIONS

5-HT: 5-Hydroxytryptamine

AD: Alzheimer's disease

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANS: Autonomic nervous system

BD: Bipolar disorder

BDNF: Brain derived neurotrophic factor

CNS: Central nervous system

CRF: Corticotropin releasing factor

CRH: Corticotropin releasing hormone

CRP: C-reactive protein

DAO: Diamine oxidase

E: Epinephrine

EPM: Elevated plus maze

GABA: Gamma aminobutyric acid

GDNF: Glial cell line–derived neurotrophic factor

GR: Glucocorticoid receptor

HPA: Hypothalamic – pituitary – adrenal

IL: Interleukin

i.p: Intraperitonially

LC: Locus coeruleus

LTD: Long term depression

LTP: Long term potentiation

MB: Marble burying

MDD: Major depressive disroder

MR: Mineralocorticoid receptors

NE: Norepinephrine

NF-kB: Nuclear factor kappa B

NGF: Nerve growth factor

NMDA: N-methyl-D-aspartate

NOR: New object recognition

NOS: Nitric oxide synthase

NT: Neurotrophin

OCD: Obsessive compulsive disorder

OF: Open field

p75 NTR: P75 neurotrophin low-affinity receptor

PCR: Polymerase chain reaction

PFC: Prefrontal cortex

PTSD: Post-traumatic stress disorder

PVN: Para ventricular nucleus

SAM: Sympathetic-adreno-medullar

SI: Chronic social isolation

SSRI: Selective serotonin reuptake inhibitor

TLR: Toll-like receptors

TNF: Tumor necrosis factor

Trk: Tropomyosin-related kinase

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1. ÖZET

Kronik sosyal izolasyon modelinde agmatinin etkilerinin araştırılması

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Anabilim Dalı: Farmakoloji

Amaç: Agmatin santral sinir sisteminde yaygın bulunan, nörotransmiter/nöromodülatör fonksiyonları olan endojen bir maddedir. Daha önce antidepresan ve anksiyolitik etkileri araştırılmış olan agmatinin bu çalışmada sıçanlarda kronik sosyal izolasyon (Sİ) modelinde etkilerinin araştırılması amaçlanmıştır.

Gereç ve yöntem: Sprague Dawley sıçanlar control, Sİ, Sİ+Fluoksetin (FLU) ve Sİ+Agmatin (AGM) 4 gruba (n=8/grup) ayrılmıştır. Hayvanlar tek olarak kafeslere alınarak 5 hafta süre ile izole edilmişler, hemen ardından kontrol ve Sİ gruplarına serum fizyolojik, Sİ+FLU ve Sİ+AGM gruplarına ise 15mg/kg FLU veya 40mg/kg AGM 10 gün süreyle günde tek doz olacak şekilde intraperitoneal olarak verilmiştir. Açık alan (open field; OF), yükseltilmiş artı labirent (elevated plus maze; EPM), yeni obje tanıma (novel object recognition; NOR) ve bilye gömme (marble burying; MB) testleri yapılmıştır. Tüm hayvanlar dekapite edilerek moleküler analizler için prefrontal korteks diseksiyonu yapılmıştır.

Bulgular: Sİ grubunda kontrol grubu ile karşılaştırıldığında OF testinde toplam aktivitede azalma, EPM testinde kapalı kollara geçiş sayısını ve süresini arttırmış, NOR testinde ayırd etme indeksinde bozulma ve MB testinde bilye gömmeyi artırmıştır. Bu değişikliklerin FLU tedavi grubunda kısmen, AGM tedavi grubunda ise tamamen geri döndüğü görülmüştür. Sİ grubunda IL-1B ve NF-kB mRNA ekspresyonlarında artış olduğu bu artışın FLU tedavi grubunda kısmen, AGM tedavi grubunda ise tamamen baskılandığı görülmüştür.

Sonuçlar: Bu çalışmanın sonuçları AGM'nin Sİ ile meydana gelen değişiklikler üzerinde etkili olduğunu ve bu etkiyi kısmen proinflamatuar sitokinlerin üretimini baskılayarak yapabileceğini göstermiştir.

Anahtar sözcükler: Agmatin, fluoksetin, sosyal izolasyon, proinflamatuar sitokinler

2. ABSTRACT

Investigating effects of agmatine in chronic social isolation model

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Aim of study: Agmatine is an endogenous molecule that is widely available in the central nervous system and has neurotransmitter/neuromodulator functions. Previous studies researched the antidepressant and anxiolytic effects of agmatine so this study aimed to investigate its effects on chronic social isolation model (SI).

Material and methods: Sprague Dawley rats were separated to 4 groups (n=8/group) as control, SI, SI+Fluoxetine (FLU) and SI+Agmatine (AGM). Animals were isolated in individual cages for 5 weeks. Right after that, control and and SI groups were given serum physiologic, while SI+FLU and SI+AGM groups were given 15mg / kg FLU or 40mg / kg AGM for 10 days as one daily intraperitoneal dose. Open field (OF), elevated plus maze (EPM), novel objects recognition (NOR) and marble burying (MB) tests were applied. All animals were decapitated and prefrontal cortex dissection was performed for molecular analysis.

Results: SI group when compared to the control group showed less total activity in OF test, more entrances and time spent into closed arms in EPM test, corruption in the discrimination index in NOR test and exessive burying in MB test. These changes were partially suppressed in FLU therapy group while totally suppressed in AGM therapy group. The elevated expression of IL-1B and NF-kB mRNA shown in SI group was also suppressed partially in FLU therapy group and totally in AGM therapy group.

Conclusion: This study shows that agmatine expresses effects against changes caused by SI and these effects may be partially caused by the suppression of proinflammatory cytokines production.

Keywords: Agmatine, fluoxetine, social isolation, proinflammatory cytokines

3. INTRODUCTION and AIM

Stress is a risk factor for several disabilities and forms a severe economic burden. World Health Organization expected that stress-related disorders, besides other types of mental illness are going to be the 2nd cause of disability in 2020. For instance, job stress alone is thought to cause hundreds of billions of US dollars annual loss in the United States (Lucassen et al., 2013).

Stress can lead to the ctivation of HPA (hypothalamic - pituitary - adrenal) axis (Moreira et al., 2016). Moreover, it is also associated with alterations in other physiological systems including mainly the autonomic nervous system (ANS) as well as the immune and circadian systems (Tsigos et al., 2016). Psychosocial stress is of no exception. Research showed that psychosocial stress can also seriously cause or worsen mental disorders (Schiavone et al., 2016). Social species depend on sets of social networks that associate deeply with behavioral, neural, cellular, molecular and genetic structures of these creatures (Cacioppo et al., 2011). Social isolation is well known and widely studied as a type of psychosocial stress and is reported to relate with a broad range of mental and physical dysfunctions in humans as well as other social species (Cacioppo et al., 2011; Cacioppo et al., 2015; Beery and Kaufer, 2014). For instance, social isolation was shown to be a risk factor for hypertension, metabolic syndrome, increased activity of the HPA axis, alterations in the function of the immunity system (Cacioppo JT and Cacioppo S, 2014) decreased cognitive function, elevated negativeness, sooner cognitive reduction, (Cacioppo and Hawkley, 2009) and depression (Gómez-Galán et al., 2016).

Agmatine, is a cationic amine (Molderings and Haerisch, 2012) neuromodulator that regulates several neurotransmitters and signaling systems, with neuroprotective effects that were suggested to be mediated by decreasing in oxidative damage, neuroinflammation and proapoptotic signaling (Neis et al., 2017). Among the established functions of agmatine we can find: blocking N-methyl-D-aspartate (NMDA) receptors, competitively inhibiting nitric oxide synthase (NOS) and acting as a neurotransmitter on imidazoline and α2-adrenergic receptors (Kim et al., 2017). This multifunctional amine, showed also anti-neurotoxic and antidepressant actions in the brain (Benitez et al., 2018), and was proposed to have anxiolytic effects

(Aricioglu, 2016) in addition to a protective role against morphine withdrawal syndrome (Aricioglu and Sav, 2016a; Aricioglu and Sav, 2016b), moreover, it was suggested to have a potential role as a regulator in post-traumatic stress disorder (PTSD) (Aricioglu, 2016). Agmatine is widely distributed in mammalian tissues. After it was observed in the rat brain, it was found in higher concentrations in other rat tissues like stomach, intestine, spleen and liver. Moreover, it is also detectable in human plasma where higher values were observed in depressed people (Grillo and Colombatto, 2004).

Preclinical research has a clear importance in the field of social isolation research, because of the difficulty of controlling and designing human studies as well as the long life span of humans compared to other social species like rodents. Preclinical studies showed that chronic social isolation led to several neurochemical and neuroendocrinal changes that cause many alterations in interleukin-1betta (IL-1B), IL-6 protein and cytosolic/nuclear factor kappa B (NF-kB) expressions in addition to changes in the activity of super oxide dismutases and reactive oxygen species system. SSRI (Selective serotonin reuptake inhibitor) fluoxetine was proven to be an effective and useful agent to alleviate these changes (Filipović et al., 2011; Zlatković et al., 2014).

In this study, we would employ the social isolation model in an attempt to investigate the potential therapeutic effect of agmatine on changes in behavior, attention and some proinflammatory cytokines levels that occur after applying this chronic stress model in rats and how they compare to the results we obtain when using fluoxetine.

4. GENERAL INFORMATION

4.1. Stress

Stress is taking a huge interest today in neuroscientific and psychiatric research. While the original aim of organism's fight or flight response to stress is to maintaine the homeostasis and preserve the existence of the creature, excessive stress may lead to multiple physical and psychological disorders (including hypertension, diabetes, Alzheimer's, depression, anxiety and post traumatic disorder). Moreover, stress with its impact on health and life quality acquires a special importance in the financial status of society. In an interesting report released by the American Psychological Association, mental health experts carried out a study on stress and health among certain socioeconomic, racial, and ethnic groups to show the long-term effects of stress. According to the report, men who are at the top 1 percent of income scales enjoy a 15 years longer life than their counterparts at the lowest 1 percent. In ladies, the difference is 10 years of longevity. Authors also said accidents, absenteeism, employee turnover, diminished productivity, and direct medical, legal, and insurance troubles that stress causes cost the United States $300 billion every year (https://www.apa.org/pi/health-disparities/resources/stress-report.pdf, Date of access: 18 February 2019).

4.1.1. History of stress research

Although the concept of stress seems popular in modern psychological and neuroscientific research, the start of early stress research was actually in the 19th century in laboratories of medicine and physiology. The word “stress”, originally derived from the Latin verb *strectus* that means *to draw tight*, and used in a wide range of articles in fields like biology, medicine, psychology and neuroscience in addition to sociology and economy. It is better to start any meaningful new health-related research of stress with understanding first steps in the

field that formed the basis of stress as a concept in these researches (Robinson, 2018).

The French famous physiologist Claude Bernard (1813-1878) stated that complex organisms are able to maintain their internal medium [extracellular fluid] fairly constant against challenges from the external world (Cliff et al., 2015). About 50 years later, the Harvard physiologist Walter Cannon (1871-1945) described and extended Bernard's work by coining the word "homeostasis" combining two Latin words ὅμος (hómos,"similar")+ ιστημι (histēmi,"standing still")/*stasis* (from *στάσις*). In his successful book *The Wisdom of the Body*, Cannon listed four core concepts that defined his idea of homeostasis:

1. Constancy has to be kept in place and maintained by effective mechanisms.
2. Steady-state conditions require that any alteration or attempt of changing the current situation would be automatically met by resisting forces.
3. In order to maintain homeostasis, a functioning regulating system made of various cooperating mechanisms is needed. These mechanisms may work simultaneously or successively.
4. Homeostasis can not be the result of mere chance. It actually comes from organized self-government (Davies, 2016).

About Eight decades ago, *Nature* published a note from Hans Selye entitled 'A Syndrome produced by Diverse Nocuous Agents'. Selye noticed that typical symptoms appear in experimental rats which were exposed to an acute and non-specific injury such as thermal injury, mechanical injury, or pharmacological sub-lethal doses of some dangerous drugs. Selye coined the term "general adaptation syndrome" to describe the aforementioned condition where the same clinical picture seemed to arise regardless of the nature of the damaging agent or the pharmacological type of the drug used. The clinical picture that Selye described includes hypothermia, decreased muscle tone, a smaller thymus; spleen; lymph nodes and liver, acute erosions of the digestive tract, larger adrenal glands and histological alterations in the adrenal medullary chromaffin cells as well as increased secretion of pituitary. In other words, we can say that Selye was describing things as stress-induced immunosuppression, peptic ulceration and the activation of both HPA axis

and the sympathomedullary system, which are considered the most important two vertebrate stress response systems (Fink, 2017).

