# ROLE OF P2X7 RECEPTORS IN NEURONAL INJURY AFTER OPTIC NERVE TRANSECTION AND FOCAL CEREBRAL ISCHEMIA IN MICE

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ROLE OF P2X7 RECEPTORS IN NEURONAL INJURY AFTER OPTIC NERVE TRANSECTION AND FOCAL CEREBRAL ISCHEMIA IN MICE

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# ABSTRACT


## ROLE OF P2X7 RECEPTORS IN NEURONAL INJURY AFTER OPTIC NERVE TRANSECTION AND FOCAL CEREBRAL ISCHEMIA IN MICE

$P2X_7$ receptors belong to the purinergic receptor superfamily that have two distrinct features. These widely expressed channels in the peripheral and central nervous system act as cation channels in response to brief activation by extracellular ATP. In addition, after prolonged activation they form large pores in the plasma membrane that allow the passage of molecules upto 900 Da. $P2X_7$ receptors were associated with regulation of cation trafficking, inflammation and ATP-mediated cell death following an ischemic insult *in vivo*. Due to the conflicting reports in the literature about the function of the $P2X_7$ receptors during injury, it was aimed to investigate the impacts of activation *vs*. inhibition of the receptor on neuronal cell death as well as on the downstream signaling cascades after middle cerebral artery occlusion (MCAo) as well as optic nerve (ON) transection in mice. In this thesis, it was demonstrated that inhibition of $P2X_7$ receptors with Brilliant Blue G (BBG) improved neuronal survival and motor functions and decreased infarct volume, brain swelling and neurological deficit scores as well as DNA fragmentation after MCAo and ON transection. On the other hand, activation of $P2X_7$ receptors with BzATP did not increase the cellular damage caused by ischemia alone as compared with vehicle-treated control animals. Impacts of activation or inhibition of the receptor on protein expression were also extensively studied and the results suggested that the inability of BzATP to further worsen the pathophysiological events occurred following an injury was attributed to the differential activation of survival kinase mediated pathways. *In vitro* analyses of the effects of BzATP on intracellular calcium levels provided supporting data. In conclusion, it can be reported from these data that clinical implementation of $P2X_7$ receptor antagonists can provide beneficial outcomes not only in acute ischemic stroke patients, but also in other neurodegenerative disease patients.

# ÖZET


## FAREDE P2X7 RESEPTÖRLERİNİN OPTİK SİNİR KESİSİ VE FOKAL SEREBRAL İSKEMİ SONRASI SİNİR HASARINDAKİ ROLÜ

$P2X_7$ reseptörleri pürinerjik reseptör ana ailesine bağlı, iki farklı özelliğe sahip reseptörlerdir. Periferal ve merkezi sinir sisteminde yaygın bir şekilde anlatımı olan bu reseptörler, ekstrasellüler ATP tarafından kısa bir süreyle uyarılmalarının ardından katyon kanalı olarak görev yapmaktadır. Buna ilaveten, uzun süreli aktivasyon sonucunda plazma membranında, 900 Da'ya kadar çıkabilen moleküllerin geçişine izin veren büyük porlar oluşturmaktadır. $P2X_7$ reseptörleri, in vivo iskemik hasar sonrası katyon geçişi, enflamasyon ve ATP kaynaklı hücre ölümünün düzenlenmesiyle ilişkilendirilmiştir. Literatürde, hasar sonrası reseptörün görevi hakkında yer alan tartışmalı raporlar nedeniyle, reseptörün aktivasyonunun ve inhibisyonunun orta serebral arter oklüzyonu (MCAo) ve optik sinir (ON) hasarı sonrasında nöronal hücre sağkalımı ve sinyal kaskadları üzerine etkilerinin incelenmesi amaçlanmıştır. Bu tez kapsamında, farelerde MCAo ve ON hasarı sonrasında $P2X_7$ reseptörlerinin Brilliant Blue G (BBG) ile inhibisyonunun nöronal sağkalım ile motor fonksiyonları arttırdığı ve enfarktüs hacmi, beyin ödemi ve nörolojik hasar skorlarının yanı sıra DNA fragmantasyonunu azalttığı gösterilmiştir. Öte yandan, $P2X_7$ reseptörlerinin BzATP ile aktivasyonu, sadece iskemiden kaynaklanan hücresel hasarı arttırmamıştır. Reseptör aktivasyonu ve inhibisyonunun protein seviyeleri üzerine etkileri de kapsamlı olarak çalışılmıştır ve sonuçlar, BzATP'nin hasar sonrası ortaya çıkan patofizyolojik olayları daha kötüye görmemesinin farklı sağkalım kinaz aracılı yolakların aktivasyonu nedeniyle olduğuna işaret etmiştir. BzATP'nin intrasellüler kalsiyum düzeyleri üzerine etkilerinin *in vitro* incelemesi, bu verileri doğrulamıştır. Sonuç olarak, elde edilen bu verilerden, $P2X_7$ reseptör antagonistlerinin klinik uygulamasının sadece akut iskemi hastalarında değil, diğer nörodejeneratif hastalıklara sahip kişilerde de faydalı sonuçlar sağlayabileceği rapor edilebilir.

# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# LIST OF SYMBOLS/ABBREVIATIONS

| | |
|---|---|
| ADP | Adenosine diphosphate |
| AMP | Adenosine 5’-monophosphate |
| AMPA | A-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| ATP | Adenosine triphosphate |
| BGG | Brilliant blue G |
| BLT1 | Leukotriene 4 type 1 receptor |
| BzATP | Benzoyl-ATP |
| CF | Cystic fibrosis |
| CNS | Central nervous system |
| EST | Expressed sequence tag |
| FPP | Farnesyl pyrophosphate |
| IL-1β | Interleukin 1-beta |
| IUPHAR | International Unionof Basic and Clinical Pharmacology |
| $IP_3$ | Inositol 1,4,5-triphosphate |
| LPA | Lysophosphaditic acid |
| LPAR6 | Lysophosphaditic acid receptor 6 |
| LTB4 | Leukotriene B4 |
| MCAo | Middle cerebral artery occlusion |
| MLCK | Mysoin light chain kinase |
| NMDA | N-methyl-D-aspartate |
| NSC | Neural stem cell |
| PLC | Phospholipase C |
| PLD | Phospholipase D |
| RGC | Retinal ganglion cell |
| UDP | Uridine diphosphate |
| UTP | Uridine triphosphate |

# 1. INTRODUCTION

## 1.1. ISCHEMIC STROKE

A reduction in the cerebral blood flow, whether transient or permanent, mainly due to an occlusion in one of the major brain arteries leads to a condition called “ischemic stroke”. It is the third leading cause of death in the developed countries with a mortality rate of around 30 per cent [1]. Due to the complex series of pathophysiological events which occur during stroke, such as excitotoxicity, apoptosis, peri-infarct depolarizations or inflammation, the extent of damaged tissue is vastly increased. In general, stroke is categorized into two subgroups: “ischemic stroke” in which the arteries that supply the brain are occluded; or “hemorrhagic stroke” in which leakage of blood into the brain from a ruptured artery causes swelling and pressure inside the skull [2]. Among these two, ischemic stroke occurs more frequently than hemorrhagic stroke and therefore, it is more frequently targeted in experimental studies [2]. In the ischemic stroke, artery occlusion which results in limited or no oxygen or glucose supply to a brain region can stem from a thrombosis or an embolism. The central part of the brain region affected from the occlusion of a particular artery during ischemic stroke is called “core”, while the tissue surrounding this so-called core region is designated as “penumbra” (Fig. 1) [1]. In the core region, blood flow is severely depleted and immediate cell death occurs as a result of energy depletion following an ischemic insult. However, in the peripheries of that region, i.e. in the penumbra, blood flow is not as severely depleted. In fact, collateral blood vessels continue to supply this region and therefore, the affected brain tissue can be protected from cell death because of the mechanisms involved are happening slower in this area.

Brain tissue uses significantly high amounts of oxygen and glucose and produces energy from oxidative phosphorylation [1]. It was hypothesized that neurons use more than half of the energy supply to maintain their membrane $Na^+/K^+$-ATPases [3]. Once the blood supply is restricted, the energy that the neuronal and glial cells require to maintain their membrane potential cannot be produced and as a result, cells are depolarized due to the loss of membrane potential [4]. Membrane depolarization causes the activation of voltage-

dependent Ca2+ channels, which in turn release excitatory amino acids such as glutamate to the extracellular space [1]. Moreover, reuptake of these amino acids are impaired due to energy depletion, and hence, these molecules accumulate further in the extracellular space, causing glutamatergic excitotoxicity as summarized in Figure 2. It was postulated that glutamate levels in the ischemic penumbra region can increase up to 80 times the normal physiological values [1].

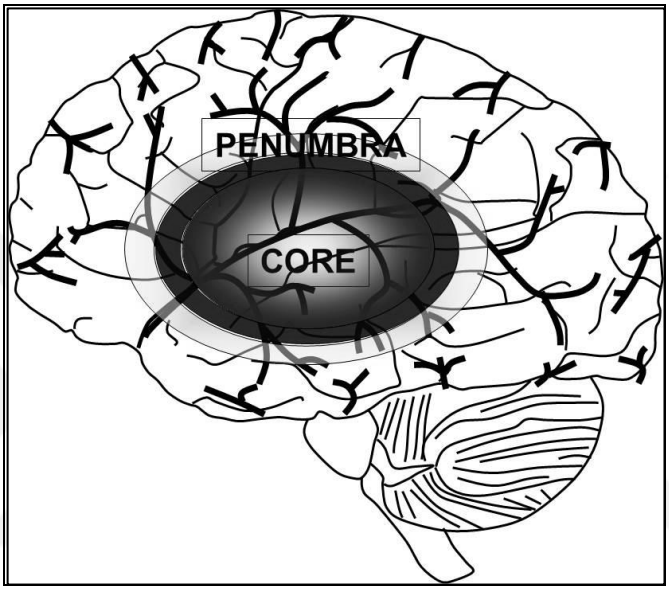


Figure 1.1. Representation of ischemic core and penumbra regions.

Another effect of energy depletion in the ischemic region is seen on the $Ca^{2+}$-ATPase that prevents high intracellular $Ca^{2+}$ levels and helps to keep these levels low [5]. Following an ischemic injury, intracellular $Ca^{2+}$ levels significantly increase and result in the activation of several enzymes and signaling pathways [4]. In particular, activation of degrading enzymes rapidly catabolize intracellular and extracellular proteins. It was also reported that high $Ca^{2+}$ levels caused depolarization of mitochondrial outer membrane and eventually apoptotic cell death [6]. Another reason of increased $Ca^{2+}$ levels is linked to the accumulation of glutamate in the extracellular space. It was reported that as the glutamate concentrations rise in the extracellular space, N-methyl-D-aspartate (NMDA) and a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are activated [7]. Activated NMDA receptors, in turn, further increases the intracellular $Ca^{2+}$ overload. Although AMPA receptors are not permeable to $Ca^{2+}$ under normal physiological conditions, during an ischemic injury the receptor becomes $Ca^{2+}$ permeable [7].

Furthermore, increased $Ca^{2+}$ levels were associated with leakage of mitochondrial membrane and subsequent free radical formation [8]. This in turn, activates mechanisms that are responsible for infiltration of leukocytes and activation of microglial cells [8]. Moreover, influx of ions, such as $Na^{+}$ and $Cl^{-}$ as well as fluid in the ischemic region results in cytotoxic cellular edema [1, 9]. Combination of these events result in the activation of eventual apoptotic cell death mechanisms in the affected cells [8].

