INVASTIGATING THE EFFECT OF P2X7 RECEPTORS ON FOCAL CEREBRAL ISCHEMIA

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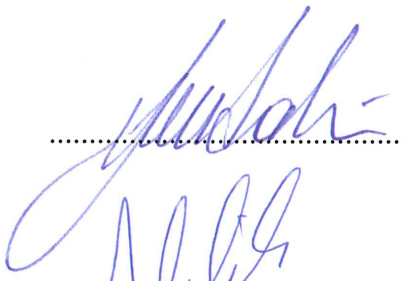
Prof. Dr. Fikrettin Şahin ..... 
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"this thesis is dedicated
to my family…"

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ABSTRACT

INVESTIGATING THE EFFECT OF P2X7 RECEPTORS ON FOCAL CEREBRAL ISCHEMIA

P2X7 receptors are cationic-selective ion channels gated by extracellular ATP and expressed throughout the peripheral and central nervous systems. P2X7 receptors are involved in the regulation of receptor trafficking, inflammation and ATP-mediated cell death. In the present study, we aimed to examine the impacts of P2X7 receptors in the development of neuronal cell death after middle cerebral artery occlusion (MCAo) in mice.

In the present studies, we show that the activation of P2X7 receptors with 250 μM BzATP does not increase the cellular damage significantly, as compared with the vehicle-treated control animals after MCAo in which slow and rapid neuronal cell death are observed. However, inhibition of P2X7 receptors by 10 mM Brilliant Blue G (BBG) improved neuronal survival. Furthermore, inhibition of P2X7 receptors decreased infarct volume, brain swelling and neurological scores 90 min after MCAo and 24 hours reperfusion. In addition, inhibition of P2X7 receptors decreased DNA fragmentation and increased neuronal survival after 30 min of MCAo which was associated with increased phosphorylation of survival kinases.

Here, we provide evidence for a potential role of P2X7 receptors in mediation of neuronal cell death. We predict that clinical implementation of P2X7 receptor antagonists can be beneficial after neurodegenerative disorders.

ÖZET

P2X7 RESEPTÖRLERİNİN BEYİN FELCİ ÜZERİNE ETKİLERİ VE ROLÜ

Periferik ve merkezi sinir sisteminde önemli rol oynayan purinerjik P2X7 reseptörleri ATP ile uyarılabilen katyonik ligand bağımlı iyon kanallarıdır. Bu reseptorler ATP bağlantılı hücre ölümünde, yangıda ve reseptör trafiğinin düzenlenmesinde önemli bir rol oynamaktadır. Bu çalışmada, P2X7 reseptörlerinin orta serebral arter oklüzyonu sonrası nöronal hücre ölümündeki rolü ve etkileri fare modeli kullanılarak incelendi.

Bu çalışmada reseptör agonisti olan BzATP, 250 μM dozunda kullanıldı fakat kontrol grubuna kıyasla hücresel hasarı anlamlı bir şekilde arttıramamıştır. P2x7 reseptörünün P2X7 reseptör antagonisti olan BBG (10mM) ile inhibe edilmesi ise beyindeki nöronların sağ kalımını anlamlı olarak arttırmıştır. BBG kullanılan 90 dakikalık serebral iskemi takiben 24 saat reperfüzyon sonunda yapılan nörolojik skorlamada, beyin ödemi ve infarkt hacminde anlamlı bir azalma tespit edilmiştir. Ek olarak, 30 dakikalık serebral iskemi takiben 72 saat reperfüzyon sonunda yaşamsal kinazlarının artmasıyla DNA parçalanması azalarak nöronal hücre sağkalımı artmıştır.

Bu çalışmada P2X7 reseptörlerinin nöronal hücre ölümündeki potansiyeli ve etkinliği gösterilmiştir. Elde ettiğimiz bulgular, ileride bu reseptör antagonistlerinin klinikte nörodejenerativ hastalıkların tedavisinde kullanılabileceğini göstermektedir.

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LIST OF SYMBOLS / ABBREVIATIONS

AP-1	Activator Protein
BBG	Brilliant Blue G
CBF	Cerebral Blood Flow
CCPA	Cyclopentyladenosine
CNS	Central Nervous System
COX-2	Cyclooxygenase-2
CPT	Cyclopentyltheophylline
CREB	cAMP Response Element Binding Protein
CV	Cresyl Violet
DPCPX	Dipropylcyclopentylxanthine
ELAM-1	Endothelial Leukocyte Adhesion Molecule-1
ER	Endoplasmic Reticulum
ICAM-1	Intracellular Adhesion Molecule-1
IgG	Immunoglobulin G
IL-1β	Interleukin-1 Beta
IL-6	Interleukin-6
iNOS	Inducible Nitric Oxide Synthase
IP	Inositol Triphosphate
LDF	Laser Doppler Flowmetry
MAPK	Mitogen Activated Protein Kinase
MCA	Middle Cerebral Artery
MCAo	Middle Cerebral Artery Occlusion
NF-κB	Nuclear Factor Kappa B
NOS	Nitric Oxide Synthase
Ox-ATP	Oxidized-ATP
P2X7R	P2X7 Receptor
PLA_2	Phospholipase A_2
PLD	Phospholipase D
PNS	Peripheral Nervous System
PPADS	Pyridoxal-Phosphate-6-Azophenyl-2',4'-Disulfonate

Px-1	Pannexin-1
ROI	Reactive Oxygen Intermediates
ROS	Reactive Oxygen Species
TNF-α	Tumor Necrosis Factor Alpha
tPA	Tissue Plasminogen Activator

1. INTRODUCTION

According to the World Health Organization (WHO), one of every 400 people confronted with stroke each year. After cardiovascular disease and cancer, stroke is designated to be the third most common cause of death in developed countries. Consequently, throughout the world, about 5.5 million people died from stroke in 1999 [1].

Stroke has several modifiable and non-modifiable risk factors. The most common non-modifiable causes of stroke are age (over 55 years), ethnicity (African Americans and Chinese), gender (men have a 30% greater risk) and genetic factors. However, modifiable risk factors that can be modified by medical treatment and life-style alteration are elevated arterial blood pressure, unhealthy diet, smoking, diabetes, physical inactivity and drug abuse [1].

Approximately 80% of all stroke cases are caused by middle cerebral artery occlusion (MCAo), the rest of the cases are resulted from a rupture in the brain blood vessels. As a consequence of the damaged blood vessels, intracranial blood pressure is increased and caused bleeding within the brain and its ventricles, which latterly leads to brain injury by distorting, compressing and tearing the surrounding tissue [2].

Brain injury after ischemic stroke develops from a several complex series of pathophysiological events including apoptotic cell death evolving within hours or even days [3]. Moreover, recent studies show that purinergic signaling pathway has an essential role in neurodegenerative diseases, such as ischemic stroke [4, 5].

P2X7 receptors are also known as purinergic receptors that mediate neuronal cell death, inflammation and cellular receptor trafficking [6]. P2X7 receptors are ATP sensitive Ca^{2+} permeable ion-gated channels, which play an important role in cerebral ischemia. BzATP (2'(3')-O- (4-Benzoylbenzoyl) adenosine--5'-triphosphate tri (triethylammonium) salt) is a P2X7 receptor agonist which possesses an exhibition potency 5-10 fold greater than ATP. On the other hand, BBG (Brilliant Blue G) is found to be the most potential antagonist of P2X7 receptor [6, 8].

In this study, BBG (antagonist), BzATP (agonist) and BBG-BzATP treatments were included with an animal model of 30 and 90 min focal cerebral ischemia in order to examine the potential roles of P2X7 receptors in neuronal cell death

2. THEORETICAL BACKGROUND

This chapter provides you with general information about cerebral ischemia (stroke) and purinergic receptors, especially P2X7 receptors. In this chapter, general information on cerebral ischemia (stroke) and purinergic receptors, especially P2X7 receptors, are explained and presented briefly.

2.1. CEREBRAL ISCHEMIA (STROKE)

Cerebral ischemia, also designated as stroke, is a medical condition in which blood supply to an organ or a tissue is reduced permanently or transiently. Brain injury after ischemic stroke develops from a several complex series of pathophysiological events including apoptotic cell death evolving over hours or even days [3]. During under stroke conditions, reduced blood flow evokes oxygen and nutrient deprivation, leading to tissue damage and ischemia [3, 9]. After the MCAo, injured territory is divided into two regions (Figure 2.1). Blood flow and ATP levels are significantly depleted within approximately 3 minutes in the center or core region of the ischemic region. As a result, metabolic failure and cell death occurs in minutes. Nevertheless, transition area or penumbra regionactively metabolizes glucose and challenges the ionic disturbance so that cell death mechanism works slowly in this region. Thus, the penumbra has better blood flow than the core region, which is respectively why many studies are focused on this territory [9, 10, 11].

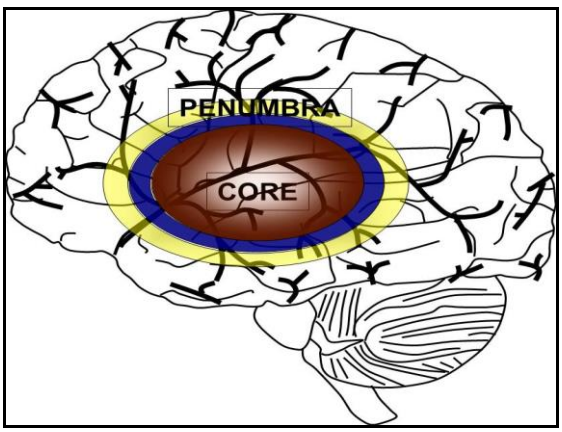

Figure 2.1. Core and penumbra territory after cerebral ischemia.

The level of oxygen and glucose uptake is extremely high in brain tissue in comparison with the other tissues. The energy production is specifically completed with oxidative phosphorylation [3, 12]. During the acute phase of stroke, neuronal membrane polarization is disturbed and the glutamate, which is the fundamental excitatory neurotransmitter throughout the central nervous system (CNS), is transferred into the extracellular synaptic space, as a result of the energy decrease through the clogging of blood pressure [12, 13]. Herein, the disturbance in glutamate uptake mechanisms leading to the accumulation of glutamate is termed as glutamate neurototoxicity (excitotoxity) in stroke pathophysiology [3, 14]. In the damaged area, glutamate levels could reach nearly 80 times higher than the baseline levels [3, 15].