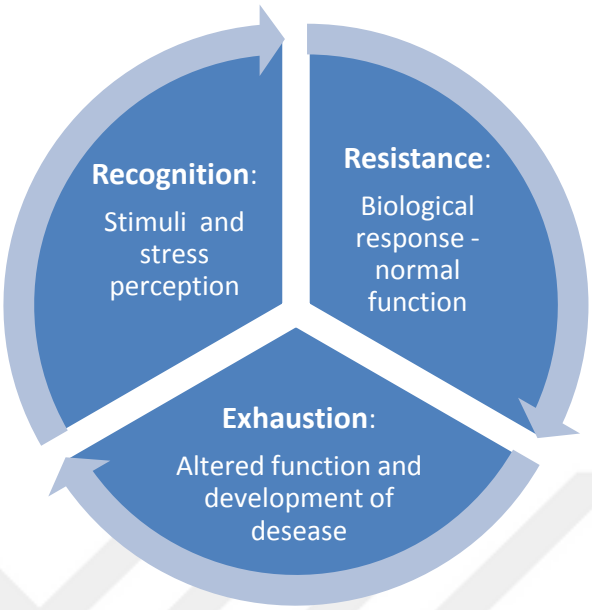

Figure 1. Phases of general adaptation syndrome (Brown EJ &Vosloo A, 2017).

Selye hypothesized that a "first mediator" in the hypothalamus is leading these physiologic changes. Two decades later, the Nobel prize winner, Roger Guillemin, who was one of Selye's PhD students, announced in 1955 the existence of a hypothalamic factor that induced adrenocorticotropic hormone release from the rat pituitary and called it corticotropin releasing factor (CRF) (Tache, 2014). Selye, considered as the father of stress research was a pioneer in the field and probably gave the very first definition of stress as a biological response. He viewed stress within the framework of homeostasis as a *"nonspecific response of the body to any demand made upon it*". He also believed that researching stress and trying to understand its various ramifications in health, sickness, philosophy and the society at large is the most meaningful and noble of scientific pursuits stating: "Stress in heath and disease is medically, sociologically, and philosophically the most meaningful subject for humanity that I can think of" (Somogyi A et al., 2012; Godoy et al., 2018).

4.1.2. HPA and SAM axes in stress response

Organisms use a complicated network within brain and body to manage what is cold "a stressful situation". When an event is detected or interpreted as a "stressor" by the specialized wide diverted stress response structure in the brain, the two major stress system constituents is activated so they release their final biochemical mediators. These two major constituents are the sympathetic-adreno-medullar (SAM) axis, that releases epinephrine and norepinephrine in addition to the HPA axis, that releases glucocorticoids. By the activation of these two axes, the body produces very rapidly (within seconds) a suitable response that could last up to days in an attempt to return to homeostasis. To achieve this, the stress response involves energy mobilization, metabolic changes, inducing immunity and suppressing the digestive and reproductive systems, alterations in the pro-inflammatory signaling in addition to short and long-term effects in the brain including changes in cellular excitability and plasticity (Godoy et al., 2018; kaur et al., 2013). In the first phase of stress response, the SAM axis is activated providing fast physiological adaptation that supports responding to the event (De Kloet et al., 2005; Joëls and Baram, 2009). Accordingly, the HPA axis is activated by the second phase involving hormonal mechanisms that are considered slow in comparison with the synapse-level activation of the SAM axis and thus would lead to long-lasting responses. Nevertheless, parasympathetic system can also be activated during stress.

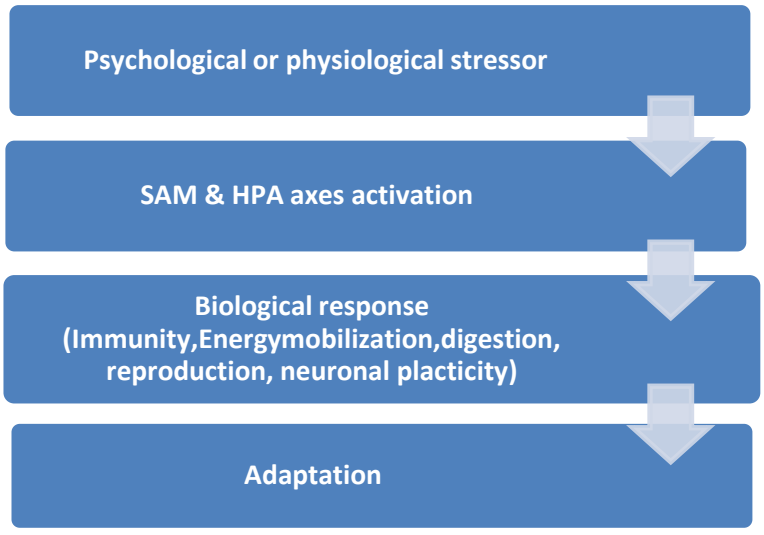

Figure 2. Stress system (Godoy LD et al, 2018).

First referred by Cannon as “fight or flight response”, the inducement of this system results in activation of preganglionic sympathetic neurons which in turn project to end organs and the adrenal medulla through the ganglia of the autonomic nervous system. Subsequently, an increasing appears in circulating levels of epinephrine (mainly from the adrenal medulla) and norepinephrine (mainly from sympathetic nerves), heart rate and contraction power, peripheral vasoconstriction and energy mobilization. For the HPA axis, the exposure to stressor, results in the activation of the hypothalamic para ventricular nucleus (PVN) to secrete corticotropin releasing hormone (CRH) that induces the secretion of ACTH by the anterior pituitary. Stimulated by ACTH, the adrenal cortex in turn makes and releases glucocorticoids that will mediate the mobilization of energy (Herman and Ulrich-Lai, 2009).

For the SAM axis, the stress response for stressors includes a rapid adaptation mediated mainly by catecholamines (epinephrine& norepinephrine). There are direct projections from the brain circuity responsible for this autonomic adaptation to pre-ganglionic sympathetic nerves that in turn connect to post-ganglionic nerves to transmit signals from the brain to the target tissue. In fact, some pre-ganglionic neurons do not have any synapses with a post ganglionic neuron, instead they synapse right ahead with chromaffin tissue in the adrenal medulla that secrete epinephrine (E) and norepinephrine (NE) which in turn increase the strength of sympathetic stress response. The parasympathetic arm of ANS regulates the progress and duration of autonomic response. The spinal cord and brain stem and give rise to Pre-ganglionic parasympathetic neurons, which in their turn synapse with post-ganglionic neurons on the target organs. Post-parasympathetic ganglionic neurons work on muscarinic receptors and activate them by releasing acetylcholine.

The rise in circulating norepinephrine and epinephrine triggers a series of reactions which orient the whole organism to a “fight-or-flight” mode. In practical terms this means staying alert for potential dangers, speeding up some metabolic pathways such as glycogenolysis and lipolysis, all alongside notable changes in the cardiovascular system (Godoy et al., 2018).

The central noradrenergic system, specifically the Locus coeruleus (LC) interconnects with different areas involved in the stress response, such as the hippocampus, temporal neocortex and amygdala. Research shows a special

importance of the LC in response to acute stress (Myers et al., 2017). It should also be mentioned that chronically activating LC is thought to be involved in the developing stress-related abnormal behaviors. Interestingly, LC works co-ordinately with the HPA axis (Godoy et al., 2018). While the HPA axis can be modulated by direct projections from LC to the PVN (Armario et al., 2012), CRH is also thought to influence the activity of LC through afferent projections running from amygdala and the brainstem nuclei (Jia et al., 2015; McCall et al., 2015).

The activation of HPA axis starts in the hypothalamic PVN which secretes CRH that triggers the release of ACTH by anterior pituitary. ACTH in turn stimulates the middle layer of the adrenal gland (named fasciculata) to secrete glucocorticoids (Godoy et al., 2018). Cortisol is the most important glucocorticoid in our bodies, and its animal counterpart in rodents is corticosterone (de Kloet, 2013). Interestingly, glucocorticoids also act on PVN, the anterior pituitary and other brain structures; including the hypothalamus, manipulating the activity of the HPA axis by a mechanism called negative feedback loops (Godoy et al., 2018; McEwen et al., 1968).

Glucocorticoids are steroids which enjoy a high cell membrane permeability. They move from the synthesizing cells to the blood and from there on they can reach any cell in the body. Cortisol (corticosterone in rodents) works on two kinds of receptors in the brain, namely glucocorticoid receptors (GR) and the mineralocorticoid receptors (MR). Various mechanisms are present in the response cascades related to these receptors including genomic and non-genomic mechanisms.

Glucocorticoid receptors' levels seem to vary depending on the environment and on life experiences, including acute and chronic stressors. This variation seems to involve a mechanism of epigenetics where DNA chromatin gets altered while the DNA sequence remains untouched. (Godoy et al., 2018). An example of the aforementioned environmental influences was shown in an experiment on pups. The amount of care pups receive from their dams seemed to influence the amount of glucocorticoid receptors in their hippocampus. Interestingly, mechanism behind this variation was proven to be that pups who received less care had more hippocampal NC3R1 (a nuclear GR gene) methylation compared to other pups that were well taken care of (Weaver et al., 2004).

Table 1. Effects of HPA axis activation (Godoy LD et al., 2018).

The effects of the glucocorticoid hormones and HPA axis activation
Increased glucose production
Exacerbation of gastric irritation
Increased urea production
Increased release of free fatty acids into systemic circulation
Increased susceptibility to atherosclerotic processes
Increased susceptibility to nonthrombotic myocardial necrosis
susceptibility to nonthrombotic myocardial necrosis
Thymicolymphatic atrophy (in animals only)
Supression of immune mechanisms
Exacerbation of herpes simplex
Increased ketone production
Appetite suppression
Associated feeling of depression, hopelessness and loss of control

4.1.3. Stress and neurotransmission

Stress evokes different alterations in neurotransmission. Research showed that acute, controllable physical stress promotes dopamine levels in ventral striatum. Interestingly, that was not the case in chronic uncontrollable same stress where dopamine release was attenuated. Accumulating body of research also suggested that availability and secretion of norepinephrine increase in response to stress inside brain areas like LC and hippocampus (Tsigos and Chrousos, 2002). Additionally, serotonin receptors (5-HT1A) were found to be down-regulated after stress in some brain areas such as the hippocampus and the cortex (Flugge, 1995). Another connection between stress and serotonin appears in increased responsiveness to stress caused by significantly decreased serotonin levels (Kumar et al., 2013). Stress has also been

stated to dysregulate GABAergic neurotransmission, suggesting a role of GABA in behavioral and biochemical changes following stress (Quintero et al., 2011).

4.1.4. Stress and metabolism

Stress response causes multiple hormonal and behavioral alterations in order to increase the ability of the organism to survive (Chrousos, 2000). CRH - increased by stress - inhibits gonadotropin-releasing hormone, growth hormone, thyrotropin-releasing hormone, and thyrotropin secretion during stress. Thus, it suppresses reproduction, growth, and thyroid function. It's thought that the chronic activation of stress system increases visceral adiposity, decreases lean body (muscle and bone) mass and suppresses osteoblastic activity (Tsigos and Chrousos, 2002). Glucocorticoids - also increased by stress - decrease pituitary gonadotropin, growth hormone, and thyrotropin secretion and induce resistance in target tissues to sex steroids and growth factors (Chrousos and Gold, 1992). Additionally, glucocorticoids affect bones, by inhibiting osteoblastic activity and inducing osteoporosis (Chrousos, 2000). Interestingly, obese individuals with psychiatric problems those had perception of 'uncontrollable' stress frequently have mild hypercortisolism (Tsigos and Chrousos, 2002).

4.2. Social isolation stress

Social isolation is considered to be a risk factor for a wide ranged morbidity and mortality. Early evidence for that was shown in epidemiological studies which defined social isolation objectively depending on factors such as the lack of spouse, making less contact with friends and family (House et al., 1988). It is well-established now that social isolation is a potent stressor in humans as well as animals, thus, it leads to alterations in behavioral reactions against other future stressors (Jones et al., 2011; Liu et al., 2010). The brain is the main organ for creating and organizing social relationships (Cacioppo and Berntson, 1992). Interestingly, social isolation (SI) stress affects different regions and functions of the brain and CNS including hippocampus, cortex, serotonin, dopamine, GABA, glutamate,

epinephrine, netrergic and opioid systems. Moreover, SI leads to alterations in the endocrine system, activation of HPA and SAM axes as well as disruptions to other systems and functions in the body such as oxidative and nitrosative stress, inflammatory factors and neurotrophic factors (Dehpour et al., 2018). Having that broad effect on both CNS and body, SI is reported to cause several health problems including anxiety, depression, daytime fatigue, inflammation and cardiovascular disease (Bhatti and Haq, 2017; Cacioppo et al., 2016).