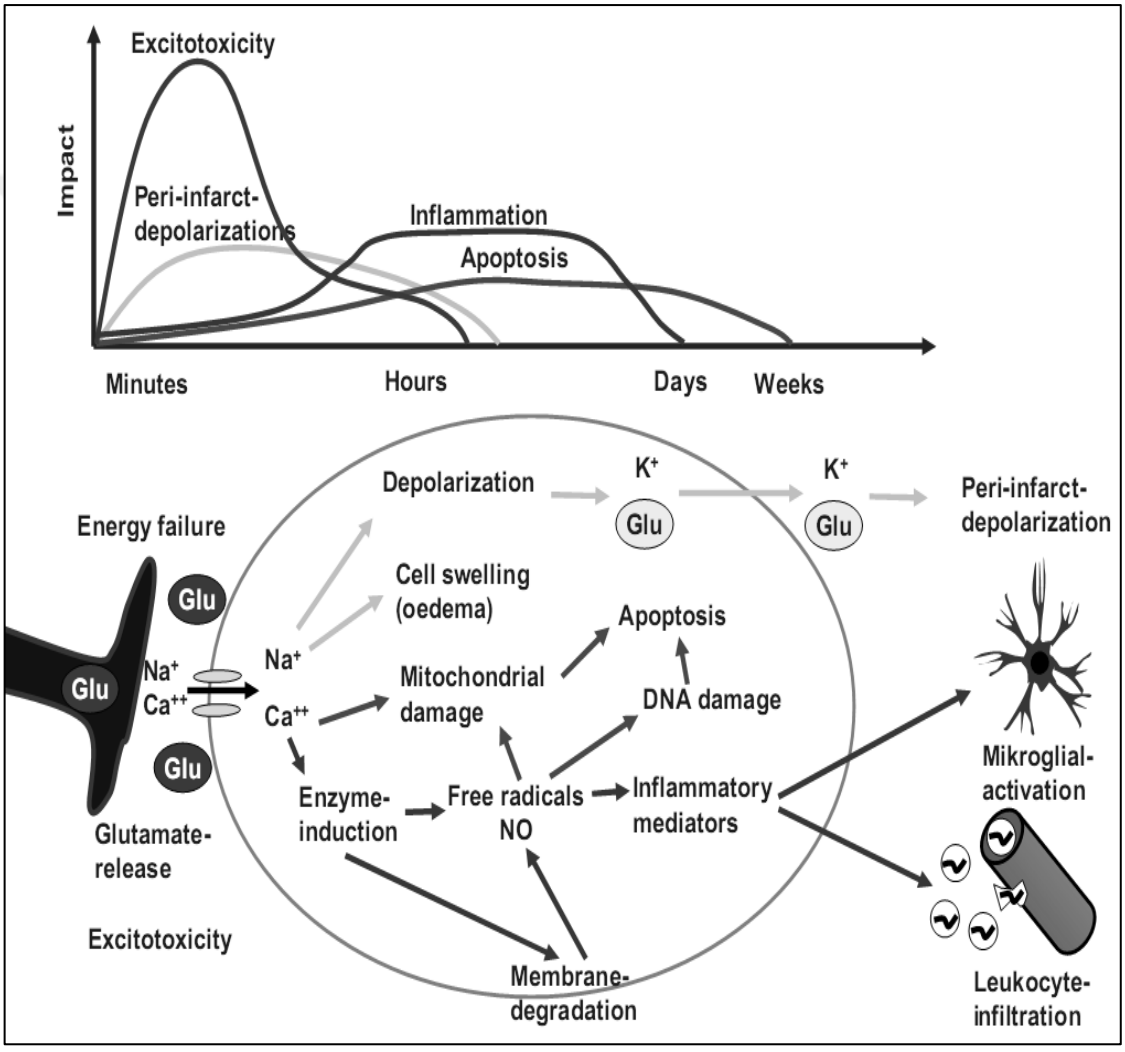


Figure 1.2. Mechanisms involved in ischemic stroke (adapted from Dirnagl *et al*. 1999 [1]).

Considering the inflammation resulted from ischemic injury, microglial activation and release of proinflammatory cytokines from these cells were extensively studied. Following the ischemic injury, it was reported that microglial cells were activated within the first 24 hours [1]. It was also reported that microglia secreted interleukin-1 (IL-1) after the onset of

transient or permanent ischemia [5]. IL-1 secretion was shown to increase cellular damage after ischemia [10].

The mechanisms mentioned above are not the absolute fate of these cells in the affected region in the brain. In fact, even though the cells in the core region rapidly deteriorate following an insult, the cells in the penumbra region can still be protected from apoptotic cell death. It was observed that if the ischemic stroke is not treated, cells of the penumbra slowly degenerate over a long period of time due to the ongoing inflammatory events [1]. Therefore, the treatment strategies for ischemic stroke primarily depend on the salvation of the cells of penumbra. Moreover, the formation of core and penumbra areas following an ischemic injury was also reported in human ischemic stroke survivors [11, 12].

## 1.2. PURINERGIC RECEPTORS

The role of purines as extracellular signaling molecules in several biological processes has been suggested for the first time in the early 1930s. In that study, adenosine and adenosine 5'-monophosphate (AMP) that were obtained from the heart muscle were associated with several implications, such as blockage of the electrical systems in the heart, dilatation of arteries or decrements in blood pressure [13]. When Gillespie reported a more potent effect by ATP compared to adenosine or AMP, it became clear that adenosine, AMP or ATP could not only have different roles in biological processes, but also have their own plasma membrane receptors [14]. Thereafter, effects of adenosine or ATP on a number of biological processes were extensively studied and documented by several researchers. By the end of 1950s, the role of ATP as a neurotransmitter had been reported in different studies [15, 16]. Purinergic receptors, however, were first identified in late 1970s by Geoffrey Burnstock [17, 18]. He also divided these receptors into two groups: P1 receptors (for adenosine) and P2 receptors (for ATP/ADP). Shortly afterwards, two subclasses of P1 receptors were identified [19, 20] and two subclasses of P2 receptors were suggested [21]. Adenosine or P1 receptors were further categorized into four groups; A1, A2A, A2B, and A3, depending on their molecular structures. Although all of these four receptors couple to G proteins, there are differences in their pharmacological characteristics and expressions in different tissues. In early 1990s, Abbracchio and Burnstock suggested that the two P2

subclasses should be named as P2X and P2Y receptors, based on the differences in their molecular structures and transduction mechanisms [22]. According to this division, P2X receptor subfamily consists of ligand-gated cation channels that play a role in fast excitatory neurotransmission, while P2Y subfamily is composed of G protein-coupled receptors that trigger the activation of cyclic AMP (cAMP) or inositol trisphosphate ($IP_3$) molecules [23]. Important features of P1 and P2 receptors are summarized in Table 1.1.

Before further discussion, it should be emphasized that ATP and ADP are rapidly converted to adenosine by physiological mechanisms. This indicates that there may be interactions between these receptors and their downstream signaling pathways because P1 and P2 receptors are generally expressed together in the cells.

Table 1.1. Summary of the important differences between P1 and P2 purinergic receptors.

| **Purinergic Receptor Super Family** | | | |
|---|---|---|---|
| **Family** | **P1 Receptors** | **P2 Receptors** | |
| **Subfamily** | - | P2X Receptors | P2Y Receptors |
| **Receptor Subtypes** | A1, A2A, A2B, A3 | P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14 | P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, P2X7 |
| **Structure** | G-protein coupled receptor | G-protein coupled receptor | Ligand-gated ion channel |

It is now well documented that purinergic receptors are expressed in a number of nervous system cell types and their roles include several neuronal mechanisms, such as nervous system homeostasis and regulation of cell function [24, 25]. Purinergic receptors expressed in tissues other than neuronal tissue have critical roles in exocrine and endocrine secretion,

immune responses, inflammation, pain, platelet aggregation and endothelial-mediated vasodilatation [26, 27].

## 1.3. P1 RECEPTORS

As of today, there are four different P1 receptor subtypes known: A1, A2A, A2B and A3 [28]. All have relatively short N- (7 – 13 residues long) and C-termini (32 – 120 residues long), compared to other purinergic receptor subfamilies. Transmembrane domains of human P1 receptors contain around 39 – 61 per cent similarity with each other and immediately before the fourth transmembrane domain, there is an intron sequence within the coding region [29]. Per cent similarity of the sequences of these receptors are higher in transmembrane domains, compared to regions around N- or C-termini. It is believed that the ligand binding and specificity are determined by the amino acid residues of the transmembrane regions and the residues that have relatively more variable sequences play the least role in ligand specificity [30].

All four of the P1 receptors couple to G proteins and similar to other such receptors, there are seven transmembrane domains in their structures. It is believed that the N-terminals of these receptors are on the extracellular side, whereas the C-terminals are on the cytoplasmic side [29].

P1 receptors are widely expressed in a number of tissues, such as CNS, heart, smooth muscle, testis, kidney, adipocytes, platelets or leukocytes. $A_1$ is the most dominant P1 receptor in the CNS. Gustafsson [31] suggested a division for $A_1$ receptors, such that the ones with high affinity binding sites for adenosine agonists and antagonists are to be grouped as $A_{1a}$, while the ones with low affinity binding sites are to be called $A_{1b}$ receptors. Despite of the observation of high and low affinity binding to $A_1$ receptors, molecular studies failed to prove the existence of such subtypes. Therefore, it has been hypothesized that these differences are not because of the existence of different subtypes, but rather, because of the existence of high and low affinity states of the same receptor [32].

A1 receptors couple to a number of G proteins of the Gi/o family [33]. As the diversity of G proteins which A1 receptor can couple to increases, the range of signaling pathways which the receptor can interact with increases. Of the several signaling pathways that A1 receptors can interact with, inhibition of cAMP through its coupling to adenylate cyclase seems to be the most prominent and widely studied pathway [21]. Activation of phospholipase C (PLC) and phospholipase D (PLD) by A1 receptors was also demonstrated in in vitro studies [34-37]. In the case of $A_1$ receptor-mediated PLC activation, elevated inositol 1,4,5-triphosphate ($IP_3$) levels and in turn, elevated cytoplasmic $Ca^{2+}$ levels were described [34]. Subsequently, increase in $Ca^{2+}$ levels can promote activation of several downstream signaling pathways.

In addition to G protein, A1 receptors can also couple to ATP-sensitive $K^+$ channels ($K_{ATP}$ channels) [38]. In neurons, A1 receptor coupling to $K_{ATP}$ channels has been linked to ischemic preconditioning [39]. Transgenic knockout mice studies indicated that $A_1$ plays roles in several mechanisms, but these roles are not critical since the development process of knockout mice was not interrupted. Interestingly, even though elimination of P1 receptors did not cause developmental problems in mice, overexpression of $A_1$ or $A_3$ subtypes was shown to exert a cardioprotective effect in transgenic mice [40].

$A_{2A}$ receptors are expressed in immune tissues, platelets, CNS tissues as well as vascular smooth muscle cells and endothelium [32]. In the CNS, $A_{2A}$ receptors are widely expressed in the regions that are considered to be “dopamine-rich” (particularly in the striatum, nucleus accumbens and olfactory tubercle [41]. Evidence available from the literature suggests that the role of $A_{2A}$ receptors in CNS is to aid neurotransmitter release. As with $A_1$ receptors, adenylate cyclase activation is the most prominent signal transduction pathway for $A_{2A}$ receptors, as well. This pathway seems to be responsible for the abovementioned role of $A_{2A}$ receptors in CNS [42]. On the other hand, $A_{2B}$ receptors are homogenously (but in rather low levels) expressed in most cell types [42]. This subtype of purinergic receptors has been associated with chronic pain behavior and neuronal hypersensitivity in pharmacological studies in which $A_{2B}$ antagonists exerted analgesic effects [43].

Forth member of P1 receptors, $A_3$ receptor, has high sequence homology among different species. Conservation of sequences, particularly the sequence of transmembrane regions,

seems to be common for all four of P1 receptors. As with $A_1$, $A_{2A}$ and $A_{2B}$ receptors, $A_3$ receptor has also been shown to induce signaling pathways through PLC. Subsequent elevation of $IP_3$ levels and intracellular $Ca^{2+}$ levels were linked to stimulation of PLC [44]. Although A3 receptor is expressed in a wide range of tissues, its function has not yet to be determined. RT-PCR studies located $A_3$ mRNA in several tissues, such as testis, lung, kidneys, placenta, heart, brain, spleen, liver, uterus, bladder and intestines [45]. A role in inflammation was suggested in studies which showed the release of allergic mediators was through the activation of this receptor [46]. In cardiac ischemia studies, protective effect of adenosine during ischemic preconditioning was attributed to the stimulation of $A_3$ receptors due to the complete prevention of preconditioning by $A_3$ antagonists. In addition, the number of apoptotic cardiac cells in cardiac ischemia was alleviated using $A_3$ agonists [47]. Moreover, $A_3$ mRNA is also widely distributed in the CNS [48] and that this expression may provide protective effects during ischemia [49].

## 1.4. P2Y RECEPTORS

After Geoffrey Burnstock first defined the term “purinergic receptors” in late 1970s, extensive research has been conducted on that topic and finally, the heterogeneity among the known members of P2 receptor family was so significant that it had to be divided into two major subfamilies according to their signal transduction mechanisms as well as nucleotide sequence similarities of these receptors [23]. According to this classification, P2X receptor subfamily consists of ligand-gated cation channels, whereas P2Y receptor subfamily consists of G protein-coupled purinoceptors. Thereafter, his classification was accepted worldwide and new members of these families were classified accordingly.

P2Y receptors are known to possess distinct extracellular N-terminal sequences with potential glycosylation sites and to be activated by a wide range of ligands, such as ATP, UTP, ADP, UDP or UDP-glucose. Amino acid sequences range from 308 to 377 and their masses from 41 to 53 kDa [27, 40]. In general, activation of P2Y receptors results in $IP_3$ formation and subsequent increase in intracellular $Ca^{2+}$ levels through PLC signaling mechanism. It is important to note that because it depends on the ionic conductivity and

related secondary messengers to activate a cellular response, P2Y receptors are slower to produce a response than P2X receptors.