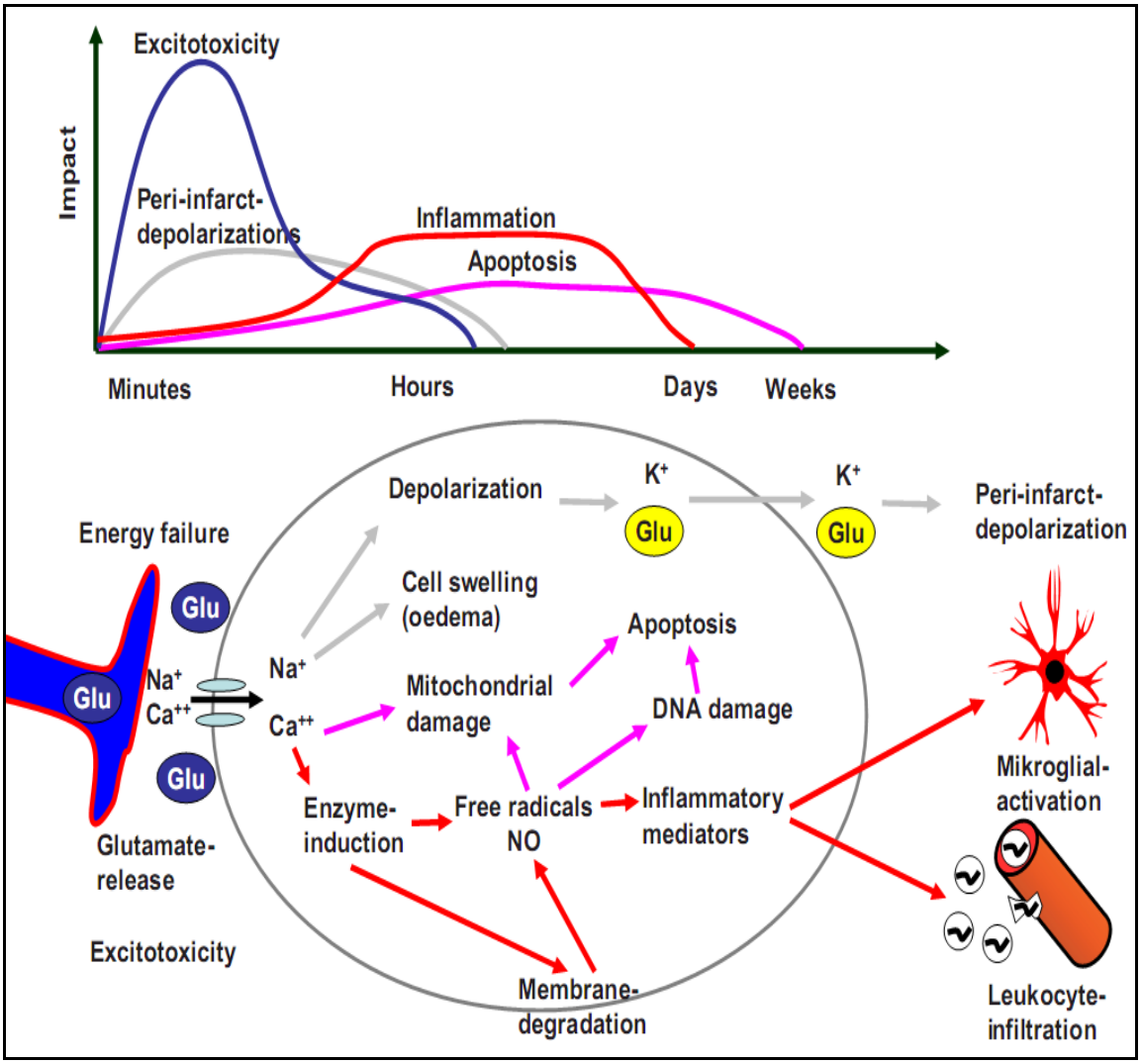

Figure 2.2. Pathophysiological events after ischemic stroke.

The post-synaptic neurons are stimulated through glutamate and consequently depolarization is occurred as sodium, Na^+, and calcium, Ca^{2+}, ions enter the neurons (Figure 2.2) [3, 14, 16]. Consequently, glutamate and potassium, K^+, ions are diffused to the extracellular space. This process initiates wavy excitation trait of neurons from the stroke field to the periphery, known as "peri-infarct depolarization" [17, 18, 19]. The wavy stimulation of neurons is responsible for about 20% of the stroke damage [20].

On the other hand, the Na^{+}', Cl^- and fluid that entered the neurons after stimulation induce cytotoxic cellular edema [3, 17]. Following glutamate neurotoxicity, the increased level of universal intracellular messenger calcium ions activates some enzymes, which results in membrane destruction and free radical formation [21]. Latterly, the stimulation of this mechanism composes leukocyte infiltration and microglia activation. For the final outcome, calcium ions and free radicals lead to the caspase-mediated cell death (apoptosis), via DNA and mitochondrial damage [21, 22].

Another important aspect of the ischemic damage generated in neural tissue is the inflammation in the damaged area. Microglias, which are resident macrophages of the CNS, become activated within 24 hours after the onset of ischemia and start secreting inflammatory cytokines. Moreover, vessels around the infarct zone express adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1) and endothelial leukocyte adhesion molecule-1 (ELAM-1) in the very early phases of ischemia [3, 13]. Furthermore, monocyte chemo attractant protein-1 and macrophage inflammatory protein-1α in the core area of the ischemia damage [14]. These adhesion molecules cause infiltration of neutrophils, macrophages and monocytes into the damaged area inducing more damage and inflammation. Nitric oxide produced by these cells also contributes to the formation of free radicals exacerbating the damage [23].Although acute phase of stroke stands for the most intensive pathopysiological process, apoptotic cells are still observed by the side of stroke field and contralateral hemisphere, indicated after months of stroke experiments within the chronic period [24]. Retrograde axonal degeneration, neurotransmitter level changes, neurogenesis, angiogenesis and synaptic plasticity events occur upon apoptotic cell death following stroke [24, 25, 26].

Until now the only obtainable treatment for ischemic stroke is acknowledged to be intravenous thrombolysis with tissue plasminogen activator (tPA), when applied within 3

hours after stroke onset [27]. Because neuronal apoptosis occurs 2 to 3 days after ischemia and neurons have a limited regeneration potential, addition of neuroprotective agents to the conventional treatment is likely to improve recovery and brain plasticity in stroke patients [28].

2.2. PURINERGIC SIGNALLING

Purinergic receptors (or purinoceptors) play extremely important roles in central nervous system (CNS) and peripheral nervous system (PNS). The members of plasma membrane molecules are found in almost all mammalian tissues [29]. According to the concept of purinergic signaling pathway, extracellular purines such as ATP (Adenosine Triphosphate), adenosine and pyrimidine act as extracellular signaling molecules [30,31]. There are 3 different receptor classes; metabotropic P1 receptors that activated via adenosine and P2 family which are subdivided into P2Y metabotropic and ionotropic P2X sub-classes [30,32].

Purinergic signaling plays radically significant role in many neuronal and non-neuronal mechanisms such as; inflammation, pain management, endothelial-mediated vasodilatation, and immune responses. In addition, purinergic signalling plays a critical role for the regulation of cellular receptor trafficking, ATP-mediated cell death, cell proliferation and differentiation [6, 32, 33].

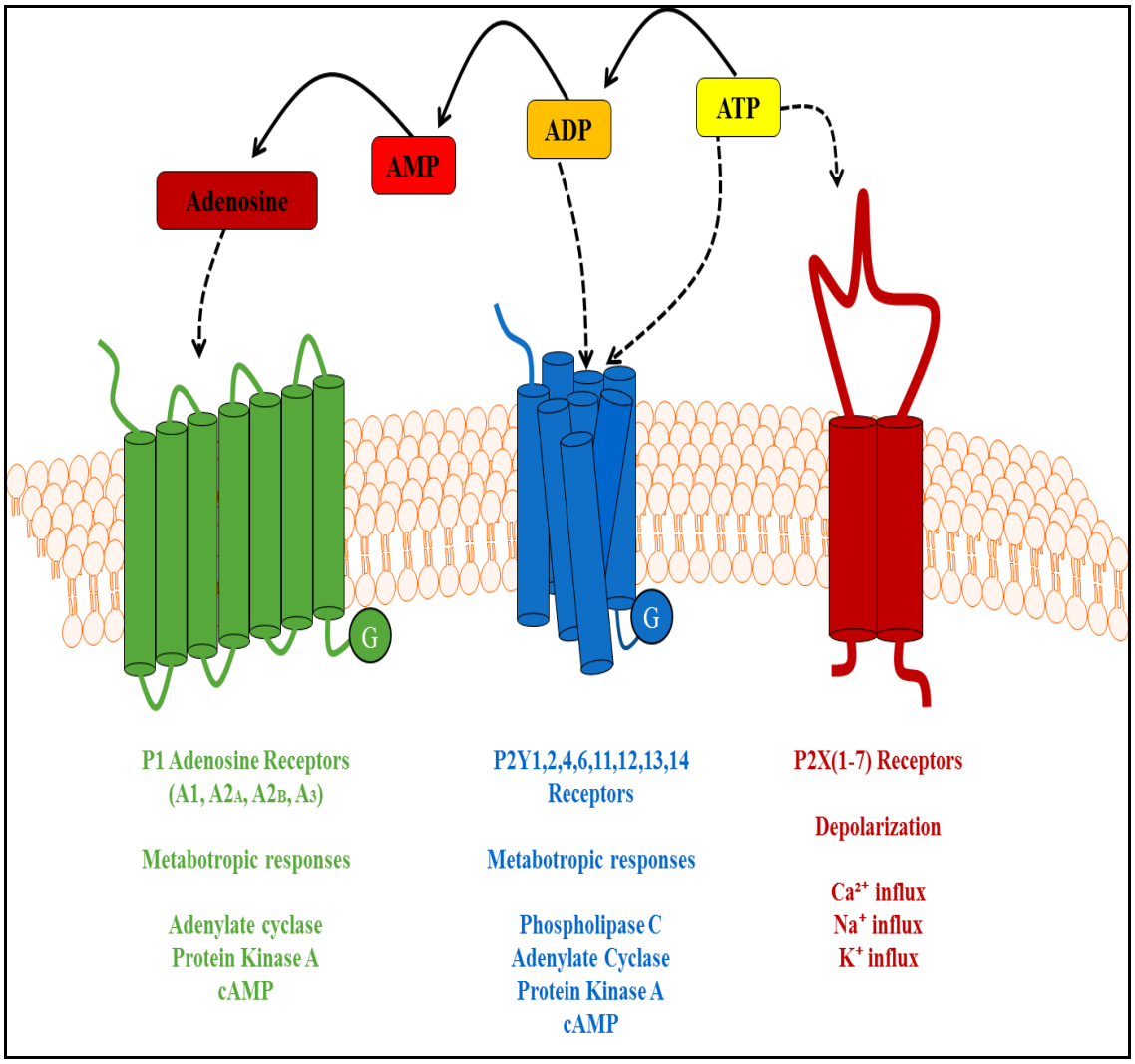

Figure 2.3. The Purinergic receptor family.

Extracellular ATP (Adenosine Three Phosphate) is the agonist of both P2Y and P2X receptors and also the substrate for ectonucleotidases that mediate the breakdown of ATP to ADP and then generates adenosine [6, 32]. Adenosine activates the G protein coupled P1 adenosine receptors (A_1, A_{2A}, A_{2B} and A_3). They are responsible for regulation and production of cAMP. P2X receptors (P2X1 to P2X7) are cationic-selective ion gated channels that have even selective for Na^+, K^+ and significantly permeable to Ca^{2+}. P2Y receptors (P2Y (1, 2, 46, 11, 12, 13, 14)) are G-protein coupled metabotropic receptors, which play an important role for phospholipase C activation, generation of inositol trisphosphate and elevation of intracellular Ca^{2+}. In addition, P2Y receptors also possess essential role for adenylate cyclase activation [6, 29, 34].

2.3. P1 or ADENOSINE RECEPTORS

The P1 class of purino receptors are G protein-coupled receptors in which they compose seven trans-membrane ™ domains and approximately 21 to 28 hydrophobic amino acid structure composing α-helix. G protein-coupled receptors include four different subtypes: A_1, A_{2A}, A_{2B} and A_3 in humans and precisely each of them vary in their functions. [35]. Regulation of the other neurotransmitters in brain such as glutamate and dopamine could be regulated by A_1 and A_{2A} receptors. In addition, A_{2B} and A_3 have essential roles in inflammation and immune response processes [35]. Moreover, P1 receptors play important role in adenylate cyclase signaling pathways in which A_1 and A_3 receptor activation lead to inhibition of adenylate cyclase [35, 36].