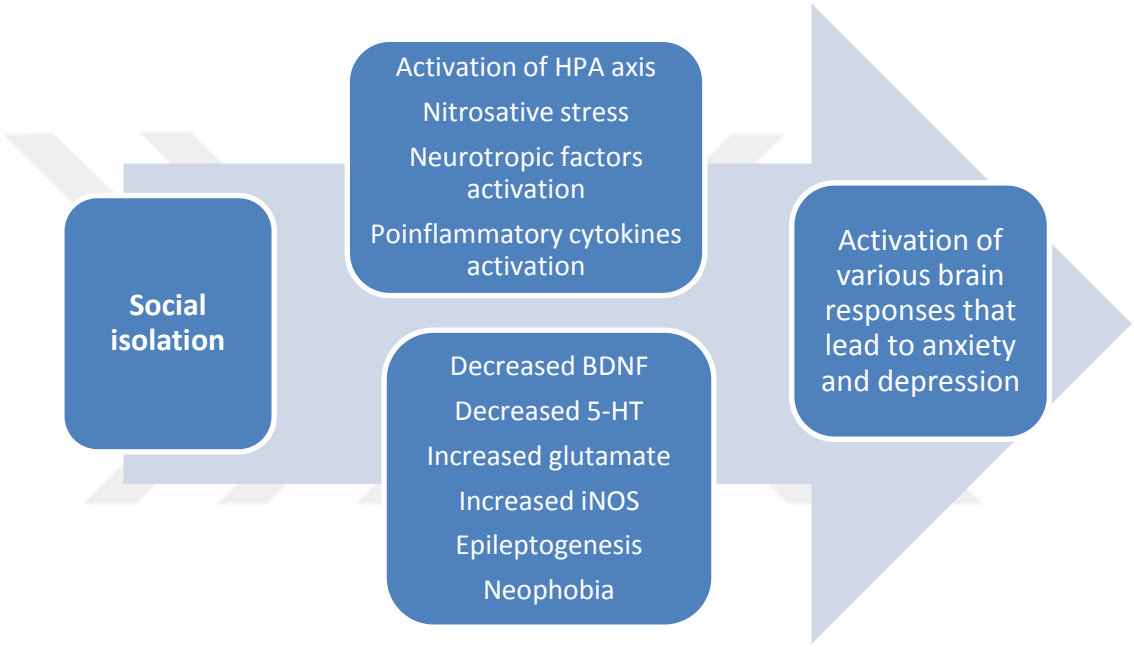

Figure 3. Stocial isolation effects on CNS. (Mumtaz F et al, 2018).

4.2.1. Social isolation effect on neuroplasticity and neurotrophic factors

Neurotrophic factors and associated signaling pathways form an essential corner in neuroplasticity and so on control the actions of which the nervous system responds to intrinsic and extrinsic stimuli by reorganizing its function, structure and connections (Levy et al., 2018).

The first classified member of neurotrophic factors was the nerve growth factor (NGF) (Korsching, 1993). After about 30 years, the brain derived neurotrophic factor (BDNF) was isolated by Brade et al. (1982) from the brain of a pig to become

another member of neurotrophic family that also contains neurotrophin (NT)-3, neurotrophin-4/5, NGF and neurotrophin-6,7 whereas neurotrophin-6,7 do not exist in mammals (Barbacid, 1995; Gotz et al., 1994; Nilsson et al., 1998).

Table 2. Classification of neurotrophic factors (Razavi S et al; 2015).

Neurotrophic Factors		
Nerve growth factor (NGF) family	**Glial cell line–derived neurotrophic factor (GDNF) family**	**Neuropoietic cytokines**
NGF, BDNF, NT-3, NT-4 (NT-4/5 or NT-5) and NT-6	GDNF, neurturin, persephin and artemin	Ciliary neurotrophic factor and leukemia inhibitory factor

However, studies of neurotrophic factors regarding social isolation has focused on BDNF only so we will discuss it in detail. Two forms of BDNF peptides are available: pro-BDNF of which proteolytic cleavage results in the other mature form of BDNF. BDNF is distributed widely in the brain and expressed in the hippocampus at its highest levels (Hofer et al., 1990). Although astrocytes uptake and can also synthesize BDNF, BDNF is fundamentally expressed by neurons (Zafra et al., 1991). Neurons in hippocampus may contain BDNF mRNA in somata and proximal dendrites. Somata BDNF plays an effective role in the formation of dendritic spine, while spine pruning and head growth relies on dendritic spine formation (Orefice et al., 2013).

Elevated levels of K^+, glutamate or BDNF may increase BDNF secretion from hippocampal neurons. This secretion level is also thought to depend on an increase in the intracellular Ca^{2+} levels (Goodman et al., 1996). Neurotrophins seem to express their effects by binding to one of three classes of tropomyosin-related kinase (Trk) receptors, and these are called TrkA, TrkB and TrkC (Binder and Scharfman, 2004). These receptors manifest varying binding affinities for different neurotrophins. BDNF goes through a dual receptor scheme; while it binds to the p75 neurotrophin low-affinity receptor (p75 NTR), it has high affinity to TrkB receptors (Chao, 2003; Cowansage at al., 2010). Contrariwise, the high affinity of pro-BDNF is reported towards the p75 NTR (Woo et al., 2005). BDNF may affect both pre- and post-

synaptic sites because TrkB receptors are available on both sites (Yoshii and Constantine-Paton, 2010).

When BDNF binds to TrkB, the dimeraization and autophosphorylation of many tyrosine residues is induced, activating different pathways which leads to neuron regeneration, survival and repair. In contrast, when pro-BDNF triggers p75 NTR, it activates c-Jun N-terminal kinase and NF-κB (Gentry et al., 2004) and these two transcriptional factors are linked to apoptotic signaling (Boulle et al., 2012; Park and Poo, 2013). The release of many neurotransmitters is affected by BDNF while synthesis of BDNF itself is also regulated by several neurotransmitters. For instance, glutamate plays a regulating role in BDNF expression where the expression of BDNF mRNA in the hippocampus is increased by activating NMDA receptors (Caldeira et al., 2007). Additionally, target-selective potentiation of gamma-aminobutyric acid (GABA)ergic presynaptic terminals is also promoted by BDNF. Moreover, selective serotonin reuptake inhibitors promote a rise in BDNF in rats' hippocampus proposing the relation of serotonin and BDNF mRNA (Molteni et al., 2010; Ohba et al., 2005).

BDNF expression was suggested to be regulated by 5-HT in an acute psychological stress rat model (Jiang et al., 2106) . Nevertheless, hippocampal BDNF expression is regulated by cholinergic activity in the medial septal nuclei, whereas BDNF and NGF mRNA levels are increased by the stimulation of that cholinergic pathway through activating D1 receptors (Ohta et al., 2010). The harmonized dual functioning system leads to the Yin-Yang hypothesis about neurotrophic action. In details, the activation of TrkB receptors by BDNF contributes to synaptic potentiation and cell survival, while the activation of p75 NTR by pro-BDNF induces synaptic depression as well as dendritic endings reduction and cell death (Lu, 2003; Lu et al., 2005).

BDNF plays a proven role in cognition and emotion (Rakofsky et al., 2012), spatial and contextual learning (Hall et al., 200; Mizuno et al., 2003) mainly by regulating serotonergic neurons' activity and development (Martinowich and Lu, 2007). At Schaffer collateral-CA1 synapses, BDNF may modulate long-term depression (LTD) in addition to long term potentiation (LTP) (Ikegaya et al., 2002; XU et al., 2000), thus influence the memory formation by strengthening synapses (Mizuno et al., 2003; Shors and Matzel, 1997). Pro-BDNF, has also been shown to facilitate

hippocampal LTD that is dependent on the NMDA receptor by activating p75 NTR. Activity-dependent plasticity in adult synapses and synaptic transmission form a base for BDNF regulating role (Bramham and Messaoudi, 2005; Woo et al., 2005). Inhibitory and excitatory synapses' formation and maturation is also induced and supported by BDNF (Lu, 2003). Nevertheless, BDNF also supports neuronal prolongation and survival of neurons. Thus, decreased BDNF signaling and reduced neuronal density in dentate granule and pyramidal CA1 cell layers of the hippocampus where reported in genetically modified mice of TrkB and TrkB/TrkC receptors (von Bohlen and Halbach et al., 2005), forasmuch, increased apoptosis was found in mice that is deficient of TrkB receptors (Minichiello et al., 1999).

Studies have shown that social isolation led to decreased BDNF expression in most cases (Zaletel et al., 2017). For example, Scaccianoceet et al. (2006) noticed a reduction in hippocampal BDNF in Sprague-Dawley rats after 8 weeks of social isolation, and that social isolation had no effect corticosterone concentrations, leading to the fact that BDNF reduction was glucocorticoids-independent.

In fact, this reduction of BDNF maybe induced by epigenetic factors. Some cytokines like interleukin 1β (IL- 1β) can regulate BDNF expression. In one interesting study, post-social isolation intrahippocampal injection of IL-1 receptor antagonist, blocked the reduction of hippocampal BDNF mRNA which means that increased IL- 1β levels induced by social isolation may lead to decreased BDNF (maybe by activating NF-κB pathway) (Barrientos et al., 2003). This explanation of BDNF reduction has been also confirmed by results extracted from studying rat hippocampus slices showing that IL- 1β is associated with decreased TrkB-regulated BDNF activity in addition to the reduced levels of some proteins that are necessary for the stabilization of LTP (Tong et al., 2012). Interestingly, these results would help to understand the underlying mechanism responsible of cognitive decline following social isolation (Pugh et al., 1999).

4.2.2. Social isolation stress, cytokines and immune-inflammatory response

Stress response, provoked by psychological, social or environmental stressors induces a variety of physiological changes including alterations in the function of

endocrine and nervous system activating SAM and HPA axes (Sterling and Eyer, 1988). A growing body of modern research shows that (prolonged or severe) stress leads to increase the risk of physical and psychiatric disorders. Stress is stated to be a common risk factor of 75-90% diseases including diseases with the most burden like cardiovascular disease, psychiatric and neurodegenerative disorders (Cohen et al., 2007). Although classical approaches to understand mechanisms behind of stress related diseases emphasized the role of HPA and SAM axes, these two systems affect target systems indirectly so understanding the etiology of those diseases is still arguable. Taking into account the well-established role of stress in producing an inflammatory response, inflammation, is a new suggested physiological mechanism that seems promising (Rohleder, 2014; Calcia et al., 2016).

Inflammation, classically known as the essential response to infections and injuries to protect homeostasis of tissues, has been found recently to be the common molecular pathway to generate or harden chronic diseases including those related to stress (Scrivo et al., 2011). Stress response leads to increased levels of glucocorticoids by activating the HPA axis. Glucocorticoids are known of their immuno-suppressive and anti-inflammatory effect of which they have been found to inhibit proliferation of lymphocytes in addition to cytotoxicity (Sorrells et al., 2009).

Cytokines - peptides that are active in the nervous system as well as the immune system (Rothwell and Hopkins, 1995) - play an important role in brain altering mood, cognitive function, endocrine and behavioral responses (Dantzer and Kelly, 2007). Research showed that glucocorticoids decrease the levels of many pro-inflammatory cytokines including interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) and also enhance the expression of anti-inflammatory cytokines such as TNF-β and IL-10 (Sorrells et al., 2009). Studies has also stated that stress can induce a pro-inflammatory response that includes IL-6, IL-1β, TNFα, nuclear factor kappa B (NF-κB) and C-reactive protein (CRP). However, inflammatory mechanisms are related with stressors' type and potency. Acute stressors seem to enhance immune function, whereas chronic stressors suppress the immune system, while acute stressors seem to induce it. Additionally, severe stressors lead to imbalanced of inflammation and anti-inflammation status by over-activating the immune system (Miller et al., 2009).

Acquired immunity is mediated by lymphocytes, namely T and B cells, and it involves elements of the innate response as well as specific antigen recognition. This type of immunity is characterized by a high degree of specificity and diversity in both recognition and "memory". All lymphocytes possess glucocorticoid and adrenergic receptors, although the density and sensitivity of adrenergic receptors differs between lymphocytes, and the response to stress of these cell subsets varies accordingly. For instance, B cells are expressed at high densities but have a lower affinity for alpha 2-adrenergic receptors, whereas T cells are expressed at low densities (Cruces et al., 2014).

Social isolation is also a stressor that leads to chronic activation of HPA and sympathetic nervous system (Cacioppo and Hawkley, 2003) and promotes inflammation (Hermes et al., 2006). In fact, there is a crosstalk between the immune system and HPA axis. HPA axis can be activated by immune cells through interlukines and other cytokines. In contrary, HPA axis can also regulate the activity of several types of immune cells such as B cells, T cells and macrophages. Additionally, glucocorticoids released by HPA axis activation also decrease cytokines' levels (Dumbell et al., 2016). Social isolation was found to be associated with heightened systemic inflammation and increased circulating blood levels of inflammatory markers (Heffner et al., 2011). For example, socially isolated individuals showed higher levels of CRP in two studies (Ford et al., 2006; Loucks et al., 2006a) and cytokine IL-6 in a third study (Loucks et al., 2006b). Different studies found that social isolation stress lead to higher levels of IL-1β in addition to TNF-α and decreased concentrations of IL-2 and IL-4 (Khan et al, 2018). Repeated social stress in mice also seems to increase levels of splenic macrophages and monocytes, while psychological stressors (like social isolation) increase the production of proinflammatory cytokines and the expression of toll-like receptors (TLR 2 and 4) in them, and thus enhance their microbicidal effects in vitro (Bailey et al., 2007). Nevertheless, psychological stress can also lead to modulations in acquired immunity by altering B cells capacity to produce antibodies (Cruses et al., 2014).