To date, there are eight mammalian P2Y receptor subtypes identified: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ [50]. There are gaps in the numbering of P2Y receptors, because it was later recognized that although some members were initially classified in P2Y receptor family due to sequence homology, they could not be stimulated by nucleotides, nor these nucleotides could trigger secondary messengers *in vitro* [51]. One such receptor is $P2Y_5$, whose sequence had similarities to other P2Y receptors (though only around 30 per cent similarity) and it could bind to [$^{35}$S]dATPαS [52]. However, further *in vitro* data did not support the initial findings and failed to prove the nucleotide binding to the receptor. Recently, it was discovered that P2Y5 receptor is activated by lysophosphatidic acid (LPA) [53] and farnesyl pyrophosphate (FPP) [54]. Upon binding, LPA activates the receptor and increases intracellular $Ca^{2+}$ levels and subsequent ERK1/2 phosphorylation [53]. LPA is known to reduce intestinal cell adhesion while increasing the migration rate of these cells [55] and $P2Y_5$ was proposed to play a significant role in maintaining of the integrity of the intestinal epithelia [54]. Due to the current evidence, $P2Y_5$ is now renamed as "Lysophosphaditic acid receptor 6 (LPAR6)". Another missing number in mammalian P2Y receptors is $P2Y_3$. This receptor is only expressed in avian species and is proposed to be an ortholog of mammalian $p2Y_6$ [56]. In birds, $P2Y_3$ is expressed in several tissues including brain, spinal cord, kidney, spleen and lung; however, its mRNA was not shown in other peripheral tissues [57]. It is mainly activated by UDP and couples to phospholipase C upon activation. UTP and ADP were also shown to activate this receptor [57]. $P2Y_7$ is another receptor which is not found in mammalian P2Y receptor family. This receptor was initially cloned from a human erythtoleukemia cDNA library and immediately expressed in several cell lines *in vitro*. When it was first expressed in COS-7 cell line, it was suggested to bind to ATP [58]. However, further research showed that nucleotides did not increase intracellular $Ca^{2+}$ levels in $P2Y_7$ overexpressing cells, hence cannot activate this receptor. Later, it was discovered that $P2Y_7$ can be activated by leukotriene B4 (LTB4) and consequently renamed as "leukotriene B4 type 1 receptor (BLT1)" [59]. It is expressed in humans, mice and rats and mainly found in peripheral leukocytes as well as peritoneal macrophages, but to a lesser extent it is also expressed in brain, heart, skeletal muscle and liver in humans [60]. Binding of LTB4 to

$P2Y_7$/BLT1/LTB4R1 increases the intracellular Ca2+ levels through the activation of inositol trisphosphate pathway [60]. As LTB4 is a potent lipid inflammatory mediator that is mainly produced by neutrophils and macrophages, activation of this receptor by LTB4 suggested a role in inflammatory signaling pathways. Recently, it was reported that LTB4R1 may be involved in systemic insulin resistance and diet-induced obesity in mice [61]. After LTB4R1-deficient mice were injected with insulin, a decrease in the accumulation of hepatic triglycerade and an increase in the per cent of phosphorylated Akt protein were recorded [62].

One P2Y receptor was first cloned from Xenopus embryo and assigned a role in neural plate development [62]. The receptor, named as "P2y8" by Bogdanov in 1997, was proposed to be a possible species homolog to mammalian $P2Y_4$. This receptor is activated by ATP, UTP, GTP, ITP and CTP with comparable potencies, but inorganic polyphosphates cannot activate the receptor [62]. Although its expression in undifferentiated human promyelocytic leukemia cell line, HL60, was demonstrated [63], it is not accepted as a member of the mammalian P2Y receptor family by the International Union of Basic and Clinical Pharmacology (IUPHAR), probably because it is still considered as a non-mammalian ortholog. It is an "orphan receptor" and its role in humans (for instance, in HL60 cells where it is moderately expressed) is currently unknown. $P2Y_9$ receptor was first identified during an analysis of the expressed sequence tag (EST) database [64] and cloned from human brain cDNA library [65]. In 2003, it was reported to be activated by LPA and hence, renamed as "LPA receptor 4 (LPAR4) as it was the fourth identified LPA receptor at that time [66]. LPA is a naturally occurring lipid mediator which is found in all types of cells and tissues in humans, exerts its effects on various cell functions via G-protein coupled LPA receptors, including LPAR4 and aforementioned LPAR6 ($P2Y_5$) [67]. It is not surprising that since both two receptors are activated by the same ligand, they are more closely related to each other, than any other P2Y or LPA receptors. Upon LPA binding to $P2Y_9$/LPAR4, an increase the levels of intracellular $Ca^{2+}$ and cAMP was observed [68]. Because $P2Y_9$/LPAR4 is expressed in almost all tissue types, it might be speculated that this receptor may be involved in the development of several organs in the body; however, knockout mouse studies did not point to a significant role in development. In a recent study, it was demonstrated that $P2Y_9$/LPAR4 negatively regulated the motility of fibroblasts [69]. In a study carried out in $P2Y_9$/LPAR4 knockout

mice, it was reported that upon injection of mice with 5-fluorouracil to induce myelosuppression, $P2Y_9$/LPAR4 could increase the regenerative ability of hematopoietic stem/progenitor cells [70]. A study that provides evidence for the developmental significance of $P2Y_9$/LPAR4 was also carried out in $P2Y_9$/LPAR4-deficient mice [71]. In this study, it was reported that in the absence of $P2Y_9$/LPAR4, mice had abnormalities in lymphatic vessel development during embryogenesis. The authors speculate that because its deficiency resulted in abnormal blood and lymphatic vessel development, $P2Y_9$/LPAR4 mutations in adults may cause tumor progression in these tissues. $P2Y_{10}$ had been considered as an orphan receptor until a study by Murakami et al. published in 2008 [72]. mRNA of $P2Y_{10}$ was detected in several tissues, including brain, lung, skeletal muscle, uterus and prostate and fairly in placenta; however, not detected in kidney, liver or testis. Later, it was reported to be activated by lysophosphatidylserine (LysoPS) in a TGFα shedding assay [73]. In that study, it was strongly emphasized that the receptor could only be activated by LysoPS and therefore authors claimed that it should be renamed as "LPS2". Another withdrawn P2Y receptor, $P2Y_{15}$ which has around 30-40 per cent amino acid sequence homology with other known P2Y receptors, was first introduced in 2004 and proposed to be a receptor for AMP and adenosine [74]. Authors of that study suggested that the orphan receptor GPR80 (also known as GPR99) was activated by AMP or adenosine and therefore, renamed the receptor as "$P2Y_{15}$". However, the study could not be repeated and the IUPHAR P2Y Subcommittee did not accept the suggested name largely due to the fact that the authors only used human embryonic kidney, HEK293, cell line which is known to express several P2Y receptors , that the results could be tempered by the presence of endogenous P2Y receptors and that the results could not be repeated. The IUPHAR P2Y Subcommittee also recommended that the next found P2Y receptor should be called "$P2Y_{16}$" in order to prevent any confusion. It is now known that $P2Y_{15}$ is an α-ketoglutarate receptor [75].

Of the eight accepted P2Y receptors, mammalian $P2Y_1$ metabotropic purinoceptor is activated selectively by ADP, but not by UTP, UDP, CTP or GTP; whereas in non-mammalian species, ADP and ATP can both activate $P2Y_1R$ [29]. Like other P2Y receptors, $P2Y_1$ receptor is also mainly coupled to Gq/11 proteins [29] and contains seven hydrophobic transmembrane domains and increases intracellular $Ca^{2+}$ levels through the actions of phospholipase C and inositol-1,4,5 trisphosphate. It was first cloned from a

cDNA library prepared from chick brain tissue [76]. There are two known isoforms of $P2Y_1$ receptor. Human $P2Y_1$ receptor mRNA has been shown in several tissues, including brain, spinal cord, heart, skeletal muscle, pancreas and spleen. Amino acid sequence of human $P2Y_1$ receptor is highly conserved and around 95 per cent similar to rat or mouse $P2Y_1$ receptor sequences [77]. Nearly half of the total $P2Y_1$ protein present in HEK293 cells, forms dimers under normal physiological conditions [78]. Role of $P2Y_1$ receptor in development was studied in $P2Y_1$ receptor-deficient mice and it was reported that $P2Y_1$ receptor-deficiency did not cause any abnormal development features in the animals. However, in these mice, decreased platelet aggregation [79] and increased bleeding times [80] were observed. The most potent and selective antagonist of this receptor is bisphosphate derivative 2'-deoxy-*N* 6-methyladenosine-3', 5'-bisphosphate (MRS 2179). This competitive antagonist does not bind to other P2Y receptors [81, 82]. On the other hand, the most potent and selective agonist of the receptor is *N*-methanocarba analog of 2-MeSADP (MRS2365) [83]. Like in other P2Y receptors, amino acid residues present in the transmembrane domains of the $P2Y_1$ receptor are also important in the nucleotide binding to the receptor [81]. Moreover, the conserved four cysteine residues that are located to the extracellular loops of the receptor is responsible for the transfer of the receptor to the cell membrane [84]. In an analysis of post-mortem brain sections of patients with Alzheimer's Disease demonstrated that $P2Y_1$ receptor is localized to neurofibrillary tangles, neuritic plaques and neuropil threads [85]; however, the reason behind this observation is currently unknown. In further studies that were aimed to understand the role of $P2Y_1$ receptors in neurons focused on its effects on the proliferation of neuronal stem cells (NSCs) in mice [86]. When cultured NSCs were treated with ADP, $P2Y_1$ receptors were activated and subsequently, proliferation was promoted [86]. In adults, hippocampus-dependent memory and cognition was associated with neurogenesis which was shown to stem from the proliferation of NSCs in the hippocampus [87-89]. Based on that knowledge, it can be speculated that once the role of $P2Y_1$ receptors in the proliferation of NSCs is uncovered, specific treatment strategies can be designed for clinical patients that require promotion of neurogenesis in their hippocampi, such as Alzheimer's Disease patients.

$P2Y_2$ receptor was first cloned in 1993 from a cDNA library of mouse neuroblastoma cell line, N108-15 [90] and was previously named as "P2U nucleotide receptor". Immediately after, human and rat homologs were also cloned. This receptor can be activated by ATP

and UTP at an almost equal potency levels; whereas ADP can only weakly activate the receptor [29]. It can couple to $G_{q/11}$ as well as $G_{i/o}$ proteins and increase intracellular $Ca^{2+}$ concentrations and stimulate cell-type specific PKC signaling pathways through the activation of PLCβ [91]. Independently from its activation of this signaling cascade, it was also reported that $P2Y_2$ receptor is involved in the stimulation of $Ca^{2+}$-regulated $Cl^-$ channels in human normal and cystic fibrosis (CF) nasal epithelia [92]. As the activation of the receptor increases chloride secretion and inhibits sodium transport and consequently, increased the liquid transport across the apical surface [93], possible impact of polymorphisms and haplotypes of $P2Y_2$ receptor on the severity of CF disease was investigated [94]. In that study, haplotypes of the receptor was reported to have an impact on the level of intracellular $Ca^{2+}$-release and therefore, it was hypothesized that the receptor could be targeted in novel therapeutical approaches against CF disease. Furthermore, impaired nucleotide-directed ion secretion was reported in a study carried out in a $P2Y_2$-deficient mouse [95]. In line with these knowledge, new $P2Y_2$ receptor agonists are currently being synthesized and clinical studies in CF patients are planned using these agonists [96]. Moreover, in a rat liver cell line, it was shown that $P2Y_2$-like receptors mediated the activation of cation, potassium and chloride currents upon stimulation with ATP, using whole cell patch-clamp techniques [97]. Expression of this receptor type has been found in several tissue types, including brain and aortic smooth muscle tissue [29]. It was reported that in human coronary artery endothelial cells (HCAEC), activation of $P2Y_2$ receptor with UTP increased the mRNA and protein levels of pro-inflammatory vascular cell adhesion molecule-1 (VCAM-1) gene and also, increased the per cent of phosphorylated vascular endothelial growth factor receptor (VEGFR)-2 protein [98]. In a study carried out in human astrocytoma cell line 1321N1 with ectopic $P2Y_2$ receptor expression indicated that activation of the receptor by UTP resulted in a significant rise in the amount of extracellular release of the soluble, non-amyloidogenic N-terminal fragment of amyloid precursor protein (sAPPα). This effect was independent from the increase in intracellular $Ca^{2+}$ levels or activation of PKC [99]. It was also postulated that this receptor might be associated with inflammation response. In rat aortic smooth muscle cells, induction with inflammasome mediators, such as interleukin (IL)-1β or tumor necrosis factor (TNF)-α caused a significant increase in the mRNA and the functional level of this receptor [100]. In parallel with this study, it was reported that IL-1β was also stimulated the $P2Y_2$ receptor transcription through the activation of nuclear factor (NF)-κB in rat

primary neuron cell culture [101]. Shortly after, NF-κB binding sequences in the promoter region of $P2Y_2$ receptor was defined and regulation of this region by NF-κB under inflammatory conditions were characterized [102].