Several types of agonists and antagonists have been identified for A_1 adenosine receptor which has been found to be ubiquitous throughout the whole body. A_1 antagonists include DPCPX (8-Cyclopentyl-1,3-dipropylxanthine) and CPT (cyclopentyltheophylline). A_1 agonist includes CCPA (2-chloro-N(6)-cyclopentyladenosine) [37].

2.4. PURINERGIC P2Y RECEPTORS

Eight different purinergic P2Y receptors have been identified such as, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors. They are G protein-coupled receptors and stimulated by ATP, ADP, UTP, UDP and UDP-glucose (Table 2.1).

Recent molecular studies show that P2Y receptors are very important for cellular receptor trafficking to the cell surface [38]. They are coupled with G proteins and trigger the activation of cAMP or IP (inositol trisphosphate). $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors generally use ADP while $P2Y_{11}$ has excessively high affinity to ATP. $P2Y_2$ uses both ATP and UTP, whereas $P2Y_4$ uses only UTP. In addition $P2Y_6$ has tendency to use UDP. Finally UDP and sugars activate the $P2Y_{14}$ receptors [38, 39].

Protein	Nucleotide
$P2Y_1$	ADP
$P2Y_2$	ATP, UTP
$P2Y_4$	UTP
$P2Y_6$	UDP
$P2Y_{11}$	ATP
$P2Y_{12}$	ADP
$P2Y_{13}$	ADP
$P2Y_{14}$	UDP-glucose

Table 2.1 Types of purinergic P2Y receptor and necessary nucleotide(s) which is (are) responsible for its stimulation [38].

2.5. PURINERGIC P2X RECEPTORS

Cationic selective ionotropic P2X receptors are ATP-gated channels, which have significant roles throughout the PNS and CNS. P2X receptors so far, have been categorized into seven subgroups (P2X1, P2X2, P2X3, P2X4, P2X5, P2X6 and P2X7), which are able to generate multimeric pores with each other both homogenously, or heterogeneously except for the P2X6 receptor [40]. The amino acid chains of the P2X receptors vary among 388 (P2X6) to 595 (P2X7) residues and they share 36-42 % sequence similarity. Two transmembrane domains and one extracellular heavy loop have been characterized in all P2X receptors. P2X receptors are extensively produced in nervous system both in neurons and glia [38, 41].

The localization of P2X receptors identified by Northern blot analysis shows that, P2X1 is dominantly found in smooth muscle cells, P2X2, P2X4 and P2X6 are generally synthesized in brain neurons and P2X3 is solely expressed in sensory neurons. Besides, P2X7 is found to be abundant in immune system cells and hepatocytes [40, 42].

P2X receptors have even permeability to K^+ and Na^+ and significant permeability to Ca^{2+}. P2X receptors play essential roles in the regulation of cardiac rhythm, renal blood

flow vascular tone, chronic pain, platelet aggregation, macrophage activation, cellular receptor trafficking, ATP-mediated cell death and neuronal-glial integration [38, 43].

2.5.1. P2X1-6 Receptors

P2X1 receptor, whose role is regulating blood pressure and homeostasis, is abundantly found in in blood vessels, smooth muscles and platelets. ATP release shortly after a vascular injury induces platelets directly via P2X1 receptors in order to promote a rapid response [44]. Investigations on P2X1 knockout mice also supported P2X1 function in platelets and its role in thrombus formation. Minimized arterial thrombosis and insufficient platelet aggregation have been observed in mice lacking P2X1 receptors [38, 45].

P2X2 demonstrates a wide expression in central nervous system components such as hippocampus and cerebellum apart from sensory and autonomic ganglia. It is thought as a part of processes including nociception, taste, peristalsis of the gut and ventilatory activity against hypoxia [46]. Although P2X2 receptor is distributed widely throughout the body, mice whose P2X2 gene was silenced shows little differences in body weight and seems histopathologically healthy when compared to wild type animals [45, 46].

P2X3 receptors are present in sensory neurons, which are responsible for the transfer of stimulus coming from pain point to center. By this means, P2X3 could be associated considerably with the emergence of migraine related pain actions [47].

The genes encoding P2X4 and P2X7 receptors are suggested as the products of gene duplication because, they are located in the same region. P2X4 receptor has numerous splice alternatives in different cell types. In many researches impact of P2X4 receptor on pathogenesis of chronic neuropathic and inflammatory pain was debated. P2X4 level shows a dramatic increase in microglia of the dorsal horn behind application of a specific model of neuropathic pain [43, 45]

P2X5 protein in mice spread broadly in the central and enteric nervous system. Additionally, in cardiac and skeletal muscle, adrenal gland, kidney and testis existence of

P2X5 receptor was discovered. It was determined that ATP dependent P2X7 channel acts as a suppressor against proliferation and triggers differentiation of skeletal muscle progenitor cells [45]. Previous histochemical studies have indicated that P2X5 gene products are synthesized in the trigeminal mesencephalic nucleus and cervical spinal cord. Also, high P2X5 receptor expression has been found in different cancer tissues, such as basal and squamous carcinomas [46].

P2X6 receptor has a role in the differentiation of mesenchymal stem cells often together with P2X4 subunit as it was found in epithelial cells of various organs (renal tubule, bronchi, thymus, umbilical vein) in addition to gland cells of the uterus and granulosa cells of the ovary [45, 46].

2.5.2. P2X7 Receptors

Extracellular ATP is a very essential signaling molecule which acts as a neurotransmitter, throughout the CNS and PNS. Several types of physiological conditions such as mechanical stress, cellular injury, and ionic disturbances in the environment or inflammation lead to significantly ATP stimulation [36]. Therefore, oscillation of huge amount of cellular ATP triggers activation microglia and astrocyte receptors and boosting critical diverse pathological cascades; such as glutamatergic excitotoxicity, oxidative damage and other cytokine-mediated signaling pathways [48].

P2X7 receptor (P2X7R) is a purinergic receptor, has specific structure and may play an important role various neurological disorders, like cerebral ischemia (stroke), spinal cord injury, neuropathic pain, Alzheimer’s disease and depression [36, 49]. The subunits of P2X7R which share a similar topology, has a large extracellular loop with the cysteine rich ATP binding site. P2X7R possess two transmembrane domain (TM1 and TM2) and intracellular carboxyl terminal domain (293 amino-acids) and amino termini (Figure 2.4) [49].

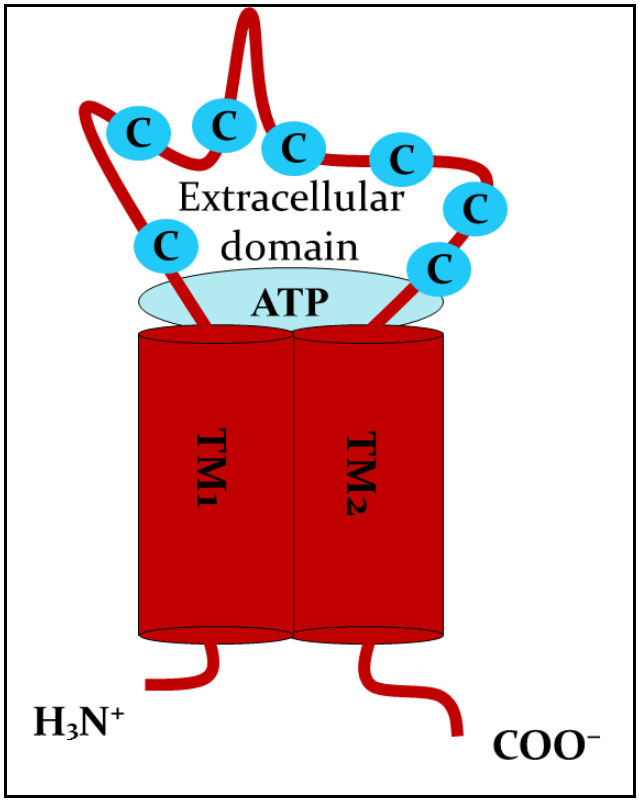

Figure 2.4. Structure of P2X7 receptor.

P2X7Rs have two different states, cationic ion gated channels and plasma membrane pores so that they are working bifunctionally. They have even permeability to Na^+ and K^+, significant permeability to Ca^{2+}. Transmembrane pore allows to macromolecules up to approximately 700-900Da in size [36]. The P2X7R can be activated by high concentration of ATP. The stimulation of P2X7R leads membrane blebbing, microglial activation, reactive oxygen species (ROS) production, and increased secretion of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6, and finally apoptosis and cell death [48].

2.5.2.1. P2X7 Receptors under Normal Physiological Conditions

Under normal physiological conditions, $P2X_{1-6}$ receptors generally locate on the membrane of neurons but P2X7Rs widespread on the membranes of astrocytes, microglia, oligodendrocytes and neurons [50, 51, 52]. In the physiological resting state, expression of P2X7Rs abundantly locates on the microglia [53].

The cellular localization of P2X7Rs in the CNS has distinct conflicts. In neurons, P2X7Rs are generally distributed on presynaptic terminal in the medulla oblongata and spinal cord, excitatory nerve terminals of CA1, CA3, the dentate gyrus and mossy fibers of the hippocampus [53].

2.5.2.2. P2X7 Receptors during Ischemia

During or after several pathological conditions like cerebral ischemia, ATP is released from cytoplasm of damaged and dying cells to extracellular space in order to nucleic acid degradation. ATP could be released various way; first of all, corruption of Na^+/K^+ pump leads to increase level of extracellular K^+ which triggers the depolarization of damaged neurons. Then overexpression of extracellular Ca^{2+} induces relaxation of ATP from organelles and autocrine process. Elevation of the level of ATP concentration in the extracellular space strongly activates the P2X7Rs and cause cellular death [48].

Intracellular level of K^+ is changing during the ischemia due to the activation of P2X7Rs. This activation leads caspase-1 activation and release of the cytokine, IL-1β which activates procaspase-1, nitric oxide synthase (NOS) and tumor necrosis factor-α (TNF-α) [48]. In addition, activation of P2X7Rs related to caspase-3 activation, phospholipase D (PLD), phospholipase A_2 (PLA_2), nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) [55, 56].

During ischemia, elevated level of cellular Ca^{2+} concentration triggers several signaling pathways by means of various types of receptor and channels, e.g. extracellular calcium sensing receptors, hemi-channels, potassium ion channels and metabotropix glutamate receptors [54]. High level of Ca^{2+} activates nucleases and proteases such as calpains (Ca^{2+} dependent proteases) which ultimately success in cell death. Meanwhile, Ca^{2+} is taken from the matrix of mitochondria reaches the maximum toxic threshold. Therefore, ions and solute Ca^{2+} flow into the cytosol and finally activate the apoptotic caspase enzyme system [36].