4.2.3. Social isolation and endocrine system

The alteration of endocrine system has been stated as an essential mechanism of which neural disorders start (Pariante and Lightman, 2008). Social isolation, as a stressor in social species causes several changes in endocrine system such as inducing HPA and SMA axes in addition to the release of glucocorticoids and catecholamines (Kvetňanský, et al., 1995).

4.3. Agmatine

Oxford dictionary says that agmatine term is derived from: a (for amino-), g (for guanidine), -ma- (for ptomaine), -ine (English suffix) with insertion of -t- supposedly for euphony (Oxford English Dictionary, 3rd ed., Oxford University Press, 2005). Agmatine gained some interest first in researching insulin substitutes. Vertebrates related agmatine research was then ignored for a long period. However, the importance of agmatine in arginine and polyamine metabolism was solely assigned to bacteria and plants these times (Bernstein and Laube, 2017). A dramatic change in agmatine research started by 1994 when Li et al. (1994) showed that agmatine, that was purified from the bovine brain as a substitute for clonidine, acts on imidazoline receptors as an endogenous ligand (Li et al., 1994). Thus, agmatine research has shown an extraordinary raise with focusing on its effects on CNS. Modern studies increasingly proofed that agmatine has wide effects on CNS functions and disorders such as neuropathic pain (Aricioglu et al., 2003b; Santos et al., 2005), neuroinjury (Gilad et al., 1996; Wag et al., 2006), mood disorders (Aricioglu and Altunbas, 2003), epilepsy (Aricioglu et al., 2003a; Luszczki et al., 2008), morphine type addiction (Aricioglu et al., 2004a; Aricioglu et al., 2004b) and cognition (Gumru et al., 2013). However, most encouriging and reassuring possible clinical roles of agmatine recently includes its effects in post-brain injury neuroprotection, anxiety, depression and pain management (Bernstein and Laube, 2017; Sahin et al., 2016).

4.3.1. Structure, metabolism and availability

Agmatine is a vasoactive metabolite of L-arginine (Török and Zemančíková, 2016) which is synthesized by decarboxylation of L-arginine via arginine decarboxylase in bacteria, plants and mammalians (Berkels et al., 2004). Agmatine then is hydrolyzed by the enzyme agmatinase to putrescine and urea or converted to guanidine butyraldehyde by diamine oxidase (DAO) (Benítez et al., 2017; Dejanovic et al., 2017). Interestingly, agmatine is widely distributed in mammalian tissues. After it was observed in the rat brain, it was found in higher concentrations in other rat tissues like stomach, intestine, spleen and liver. Moreover, it is also detectable in human plasma where higher values were observed in depressed people (Colombatto and Grillo, 2004).

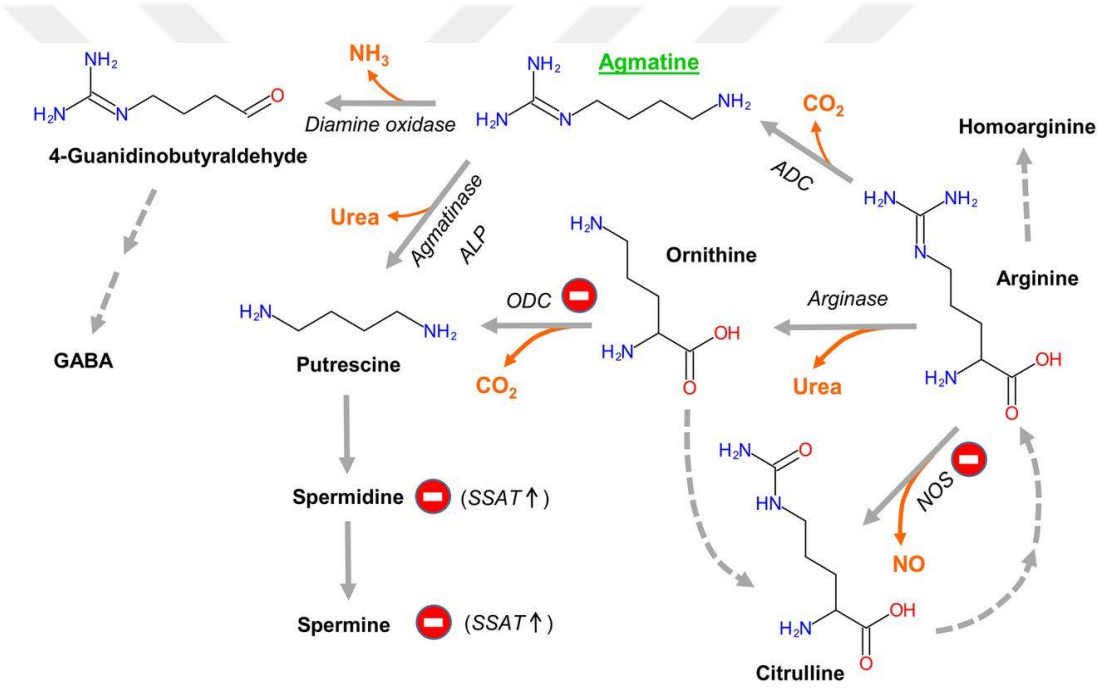

Figure 4. Agmatine metabolism pathway. (Laube G et al 2017).

4.3.2. Effects of agmatine on nervous system

Agmatine levels in brain are comparable to some neurotransmitters like dopamine (Feng et al., 1997; Raasch et al., 1995). Nevertheless, agmatine has shown neuroprotective effects against mitochondrial dysfunction, oxidative stress, excitotoxicity, apoptotic and inflammatory situations (Neis et al., 2017). Agmatine can activate imidazoline, nicotinic, 5-HT2A, 5HT3, and alpha 2-adrenergic receptors

(Piletz et al., 2013; Raasch et al., 2001; Reis and Regunathan, 2000). Moreover, it antagonizes NMDA receptors and inhibits all NOS isoforms in the brain (Reis and Regunathan, 1999).

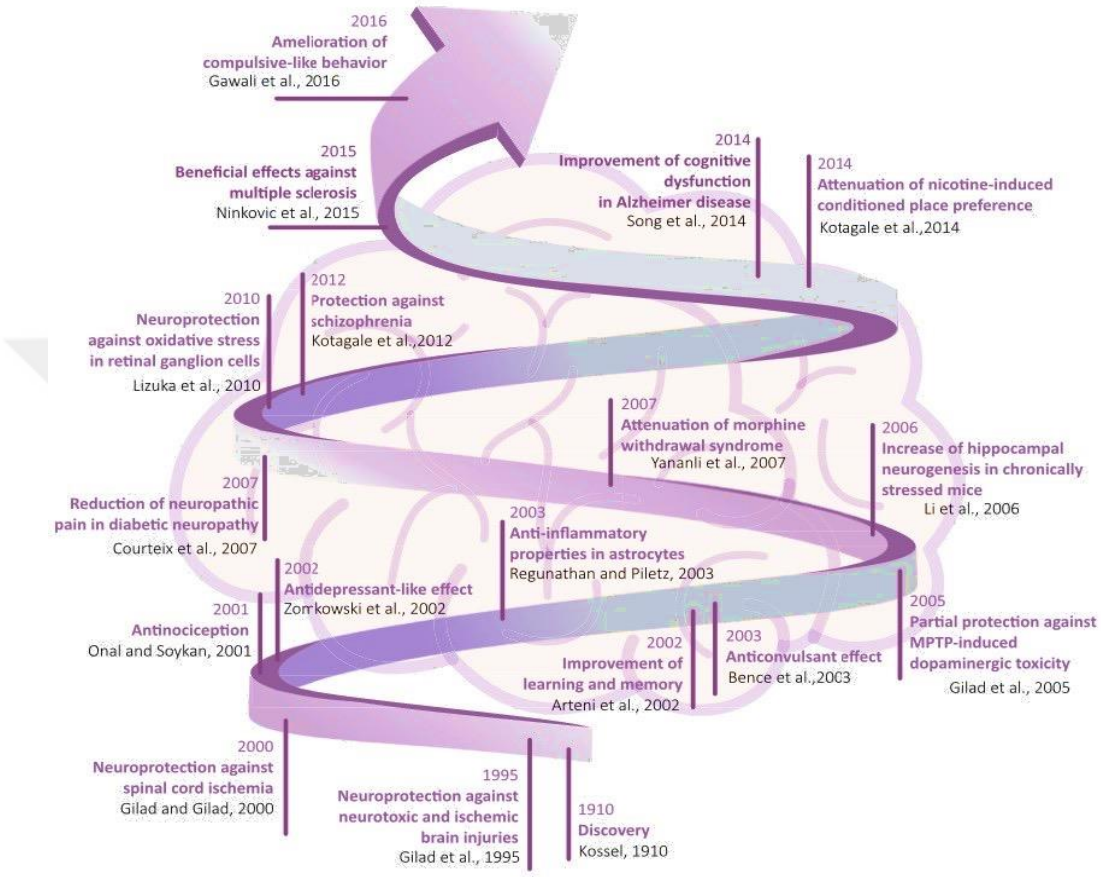

Figure 5. Effects of agmatine on nervous system along research history (Neis V et al. 2017).

4.3.3. Effects of agmatine on CNS disorders

4.3.3.1. Drug and substance addiction

Agmatine showed very promising results against substance dependency and withdrawal effects. Animal studies stated that agmatine exerted preventive effects against reduced-ethanol neurotoxicity and withdrawal (Barron et al., 2012), and it decreased mice ethanol acquisition (Sameer et al., 2013). On the other hand, agmatine was reported to improve opioid analgesia and, interestingly, inhibit

tolerance and dependency of opioids at the same time (Aricioglu et al., 2004; Su et al., 2003). Thus, the development of agmatinase inhibitors was taken in account in order to decrease dependency and withdrawal as well as supporting analgesia by brain's endogenous agmatine (Wi et al., 2008).

4.3.3.2. Alzheimer's disease (AD) and other neurodegenerative diseases

Agmatine exerted neuroprotective effects in an AD rat model where it inhibited apoptosis and other mechanisms generally associated with cognitive decline (Kang et al., 2017). Nevertheless, agmatine may have therapeutic roles in other neurodegenerative disorders. Application of the agmatine enhanced the expression of 2 matrix metalloproteases at the blood brain barrier and also inhibited superoxide dismutase activity in multiple sclerosis animal model (Ninkovic et al., 2015; Stevanovic et al., 2013). Moreover, agmatine demonstrated neuroprotective effects in a cell culture model of Parkinson's disease (Bernstein and Laube, 2017).

4.3.3.3. Agmatine and psychiatric disorders

4.3.3.3.1. Schizophrenia

Excessive agmatine levels were found in postmortem frontal cortex (Liu et al., 2016) and blood plasma (Uzbay et al., 2013) of individuals with chronic schizophrenia. However, these alterations in agmatine levels may be influenced by typical and atypical antipsychotics used in treatment as they both interact with agmatine (Kotagale et al., 2012). However, agmatine showed different results when applicated to schizophrenic animal models and it is not clear yet whether it can be suggested as future treatment option for schizophrenia (Bernstein and Laube, 2017).

4.3.3.3.2. Depression and bipolar disorder

The antidepressant mechanisms of agmatine includes antagonizing NMDA receptors, activating AMPA receptors, inhibiting NOS, activating of serotonin 5-HT1A/1B and 5-HT2 receptors, and altering the activity of neuropeptide Y system

(Bernstein and Laube, 2017; Zomkowski et al., 2002). Interestingly, agmatinase (the enzyme that mediates agmatine degradation) was found to be up-regulated in interneurons of MDD patients (Bernstein et al., 2012; Bernstein et al., 2014) while plasma agmatine concentrations showed a noticeable raise in patient with depression comparing to normal individuals (Halaris et al., 1999). Importantly, a pilot study on MDD patients showed a full recovery of depression with agmatine without side effects (Shopsin, 2013). Interestingly, two preclinical studies have shown that agmatine and lithium (commonly used for bipolar disorder) have synergistic antidepressant effects in rodents, and those effects were mediated by the NMDA receptor pathway (Bernstein and Laube, 2017).