$P2Y_4$ receptor was first cloned from human placenta tissue and shortly after, rat and mouse homologs were also cloned [103]. Unlike other P2Y receptor family members, expression of this particular receptor seems limited to a few tissue types, namely placenta and lung. Its mRNA was not found in other tissues or cell types using Northern Blot analysis [103]. However, mRNA of $p2Y_4$ receptor was also reported in intestines of mice and humans in another study [29] and rat $p2Y_4$ receptor mRNA was also identified in rat brains [29]. Compared to other nucleotides, in humans UTP activates this purinoceptor at a maximal degree; while other nucleoside diphosphates do not activate the receptor . However, in rodents ATP and UTP activate this receptor subtype at an equal potency [29]. There are two G proteins identified that couple to this receptor: $G_i$ protein and $G_{q/11}$ protein [103]. In a study of $p2Y_4$ receptor-knockout mice, it was reported that phenotypes as well as growth of the animals were indistinguishable from their wild type counterparts. The only difference observed was, however, the $Cl^-$ secretion response of the jejunal epithelium in response to UTP or ATP induction [104]. Overexpression of this receptor in human neuroblastoma cell line, SH-SY5Y, was associated with neuronal differentiation upon induction with UTP [105]. Moreover, expression of this receptor in both neuronal and glial cells of the central nervous system was reported, however the exact role of the receptor is currently not known [106].

$P2Y_6$ receptor was first cloned from rat aortic smooth muscle cell cDNA library [107] and shortly after that, its human homolog was cloned from human placenta and spleen [29]. Moreover, expression of this receptor was reported in both mRNA and protein levels in several tissues, including brain, pituitary, heart, skeletal muscle, liver, kidney, spleen and intestine [29]. $P2Y_6$ receptor is highly selective for UDP, compared to other P2Y receptor family members. ADP can only weakly activate the receptor; while ATP or UTP cannot activate it at all [108]. It was shown that $P2Y_6$ receptor was upregulated upon stimulation with UDP which was released by intestinal epithelial cells in response to inflammation and that this upregulation resulted in an increase in mRNA levels of a chemokine, CXCL8 (IL-8) independently from NF-κB signaling [109].

$P2Y_{11}$ receptor was first cloned from human placenta in 1997 [110]. Compared to other P2Y receptors, this receptor is unique in several ways. First, it is the only P2Y receptor that is preferentially activated by ATP. It has been shown that UTP or UDP cannot activate this receptor [x95]. Second, it can interact with not only $G_{q/11}$ but also with $G_s$. Last but not least, there is no other P2Y receptor family member that has an intron in its coding sequence, other than $P2Y_{11}$ receptor [110]. To date, functional $P2Y_{11}$ receptor orthologs could not be cloned from mice or rats [111]. It has been shown that upon activation by ATP, the receptor resulted in the stimulation of phospholipase C and adenylate cyclase [112]. Other than placenta, the receptor mRNA was also found in peripheral human blood leukocytes, and in granulocytes during cell differentiation [113]. In a study which included patients with acute myocardial infarction reported that the polymorphisms of this receptor was associated with an increased risk for this disease, possibly due to the formation of a polar threonine residue rather than a hydrophobic alanine residue at the end of the transmembrane domain [114].

$P2Y_{12}$ receptor was formerly designated as "$P2_T$ receptor" when it was first described in platelets in mice. However, subsequent studies revealed that this is indeed a P2Y receptor that is mainly activated by ADP [115]. Later, it was cloned from a cDNA library prepared from rat placenta cells [116]. $P2Y_{12}$ receptor mRNA was found in several tissue types, including brain, brain capillary endothelia and smooth muscle [29]. This receptor is coupled to $G_i$ protein and inhibits adenylate cyclase upon activation by ADP [115]. This receptor was implicated in platelet aggregation where it helps to amplify the platelet aggregation which was first started by another P2Y receptor, that is $P2Y_1$ receptor [116]. It is known that microglial cells express a wide variety of purinergic receptors. In a study conducted in $P2Y_{12}$ receptor-deficient mice, it was reported that microglia could not polarize or migrate through the site of injury site, suggesting that this receptor could be playing a central role in neurodegeneration due to a number of neurodegenerative disorders [117].

$P2Y_{13}$ receptor is a rather new P2Y receptor which was initially designated as the orphan receptor GPR86, was first identified in 2001 in human [118]. Human $P2Y_{13}$ receptor shares around 40 per cent sequence homology with $P2Y_{12}$ receptor and is expressed in several tissues, including brain, heart, spleen, lung, liver and bone marrow. It was indicated that the receptor is coupled to Gi/o protein [118]. $P2Y_{13}$ receptor was shown to regulate the

differentiation of bone marrow progenitor cells into osteoblasts or adipocytes and therefore, could be a useful target in treatment several diseases related with bone marrow stromal cell differentiation [119]. Moreover, it was reported that neuronal $P2Y_{13}$ as well as microglial $P2Y_{12}$ receptors might be responsible for the neuroprotection against oxydative stress [120].

$P2Y_{14}$ receptor is also a rather new member of the P2Y receptor family. It was initially designated as the orphan receptor GPR105. In 2001, mouse and rat orthologs of this receptor were cloned for the first time. The nucleotide sequence was highly conserved in mice, rats and humans [121]. The receptor can be activated by UDP-glucose, UDP-galactose and UDP-glucuronic acid; however, UTP or ATP cannot activate the $P2Y_{14}$ receptor. It was shown to couple to Gi/o protein upon activation. The receptor's mRNA was identified in several tissues in human [122].

## 1.5. P2X RECEPTORS

Unlike P2Y receptors, whose members have seven transmembrane domains and couple to G-proteins, members of P2X receptor family have only two transmembrane spanning regions (TM1 and TM2, respectively). One of the unique features of the purinoceptors of this family is that they contain both N- and C-termini in the intracellular side of the cells. These receptors are generally larger than P2Y receptors and may contain upto around 600 amino acids in length. The large extracellular loop between TM1 and TM2 has at least 10 conserved cysteine residues and at least one ATP-binding site [123]. They mediate a faster response to ATP binding than P2Y receptors, due to the fact that P2Y receptor-mediated signaling involves coupling with G proteins and secondary messengers , such as $Ca^{2+}$ [29]. P2X receptors were first cloned in 1994 from rat tissue [124, 125]. It was postulated that the receptor forms a trimer on the plasma membrane [126]; however, whether some members of this family form heterotrimers or homotrimers is still debatable. This stems from the observation that ectopically expressed P2X receptors may act differently than their endogenous counterparts. To date, there are seven mammalian P2X receptors identified: $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ [127]. P2X receptors are widely expressed in mammalian tissues, including brain and immune cells. In brain, they

are expressed in both glial and neuronal cell types. Their expression in excitable tissue was also associated with their being permeable for cations, such as $Ca^{2+}$, $Na^{+}$ and $K^{+}$ and their ability to activate voltage-gated $Ca^{2+}$ channels [127]. It was postulated that all P2X receptors can be found as homomers except P2X6 receptor; whose functional homotrimer form does not exist [128]. Several heteromers which may play a role in tissue-specific pharmacological functions were identified. For instance, $P2X_{2}/_{6}$ receptors were characterized in brain stem and $P2X_{4}/_{6}$ receptors were located in the neuronal membranes in the CNS [128].

$P2X_1$ receptor was first cloned from a cDNA library prepared from rat tissue. Shortly after, mouse and human homologs were also cloned [124, 125]. The expression of this receptor in platelets has been known for decades [29]. It is well known that a significant and sudden rise in the extracellular ATP levels occurs only after pathological conditions. It can be speculated that this high ATP levels might be responsible for the crosstalk between inflammation and thrombosis in platelets and hence, regulate this mechanism through $P2X_1$ receptor. Although heteromeric forms for $P2X_1$ receptor were identified, the only functional form in platelets is the homomeric one [129]. Once the receptor was activated by ATP, an increase in intracellular $Ca^{2+}$ levels and subsequent ERK1/2 MAPK and myosin light chain kinase (MLCK) were reported in a study conducted in human platelets [130]. Apart from platelets, this receptor is also expressed in other immune cells, including neutrophils, monocytes, macrophages, mast cells, myeloid dendritic cells and eosinophils [131]. Moreover, the existence of functional heteromeric $P2X_{1/5}$ receptor was demonstrated in the mouse astrocytes [132].

$P2X_2$ receptor was first cloned in 1994 from a rat cDNA library prepared from PC12 pheochromacytoma cell line [124]. Its mRNA expression was detected in several tissues, including brain (particularly in pituitary gland), spinal cord adrenal medulla and intestine. Although this is the only receptor found in rat adrenal medulla and high expression levels and wide range of expression was noted in rat brain, it is not expressed in heart, lung or skeletal muscle tissue [124]. In 1997, a slightly shorter but functional splice variant was reported. This variant was reported to have a lower sensitivity for antagonists than the full-length protein [133]. After a truncated form of this receptor was found in mouse cochlea, it was hypothesized that the receptor might possess a possible role in hearing [134]. Indeed, it was demonstrated that the receptor regulated sound transduction and auditory

neurotransmission [135]. Moreover, in a study carried out in transgenic mice, $P2X_2$ receptor deficiency was associated with progressive hearing loss [136].

$P2X_3$ receptor was first cloned in 1995 from a rat cDNA library prepared from dorsal root ganglion cells [137]. Like all the other P2X receptors, mRNA of this receptor was also found in sensory neurons. However, this receptor's mRNA expression is limited to sensory neurons (even in sensory neurons, the receptor has a limited expression profile that is restricted to only trigeminal, nodose and dorsal root ganglia cells) and not present in neurons of the CNS [137]. On the other hand, human P2X3 receptor mRNA was reported in spinal cord and heart tissue [138]. In 1997, a role in pain states was suggested for this receptor, due to its co-expression with transient receptor potential V1 (TRPV1) channels and this theory was confirmed in an in vivo study carried out in rats [139]. Following that study, a role in acute and chronic inflammatory pain was suggested for $P2X_3$ receptor [140]. When analyzed in mRNA and protein levels, it was demonstrated that $P2X_3$ receptor was upregulated in dorsal root ganglions and in sciatic nerves of rats after neuropathic injury [141]. In another in vivo chronic pain study in rats, silencing of $P2X_3$ receptor was reported to result in pain relief [142].

$P2X_4$ receptor was first cloned in 1995 from a rat cDNA library prepared from hippocampus tissue [143]. Shortly after, human and mouse homologs of the receptor were also cloned [26]. $P2X_4$ receptor is expressed predominantly in the neurons of cortex, hippocampus, cerebellum and spinal cord. It is also expressed in several other tissues, including lung, adrenal gland and thymus [143]. Not only in neuronal cells, $P2X_4$ receptor is also found in microglial cells. Upon spinal cord injury, an increase in the mRNA levels of this receptor was reported [144]. That study also suggested that the pain sensitivity resulted from peripheral nerve injury was mediated by microglial $P2X_4$ receptors. This finding was further supported in a study carried out in $P2X_4$ receptor-knockout mice [145].

$P2X_5$ receptor was first cloned in 1996 from a rat cDNA library [29]. Until recently, the presence of $P2X_5$ receptor in the CNS was controversial. However, in a recent study, a wide-range distribution of this purinoceptor was demonstrated in both mRNA and protein levels [146]. Although expression of this receptor was clarified, its function in the CNS is still unknown.

$P2X_6$ receptor was first cloned in 1996 from a rat cDNA library prepared from brain tissue [147]. Human homolog of the receptor was cloned from peripheral lymphocytes. To date, functional homomeric $P2X_6$ receptor was not found, instead, the receptor's heteromeric forms are functional in tissues, such as skeletal muscle and brain. $P2X_6$ receptor is expressed in several tissues, including brain, lung, uterus and ovary [29]. In the rat gastrointestinal tract, $P2X_6$ receptor was found in neurons as an heteromer of $P2X_2$ receptor, while in macrophages it formed heterotrimers with $P2X_4$ receptor [148].

## 1.6. P2X7 RECEPTORS

$P2X_7$ receptor was formerly known as "P2Z receptor" and was first cloned in 1996 from rat brain [149]. Shortly after, human and mouse homologs were also cloned [150, 151]. It is expressed in a wide variety of cell types, including neuronal and glial cells, mast cells, macrophages and monocytes [152]. Based on its expression profile in the body, it was speculated that the receptor plays a central role in neuroinflammation. The unique properties of this receptor seem to support this hypothesis. These properties include that this receptor is the largest receptor among the purinergic receptor super family and has a long intracellular C-terminal domain that was proposed to be important in its role in inflammation [153]. The extracellular loop between two transmembrane domains of this receptor is around 600 amino acid-long. This is more than any other P2X receptors whose extracellular loops are only around 300 amino acids. Unlike nanomolar ATP concentrations that are enough to stimulate the other members of P2X receptor family, $P2X_7$ receptor requires millimolar concentrations of ATP to be activated [154]. Another unique feature of the receptor is that brief stimulation with ATP opens the receptor and results in $Ca^{2+}$ and $Na^{+}$ influx, whereas during prolonged stimulation the receptor forms a pore that allows the passage of large molecules of upto 900 Da molecular weight and eventually causes cell death [155]. In 2006, it was suggested that this response of $P2X_7$ receptors upon ATP induction involved that activation of pannnexin-1 hemichannels [156]. In that study, it was also reported that ATP-induced pannexin1-$P2X_7$ receptor activation resulted in the release of interleukin (IL)-1β to the cell medium *in vitro*. To make matters worse, when pannexin1-$P2X_7$ receptor complex was activated, pannexin1 hemichannel results in the extracellular release of ATP. Consequently, this extracellular ATP further

activates $P2X_7$ receptors [155]. Although the theory that pannexin hemichannels mediate pore formation of $P2X_7$ receptors in response to several types of cellular injury gained a lot of attention and support, there are also conflicting studies in which the hemichannel was not required for $P2X_7$ receptor pore formation [157]. It is tempting to speculate that the involvement of pannexin1 hemichannels in the receptor's pore formation response to injury may be dependent on the cell type that they are present and may differ according to the type of the injury. If that is indeed the case, one can also speculate that the downstream inflammatory response obtained would differ under different conditions, as well.