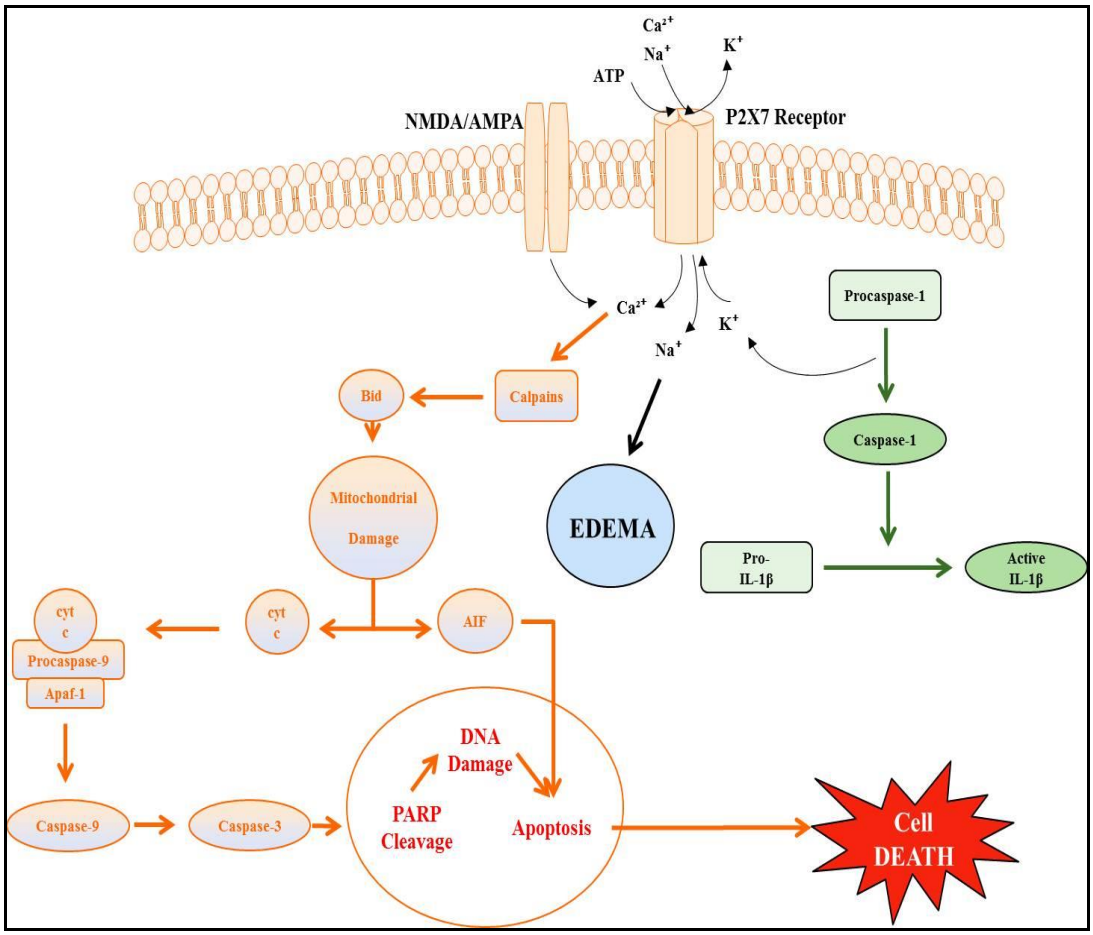

Figure 2.5. Signal transduction components of P2X7 receptor.

Activation of P2X7R via ATP causes channels opening on the membrane which permeable to Na^+, K^+ and significantly Ca^{2+}. Elevated level of Ca^{2+} or stimulation of caspase 8 from extrinsic pathway activates calpains. Activation of calpain triggers cleaving BID to tBID [3, 25, 27]. Then, tBID induces conformational changes in other proapoptotic proteins such as Bak, Bax, Bad, and Bcl-XL. Activation of apoptotic proteins lead to opening of mitochondrial membrane pores and cytocyrome c (Cytc) is released. Released Cytc binds Apaf-1 (protein-activating factor-1) and procaspase-9 to form an "apoptosome" complex which activates caspase-9 and later caspase-3. Activated caspase-3 triggers activation of PARP (poly (ADP-ribose) polymerase) which leads to DNA damage and apoptosis. In addition activation of PLA_2 and cyclooxygenase generates several free radical species that contributes membrane damage. Moreover, activation of P2X7 receptor leads efflux of K^+ ions from cell which converts pro-IL-1β to mature IL-1β and release from the cell. (Figure 2.5) [55, 56].

Pannexins are massive protein membrane channels which robustly expressed in the brain [57]. They provide synchronization between hippocampus and cortex. Under ischemic conditions, P2X7Rs are responsible for regulation of pannexin-1 (Px-1) and influx of Ca^{2+} from extracellular space [58, 59].

2.5.2.3. P2X7 Receptor Antagonists

Recent studies claim that P2X7R system can stimulate neurodegenerative diseases. Suppression of this purinergic receptor could be a medicinal treatment for the neurodegenerative process.

Increasing number of P2X7R antagonists have been identified so for such as PPADS Tetrasodium salt, Oxidized ATP, Suramin, KN62, Coomassie Brilliant Blue G (BBG), AZ 116453743, AZ Compound, A-438079, and A-740003 [60, 61].

PPADS tetrasodium salt (pyridoxalphosphate-6-azopheny-2',4'-disulfonate) and suramin (polysulfonated naphthylurea anticancer angent) are noncompetitive P2X7R antagonists which block P2X7R with low affinity [60].

BBG is the most selective and noncompetitive antagonist which has approximately 40-60 fold greater selectivity and potency for human P2X7R. BBG is originally obtained from a blue food dye [60]. BBG is considered to be safe and healthy and it was also approved to use BBG in food ingredients. In addition it is safe in healthy for people and also is approved for foodstuff use. BBG could decrease the activity of P2X7R and it has low toxicity. Although BBG could cross the blood brain barrier (BBB), ox-ATP could not cross the BBB. Recent studies showed that usage of BBG for P2X7R antagonist is the safe and it could serve as a therapeutic target for the treatment of neurodegenerative disorders. BBG reduces the electrical responses, Ca^{2+} influx and neuronal death [43].

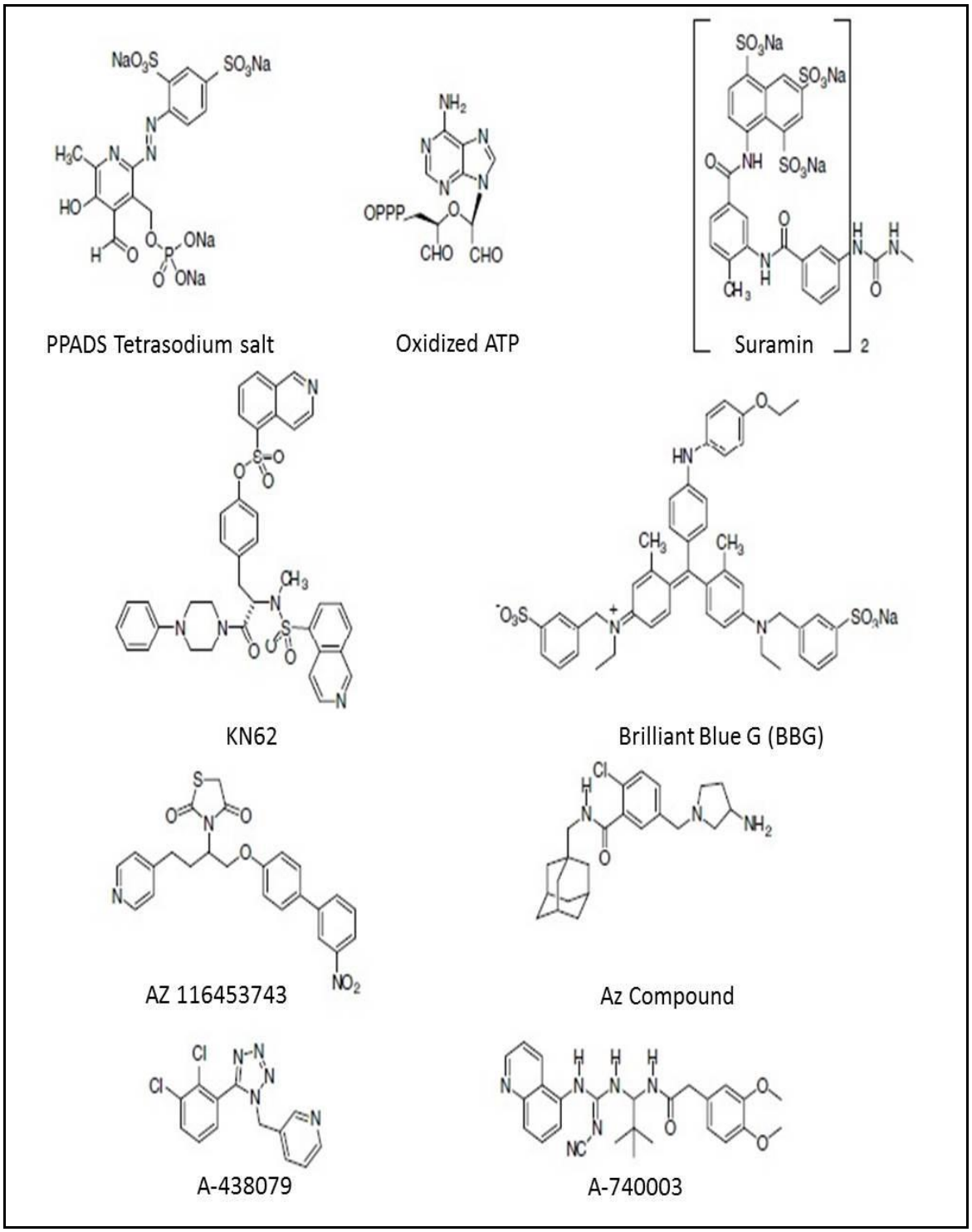

Figure 2.6. Chemical structures of potential P2X7R antagonists.

Ox-ATP (2',3'-dialdehyde) is an irreversible inhibitor of P2X7R, has a greater potency not only P2X receptors but also P2Y receptor family. In addition it has different pharmacological actions such as P2X1 and P2X2 receptor inhibition, NF-κB and cytokine

release. It is very similar to BBG [62].

KN-62 is another type of antagonist, effective for human P2X7Rs but inadequate activity at the rat P2X7Rs. Additively; it may useful for the pharmacological identification of P2X7Rs. It is also known as a selective CAM-kinase-II inhibitor and also attenuates the elevation of NOS serine phosphorylation induced by animal cerebral ischemia model [63].

AZ11645373 (3- (1- (3'-nitrobiphenyl-4yloxy)-4- (pyridine-4-yl) butan-2-yl) thiazolidine-2,4-dione) potentially blocks human P2X7R-mediated cation influx and IL-1β release. Nevertheless, AZ11645373 demonstrates lacking activity at the rat P2X7Rs like KN-62 so that usage of AZ11645373 and KN-62 limiting to examine P2X7R function in animal models [64].

A-438079,3- ((5-(2,3-dichlorophenyl)-1H-tetrazol-1-y)methyl pyridine) is a reversible competitive blocker of P2X7R. It distributes the Bz-ATP stimulated intracellular Ca^{2+} concentration. In addition, it is responsible for inhibition of Bz-ATP stimulated IL-1β release and pore formation [60, 61].

A-740003 ((N-(1-{[(cyanoimino)(5-quinolinylamino) methyl]amino}-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl)acetamide) is also another type of P2X7R antagonist, has strong affinity for both human and rat P2X7Rs. In addition, like A-438079, A730003 blocked P2X7R- mediated changes in intracellular Ca^{2+} concentration [65].

2.5.2.4. P2X7 Receptor Agonists

In brief, agonist activation leads rapid and reversible membrane channel opening which is permeable to Na^{+}, K^{+} and Ca^{2+}[66]. Homomeric P2X7Rs are activated via high concentration of ATP and Bz-ATP (2',3'-O-(4-benzoylbenzoyl)-ATP), which has greater potency (approximately 10-30 fold) than ATP [67]. Bz-ATP is not only a selective P2X7R agonist but also bind to other P2X receptors such as P2X1, P2X2 and P2X3 with same potency. ATP and Bz-ATP also stimulate the generation of ROI (reactive oxygen intermediates) due to the elevation of intracellular Ca^{2+}, but BBG and ox-ATP prevent this effect [50].