4.3.3.3.3. Anxiety

Subsequent to the discovery of agmatine's antidepressant-like effects, it was proposed that agmatine may also have anxiolytic-like effects comparable to those shown by selective NMDA antagonists and NOS inhibitors and alpha-2 adrenoceptor agonists (Bernstein and Laube, 2017). Aricioglu & Altunbas (2003) have tested agmatine effects on rats and stated that agmatine significantly decreased immobility duration in forced swimming test similar to imipramine and that agmatine and imipramine had also significant effects in the EPM test. A following study in rodents, have also shown similar anxiolytic properties of agmatine using several behavioral tests and ways to apply agmatine where agmatine demonstrated clear anxiolytic effects in all tests (Gong et al., 2006). However, a recent study found that agmatine attenuated anxiety caused by chronic unpredictable mild stress in OF and EPM tests by altering the nitric oxide signaling pathway (Gawali et al., 2017).

4.3.3.3.4. Other CNS disorders

Agmatine reduced behaviors related with pain in animal (rodents) models of neuropathic sensory hypersensitivity, an effect that was mediated by modulating NMDA receptors and nitric oxide system. Nevertheless, it exerted improving effect on learning and memory and also showed a protective effect by inhibiting some

pathological cell changes following injury in traumatic brain injuries and spinal cord injuries (Bernstein and Laube, 2017).

4.3.4. Agmatine as a potential stress pharmacological treatment

Chronic stress is known to be a common risk factor of depression and anxiety. Modern research showed that agmatine has antidepressant-like and anti-stress effects (Aricioglu & Regunathan, 2005; Arisoy et al., 2020; Piletz et al, 2013; Sahin Ozkartal et al., 2019). Agmatine was found to reverse anxiety-like behavior and depressive behavior in rats (Aricioglu, 2016) and have a remarkable effect on depression and anxiety-like behavior in mice through nitrergic pathway, that was suggested to be linked to altering BDNF and corticosterone concentrations (Gawali et al., 2017). Nevertheless, animal research showed that agmatine decreased immobility time in forced swimming test and increased time spent in open arms in EPM expressing antidepressant-like and anxiolytic-like effects (Aricioglu and Altunbas, 2003).

Morover, studies showed that exogenous agmatine regulates increased levels of glucocorticoids and glutamate efflux in brain regions modulated by stress response. It was also stated by researchers that agmatine has neuroprotective effects attenuating glucocorticoids and glutamate-induced cell damage in primary neuronal cultures of hippocampus (Taksande et al., 2013). Interestingly, endogenous agamtine levels also showed elevation following an acute stress model and that alteration was proposed to be associated with stress response regarding agmatine's neuroprotective effects (Aricioglu et al., 2003). Agmatine showed also elevated levels in patients under stress and depression in one study (Arisoy et al., 2020). Considering also the above-mentioned effects of agmatine on many stress-related diseases, it is suggested that exogenous agmatine may be a future effective therapy against stress-triggered dysfunctions and disorders.

4.3.5. Agmatine neuroinflammatory effects

Agmatine was reported to have several neuroinflammatory effects. Recent studies showed that agmatine have decreasing effect on proinflammatory factors such as

TNF-a (Binnetoglu et al., 2019) and IL-1B (Sahin Ozkartal et al., 2019). For instance, it expressed decreasing effect on TNF-a and IL-1B levels in Parkinson's disease rat model (El-sayed et al., 2019). Moreover, it showed a protective role in mice against lung injury and led to decreased levels of TNF-a, IL-6 and IL-1B in addition to inhibition of NF-kB pathway in lungs (Neis et al., 2017). Agmatine also reduced pro-inflammatory cytokine levels in both brain and serum in one rat study (Sahin Ozkartal et al, 2019). Nevertheless, agmatine expressed the ability to modulate activation of macrophages during inflammations. Intriguingly, the ability of agmatine to promote lipopolysaccharide-treated neural progenitor cells to neural stem cells by modulating IL-B expression suggested that agmatine is a potential pharmacological therapy for neuroinflammatory disorders (Neis et al., 2017).

5. MATERIAL and METHODS

5.1. Materials

Fluoxetine (FLU) (15mg/kg) and agmatine (AGM) (40mg/kg) were disssolved in saline and injected once a day for 9 days in a volume of 0,1 ml/100 g intraperitonially (i.p).

5.2. Animals and housing

After the proposal of study was approved (approval code: 63.217.mar, by date: 11-09-2017) by the Animal Research Ethics Committee of Marmara University, 32 male Sprague Dawley rats (8-12 weeks g) were housed at controlled temperature of (22 ±1 °C), 12/12 light and dark cycle room conditions. Animals were fed ad libitum bait and water and behavioral tests were applied at the light phase of light/dark cycle. Animals were spontaneously divided into 4 groups as follows:

Control group (n=8): animals were raised in standard two cages (4 animals for every cage) and intraperitonially i.p injected only by saline 1mg/kg once a day for 9 days after 5 weeks.

SI group (n=8): animals were raised in isolated standard cages (1 animal for every cage) and i.p injected only by saline 1mg/kg once a day for 9 days after 5 weeks.

SI + FLU group (n=8): animals were raised in isolated standard cages (1 animal for every cage) and i.p injected by fluoxetine (15mg/kg) solved in water 2mg/kg once a day for 9 days after 5 weeks.

SI + AGM group (n=8): animals were raised in isolated standard cages (1 animal for every cage) and i.p injected by agmatine (40mg/kg) solved in saline 1mg/kg once a day for 9 days after 5 weeks.

5.3. Social isolation protocol

The SI model was applied according to Garzon and Del Rio (1981). Rats exposed to SI model were left single in their cages and kept there for 6 weeks without any contact except cleaning times. By the end of week 5, isolated rats were divided into 3 groups, a group was treated with agmatine, another group that was treated with fluoxetine and one was given only physiologic serum as a control.

Figure 6. Isolated rats reared in individual cages.

5.4. Experimental design and treatments

After SI protocol was applied to the isolated groups for 5 weeks, SI+AGM and SI+ FLU groups were i.p injected with fluoxetine and agmatine respectively for one week by the dose described above while saline was injected to control and SI groups at the same time. Then, behavioral tests were conducted for two days while keeping on giving the same injection to every animal. Injecting animals was carried on every day at the same time and 1-hour break was leaved between injection and behavioral tests, injection and behavioral test were carried on at the light time of light/dark cycle of animals. At the end of behavioral tests animals were decapitated and prefrontal cortex dissection was performed for molecular analysis.

5.5. Behavioral tests

5.5.1. OF test

The open field test is a widely accepted behavioral test that does not require animals to acquire novel skills (Chen et al., 2013). The test was initially started in 1934 by Hall CS (1934) to measure emotionality in rodents.

Eventually, it has been one of the most used behavioral tests in animal psychology (Seibenhener and Wooten, 2015). It is commonly used to assess anxiety-like and exploratory behaviors and can show how drugs affect several body systems such as CNS. This test has several advantages such as the ability to assess locomotor and behavioral activity easily, carrying out the test without handling animals, being a noninvasive procedure that can be repeated many times during the study and the ability of testing multiple animals at the same time (Tatem et al., 2014). Locomotor activity test was applied with special activity cage system. Cage is (41x44x32 cm), made from Plexiglas and the activity of every animal was video-recorded for 5 minutes then animal's horizontal and vertical movements were calculated. The movement surface was cleaned after every animal with ethanol 75%.

Figure 7. OF test procedure

5.5.2. EPM test

The elevated plus maze test was described by Handley and Mithani (1984). Beyond its use in studying anxiolytic and anxiogenic effects, it can also be used in studying drugs of abuse, brain sites and mechanisms (Frye and Walf, 2007). The system we used to assess animals' behavior was composed of plus shaped four hands (everyone is 50x10 cm), 2 closed (surrounded by walls) & 2 open, that were elevated to 40 cm height. Every rat was smoothly placed at the (10x10 cm) center of the maze facing one of the open hands and its actions were recorded with a video camera for 5 minutes so we could calculate total time it spent inside and number of approaches to enter the open hands with the same calculations done for closed hands. The maze was cleaned before every experiment with ethanol 75%.

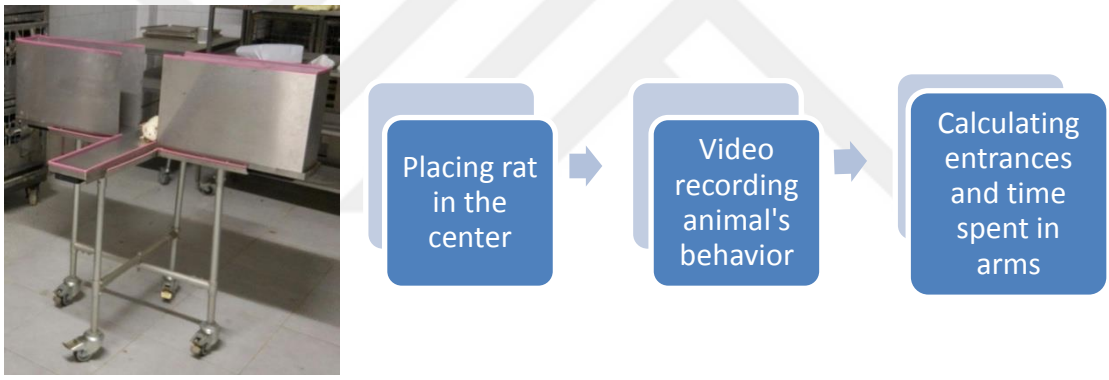

Figure 8. EPM test apparatus and procedure

5.5.3. NOR test

Object recognition memory tests are used widely to assess declarative memory. The new object recognition test is a relatively useful sensitive method that can be applied relatively rapidly for evaluating agents regarding cognition enhancing (DiCamillo and Mathiasen, 2010).

The test was originally described by Ennaceur and Delacour (Ennaceur and Meliani, 1988) and done with black (50x50x30 cm) Plexiglas surface in a semi-dark medium. Surface and objects were always cleaned by wiping with ethanol before taking a new

animal to the test. Test took two days, one for familiarization, and one for testing. In the first day, all animals were placed to the Plexiglas surface in groups of 4 animals without any object for 60 minutes. In the next day, there were two periods (everyone is 3 minutes) with a 60 minutes break between them. In the first period T1, two similar objects were placed together to the test surface where the animal was free to move and discover them. In the next period T2, the same was done but with one of the 2 objects changed. The two periods T1 and T2 were video-recorded and the animal's interest in both of two objects during T2 was scored. The ratio of animal's interest in the old object to its interest in the new one is accepted as a measure of animal's learning and cognitive function (Besheer and Bevins, 2006).

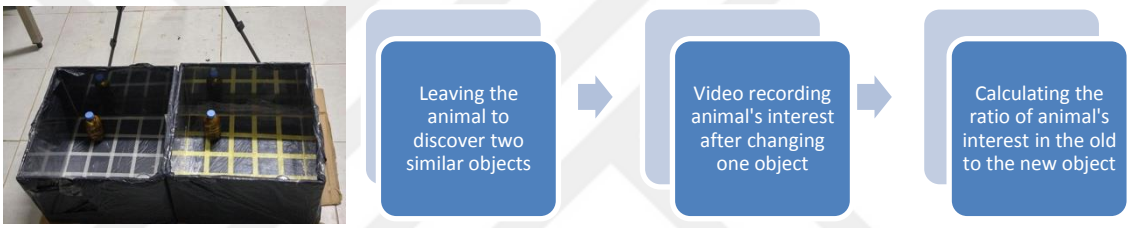

Figure 9. Cages used in NOR test and the procedure

5.5.4. MB test

MB test is a model used for anxious and obsessive behaviors. Defensive burying was introduced by Pinel and Treit (1978). MB test is also used to assess expected activity of new antidepressants, anxiolytics and antipsychotic drugs. Evidence suggesting the applicability of marble burying as a preclinical test of anxiety basically came from studies showing a decrease in marble burying by the use of benzodiazepines (e.g., diazepam) (Jimenez-Gomez et al., 2011). Besides being a useful test for agents suggested to be used in obsessive compulsive disorder (OCD), MB test is also applied to check responses to treatments regarding impulsiveness, autism and co-morbid dementia symptoms. MB test was found to be sensitive for traditional anxiolytics, several classes of serotonergic and noradrenergic antidepressants such as

imipramine and citalopram, glutamatergic agents in addition to both traditional and atypical antipsychotics.

The test was applied in (42x26x15 cm) plexiglass cages with fixed 5 cm height layer of sawdust. 20 marbles were placed symmetrically with 4 cm distance between every 2 marbles, then the number of marbles that had been moved and/or buried by a ratio of 2/3 during the 30 minutes test period was calculated (Angoa-Perez et al., 2013).