Several signaling pathways were associated with $P2X_7$ receptor, including Rho-dependent, Akt, ERK1/2 or JNK pathways [155]. For instance, it was reported that when astrocytes were stimulated with ATP or with a selective $P2X_7$ agonist, benzoyl-benzoyl ATP (BzATP), activated $P2X_7$ receptor resulted in the upregulation of monocyte chemoattractant protein-1 (MCP-1) through ERK1/2 and p38 pathways [158]. Since MCP-1 protein is involved in the infiltration of leukocytes into the CNS upon inflammation, a possible link between this receptor and inflammation was suggested [158]. In another study in astrocytes demonstrated that when this receptor was activated by ATP or BzATP, the pore that it formed lead to the release of glutamate and aspartate neurotransmitters [159]. As discussed above, pannexin1-mediated cytokine release through $P2X_7$ receptor is an important part of the inflammation process. It was recently reported that patients with a loss-of-function mutation in their $P2X_7$ receptor gene were protected against the detrimental effects of high levels of ATP [160].

Controversial results have been reported on the role of $P2X_7$ receptor in cellular survival and death. One widely accepted theory suggests that when the receptor was activated by a brief stimulant, it functions as a cation channel and acts in favor of survival; whereas when it was activated by a sustained stimulant, it becomes a large pore and promotes death. On the other hand, it was also reported that $P2X_7$ receptor was upregulated in the brain following an insult which triggers inflammatory response [155]. It was later shown that increase in the levels of $P2X_7$ receptor expression alone, without any other insult to the brain, activated microglial cells and enhanced their proliferation [161]. It was postulated that microglia-activating effect of overexpression of the receptor was through the receptor's pore-forming capacity. Therefore, the mechanism behind $P2X_7$ receptor-mediated neuroinflammation was suggested such that, following an insult, extracellular

ATP levels rise to millimolar levels, which in turn activated the receptor and subsequent microglia activation through IL-1β signaling pathway [162]. Interestingly, it was also reported that microglial $P2X_7$ receptor activation was responsible for the clearance of cellular debris after brain injury and thus, was a part of the endogenous repairing process in the brain [163].

## 1.7. PURINERGIC RECEPTORS IN ISCHEMIA

An increase in ATP concentration in the extracellular space following cerebral ischemia was first shown in 1999 [164]. This increase results from several mechanisms. For instance, exocytosis of ATP from microglial cells and astrocytes through mechanisms involving pannexin1 hemichannels were reported [165, 166]. Following an ischemic injury, depolarization of neurons also results in the release of ATP to the extracellular space from neuronal vesicles [167]. Moreover, as discussed in the previous section, with the help of pannexin1 hemichannels activated $P2X_7$ receptors form large pores on the cell membrane that eventually increases the extracellular ATP concentration [155].

All members of the purinergic receptor superfamily were studied in ischemic stroke. Of the P1 receptor family, $A_1$ receptors were attributed a neuroprotective role in early studies in this field [168, 169]. It was also shown that activation of $A_1$ receptors by selective activators in *in vitro* ischemia models was improved neuronal survival and decreased the number of apoptotic neurons [170]. In *in vivo* global cerebral ischemia models, using $A_1$ receptor-specific agonists also reduced the number of apoptotic neurons [171]. As expected, $A_1$ receptor antagonists showed an opposite effect and worsened the outcomes in *in vivo* studies [172]. Although the results obtained by $A_1$ receptor agonists seemed promising, when these chemicals were used in animals, they caused several side-effects, including bradycardia and hypotension [173].

It was demonstrated that post-ischemic administration of a selective $A_{2A}$ receptor antagonist reduced neuronal loss in vivo [174]. In this study, the antagonist was used 24 hours after ischemic onset. To further demonstrate the detrimental effect of this receptor, knock-out mice were subjected to transient middle cerebral artery occlusion (MCAo) model of ischemia. The results obtained from that study indicated that infarct volumes

were significantly lower in $A_{2A}$ receptor knock-out mice [175]. It is believed that $A_{2A}$ receptor antagonists mediate the regulation of glutamatergic transmission and hence, excitotoxicity following an ischemic insult and therefore, present a neuroprotective effect. Interestingly, an agonist of the receptor was also shown to provide protective effects in terms of neuronal survival after brain trauma and focal cerebral ischemia [176]. It was also reported in that study that $A_{2A}$ receptor activation had antioxidant and antiinflammatory effects. On the other hand, because the $A_{2B}$ receptors are relatively less abundant in the brain compared to other P1 receptors, studies on the role of this receptor in ischemia are limited. However, it was demonstrated that $A_{2B}$ receptor antagonists had favorable effects on cellular survival. Recently, it was speculated that following ischemia, activation of neuronal $A_{2B}$ receptors might be detrimental, whereas when the receptor located in endothelial or blood cells are activated, they might provide antiinflammatory effects [177].

Using *in vivo* ischemia models and transgenic animals, the role of $A_3$ receptors in ischemia was studied. According to the data obtained, activation of this receptor using agonists resulted in decreased infarct size and increased amount of neuronal survival [178]. Moreover, $A_3$ receptor knock-out animals had increased infarct size compared to wild-type controls [178]. Interestingly, conflicting results were obtained with $A_3$ receptor antagonists in several studies. A possible explanation to this, could be the differences in ischemia conditions in different studies. It could also be possible that this receptor stimulates survival or death signals depending on the severity of the stimulus. For instance, a mild ischemic attack might activate $A_3$ receptor-mediated survival pathways, while a severe injury and subsequent sustained $A_3$ receptor activation might lead to cell death.

Of the P2Y receptors, $P2Y_1$ receptor is widely expressed in the brain [155] and increased expression of this receptor was reported after ischemic injury [179]. In the literature, conflicting data on the role of $P2Y_1$ receptors in ischemia exist. In early studies, use of antagonists of the receptor was shown to exert protective effects [180]. Shortly after, it was reported that $P2Y_1$ receptor agonist reduced the infarct volume in *in vivo* ischemia models [181]. The conflicting data could be attributed to the difference in experimental conditions between studies, as mentioned above for $A_3$ receptors. For $P2Y_1$ receptors; however, this controversy could stem from the fact that this receptor is expressed in several cell types in the CNS and that the results obtained by agonist and antagonist administrations depend on their differential effects on different cell types. In a study focused on $P2Y_{12}$ receptors, it

was reported that $P2Y_{12}$ receptor knock-out mice had better outcomes following an ischemic injury compared to wild-type animals [182]. This effect was attributed to the inability of microglial cell migration, due to the fact that $P2Y_{12}$ receptor is mainly located in microglia. This observation was also supported in studies where an antagonist was used. It was reported that a $P2Y_{12}$ receptor antagonist reduced inflammation and activation of immune cells [183].

In general, expression of P2X receptors is upregulated following an injury in the CNS [155]. As discussed in the section above, $P2X_4$ receptor is the predominant P2X receptor type in the brain and it is expressed in microglial cells, but not in neurons. After an ischemic injury in the brain, upregulation of the receptor in microglial cells was reported to occur in a delayed fashion and hence, it was attributed to the receptor's involvement in the secondary injury mechanisms [184]. As with other P2X receptors, activation of $P2X_4$ receptor results in an increase in the levels of intracellular $Ca^{2+}$. This, in turn, causes depolarization resulting in an increase in ATP which stimulates microglial migration as well as the release of proinflammatory cytokines [184]. Therefore, this receptor may be an important player in the neuroinflammation process following brain injury.

## 1.8. P2X7 RECEPTORS IN ISCHEMIA

$P2X_7$ receptors are upregulated after cerebral ischemia. Although a key role for this receptor in ischemia is well accepted, there are conflicting data in the literature regarding its effects on survival. It is widely accepted that $P2X_7$ receptor is upregulated in the brain following an insult [155], however, it is still under debate which cell types (neurons or microglial cells) overexpress this receptor. In several of the reported studies, the selective and potent $P2X_7$ receptor antagonist Brilliant Blue G (BBG) was chosen. It was reported that administration of BBG resulted in a reduction in glutamate release, i. e. glutamatergic excitotoxicity [185]. *In vivo* studies of BBG not only supported these data, but also showed that BBG reduced neuronal damage and infarct size [186]. Moreover, it was also shown that BBG reduced microglial cell death after oxygen-glucose deprivation [187]. In transient global ischemia model, it was reported that BBG resulted in a decrease in neuronal cell death, an increase in the number of viable neurons and a decreased infract

volume [188, 189]. In the light of these data, BBG was chosen as the antagonist of $P2X_7$ receptor in this project.

On the other hand, BzATP was reported to selectively activate $P2X_7$ receptor at the time of this project was started [186]. In a study by Arbeloa, it was reported that BBG decreased the amount of $Ca^{2+}$ influx that was caused by BzATP administration [188]. It can be speculated that the favorable effect attributed to BBG-mediated inhibition of the receptor may involve several different mechanisms and signaling pathways due to the distinct characteristic of this receptor. $P2X_7$ receptor is known to have two different states; in one it serves as a cation channel upon brief stimulation and in another it forms large plasma membrane pores due to prolonged stimulation [155]. Notably, it is not possible to desensitize the receptor after it forms a large pore [154]. Moreover, once the receptor is activated with a prolonged stimulus, overload of $Ca^{2+}$ ions in the intracellular space results in the activation of different apoptotic mechanisms, disintegration of the cytoskeleton, and subsequent membrane blebbing and cell lysis. Therefore, it was postulated that excess ATP levels following an ischemic insult can cause the sustained activation of the receptor, and thereby result in cell death in neurons [156]. In the light of these data, it was hypothesized in this study that targeting $P2X_7$ receptors could provide further insights to the pathophysiology and treatment strategies in ischemic stroke. To this end, a widely used ischemic stroke model, middle cerebral artery occlusion (MCAo) was chosen in mice. In addition to ischemia, an optic nerve transection model was also chosen. Retina can be considered as an extension of the CNS. It can also activate similar pathophysiological processes in response to injury to that of brain and spinal cord. However, compared to the CNS, degenerative processes in retina last a prolonged time period which provide researchers a broadened timeframe to understand the entire process through noninvasive monitoring methods as well as to prevent the unwanted outcomes of the pathological environment. Thus, the present study was carried out to investigate the involvement of activation and inhibition of $P2X_7$ purinergic receptors in *in vivo* acute and sub-acute ischemic injury as well as in retinal injury models. In addition, it was also aimed to investigate the downstream signaling pathways and molecular mechanisms to provide a clearer understanding to the controversial role of $P2X_7$ receptor following an injury.

# 2. MATERIALS AND METHODS

## 2.1. MATERIALS

Materials used in this thesis are given in Table 2.1.

Table 2.1. List of materials used in this study.

| Name of the material | Brand | Concentration and comments |
|---|---|---|
| 8.0 nylon monofilament | Ethilon | For MCAo application |
| Brilliant Blue G (BBG) | Sigma, B0770 | 10 mg/kg dose in SF used for icv application |
| Bovine Serum Albumin (BSA) | Sigma, A9418 | 1 mg/mL in dH2O was prepared |
| BzATP | Santa Cruz, sc-203862 | 5 mg/kg and 20 mg/kg doses were used for icv application |
| Chloral hydrate | | 400 mg/kg for icv application, 7 per cent |
| Cresyl violet | Sigma, C5042 | For brain swelling and infarct volume analyses |
| DAPI | Sigma, D9542 | Prepared in Methanol, 1 mM dose was used for nuclei staining |
| DMEM, high glucose | Gibco, 61965026 | For isolation and culturing of RGCs |
| Fluorogold | Santa Cruz, sc-358883 | For labeling RGCs, 0.7 uM was used |
| Fura-2AM | Invitrogen, F1201 | For intracellular $Ca^{2+}$ concentration analyses, diluted in HBSS, 5 uM dose was used |
| Hank's Balanced Sodium Salt (HBSS) | Sigma, H6648 | For dissolving materials such as Fura-2AM |
| Hydrogen Peroxide ($H_2O_2$) | Sigma, H1009 | 0.3 per cent in PBS was prepared |
| In Situ Cell Death Detection Kit | Roche | For TUNEL assay |
| Isofluorane | ADEKA | 1 per cent used for anesthetic |
| Methanol, 99.8 per cent | Sigma, 322415 | Used for dilution of chemicals, such as DAPI |
| Neurobasal A medium | Gibco, Invitrogen | For isolation and culturing of primary cortical neurons |
| NuPage SDS-PAGE gel | Invitrogen, NW00100BOX | 4 – 12 per cent Bis-Tris gel was used for Western blotting |
| Paraformaldehyde (PFA) | Sigma, P6148 | 4 per cent in PBS was used for fixation in IF |
| Papain | Sigma, P4762 | 1 M in PBS, used for dissociation of tissues |
| PVDF membrane | Invitrogen, | For Western blotting |

| | | |
|---|---|---|
| | IB401001 | |
| Triton X-100 | Sigma, T8787 | For membrane permeabilization in IF |

Antibodies used in this thesis are given in Table 2.2.