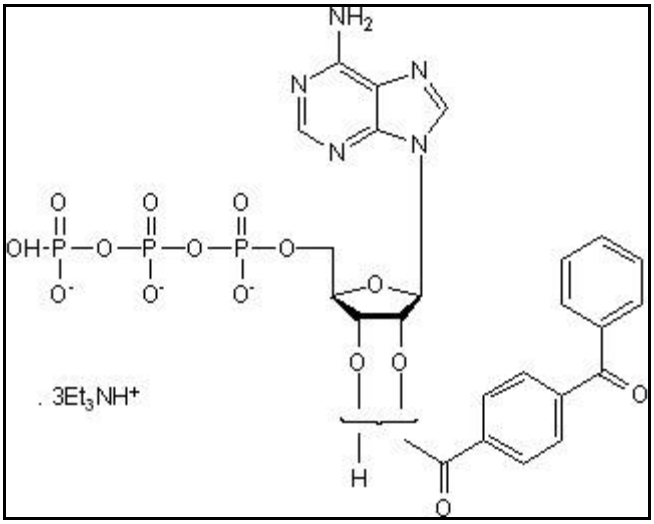

Figure 2.7. Chemical structure of BzATP.

There are also different agonists for P2X7Rs such as ADP (Adenosine diphosphate) and AMP (Adenosine monophosphate) which have lower affinities to this receptor than ATP itself. In addition 2-MethylthioATP (2-Methylthioadenosine-5'-triphosphate tetrasodium salt), ATP-γ-S (Adenosine 5'-[γ-thio] triphosphate tetralithium salt), ADP-β-S (Adenosine-5'-O-(2-thiodiphosphate) and α,β-meATP (α,βmethyleneATP) are weak agonists for P2X7Rs [61].

3. MATERIALS & METHODS

3.1. EXPERIMENTAL SETUP AND GROUPS

All experimental procedures were conducted with governmental approval according to local guidelines for the care and use of laboratory animals. All animals were kept under regular lighting conditions as 12 hours darkness and 12 hours light. Adult male C57BL/6j mice (21-25 g) randomly assigned to the following experimental groups: including; 30 min MCA occlusion 72 hour reperfusion or 90 min MCA occlusion 24 hour reperfusion was submitted to vehicle (normal saline) (n=7) or 10 mg/kg P2X7 receptor antagonist Brilliant Blue G (BBG) (n=7) or 5 mg/kg P2X7 receptor agonist 2'(3')-O-(4-Benzoylbenzoyl) adenosine--5'-triphosphate tri (triethylammonium) salt (BzATP) (n=7) or both (10 mg/kg BBG and 5 mg/kg BzATP) (n=7).

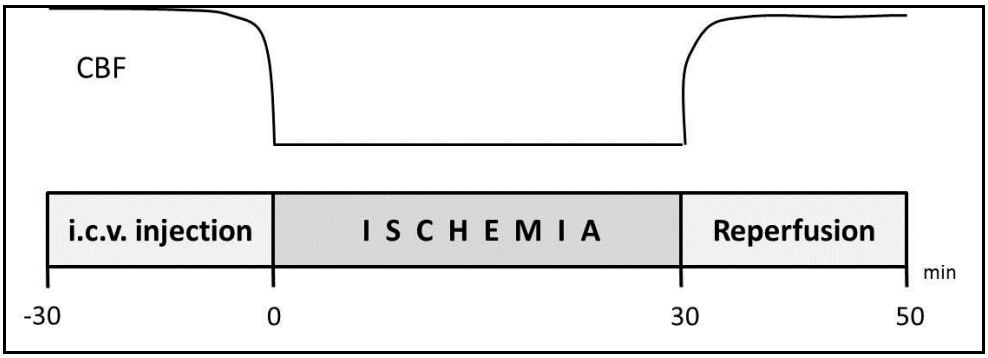

Figure 3.1. Representation of 30 min MCAo experimental setup.

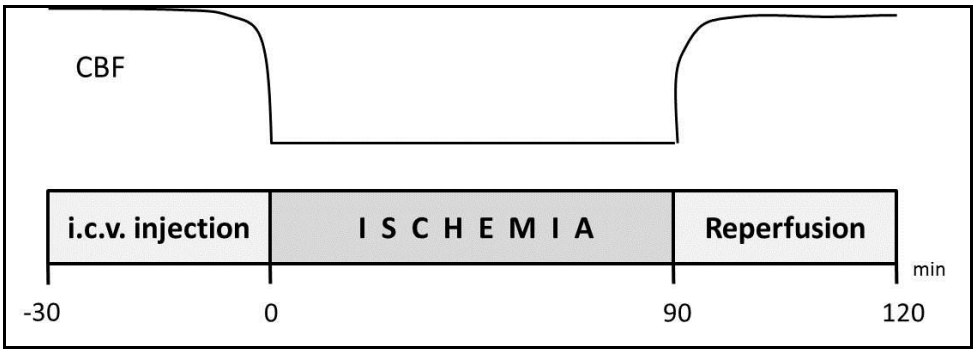

Figure 3.2. Representation of 90 min MCAo experimental setup.

3.2. INTRACEREBROVENTRICULAR (i.c.v.) INJECTION

Animals were anesthetized with 400 mg/kg chloral hydrate (intraperitonaelly) (23100; Sigma-Aldrich). Skull of the mice will be drilled and mice will be treated with vehicle (normal saline) or 10 mg/kg Brilliant Blue G (BBG) (sc-203733; Santa Cruz Biotechnology) or 5 mg/kg 2'(3')-O-(4-Benzoylbenzoyl)adenosine--5'-triphosphate tri(triethylammonium) salt (BzATP) (sc-203862; Santa Cruz Biotechnology) or both 10 mg/kg BBG and 5 mg/kg BzATP was injected introcerebroventricullarly via micro-syringe pump controller (Micro 4; World Precision Instrument) 30 minutes before MCA occlusion

3.2. INDUCTION OF CEREBRAL ISCHEMIA & REPERFUSION

Adult male C57Bl6 mice (8-10 weeks, 21-25g) were anesthetized with 1% isofluorane (30% O_2, remainder N_2O). Rectal temperature (36.5 and 37.0^oC) was controlled using feedback-controlled heating system (MAY instruments, Ankara, Turkey). During the MCA occlusion and reperfusion, cerebral blood flow (CBF) was monitored via Laser Doppler Flowmetry (LDF) using a flexible 0.5mm fiber optic probe (Perimed, Sweden) which was attached with tissue adhesive to the intact skull overlying the MCA territory (2mm posterior and 6mm lateral from the bregma). Focal cerebral ischemia was induced using an intraluminal filament technique. After a small midline neck incision, left common and external carotid arteries was isolated and ligated. Internal carotid artery was temporally ligated using a microvascular clip (FE691; Aesculap, Germany). A 180-190 µm silicon coated (Xantropen; Bayer Dental, Japan) 8.0 nylon monofilament (Ethilon; Ethicon, Germany) was inserted through a small incision into the common carotid artery and advance 9 mm distal to the carotid bifurcation for middle cerebral artery (MCA) occlusion.

Thirty minutes (72h reperfusion) or ninety minutes (24h reperfusion) after onset of ischemia, reperfusion was initiated by with-drawl of the thread. After that LDF recordings were continued for 30 min for control of the reperfusion. Anesthesia was then discontinued and mice were placed own cages.

After 24 hour (90 min MCA occlusion) or 72 hour (30 min MCA occlusion) of reperfusion, mice were re-anesthetized and decapitated. Brains were quickly removed and

frozen with dry ice. Thereafter, brains will be cut on a cryostat (CM1850-UV; Leica, Germany) into 18µm coronal sections.

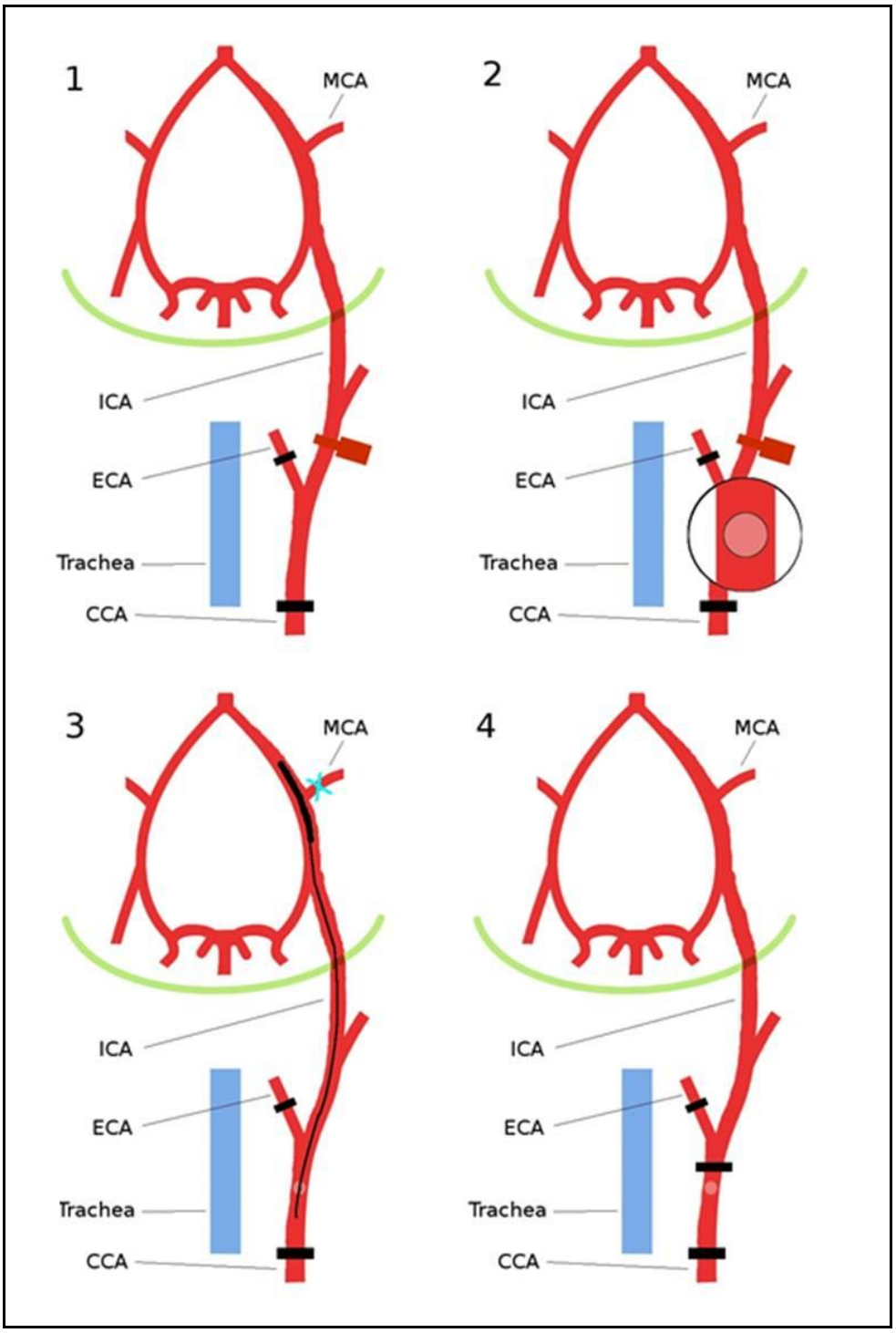

Figure 3.3. Graphical representation of the surgical operation for the induction of cerebral ischemia and reperfusion.

3.3. NEUROLOGICAL DEFICIT SCORES

Neurological score were observed 24 hours after for 90 min MCA occlusion using the following scores: 0 = normal function; 1 = flexion of torso and of the contralateral forelimb upon lifting of the animal by the tail; 2 = circling to the contralateral side but normal posture at rest; 3 = reclination to the contralateral side at rest; 4 = absence of spontaneous motor activity.