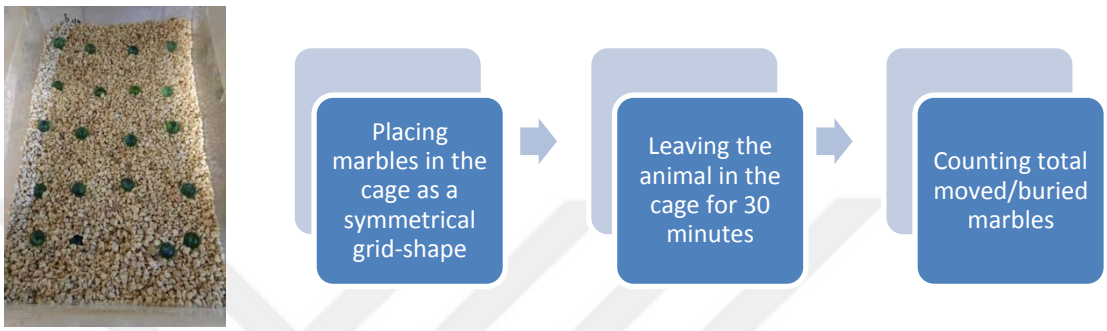

Figure 10. MB test steps. Buried or moved marbles reflect animal's anxiety-like behavior.

5.6. Collection of tissue samples

Rats were sacrificed by rapid decapitation after behavioural tests. Macroscopic brain dissection was performed on ice, immediately. After brain dissection, PFC brain tissues were collected for gene expression analysis of proinflammatory cytokines (IL-1β and NF-kB). All tissue samples were divided into two and collected into liquid nitrogen, then stored separately at -80 ˚C until gene expression analysis later.

5.7. Real-time PCR analysis of gene expressions

PFC tissue samples of rats were homogenized with Qiagen Tissue Ruptor®. RNA was isolated from tissue homogenates by using RNA isolation commercial kit (MRC® RNAzol RT, RN190). Following isolation, purity and the concentration of RNA samples were detected with Thermo Scientific Nanodrop 2000® and determined by the O.D. ratios of 260/280 and 260/230. Reverse transcription was performed from 2 µl RNA samples (100 ng/ml) with commercial kit (Jena Bioscience®, PCR511) according to the manufacturer's protocol using Bio-Rad®

termal cycler. Real-time PCR was performed with Agilent Stratagene 3005P® by using PCR master kit (Jena Bioscience®, qPCR GreenMaster with UNG/lowROX kit, PCR306) added with cDNA samples (2 µl) and primers. β-actin was used as the internal housekeeping control gene and the relative quantification of genes of interest was calculated from $2^{(-ddCT)}$ method.

5.8. Statistical Analysis

Statistical analysis was performed using GraphPad Prism® 5 program (GraphPad Software Inc., La Jolla, CA, USA). One-way analysis of variance (ANOVA) followed by post-hoc Tukey's HSD test was used for statistical analysis. All data were presented as the means of ± S.E.M. $p<0.05$ was regarded as the value of statistical significance.

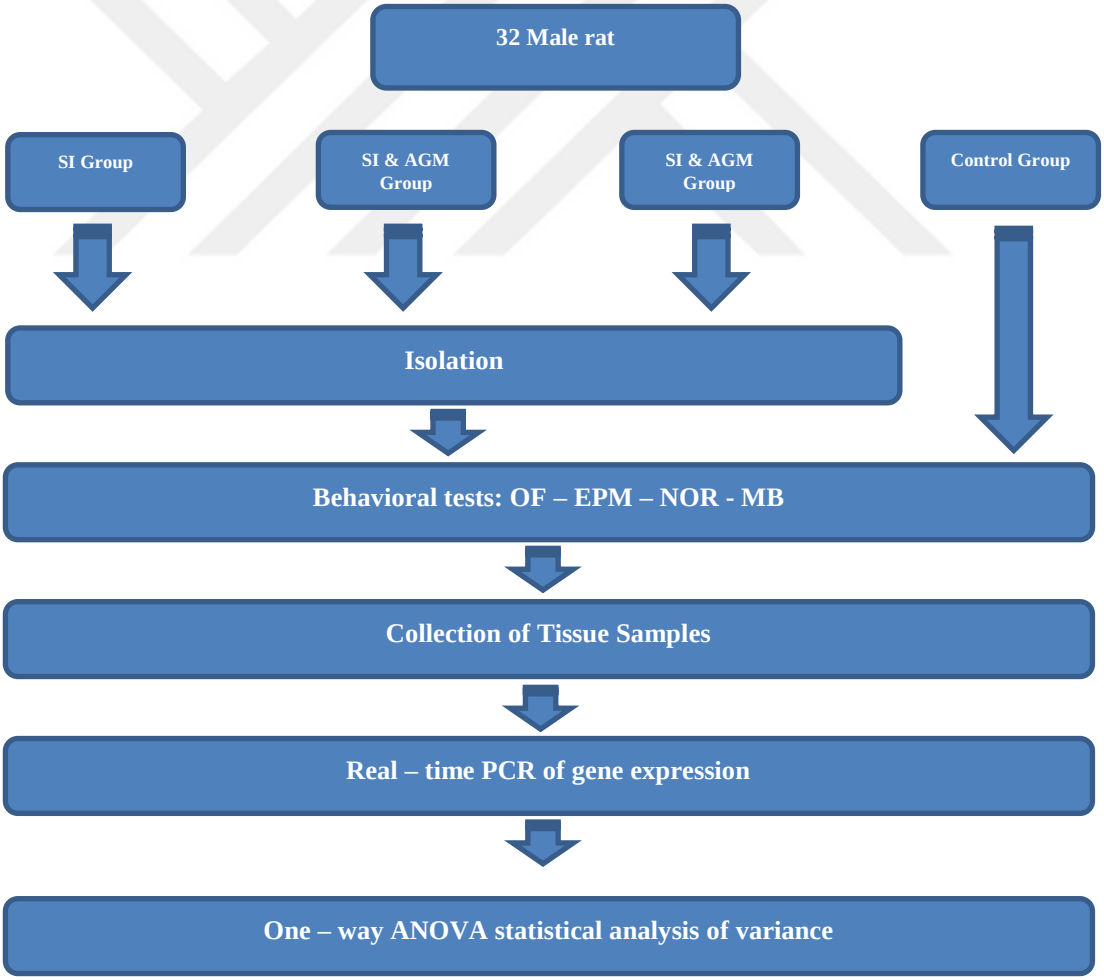

Figure 11. Complete experiment's flow chart.

6. RESULTS

6.1. Body weight

Body weight was weekly recorded throughout the experiment. Before starting the SI procedure, there were no significant differences between the groups at Week 0 (Table 3). The body weight in SI was significantly lower compared to control group at 3rd, 4th ($p<0,05$) and 5th weeks. Not with FLU treatment but with AGM these changes were significantly reversed.

Table 3. Weekly body weight changes.

GROUPS/ WEEKS	WEEK 0	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5
CONTROL	250±28	272±19	295±25	310±38	339±22	352±32
SI	264±19	270±24	279±20	285±18*	296±15**	304±21**
SI+FLU	245±23	267±29	280±30	291±32	309±18	321±20
SI+AGM	252±12	275±17	299±12	316±25*	348±20*	362±18**

SI: Social isolation; FLU: Fluoxetine, AGM: Agmatine

6.2. Behavioral Data

6.2.1. Locomotor activity results

SI-treated rats developed reduced locomotor activity ($p<0.001$) compared to control group which was significantly reversed by fluoxetine ($p<0.01$) and agmatine ($p<0.001$) treatment (Figure 12).

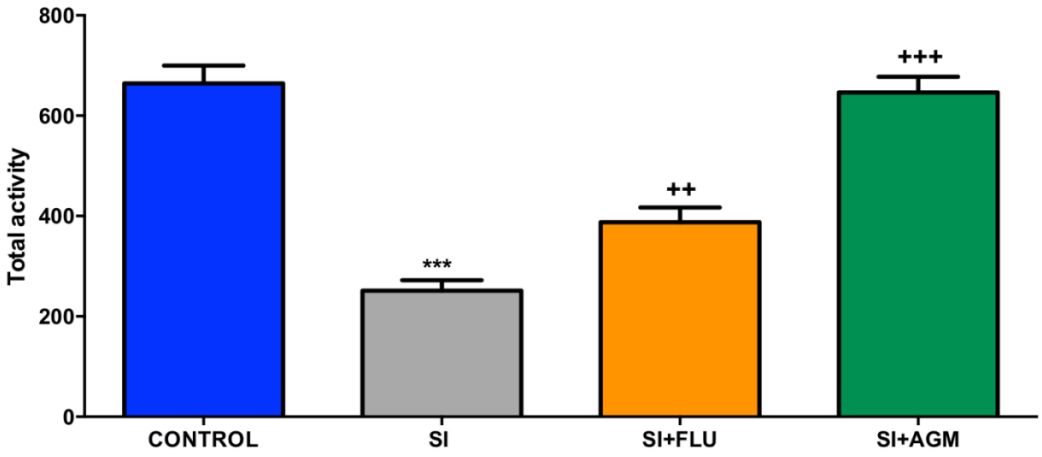

Figure 12. The effect of SI and treatments on total activity in OF test. All data are expressed as mean ± S.E.M. ***p<0.01, vs. Control group $^{++}$p<0.01 and +++p<0.001 vs. SI group. SI; social isolation, AGM; agmatine (40 mg/kg/day; i.p.), FLU; fluoxetine (15 mg/kg/day, i.p.).

6.2.2. EPM Results

Time spent on closed arms in EPM was significantly longer in SI group compared to control (p<0.001) as a reflection of depressive-like behavior which was significantly decreased both with fluoxetine and agmatine treatments (p<0.001). (Figure 13).

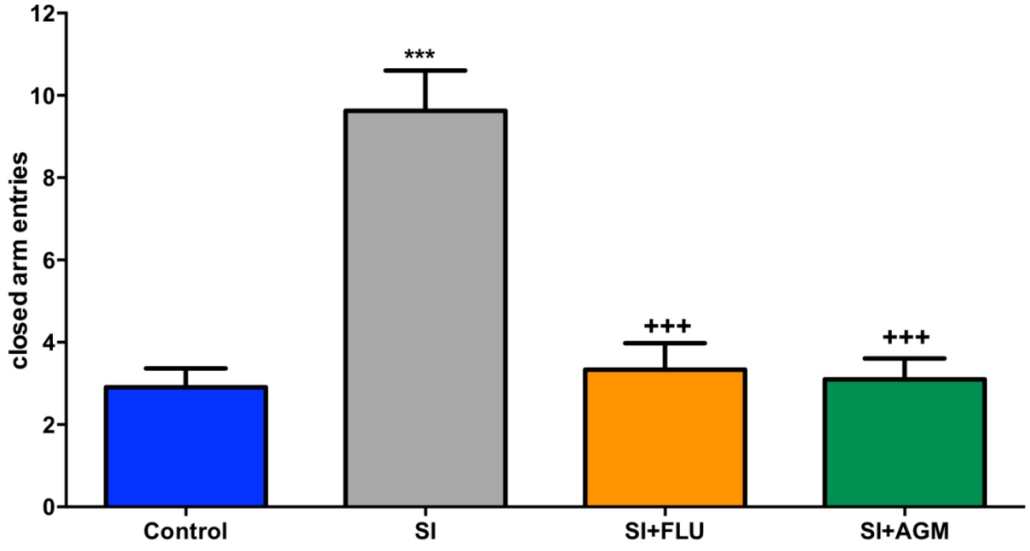

Figure 13. The effect of SI and treatments on closed arm entries in EPM test. All data are expressed as mean ± S.E.M. ***p<0.01, vs. Control group and +++p<0.001 vs. SI group. SI; social isolation, AGM; agmatine (40 mg/kg/day; i.p.), FLU; fluoxetine (15 mg/kg/day, i.p.).

6.2.3. MB test results

SI procedure caused significant suppression number of marble buried in MB tests with control group ($p<0.01$). Fluoxetine ($p<0.01$) and agmatine ($p<0.001$) treatment significantly reversed these changes compared with SI group. (Figure 14).