Table 2.2. List of antibodies used in this study.

| Name of the antibody | Brand | Dilution |
|---|---|---|
| β-Actin, mouse | Cell Signaling, #4967 | 1:1000 in 5 per cent non-fat milk/TBS |
| β-Actin, rabbit | Cell Signaling, #4970 | 1:1000 in 5 per cent non-fat milk/TBS |
| BAX | Abcam, ab32503 | 1:1000 in 5 per cent non-fat milk/TBS |
| Caspase-2 | Abcam, ab32021 | 1:1000 in 5 per cent non-fat milk/TBS |
| Caspase-9 | Abcam, ab32539 | 1:1000 in 5 per cent non-fat milk/TBS |
| GFAP-AlexaFluor555 conjugated | Cell Signaling, #3656 | 1:100 in 0.1 M PBS |
| GSK-3α | Cell Signaling, #4818 | 1:1000 in 5 per cent non-fat milk/TBS |
| GSK-3β | Cell Signaling, #12456 | 1:1000 in 5 per cent non-fat milk/TBS |
| NeuN | Chemicon, MAB377 | 1:100 in 0.1 M PBS |
| p-AKT | Cell Signaling, #4060 | 1:1000 in 5 per cent non-fat milk/TBS |
| p-ERK-1/2 | Cell Signaling, #9101 | 1:1000 in 5 per cent non-fat milk/TBS |
| p-GSK3-α/β | Cell Signaling, #8566 | 1:1000 in 5 per cent non-fat milk/TBS |
| p-JNK | Cell Signaling, #9255 | 1:1000 in 5 per cent non-fat milk/TBS |
| p-PTEN | Cell Signaling, #9554 | 1:1000 in 5 per cent non-fat milk/TBS |
| P2X7 Receptor | Abcam, ab106246 | 1:100 in 0.1 M PBS |
| Donkey anti-goat secondary (Peroxidase conjugated) | Santa Cruz, sc-2033 | 1:2000 in 5 per cent non-fat milk/TBS |
| Goat anti-mouse secondary (Peroxidase conjugated) | Santa Cruz, sc-2005 | 1:2000 in 5 per cent non-fat milk/TBS |
| Goat anti-rabbit secondary (Peroxidase conjugated) | Santa Cruz, sc-2030 | 1:2000 in 5 per cent non-fat milk/TBS |

## 2.2. METHODS

### 2.2.1. Experimental Setup and Groups

All experimental procedures were conducted with governmental approval according to local guidelines for the care and use of laboratory animals. Animals of all groups were kept under regular lighting conditions as 12 hour-darkness and 12 hour-light. Adult male C57BL/6j mice (21-25 g) were used in this study. Animals were randomly assigned to the following experimental groups where they received vehicle (0.9 per cent saline) (n=7) or 10 mg/kg $P2X_7$ receptor antagonist Brilliant Blue G (BBG) (n=7) or 5 mg/kg $P2X_7$ 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) in two experimental setups: (i) 30 min MCA occlusion 72 hour reperfusion or (ii) 90 min MCA occlusion 24 hour reperfusion.

### 2.2.2. Intracerebroventricular (i.c.v.) Injection

For i.c.v. injections, animals were anesthetized with 400 mg/kg chloral hydrate (intraperitonaelly; i.p.). Skulls were drilled and animals were treated with either vehicle (0.9 per cent saline) or 10 mg/kg BBG or 5 mg/kg BzATP; or both 10 mg/kg BBG and 5 mg/kg BzATP were i.c.v. injected using a micro-syringe pump controller (Micro 4; World Precision Instrument) 30 minutes before MCA occlusion.

### 2.2.3. Induction of Cerebral Ischemia and Reperfusion

Animals were anesthetized with 1 per cent isofluorane (30 per cent $O_2$, remainder $N_2O$). Rectal temperatures (36.5 and 37.0 $^oC$) were controlled using a feedback-controlled heating system (MAY instruments). During the MCA occlusion and reperfusion, cerebral blood flow (CBF) was monitored via Laser Doppler Flowmetry (LDF) using a flexible 0.5 mm fiber optic probe (Perimed, Sweden) which was attached with tissue adhesive to the intact skull overlying the MCA territory (2 mm posterior and 6 mm 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 were isolated and ligated. Internal carotid artery will temporally ligate 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 (72 h reperfusion) or ninety minutes (24h reperfusion) after the 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) into 18µm coronal sections.

### 2.2.4. Retrograde Labeling of Retinal Ganglion Cells (Rgcs) and Optic Nerve (ON) Transection

For retrograde labeling of Retinal Ganglion Cells (RGCs), animals (12–14 weeks) were i.p. anesthetized with 7 per cent chloral hydrate. The superior colliculi were exposed via a bur hole that was drilled into the pericranium 0.7 mm lateral to the sagittal suture and 3 mm posterior to the bregma. A Hamilton syringe was inserted 2.0 mm beneath the brain surface, and 0.7 µl of fluorogold (infusion rate 0.7 µl/min) was injected stereotactically into both superior colliculi. After infusion, the injection needle remained inside the tissue for 2 min to prevent fluorogold diffusion along the needle track, before the syringe was withdrawn. Four days after labeling, mice were re-anesthetized with 7 per cent chloral hydrate. After skin incision close to the superior orbital rim, the right orbita was opened, leaving the supraorbital vein intact, and the lacrimal gland was resected subtotally. After spreading the superior extraocular muscles, the right ON was transected under microscopical control approximately 0.5 mm distant to the posterior pole of the eye, taking care not to damage the retinal blood vessels. The wounds were sutured and the retinal blood supply was verified by fundoscopy. Fourteen days after ON transection, mice were sacrificed with an overdose of chloral hydrate and both eyes were removed. The retinas were dissected, flat-mounted on glass slides, and fixed in 4 per cent paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) for 30 min.

### 2.2.5. Intraocular Injections

By means of a glass microelectrode with a tip outer diameter of 50 μm, 1 μl of either i) Vehicle, ii) 10 mM BBG, iii) 250 μM BzATP or iv) combination of BBG and BzATP was carefully injected into the vitreous space, puncturing the eye at the cornea-sclera junction (n=7 animals/group). Injections were done at Day 0, 4, 7, and 10 after optic nerve transection as described above. Animals were sacrificed 14 days after axotomy, and their retinae were removed, flat-mounted, and evaluated microscopically.

### 2.2.6. 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.

### 2.2.7. 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 per cent paraformaldehyde (PFA) in 0.1 M buffered phosphate-saline (PBS) and subsequently stained with cresyl violet. Nine random area of interest, 1 mm apart, each measuring 62,500 $\mu m^2$, were evaluated under light microscope. Mean values were calculated both ischemic (ipsilateral) and non-ischemic (contralateral) areas. Therefore, the percentage of surviving neurons was determined.

### 2.2.8. Cresyl Violet Staining, Infarct Volume and 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.

### 2.2.9. Analysis of Apoptotic Cells ( Tunel Staining)

In order to detection and quantification of 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 per cent 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 $\mu m^2$ under a fluorescence microscope.

### 2.2.10. Evaluation Of Rgc Survival By Stereology Of Surviving Neurons

Surviving RGCs were evaluated by confocal microscope (ZeissLSM-780) in retinal whole-mounts. RGC densities were determined by counting tracer-labeled RGCs in 12 random ROI (three areas per retinal quadrant at different eccentricities of 1/6, 3/6 and 5/6 of the retinal radius, respectively; measuring 62.500 μm2 each). Mean values were calculated for all eccentricities as well as over the whole retina.

### 2.2.11. Analysis of Protein Levels (Protein Extraction, Sds-Page and Watern Blot)

Tissue samples were obtained from ischemic striatum and overlying cortex of the animals. The tissue samples belonging to the same group were pooled, homogenized and treated with protease and phosphatase inhibitor cocktails. Protein concentrations were measured with Qubit 2.0 Fluoremeter according to the manufacturer's protocol (Invitrogen). Equal amounts of protein (20 μg) were size-fractionated by 4-12 per cent NuPAGE electrophoresis and then transferred to polyvinylidene fluoride membrane (PVDF) using iBlot Dry Blotting System (Invitrogen). Membranes were blocked in 5 per cent non-fat milk in 50 mM Tris-buffered saline containing 0.1 per cent Tween (blocking solution) for 1 h, washed in Tris-buffered saline containing 0.1 per cent Tween (TBS-T) and incubated overnight with rabbit anti-phospho-Akt (4060; Cell Signaling), mouse anti-phospho-ERK-1/-2 (9101; Cell Signaling), rabbit anti-phospho JNK-1/-2 (9255; Cell Signaling rabbit anti-phospho PTEN (9554; Cell Signaling), rabbit anti-phospho GSK3 alpha beta (8566; Cell Signaling) or rabbit anti-beta actin (4970; Cell Signaling). Each antibody was diluted 1:1000 directly in blocking solution. On the following day, membranes were incubated with peroxidase-conjugated goat anti-rabbit (Amersham, GE Health Care), or donkey anti-goat (sc-2033; Santa Cruz Biotechnology) antibodies. Protein loading was controlled by stripping and reprobing the blots with rabbit polyclonal anti β-actin antibody (4967, Cell Signaling). Blots were developed using ECL-Advanced Western Blotting Detection Kit (Amersham, GE Health Care) and visualized by the MF-ChemiBIS (DNR). The intensity of each signal was measured on a total of three digitized blots each using the Image J software program. Protein levels were analyzed densitometrically and corrected with values determined on β-actin blots and expressed as a percent of total protein.

### 2.2.12. Immunohistochemistry

For immunological staining of samples, four animals per group were transcardially perfused with 0.9 per cent NaCl. Brain tissue was frozen on dry ice and cut on a cryostat into 18 μm coronal sections. Brain sections from the level of the bregma (i.e. midstriatum) were fixed in 4 per cent PFA, rinsed, pretreated for antigen retrieval with 0.01 M citrate buffer (pH 5.0), rinsed and immersed for 1 h in 0.1 M phosphate-buffered saline containing 0.3 per cent Triton X-100 and 10 per cent normal donkey serum. Brain sections were

incubated overnight at 4 $^{o}$C with monoclonal mouse anti-NeuN (MAB377; Chemicon), monoclonal mouse anti-glial fibrillary acidic protein (GFAP) Alexa Fluor 555 conjugated (#3656; Cell Signalling), and P2X7R (ab106246, abcam) antibodies (diluted 1:100 in 0.1M phosphate-buffered saline) that were detected with Cy3 or Alexa-488 conjugated secondary antibodies. Sections were counterstained with 4'-6-diamidino-2-phenylindole (D9542, DAPI, Sigma) and evaluated under a confocal microscope (Zeiss LSM 780). Two sections were processed for each animal. Mean values were calculated for both sections for the whole striatum.

### 2.2.13. Primary Cortical Neuron Culture

Primary cortical neuron culture was prepared from brain cortices of one-day-old Balb/c mice. Cortical tissue was dissected and placed into a tube containing papain solution after a careful removal of meninges. The tissue was papain digested for 45 minutes at 4$^{o}$C. Cells were gently triturated, tissue and cell debris were removed by centrifugation and 2x10$^{5}$ cells were seeded to each poly-D lysine coated glass-bottomed 35 mm fluorodishes. Cultured cells were incubated at 37$^{o}$C in a humidified atmosphere of 95 per cent air and 5 per cent $CO_2$ in Neurobasal A medium supplemented with 1 per cent GlutaMax-1, 1 per cent antibiotic and antimycotic solution and 2 per cent B27 supplement. The medium was refreshed in every two days and at the 7$^{th}$ day *in vitro* cells were used in experiments.

### 2.2.14. *In Vitro* Calcium Imaging

Calcium imaging was performed to monitor intracellular calcium concentrations *in vitro*. For this purpose, calcium sensitive fluorescent dye Fura 2AM was prepared by dissolving in dimethylsulfoxide to measure the changes in the intracellular calcium activity in primary cortical neurons. When cells reached to 7 days in vitro, culture medium was aspirated and petri dishes were washed with Hank's Balanced Salt Solution (HBSS). Next, cells were loaded with 5 μM Fura 2AM within 1 ml HBSS containing 2 mg/ml Bovine Serum Albumin. After 30 minutes of incubation at 37°C, Fura 2AM was aspirated and cells were washed once with HBSS to eliminate non diffused Fura remnants on the culture. Finally, 1

ml DMEM high glucose Phenol Red(-) was added to the dishes and then, petri dishes were quickly placed on the spinning disk microscope (Zeiss Cell Observer Spinning Disk Confocal) stage with a small incubator apparatus. Thereafter, cells were kept at 37°C and 5 per cent $CO_2$. Flourescent measurements at 463 nm emission were taken as the ratio of the signals obtained upon excitation by 340/380 nm. Time lapse records of the first 10 minutes were taken as baseline and used to normalize the data obtained at designated time points. Eight different regions of interest (ROI) and five cells in each ROI were analyzed per each group. At the end of the measurements, cells were fixed with 4 per cent PFA and stained with NeuN to further confirm the accuracy of the assessed cells.