3.4. CRESYL VIOLET STAINING, INFARCT VOLUME & BRAIN SWELLING CALCULATIONS

Coronal brain sections from four equidistant brain levels, 2 mm apart, were stained with cresyl violet according to a standard protocol. On the sections, the border between infarcted and noninfarcted tissues was outlined using an image analysis system (Image J; National Institute of Health, Bethesda, MD, USA), and the area of infarction was assessed by subtracting the area of the nonlesioned ipsilateral hemisphere from that of the contralateral side. The volume of infarction was calculated by integration of these lesion areas. Edema was calculated as the volume difference between the ischemic and the nonischemichemisphere and expressed as a percentage of the intact hemisphere.

3.5. ANALYSIS OF APOPTOTIC CELLS VIA TUNNEL STAINING

In order to detect and quantificate the number of apoptotic cells, DNA strand breaks on the coronal brain sections were labeled via TUNEL technology (In Situ Cell Death Detection Kit; Roche, Switzerland). Cryostat sections from 30 min MCA occlusion 72 h reperfusion were fixed with 4% PFA/0.1 M PBS. Sections were stained with terminal transferase biotinylated-dUTP nick end labeling (TUNEL) and counterstained with 4’,6-diamidino-2-phenylindole (DAPI). Sections were analyzed by quantifying DNA-fragmented cells in nine adjacent regions of interest in the striatum each measuring 62,500 μm^2 under a fluorescence microscope

3.6. ANALYSIS OF SURVIVING NEURONS

From mice submitted to 30 min MCA occlusion cryostat sections from the level of bregma were fixed for 10 min at room temperature (RT) with 4% paraformaldehyde (PFA) in 0.1M buffered phosphate-saline (PBS) and subsequently stained with cresyl violet. Nine random area of interest, 1mm apart, each measuring 62,500 μm^2, will be evaluated under light microscope. Mean values were calculated both ischemic (ipsilateral) and non-ischemic (contralateral) areas, by which the percentage of surviving neurons was determined.

3.7. STATISTICS

Statistical data analyses were done with a standard software package (SPSS for Windows; SPSS Inc., Chicago, IL, USA). Differences between the groups were calculated by one-way ANOVA, followed by least significant differences tests. All values will be given as mean±SD and "n" values indicating the number of different animals analyzed. P values < 0.05 are considered significant.

4. RESULTS

4.1. LASER DOPPLER FLOWMETRY

Cerebral blood flow (CBF) was measured with Laser Doppler Flowmetry (LDF) recordings which were applied for the left MCA territory (2 mm posterior and 6 mm lateral from the bregma) during focal cerebral ischemia. Recordings were saved 10 minutes before MCA occlusion and continued during the MCA occlusion. Thereby, recordings were continued 20 min (for 30 min MCA occlusion) or 30 min (for 90 min MCA occlusion) after the onset of reperfusion.

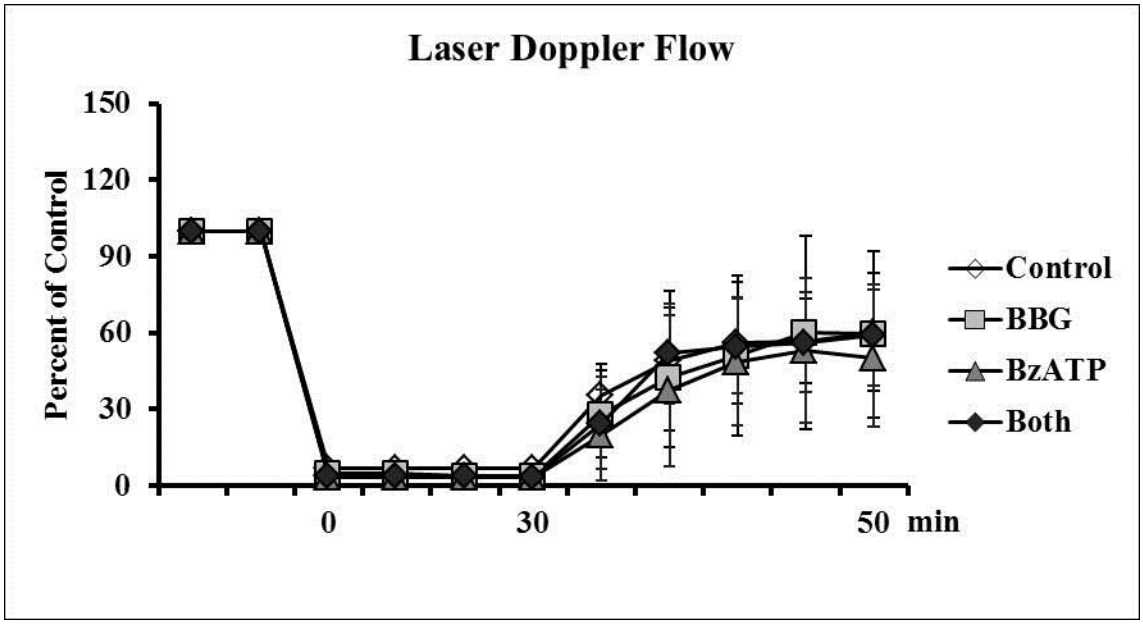

Figure 4. 1. Laser Doppler Flowmetry (LDF) result from animals submitted to 30 min MCA occlusion followed by 72 h reperfusion. Experimental groups were; Control (normal saline), 10 mM BBG (Brilliant Blue G), 250 μM BzATP (2',3'-O-(4-benzoylbenzoyl)-ATP) and 10 mM BBG and 250 μM BzATP (Mean±SD).

Results from animals submitted to 30 min MCA occlusion followed by 72 h reperfusion showed that MCA thread occlusion caused in a sharp decrease of CBF to ~5% of the pre-ischemic control values in the MCA territory. In all groups, thread retraction after 30 min was followed by a rapid restoration of blood flow. During the first 20 min of

reperfusion, CBF of the Control, BBG and Both treated groups reached ~60% of pre-ischemic levels whereas only BzATP treated group reached ~50% of pre-ischemic levels. In addition, no statistically significant difference was observed between groups by using repeated- and on-way- ANOVA analysis.

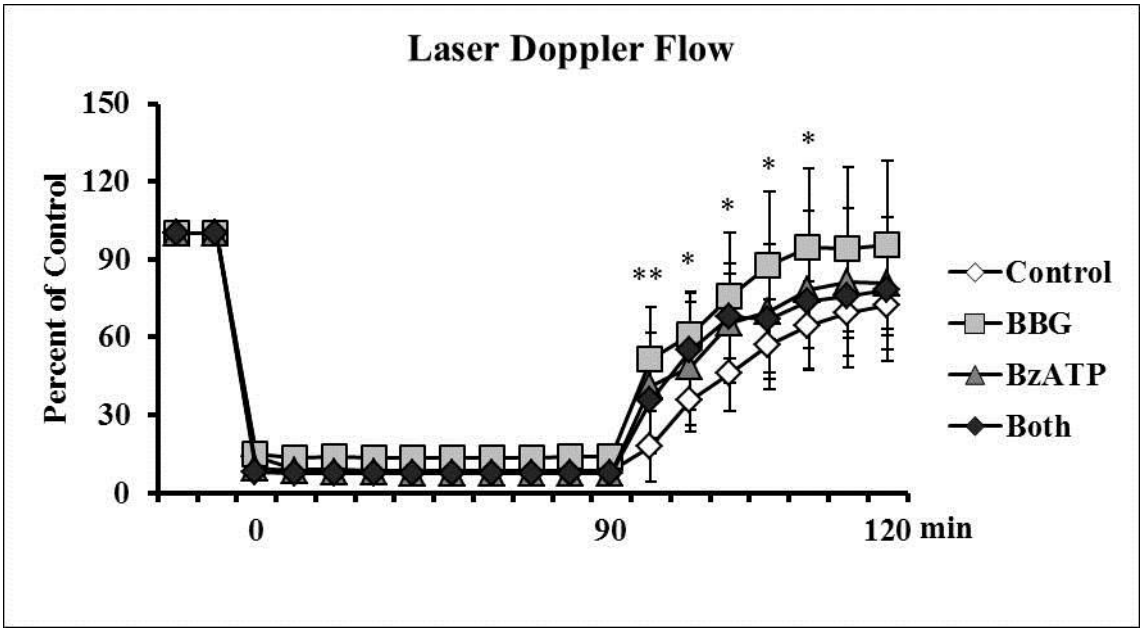

Figure 4.2. Laser Doppler Flowmetry result from 90 min MCA occlusion, 24 h reperfusion. Experimental groups were; Control (normal saline), 10 mM BBG (Brilliant Blue G), 250 μM BzATP (2',3'-O-(4-benzoylbenzoyl)-ATP) and 10 mM BBG and 250 μM BzATP (mean±SD, **p< 0.01 and *p < 0.05).

Results showed that CBF values were decreased to ~10% of the pre-ischemic control values after occlusion of the MCA, respectively. Withdrawal of the filament from MCA was resulted rapid restoration of blood flow, especially BBG treated group. Although, Control group was reached to ~70%, both and BzATP treated groups were reached ~80% of the pre-ischemic control values during the first 30 min of the reperfusion. BBG treated group was reached ~90% and also there were statistically differences at the time point of 91 ($p<0.01$), 95, 100, 105 and 110 ($p<0.05$) min, respectively.

4.2. NEUROLOGICAL SCORE

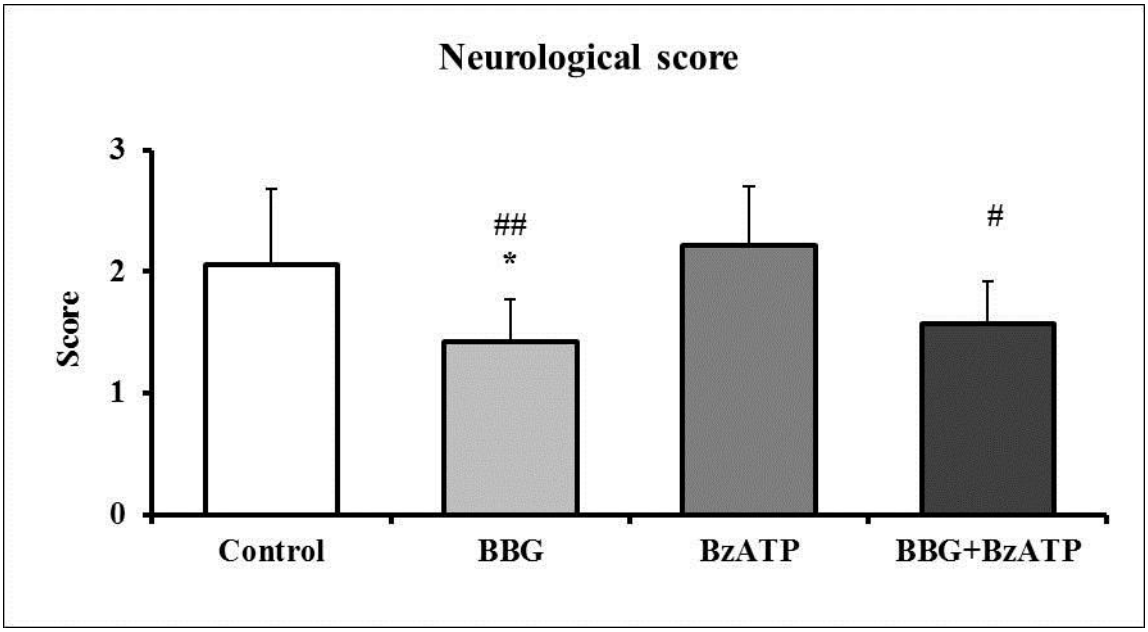

Figure 4.3. Neurological deficits score, 24 hours after 90 min MCA occlusion evaluated using a 0-4 point scale neurological scores: 0 = normal function; 1 = flexion of torso and of the contralateral forelimb upon lifting of the animal by the tail; 2 = circling to the contralateral side but normal posture at rest; 3 = reclination to the contralateral side at rest; 4 = absence of spontaneous motor activity. Experimental groups were; Control (normal saline), 10 mM BBG (Brilliant Blue G), 250 μM BzATP (2',3'-O-(4-benzoylbenzoyl)-ATP) and 10 mM BBG and 250 μM BzATP (Mean±SD; ## $p<0.01$ and #$p< 0.05$).