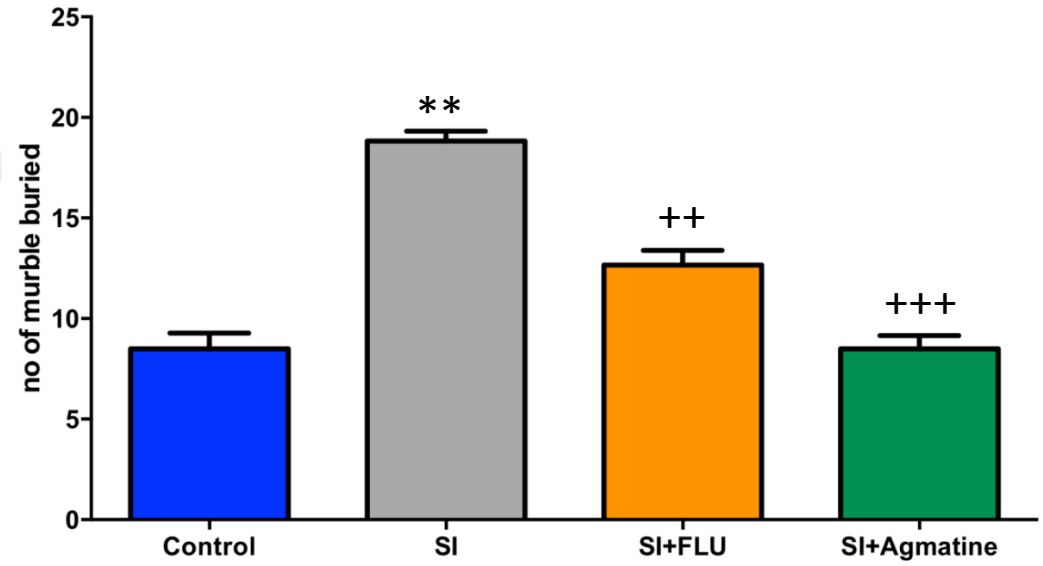

Figure 14. The effect of SI and treatments on number of marbles buried in MB test. All data are expressed as mean ± S.E.M. **p<0.01, vs. Control group $^{++}$p<0.01 and +++p<0.001 vs. SI group. SI; social isolation, AGM; agmatine (40 mg/kg/day; i.p.), FLU; fluoxetine (15 mg/kg/day, i.p.).

6.2.4. NOR test results

SI procedure caused significant disturbance with control group in terms of novel object recognition which represents attention. Therefore, discrimination index of SI group was statistically lower compared to control group ($p<0.001$). Agmatine treatment significantly reversed this effect of SI group ($p<0.01$) but not fluoxetine. (Figure 15).

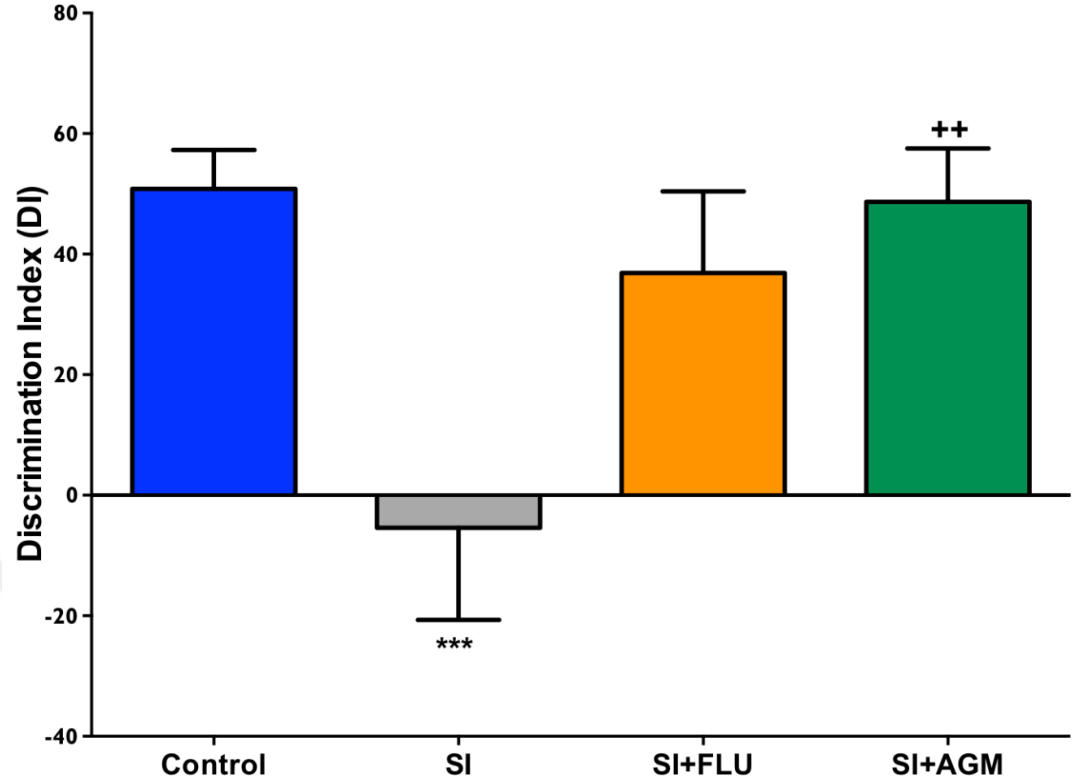

Figure 15. The effect of SI and treatments on discrimination index in NOR test. All data are expressed as mean ± S.E.M. ***p<0.01, vs. Control group and ++p<0.001 vs. SI group. SI; social isolation, AGM; agmatine (40 mg/kg/day; i.p.), FLU; fluoxetine (15 mg/kg/day, i.p.).

6.3. Molecular results

6.3.1. IL-1B mRNA levels in PFC

In this study, 5-week of SI procedure caused a significant increase in IL-1β mRNA levels in PFC of rats (Figure 11). IL-1β mRNA levels were found to be significantly increased compared to control group (p<0,05). SI-induced PFC IL-1β mRNA levels were attenuated in rats chronically treated with AGM (p<0,05) but not with FLU treatment (p>0,05). (Figure 16).

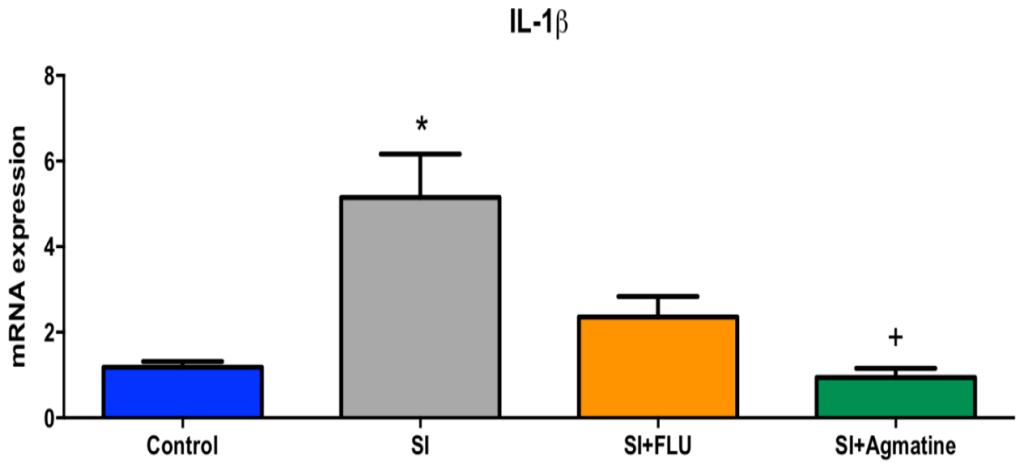

Figure 16. The effect of SI and treatments on IL-1B mRNA levels in PFC of rats. All data are expressed as mean ± S.E.M. *p<0.05, vs. Control group and +p<0.05 vs. SI group. SI; social isolation, AGM; agmatine (40 mg/kg/day; i.p.), FLU; fluoxetine (15 mg/kg/day, i.p.).

6.3.2. NF-kB mRNA levels in PFC

5-week of SI procedure caused a significant increase in NF-kB mRNA levels in PFC of rats compared to control group (Figure 12). SI-induced increase in IL-1β mRNA (p<0,01) levels were attenuated in rats chronically treated with AGM (p<0,001) and FLU (p<0,001) (Figure 17).

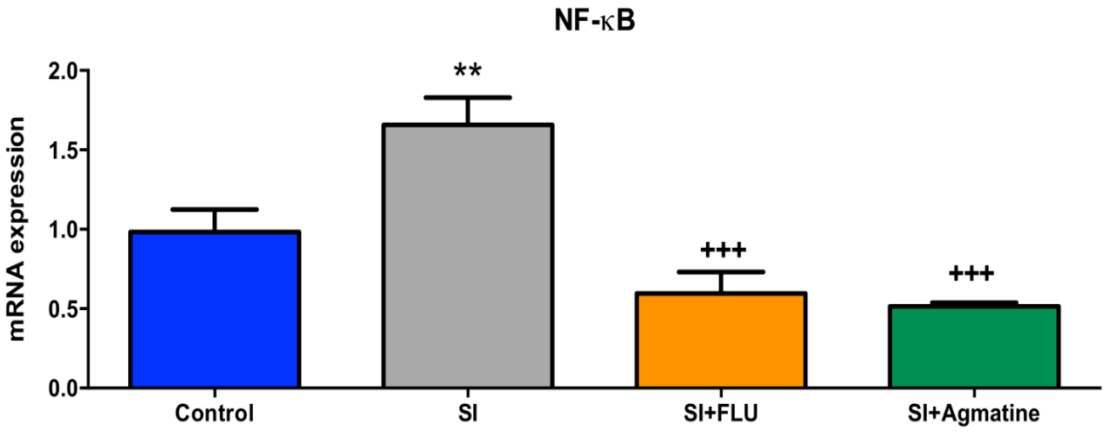

Figure 17. The effect of SI and treatments on NF-kB mRNA levels in PFC of rats. All data are expressed as mean ± S.E.M. **p<0.01, vs. Control group and +++p<0.001 vs. SI group. SI; social isolation, AGM; agmatine (40 mg/kg/day; i.p.), FLU; fluoxetine (15 mg/kg/day, i.p.).

7. DISCUSSION and CONCLUSION

The aim of our study was to test the effects of the natural neurotransmitter and/or neuromodulator agmatine on rats exposed to SI stress comparing to the common SSRI fluoxetine.

Previous studies reported that SI led to low body weights in rats compared to group reared rats (Carnevali et al., 2020; Izadi et al., 2018). Sun et al. (2016) reported that fluoxetine did not cause significant improvement in this weight decrease following SI. Similar results also were reached by Sonei et al. (2017). Similarly, our study also showed that rats exposed to SI had significantly lower body weight compared to controls and that fluoxetine did not change this weight differences significantly. Interestingly, Agmatine – and for the first time up to our knowledge – was found to significantly reverse low body weight caused by SI in our study.

Animal models of SI stress are frequently applied to investigate the mechanisms underlying neuropsychiatric disorders. Socially isolated rats express behavioral deficits in various tests, such as the OF test and EPM test (Nakagawa et al., 2019).

The OF test is well-established in measuring anxiety-like and depressive-like behaviors in preclinical studies. Several studies on socially isolated rats, such as a study done by Nakagawa et al. (2019) found that SI model rats showed anxiety-like behavior expressed by total less distance passed during OF test. In line with these results, we also found that isolated raring led to a significantly reduced locomotor activity in OF test. However, some researchers like Borges et al. (2019) reported no change in locomotor activity in the OF test after SI. These differences in results between studies maybe related to the isolation conditions (e.g. period) or used rat subspecies. The study of Borges et al. applied a one-month model of isolation comparing to 6 weeks in our study.

The EPM test is a widely applied behavioral test for assessing anxiety in rodents (Horii et al., 2018). Studies on socially isolated rats have used EPM test to examine behavioral changes. Nakagawa et al. (2019) found that socially isolated rats expressed anxiety-like behavior in EPM test. Regenass et al. (2017) also

stated that socially isolated rats showed anxiety suggesting behavior in EPM test. Similar results were also reached by Mohale et al. (2016) on SI model rats. Our study similarly found that isolated rats significantly expressed anxiety indicating behavior emphasizing previous studies.

Mathiasen and DiCamillo (2010) in addition to Shortall et al. (2018) reported that SI impaired NOR in rats. Similar results also were reached by Watson et al. (2012) and Mclntosh et al. (2013). Our study has also come to corresponding results that isolated rats expressed impaired novel object recognition comparing to the group reared controls.

Rodents show burying behavior as a defensing response to aversive stimuli. Thus, marble burying test is recently utilized in preclinical studies to investigate anxiety-like behavior (Thomas et al., 2009). Zlatkovic et al. (2014) reported that rats exposed to SI stress expressed increased burying behavior. Corresponding results were also achieved by Peric et al. (2018). Other study also stated that chronically isolated rats showed increased burying response (Vazquez-Leon et al., 2017). In like manner, our study also found that rats demonstrated excessive burying in MB test after SI.

Fluoxetine was found to be effective against behavioral changes like the changes in sucrose consumption caused by isolated rearing whether given alone (Bernes and Fornaguera, 2009) or as a combined treatment (Mikics et al., 2018). One study also found agmatine to attenuate obsessive compulsive behavior caused by social isolation in mice (Shende and Patel, 2014). Our study is the first study to test the antidepressant effects of agmatine on social isolated rats compared to fluoxetine. Several preclinical studies showed that agmatine has anxiolytic-like and antidepressant-like effects (Aricioglu, 2016; Aricioglu and Altunbas, 2003; Neis et al., 2016, Olescowicz, 2018; Piletz et al., 2013).