### 2.2.15. 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.

# 3. RESULTS

## 3.1. EXPRESSION OF P2X7 RECEPTORS IN THE BRAIN AND RETINA

It has been postulated that adenosine and ATP are used as transmitter molecules in the retina and they mediate the communication between neuronal and glial cells. Therefore, it was hypothesized that such connection could be regulated via purinergic receptors. Retinal purinergic receptor expression was first demonstrated using RT-PCR analysis of rat retinal tissue. The data provided evidence for the presence of $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$ and $P2X_7$ receptors in retina, while $P2X_1$ and $P2X_6$ receptor mRNAs were not present [190,191]. However, conflicting data in the literature were present about which cells in the different retinal cell layers were positive for $P2X_7$ receptor ($P2X_7R$) mRNA. In one study, it was reported that cells in the ganglion cell layer and inner nuclear layer were immune-positive for $P2X_7$ receptor [191]. It was also suggested that the most abundant glial cells of the retina, i.e. Müller cells, did not express $P2X_7R$ [192]. Shortly after, it was reported that human Müller glial cells were positive for $P2X_7$ receptor expression and that the receptor could be activated by ATP or BzATP [193]. In addition, the reports suggesting a retinal ganglion cell-positive expression did not use immunohistochemical analyses to demonstrate the localization of the receptor. In parallel with that, before the present work had been started, expression of $P2X_7R$ in neurons of retina had not been confirmed. Moreover, during the time this study was started, it was widely accepted that $P2X_7R$ was predominantly expressed in glial cells. Therefore, it was of utmost importance to determine the correct cell type specific localization of the receptor in the murine retina before further studies were conducted. To this end, after animals were sacrificed, eyes were removed and radial retinal sections were prepared. This allowed the observation of all the retinal layers in each slice. Next, sections were stained with antibodies against specific cell type markers, such as neuronal nuclei protein (NeuN) for neurons or glial fibrillary acidic protein (GFAP) for astroglia. NeuN, is a nuclear protein that is present in the nuclei of almost all neuron types throughout the central and peripheral nervous systems of mice [194]. GFAP, is a highly specific intermediate filament protein that is present in mature astroglial cells [195]. For all immunofluorescence analyses, two negative controls; one without primary

antibody and the other without secondary antibody were also carried out in order to eliminate the risk of false positives. It was shown that $P2X_7R$ was expressed throughout the ganglion cell layer as well as bipolar neurons of axotomized mouse retina. $P2X_7R$ immunoreactivity was also observed at the inner neuronal layer of the retina (Fig 3.1). $P2X_7R$ immunoreactivity colocalized with NeuN, indicating a neuron-specific expression of the receptor in murine retina.

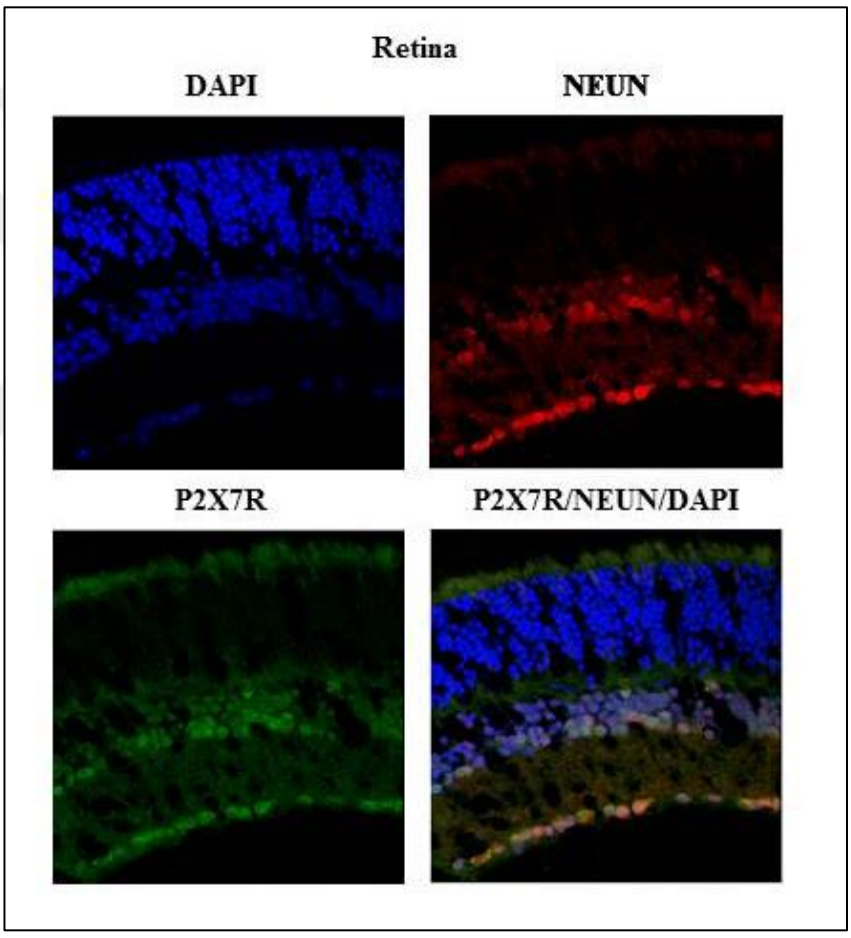


Figure 3.1. Immunoreactivity of $P2X_7R$ in mouse retina. In retinal sections, $P2X_7$ receptor immunoreactivity was colocalized with neuronal nuclei marker (NeuN) in the retinal ganglion cells and bipolar cells of the inner nuclear layer. 4',6-diamidino-2-phenylindole (DAPI) was used as a nuclear counter stain.

In the light of this result, it was felt necessary to further investigate the receptor's expression in the ischemic mouse brain, since the increase in ATP concentration was a well-known result of depolarization of neurons following cerebral ischemia and it was

highly likely that the receptor was involved in the response mechanisms of the cells against an overload in the extracellular ATP concentration [164]. To this end, animals were subjected to ischemia-reperfusion, brains were removed and coronal sections were prepared from the mouse brains. Double immunofluorescence staining of the sections with NeuN or GFAP markers along with anti-$P2X_7R$ antibody was analyzed using confocal microscopy. Similar to the results obtained in the retinal tissue, $P2X_7$ receptor expression was only determined in the neurons of the striatum (Fig 3.2.A), as demonstrated with NeuN double-immunofluorescence. After a thorough analysis of the sections, it was safe to state that a colocalization between $P2X_7R$ and GFAP was not observed, hence $P2X_7R$ was not expressed in the astroglial cells of the mouse brain (Fig 3.2.B).

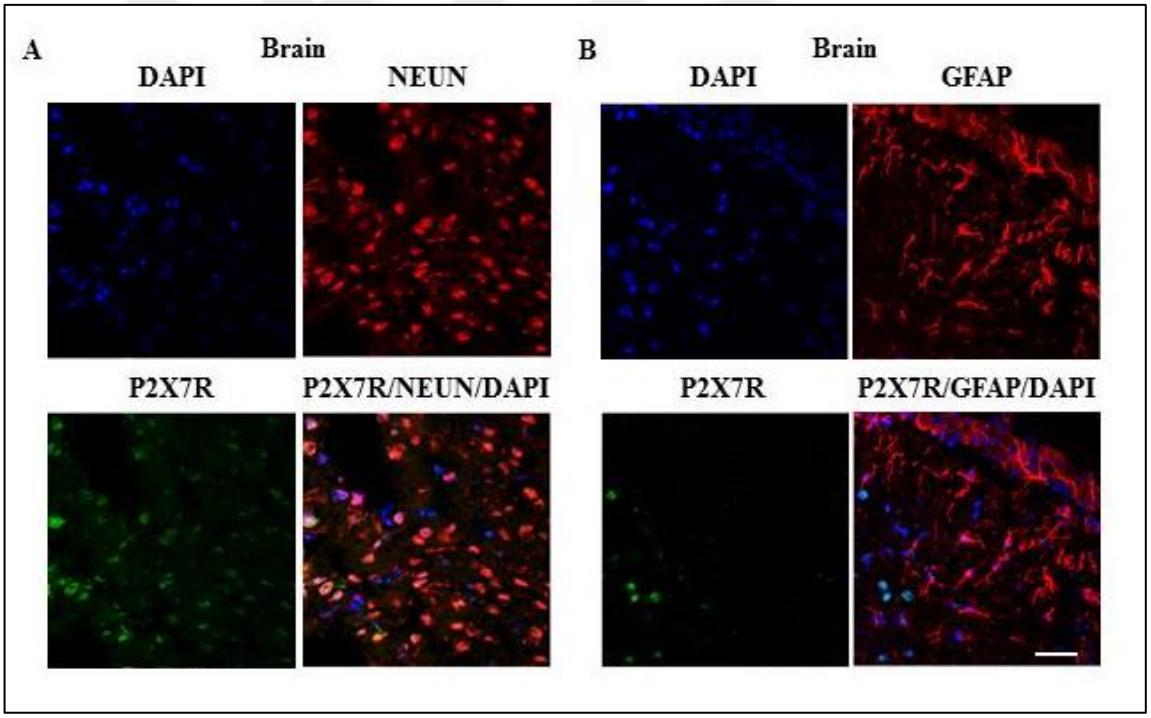


Figure 3.2. Immunoreactivity of $P2X_7R$ in mouse brain. In the ischemic brain sections, $P2X_7$ receptor expression was demonstrated in NeuN+ cells (A), but not in GFAP+ cells (B) in mouse striatum. DAPI was used as a nuclear counter stain.

### 3.2. P2X7R INHIBITION PROMOTES SURVIVAL OF AXOTOMIZED RGCS

After demonstrating a neuron-specific localization of the $P2X_7$ receptor in the mouse retina, its role in neuronal degeneration following an optic nerve (ON) transection was addressed. Due to the conflicting data in the literature, it was decided that activation as well as inhibition of the receptor must be carried out prior to ON axotomy. In order to analyze the amount of RGC survival, cells were labeled with Fluoro-Gold™ 3 days before the experiment. Fluoro-Gold™ is a fluorescent neuronal retrograde tracer that is widely used in retrograde labeling of RGCs [196].

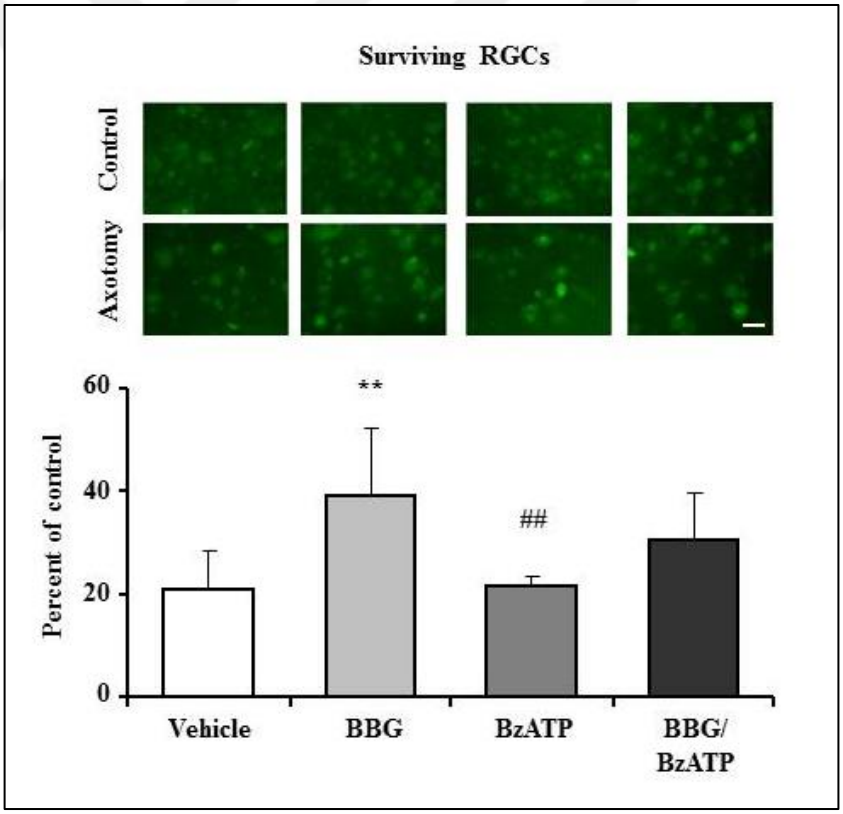


Figure 3.3. Effects of $P2X_7R$ antagonist BBG and $P2X_7R$ agonist BzATP on RGC survival in whole retina. Surviving fluorogold-prelabeled RGCs in mice submitted to optic nerve (ON) transection were counted from various areas in whole retina. Data are mean values ± S.D. (n= 7 animals/group). **p<0.01 compared with vehicle; ##p<0.01 compared with BBG treatment.