24 hours after 90 min MCA occlusion, neurological deficits related to cerebral ischemia was observed in all experimental groups. Results showed that vehicle treated control group average neurological score were 2.1±0.2 and BzATP has very near neurological score of 2.2±0.4. In addition, BBG treated group was 1.4±0.3, significantly reduced neurological deficits score with respect to control ($p = *<0.05$) and BzATP ($p = ##<0.01$) treated groups Both BBG and BzATP treated group was also 1.6±0.3 which statistically decrease the neurological score in proportion to BzATP ($p = #<0.05$).

4.3. INFARCT VOLUMES

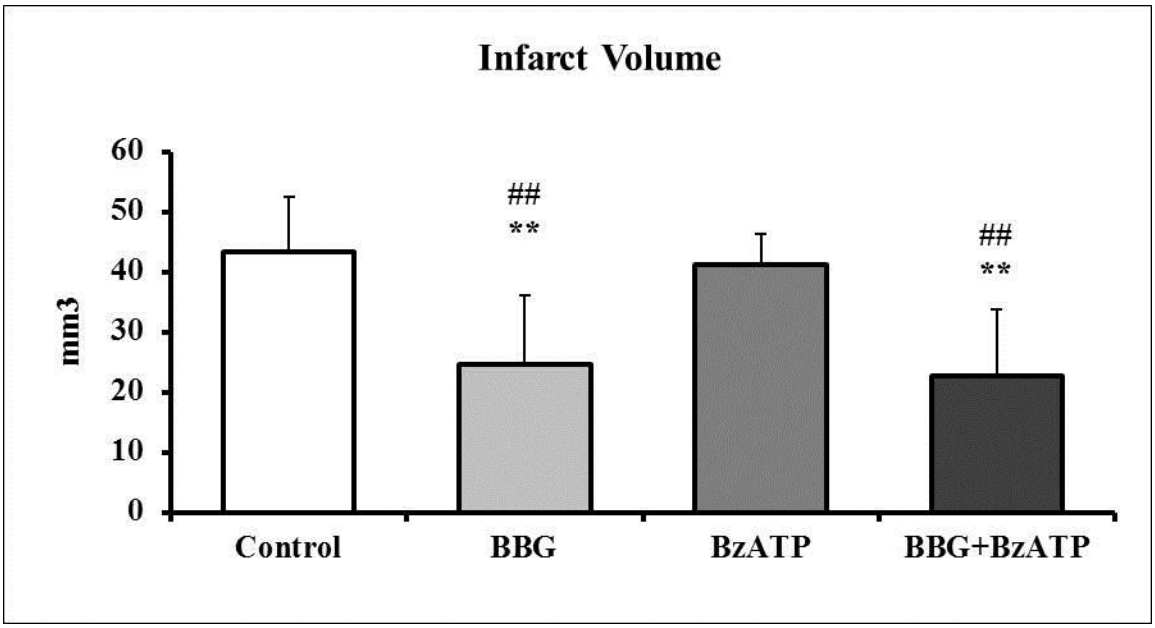

Figure 4.4. Infarct volumes of the experimental groups; Control (normal saline), 10 mM BBG (Brilliant Blue G), 250 μM BzATP (2',3'-O-(4-benzoylbenzoyl)-ATP) and 10 mM BBG and 250 μM BzATP. (Mean±SD, **;## $p < 0.01$)

In order to determine infarct volume, CV staining was applied to 90 min MCA occlusion 24 h reperfusion animal brain cross-sections and infarct volume was calculated with an image analysis system. In the vehicle treated Control group, infarct volume was 43.4±8.0 mm^3. Similarly, P2X7 receptor agonist BzATP (250 μM) was 41.2±5.0 mm^3. In the BBG (10 mM) group which was an antagonist of P2X7 receptor, infarct volume was significantly decreased to 24.7±11.0 mm^3. Finally, in BBG (10 mM) and BzATP (250 μM) treated group was 22.6±10.8 mm^3. Infarct volume was also significantly decreased in BBG and BzATP group.

The difference between BBG and control or BzATP group was found to be statistically significant ($p<0.01$). Similarly, the difference between BBG and BzATP treated group and control or BzATP group was also found to be statistically significant ($p<0.01$). However, there were no significant change observed between the Control and BzATP or BBG and both groups.

4.4. BRAIN SWELLING

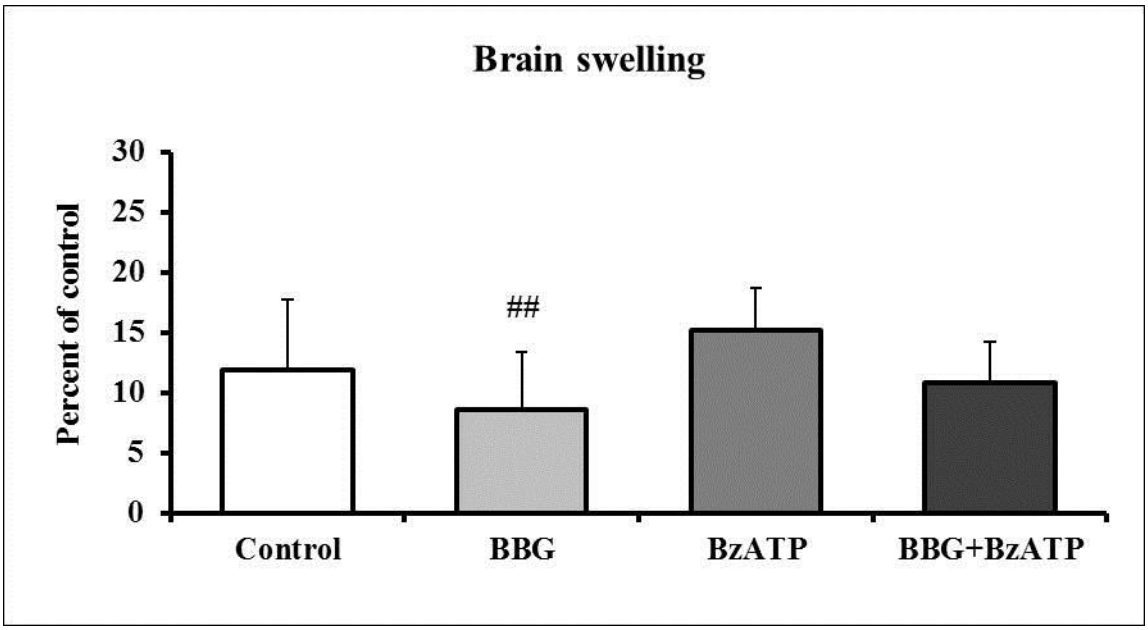

Figure 4.5. Percentage values of brain swelling. Control (normal saline), 10 mM BBG (Brilliant Blue G), 250 μM BzATP (2',3'-O-(4-benzoylbenzoyl)-ATP) and 10 mM BBG and 250 μM BzATP (Mean±SD, # $p < 0.05$).

In order to determine brain swelling, four level of equidistant brain level, 2 mm apart were stained with cresyl violet from animals submitted to 90 min MCA occlusion followed 24 h reperfusion. Then, brain swelling was calculated with an image analysis system (Image J; National Institute of Health, Bethesda, MD, USA).

The above results showed ischemic brain edema, expressed as percentage swelling of the non-ischemic hemisphere, was 12.0±5.7, 9.0±4.8, 15.0±3.5 and 11.0±3.4 in control , BBG, BzATP and both (BBG+BzATP) treated groups, respectively.

Brain edema in BzATP treated group has the highest brain swelling among the experimental groups. Although in both group (BBG+BzATP) there was a decrease the swelling, there was no significance between the groups, respectively. Brain swelling was only statistically significant between BBG and BzATP treated groups ($p<0.05$).

4.5. DNA FRAGMENTATION

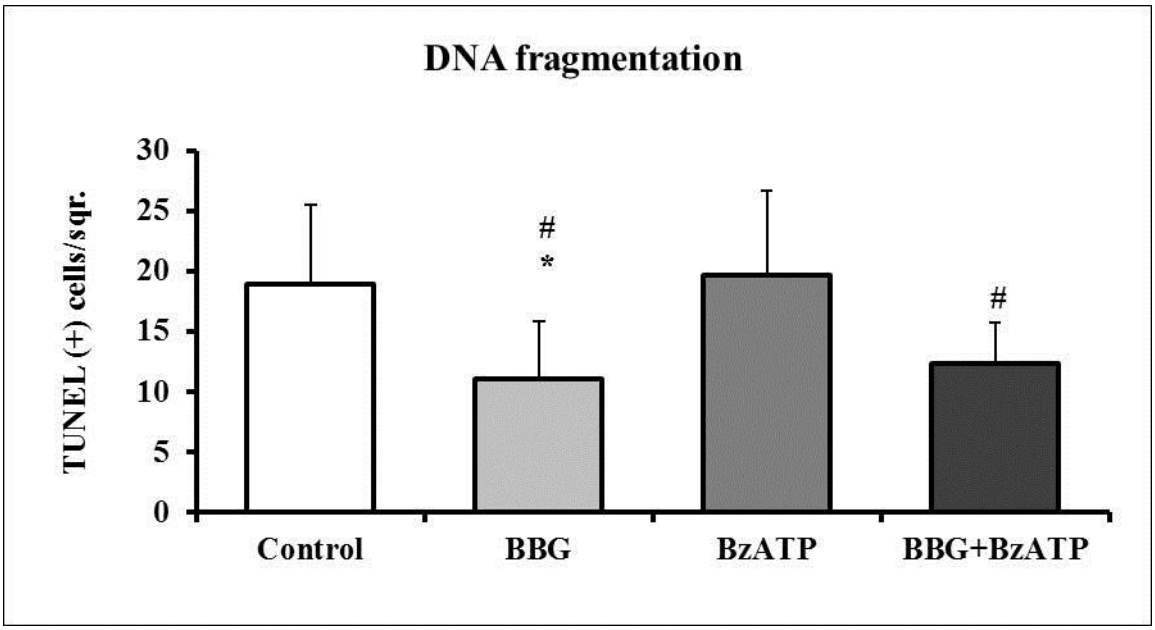

Figure 4.6. Mean density of DNA-fragmented cells in striatum from animals undergoing 30 min MCA occlusion, 72 h reperfusion. Control (normal saline), 10 mM BBG (Brilliant Blue G), 250 μM BzATP (2',3'-O-(4-benzoylbenzoyl)-ATP) and 10 mM BBG and 250 μM BzATP (Mean±SD, *, # $p < 0.05$).

From animals submitted to 30 min MCA occlusion followed 72 h repersusion, section from the bregma level were stained with terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) kit in order to quantification of number of apoptotic cell. Nine regions from the bregma level, 1mm apart, and each measuring 62,500 μm^2 were investigated.

In the graph, TUNEL-positive cells per square were given for experimental groups; the highest apoptotic cells per square were seen in BzATP group which was 19.6±7.0. Similarly, in the vehicle treated control group was 18.94±6.6. In the BBG group, the number of apoptotic cells was significantly decrease in proportion to control and BzATP treated groups (*, #<0.05). In addition, number of apoptotic cells for both groups (BBG+BzATP) was also statistically decreased with respect to BzATP treated group (#<0.05).