One study reported that agmatine exerts a significant anxiolytic effect in both rats and mice (Gong et al., 2006). Other study also found that agmatine was able to decrease depressive-like behavior and suggested that its antidepressant-like effect may be dependent on its ability to maintain the pro-/anti-oxidative homeostasis in the hippocampus (Freitas et al., 2014). According to Gawali et al. (2017),

agmatine showed marked effect on depression and anxiety-like behavior in mice through nitrergic pathway that may be related to modulation of oxidative-nitrergic stress, corticosterone and BDNF levels, in addition to anxiolytic-like behavior expressed in elevated plus maze EPM and open field test OF test. Interestingly, Sahin Ozkartal et al. (2019) reported also antidepressant-like effects of agmatine on rats exposed to chronic stress and discovered that these effects may be related to the inflammasome mechanisms. Nevertheless, other studies also stated that agmatine expressed modulatory effect on anxiety and depression and increased the time spent in the open arms in the EPM (Aricioglu, 2016; Aricioglu and Altunbas, 2003). Interestingly, Neis et al. (2016) found that a single dose of agmatine was able to reverse behavioral alterations induced in mice by chronic unpredictable stress suggesting that agmatine may have fast-acting antidepressant-like properties. Corresponding results were also achieved by Guerra de Souza et al. (2018) who found that agmatine had antidepressant-like effects on mice exposed to Alzheimer's model. Moreover, agmatine was able to reverse the corticosterone-induced depressive-like behaviors in mice in the tail suspension test as well as the deficits in hippocampal cell proliferation according to another study (Olescowicz, 2018).

In line with results reported by Aricioglu and Altunbas (2003), our study has also found both agmatine and fluoxetine to significantly increase time spent in open arms in EPM test.

A priclinical study showed that agmatine significantly decreased marble burying behavior and prevented the increase of locomotor activity following SI in mice (Shende and Patel, 2014). In this study, we have also found that agmatine significantly reduced marble burying and reversed the increase in locomotor activity caused by SI in rats. To the best of our knowledge, the significant effects of both agmatine and fluoxetine on isolated animals subjected to NOR test have been also shown for the first time in our laboratory.

IL-1β and other pro-inflammatory cytokines such as IL-6 and TNF-α are induced by psychological stress and shown to be involved in depressive behavior in rodents as well as patients suffering from depression (Koo et al., 2010). One study on rats

showed that mobility restriction stress caused elevation in IL-1β levels (Chuian et al., 2005). Another study reported that isolated rearing led to elevated IL-1β in rats (Ko and Liu, 2015), a result that was reassured by the same researchers in another study (Ko and Liu, 2016). Our study has also found that rats exposed to social isolation showed excessive levels of IL-1β and NF-kB.

Fluoxetine was stated to decrease elevated IL-1β levels in rats exposed to chronic mild stress (Lu et al., 2017). Corresponding results were also reached by Habib M et al. (2015). Fluoxetine was also reported to treat elevation of IL-1β Levels in socially isolated rats (Todorovic and Filipovic, 2017).

Interestingly, agmatine was found to modulate IL-1β levels and thus increase differentiation of neural progenitor cells to stem cells in mice brain tissue subjected to lipopolysaccharide-induced inflammation (Song et al., 2013). Other studies also reported that agmatine led to decreased IL-1β levels (Neis et al., 2017; Sahin Ozkartal et al., 2019). Our study also showed that animals expressed higher levels of IL-1β after social isolation compared to controls and that both agmatine and fluoxetine decreased IL-1β levels. According to our literature review, our study was the first to show agmatine's inhibiting effect on IL-1β in SI model.

NF-κB is activated by IL-1β in addition to other cytokines and proposed to mediate the effects of IL-1β in stress response context. Moreover, NF-κB signaling increases in healthy subjects under social stress. According to an interesting study, the anhedonic and antineurogenic effects of repeated stress are associated with the IL-1β/NF-κB signaling pathway and NF-κB blockade could present antistress and anti-inflammatory effects (Koo et al., 2010). Other preclinical studies also found that rats exposed to SI had increased NF-κB activity (Djordjevic et al., 2010) and levels (Djordjevic et al., 2015). Similar results were achieved also by our study in PCR test.

Intriguingly, a study found that rats exposed to SI did not show elevated NF-κB levels when they were given fluoxetine (Todorovic and Filipovic, 2017). A result that has been confirmed by our study.

Up to our knowledge, our study was the first study to find that agmatine reverses increased NF-κB levels caused by SI.

There were two limitations of our study. Firstly; the number of animals was not large. However, it was enough to express useful results. Secondly; available conditions at the study time made it impossible to make immunohistochemical tests that would help to research physiological and biochemical mechanisms behind SI-induced neurobehavioral symptoms and therapeutic effects of agmatine comparing to fluoxetine on these symptoms.

Importantly, for the best of our knowledge, our study was the first study to:

I) Investigate and report that agmatine reverses low body weight due to SI.

II) Investigate and report the attention-enhancing effects of agmatine due to SI.

III) Investigate and report the antidepressant-like effects of agmatine in SI model.

IV) Investigate and report the anxiolytic-like effects of agmatine in SI model.

V) Find that agmatine reverses increased IL-1β levels caused by SI.

VI) Find that agmatine reverses increased NF-κB levels caused by SI.

In fact, our findings suggest the possible role of agmatine as a treatment or preventative agent for social isolation induced depressive and anxiolytic symptoms in addition to attention impairments and that agmatine's therapeutic effects on these symptoms is mediated partially by its inhibitory effects on proinflammatory cytokines IL-1B and NF-kB.

However, further studies are also required to investigate deep molecular and physiological mechanisms underlying the behavioral effects of agmatine shown by behavioral tests stepping forward towards clinical study and probable application of agmatine to treat these symptoms.

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0237 özet numaralı **"F. Aricioglu, A. Shabani, G. Unal, H. Zortul. Effect of Agmatine on Chronic Social Isolation in Rats: Involvement of Neuroinflammation**'' başlıklı bildiriniz, ICP 2020 Bilimsel Kurulu tarafından değerlendirilmiş ve Kongre'de "**Poster Araştırma Bildirisi Sunumu**" olarak kabul edilmiştir. Bildiri sunum programı önümüzdeki günlerde ICP 2020 web sayfasında ilan edilecektir.

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• JPEG veya PPT olarak 3:4 oranında **dikey** olarak hazırlanmalıdır.
• Yüksek çözünürlüklü, minimum **1536(en) x 2048(boy)** olmalıdır.
• Belirtilen ölçülerde çalışılan Dijital E-poster'de yazılar mümkün olduğunca büyük ve okunaklı olmalıdır. Önerilen **font 12 - 22 punto arasında arial dir**.
• E-Poster üzerindeki bilgiler bir sayfaya sığmaz ise aynı ölçülerde ikinci bir sayfa yapılmalı, yapıştırılan **resim ve tabloların kalitesi** yüksek olmalıdır.
• JPEG çözünürlüğünde sorun yaşayan bildiri sahipleri isterlerse direk olarak ppt veya pptx uzantılı çalışma dosyalarını da gönderebilirler.
• JPEG ya da çalışma dosyaları faika.yapici@burkon.com adresine gönderilmelidir.
• Araştırma posterleri amaç, yöntem, bulgular ve sonuç başlıkları altında 4 bölümden oluşacak şekilde yazılmalıdır.
• Olgu posterleri giriş, olgu, tartışma başlıkları altında 3 bölümden oluşacak şekilde yazılmalıdır.
• Posterler sunulmak üzere 3 ayrı gruba ayrılacaktır. Poster sahipleri kendilerine belirtilen günde POSTERLERİNİN BAŞINDA POSTER JÜRİSİNE ve izleyicilere posterlerini sunmak üzere, ilan edilen gün ve saatlerde poster alanında bulunmaları zorunludur. Sunum saatinde posterinin başında bulunmayan kişilerin posterleri özet yayınlarında yer almayacak ve değerlendirmelerden de çıkartılacaktır.

Saygılarımızla,

Prof. Dr. M. Kemal Sayar
Kongre Başkanı

Prof. Dr. Mücahit Öztürk
Kongre Eş-Başkanı

ENCLOSURE-2

MARMARA ÜNİVERSİTESİ HAYVAN DENEYLERİ YEREL ETİK KURULU

PROJE ONAY FORMU

BAŞVURU BİLGİLERİ	**PROTOKOL KODU**	63.2017.mar	**ÇALIŞMA:** BİLİMSEL
	PROJE ADI	Kronik Sosyal İzolasyon Modelinde Agmatin'in Etkilerinin Araştırılması	
	SORUMLU ARAŞTIRMACI ÜNVANI/ ADI	Prof. Dr. Feyza ARICIOĞLU	
	ARAŞTIRMA MERKEZİ	DEHAMER	
	DESTEKLEYİCİ		
KARAR BİLGİLERİ	**TARİH: 11.09.2017** Yukarıda başvuru bilgileri verilen araştırma başvuru dosyası ve ilgili belgeler araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş ve gerçekleştirilmesinde sakınca bulunmadığı için kurulumuzca onaylanmasına oy birliği ile karar verilmiştir. Onay sonrasında yapılacak her türlü proje değişiklikleri (katılımcılar, başlık vb.) veya protokol değişikliklerinin Etik Kurula bildirilerek proje onayının yenilenmesi gerekmektedir.		
ETİK KURUL BİLGİLERİ			
ÇALIŞMA ESASI	Deney hayvanları ile yapılacak olan bilimsel araştırma, test, sağlık hizmetleri uygulamaları ve eğitim-öğretim gibi temel etkinliklerde kullanılan yöntem ve materyaller ile ilgili etik standartları gözetmek, etik ilkeler doğrultusunda görüş bildirmek, araştırma önerilerini incelemek ve sertifikası olmayanların deney hayvanı kullanmalarını engellemektir.		

ÜYELER

Ünvanı/ Adı/ Soyadı	Uzmanlık Dalı	Kurumu/ Ek Üyeliği	Onaylanan Proje ile İlişkisi	Toplantıya Katılım	İmza
Prof.Dr. Göksel ŞENER	Farmakoloji	M.Ü. Eczacılık Fakültesi ve Hayvan Deneyleri Etik Kurul Başkanı	Var Yok	Evet Hayır	
Prof.Dr. Levent KABASAKAL	Farmakoloji	M.Ü. Eczacılık Fakültesi ve Hayvan Deneyleri Etik Kurul Üyesi	Var Yok	Evet Hayır	
Prof.Dr. Ayşen YARAT	Biyokimya	M.Ü. Diş Hekimliği Fakültesi ve Hayvan Deneyleri Etik Kurul Üyesi	Var Yok	Evet Hayır	
Prof.Dr. Serap ŞİRVANCI	Histoloji Embriyoloji ABD	M.Ü. Tıp Fakültesi ve Hayvan Deneyleri Etik Kurul Üyesi	Var Yok	Evet Hayır	
Prof.Dr. Rezzan GÜLHAN	Farmakoloji	M.Ü. Tıp Fakültesi ve Hayvan Deneyleri Etik Kurul Üyesi	Var Yok	Evet Hayır	
Doç.Dr. Gürkan SERT	Tıp Tarihi ve Etik	M.Ü. Tıp Fakültesi ve Hayvan Deneyleri Etik Kurul Üyesi	Var Yok	Evet Hayır	
Vet. Dr. Dilek ÖZBEYLİ	Veteriner Hekim	M.Ü. Tıp Fakültesi ve Hayvan Deneyleri Etik Kurul Üyesi	Var Yok	Evet Hayır	
Bio. Arif GÜMÜŞ	Biyoloji	Sivil Toplum Kuruluşu Üyesi ve Kurumla ilişkisi olmayan TC vatandaşı üye	Var Yok	Evet Hayır	
Cihanşah MURATOĞLU	Serbest Danışmanlık	Kurumla ilişkisi olmayan TC vatandaşı üye	Var Yok	Evet Hayır	

CURRICULUM VITAE

Name	ALI	**Surname**	SHABANI
Place of Birth	Saudi Arabia	**Date of Birth**	23.08.1983
Nationality	Syrian	**Tel**	05050506975
E-mail	Ali.s.07@outlook.com		

Educational Level

Postgraduate/Specialization	**Name of the Institution where he/she was graduated**	**Graduation year**
Masters		
Undergraduate	Ebla University	2015
High school	Asaad Akil High school	2001

Job Experience

Duty	**Institution**	**Duration (Year - Year)**
Admenistrative assistant	Osais company	2008
Translator	Osais company	2014-2015
Project manager	USCSO	2017
Medical translator	Free-lancer	2018-2020
Co-founder	Own job	

Foreign Languages	**Reading comprehension**	**Speaking***	**Writing***
Turkish	Very good	Very good	Very good
English	Very good	Very good	Very good

Foreign Language Examination Grade#								
YDS	ÜDS	IELTS	TOEFL IBT	TOEFL PBT	TOEFL CBT	FCE	CAE	CPE
		7						

Computer Knowledge

Program	**Use proficiency**
Internet- Windows- Office	normal
Visual Basic	elementary
Disigning programs	basics