4.6. ANALYSIS OF SURVIVING NEURONS

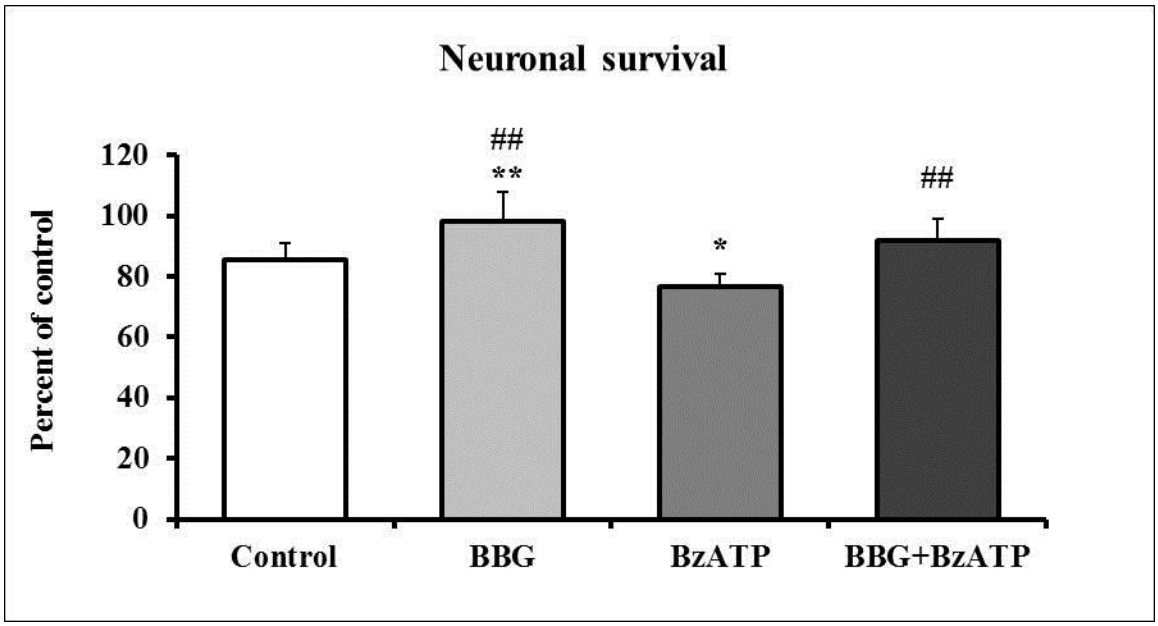

Figure 4.7. Percentage of neuronal survival in ischemic striatum. Control (normal saline), 10 mM BBG (Brilliant Blue G), 250 μM BzATP (2',3'-O-(4-benzoylbenzoyl)-ATP) and 10 mM BBG and 250 μM BzATP (Mean±SD, **, ## $p< 0.01$ and * $p< 0.05$).

To evaluate the percentage of neuronal survival, cresyl violet staining was done from animals subjected to 30 min MCA occlusion, 24 h reperfusion. Nine random area of both ipsilateral and contralateral, 1 mm apart, each measuring 62,500 μm^2 were examined under light microscope and percentage of neuronal survival was calculated with respect to contralateral hemisphere.

Results indicated that percentage of neuronal survival in the vehicle treated control group was 85.4±5.5. In the BzATP group, neuronal survival rate was significantly decreased to 76.7±4.0 ($p<0.05$). In the BBG group, percentage of neuronal survival was significantly increased to 98.3±9.8 with respect to both control and BzATP groups ($p<0.05$). In addition, treatment of both BBG and BzATP group was 91.7±7.2. Thereby, difference between the BBG+BzATP group and BzATP group revealed to be statistically significant ($p<0.05$).

5. CONCLUSION and RECOMMENDATIONS

5.1. CONCLUSION

In the last decades, after cardiovascular diseases and cancer, stroke is considered to be the main cause of death in western countries [1]. Transient or permanent occlusion of cerebral blood flow leads to complex pathophysiological processes that develop in time. Post-ischemic endogenous responses of the central nervous system go in line with an enhanced responsiveness to rehabilitative and plasticity-promoting treatments [3]. Nowadays, there are several conflicts for contribution of purinergic receptors on focal cerebral ischemia. Recent publications about purinergic receptors, especially P2X7R, have several obscurities for their role on focal cerebral ischemia. Considering the importance of purinergic receptors on neuronal ischemic research, in this study, we aimed to examine the impacts of P2X7 receptors on neuronal cell death after cerebral ischemia.

Firstly, animals were anesthetized with chloral hydrate (400mg/kg). Secondly, experimental chemicals (Normal Saline, BBG, BzATP and both (BBG+BzATP) were treated with *i.c.v.* injection on striatum before onset of the MCAo. Laterly, 30 min MCAo was started and CBF recording was saved for success of the experiment.

Recently, LDF measurements have become a necessity for the studies involving cerebral I/R injuries to ensure the reproducibility and success of the experimental stroke surgery in animals. This non-invasive technique allows instantaneous and continuous direct control for measurement of microcirculary blood flow in tissue which exhibits quantitative changes (in percentages) of blood flow so that it cannot be used for interindividual comparisons [68].

LDF results from 30 min MCAo (Figure 4.1) or 90 min MCAo (Figure 4.2) showed that there were approximately 5% and 10% reduction of CBF, respectively. Reduction rates indicated that experimental focal cerebral ischemia was completed successfully for both 30 min and 90 min MCA occlusion [68]. Thereafter, withdrawal of filament from MCA territory leaded to the reperfusion of both 30 min and 90 min MCA occlusion treated animals.

LDF result from 30 min MCAo showed no statistical differences among experimental groups during the reperfusion. Moreover, in 90 min MCAo, reperfusion rate of BBG (10 mM) was higher than BzATP (250 μM) and both (10mM BBG and 250 μM) treated groups and also statistically higher than Control treated group.

Neurological deficit scores are very important for investigating the neurological function after focal cerebral ischemia. It was performed by just one observer only. Neurological scores were observed from animals subjected to 90 min MCAo followed by 24 h reperfusion. Results (Figure 4.4) indicated that BBG and Both treated animals have better neurological function than Control and BzATP groups. Control and BzATP treated groups have the approximately same neurological score.

In order to determine infarct volume and neuronal survival cresyl violet (CV) staining was applied. CV is widely used for staining of nissl bodies which are granular endoplasmic reticulum (ER) and ribosomes. Since all cells contain ribosomes and ER, CV stained both glia and neurons. Living cells were clearly stained with brilliant cresyl but damaged cells were not stained. Therefore, CV staining was essentially useful for the calculation of infarct volume and determination of the neuronal survival. Figure 4.3 showed that infarct volume from animals submitted to 90 min MCAo followed by 24 h reperfusion. Inhibition of P2X7R was significantly reduced infarct volume with respect to activation of P2X7R. 10 mM BBG which was antagonist of P2X7R was significantly decreased infarct volume but activation of the receptor did not directly contribute to infarct development. There was a slight difference between control and BzATP treated groups. In addition, the inhibition and activation of receptor at the same time (both group) showed that P2X7R directly related to reduction of infarct volume with BBG on focal cerebral ischemia.

The intracellular (cytotoxic) and the extracellular (vasogenic) edema are responsible for the development of ischemic edema or brain swelling. In the ischemic core region, cytotoxic edema is developed within minutes due to the energy failure and anoxic membrane depolarization [3]. Ischemic neurons can be depolarized owing to the lack of energy supply and the release of K^+ and glutamate but in the core region, cells can undergo an anoxic depolarization and never repolarize [3, 69]. Energy failure causes to the

depolarization of neurons. Then, activation of glutamate receptors trigger to accumulation of intracellular Na^+ which causes the influx of water and cellular swelling [69]. Vasogenic edema causes to disruption of BBB which leads to increase of BBB permeability, movement of large proteins, and movement of intravascular fluid to the interstitial and intracellular compartments [69, 70]. In addition purinergic P2X7Rs are also permeable to Na^+ ion and they may contribute to development of brain swelling [70].

In light of this information, P2X7R is also ion gated channels which permeable to Na^+ and blocking of its activation with BBG (10mM) was reduced the brain swelling. However, activation of P2X7 receptor could contribute the formation of brain edema.

In order to determine brain edema, four level of brain level stained with CV from animals submitted to 90 min MCAo followed by 24 h reperfusion and brain swelling (Figure 4.5) were calculated with an image analysis system (Image J; National Institute of Health, Bethesda, MD, USA). Results show that BzATP (250μM) was slightly increased the edema in proportion to Control group. On the contrary, BBG (10mM) was significantly reduced the brain swelling with respect to BzATP (250μM) treated group ($p<0.01$). In addition, both group was also slightly decreased the brain swelling.

Brain tissue has a relatively high consumption of oxygen and glucose for energy production. Reduction of cerebral blood flow due to the focal impairment, obstruct the blood flow and particularly oxygen and glucose. Membrane potential is lost because of energy depletion. Meanwhile, Ca^{2+} dependent ion channels open and excitatory amino acids are released into the extracellular space. In addition binding of glutamate to NMDA and AMPA receptors also promote Ca^{2+} influx. Elevated level of Ca^{2+} or stimulation of caspase 8 from extrinsic pathway activates calpains. Activation of calpain triggers cleaving BID to tBID [3, 25, 27]. Then, tBID induces conformational changes in other proapoptotic proteins such as Bak, Bax, Bad, and Bcl-XL. Activation of apoptotic proteins lead to opening of mitochondrial membrane pores and cytocyrome c (Cytc) is released. Released Cytc binds Apaf-1 (protein-activating factor-1) and procaspase-9 to form an “apoptosome” complex which activates caspase-9 and later caspase-3. Activated caspase-3 triggers activation of PARP (poly (ADP-ribose) polymerase) which leads to DNA damage and apoptosis. In addition activation of PLA_2 and cyclooxygenase generates several free radical

species that contributes membrane damage [3, 41].

Apoptosis or programmed cell death was determined in tissue sections from bregma level via positive terminal deoxyribonucleotidyl transferase (TNT)-mediated dUTP (2′-deoxyuridine-5′-triphosphate)-digoxigenin nick end labeling (TUNEL). Results from animals objected to 30 min MCAo followed by 72 h reperfusion (Figure 4.6), showed that blocking of P2X7 receptor was reduced the DNA fragmentation. Although, BBG (10mM) was significantly reduced number of apoptotic cell, P2X7 receptor agonist BzATP (250 μM) was slightly increased the number of apoptotic cell. Both group (10mM BBG + 250 μM BzATP) was also decreased the DNA fragmentation. Inhibition or activation of P2X7R presumably contributes to apoptosis after ischemic stroke. The influx of Ca^{2} was refused with the blocking of P2X7 channels and this might decrease cellular Ca^{2+} level so affects apoptosis pathway.

Infarct volume results were also supported by neuronal survival rates (Figure 4.4). Analysis of cresyl violet stained sections showed that number of surviving neurons was significantly increased with inhibition of P2X7Rs via BBG in proportion to Control and BzATP treated groups. Both group was also statistically increased the neuronal survival with respect to BzATP but not BzATP. In addition overexpression of P2X7Rs with BzATP was caused the increase of neuronal survival.

5.2. RECOMMENDATIONS

This is the first report to our knowledge which shows a potential role of P2X7 receptors in mediation of neuronal cell death. We predict that clinical implementation of P2X7 receptor antagonists can be beneficial in the treatment of neurodegenerative disorders.

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