Injection of Fluoro-Gold™ into contralateral superior colliculus results in a reliable RGC staining, because it is a well-known fact that RGCs project to superior colliculus of the contralateral hemisphere. Therefore, in order to analyze RGC survival in control vs treatment groups, after the removal of retinae from the eyes of the sacrificed animals, green fluorescent cells can be directly counted. To pharmacologically inhibit the receptor, a selective antagonist (BBG) was chosen. BBG, is accepted as one of the most potent, selective antagonists of $P2X_7$ receptor [197]. Injection of BBG 30 minutes prior to ON axotomy resulted in a significant increase in the percent of surviving retinal ganglion cells (RGCs) in the whole retina compared to vehicle control (Fig 3.3). In all optic nerve transection experiments, only one eye was operated, whereas the other eye was used as a control. This allowed the direct comparison of the treatment administered to the eye with control tissue in the same animal. Therefore, the percent of surviving RGCs in Figures 3.3 and 3.4 were calculated using the number of RGCs in the unoperated eye as 100 per cent. Apart from the whole retinal count, different eccentricities were also evaluated in order to identify whether there was a specific pattern of RGC survival (Fig 3.4A). BBG increased the percent of surviving RGCs in all areas counted; however, only the inner and outer eccentricities were significant. The percent of surviving RGCs in the middle section was still higher than the vehicle control, yet it was not statistically significant (Fig 3.4B). To pharmacologically activate the receptor, a potent and selective agonist (BzATP) was chosen. Although recent reports indicated that this agonist might be non-selective for this receptor, at the time of the present study, BzATP was the only recommended selective $P2X_7$ receptor agonist [197]. Interestingly, comparison of the number of RGCs in BzATP-administered retinae with vehicle-treated group revealed that BzATP treatment did not cause any significant effect on the survival of neurons. However, the percent of surviving RGCs was significantly lower in BzATP-administered group when compared with BBG group. This result indicated that inhibition of $P2X_7$ receptor before optic nerve transection increased neuronal survival, whereas activation of the same receptor did not further reduce the survival rate of these cells below the vehicle control. When the antagonist (BBG) and agonist (BzATP) of the receptor were given together, the percent of survival was lower than BBG alone, but higher than BzATP alone.

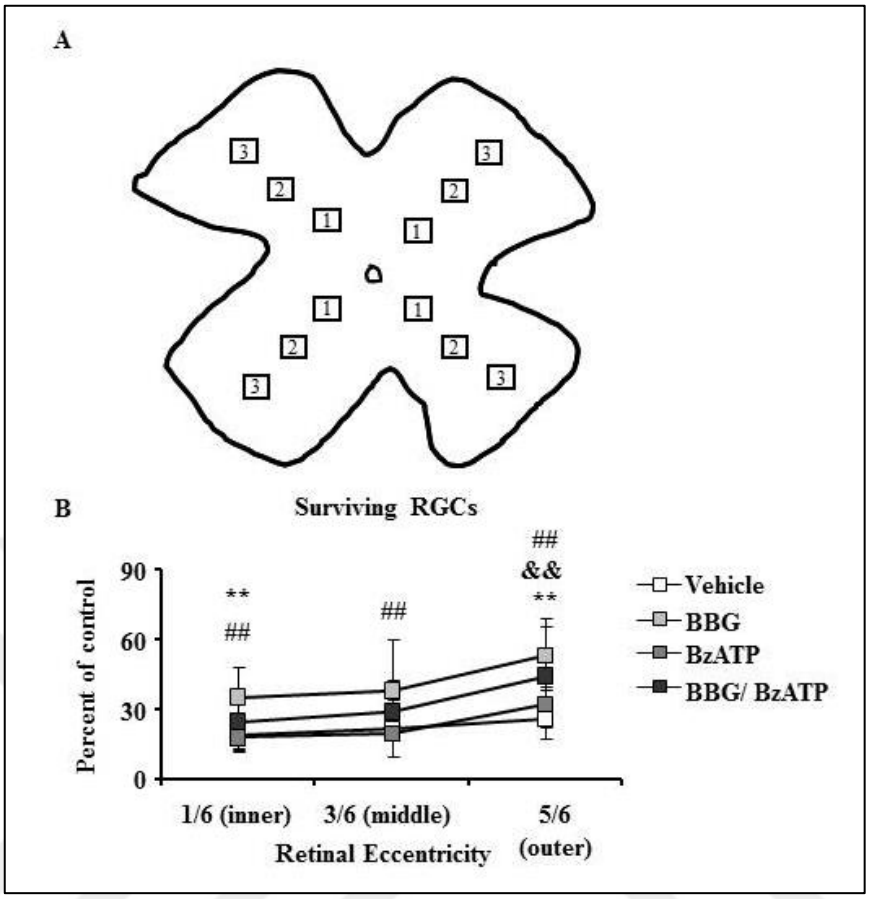


Figure 3.4. Effects of $P2X_7R$ antagonist BBG and $P2X_7R$ agonist BzATP on RGC survival in different retinal eccentricities. Data are mean values ± S.D. (n= 7 animals/group). **p<0.01 vehicle vs BBG treated group, ##p<0.01 BBG vs BzATP treated group. &&p<0.01 BzATP vs BBG/BzATP treated group.

In the light of this result, it could be speculated that BzATP-mediated activation of the receptor was only partially blocked by BBG when the two chemicals were given together and that this partial blockade was responsible for the statistically insignificant increase in the percent survival when compared with BzATP alone group.

### 3.3. P2X7R ANTAGONIST BBG PROTECTS AGAINST 90 MINUTES OF FOCAL CEREBRAL ISCHEMIA

As stated above, $P2X_7$ receptor was located in NeuN positive cells in mouse brain sections (Fig 3.2). The increase in extracellular ATP levels following an ischemic insult is well

known, as discussed in Section 1.7. $P2X_7$ receptor differs from the other purinergic receptors, as it requires millimolar ATP concentrations to be activated [154]. Such high concentrations are achieved after ischemic stroke [1]. Due to conflicting data in the literature about the role of $P2X_7$ receptor in ischemia, it was important to assess the receptor's function using a pharmacological activator and an inhibitor. First, a model of 90 minutes of ischemia with 24 hour-reperfusion was used. It was shown that in the animals subjected to 90 minutes of MCAo followed by a 24-hour reperfusion, a focal infarct in the cerebral cortex and in the underlying striatum, and a subsequent edema were observed in the vehicle-treated group in which the animals were not given the active molecule [198].

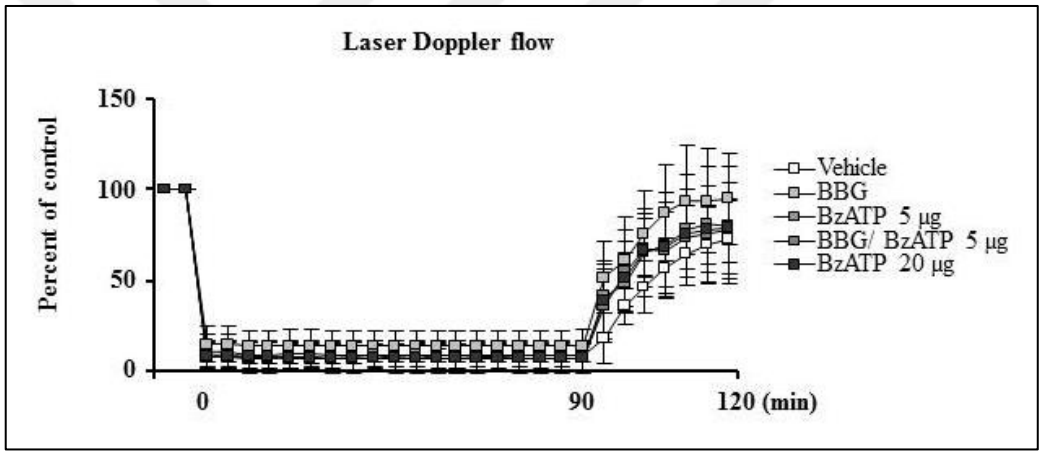


Figure 3.5. Blood flow changes after 90 min of focal cerebral ischemia. Percent decrease in blood flow during ischemia and percent increase during reperfusion was monitored for each of the operated animals.

In MCAo, blood flow in the area where the middle cerebral artery supplies is reduced to around 15-20 per cent. This decrease in the blood flow is measured real time using noninvasive Laser-Doppler flowmetry instruments during the entire MCAo process in order to monitor the success of the ischemia and subsequent reperfusion procedures and also prevent unwanted release of the occlusion in the artery [198]. The decrease in blood flow in this study was carefully monitored using Laser-Doppler flowmetry. Percent decrease in the blood flow during ischemia and percent increase during reperfusion in animals of each study group was relatively similar throughout the observed time period (Fig 3.5).The infarct volume caused by ischemia-reperfusion injury is measured using

Cresyl Violet staining [198]. This technique relies on a basic dye which stains the acidic components in the cytoplasm with blue color that can be easily observed under a light microscope. Because neurons are rich in Nissl substances (rough endoplasmic reticulum) in their cytoplasm, this dye stains intact neuronal cytoplasm as well as nucleoli in neurons [199].

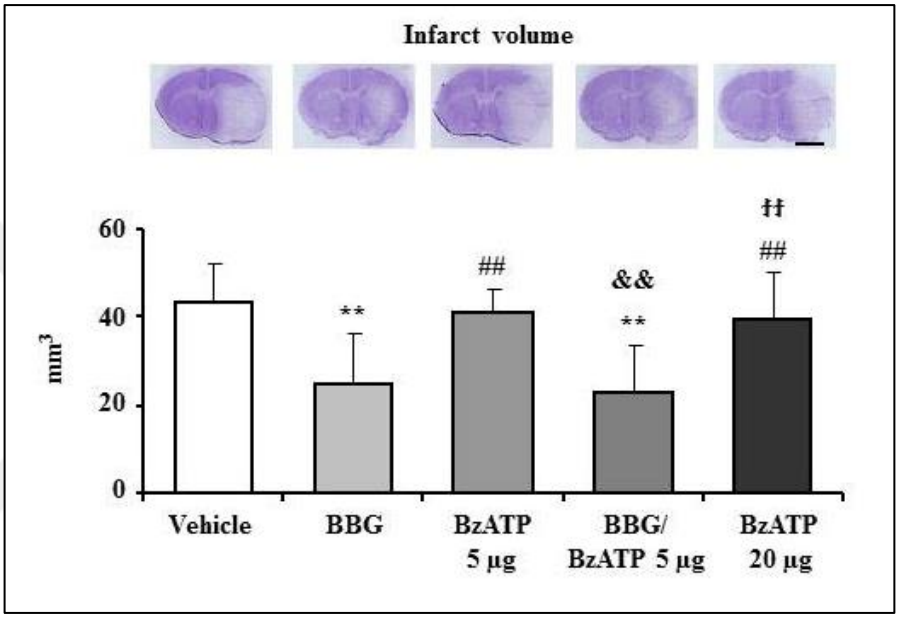


Figure 3.6. Infarct volume after 90 min of focal cerebral ischemia. Infarct volume was assessed in ischemic brain sections using cresyl violet staining. Data are mean values ± S.D. (n= 7 animals/ group). **p<0.01 compared with vehicle; ##p<0.01 compared with BBG treatment; &&p<0.01 compared with BzATP treatment; . ŧŧp<0.01 compared with BBG/BzATP 5 µg treatment.

Following an ischemic insult, micro vacuolation of the cytoplasm was reported in neurons [199]. As the Cresyl Violet dye stains neurons with blue color, the infarct area is stained with a lighter blue color due to the loss in the number of intact neurons. As seen in Fig 3.6, the contralateral hemisphere which was assumed to be “not affected” from the MCAo was stained with a darker blue color, whereas the infarct area was lighter blue in color. Thus, the volume of infarct area was measured for each animal in all groups. In BBG-treated animals, infarct volume was significantly lower than vehicle control group, indicating that the number of intact neurons in BBG group was higher than either control or BzATP

groups. Administration of BzATP did not result in a significant impact on the infarct volume when compared with control group. However, when compared with BBG, infarct volume in BzATP group was significantly higher. When the agonist and antagonist were given together, BBG reversed the effect of BzATP on infarct volume and decreased it significantly when compared with the control group. Complete reversal of the unfavorable effect of BzATP with BBG raised the question whether the dosage used was not high enough. Therefore, another group of animals was added to the study, in which a 4-fold higher BzATP dose was used. Considering the solubility of BzATP, 20 µg/animal was the highest *in vivo* dose possible and that dose was given to this group. The results obtained with higher BzATP dose was almost similar to the ones obtained with lower dose, suggesting that BBG could block the deleterious effects of BzATP on infarct volume in focal cerebral ischemia in mice (Fig 3.6).

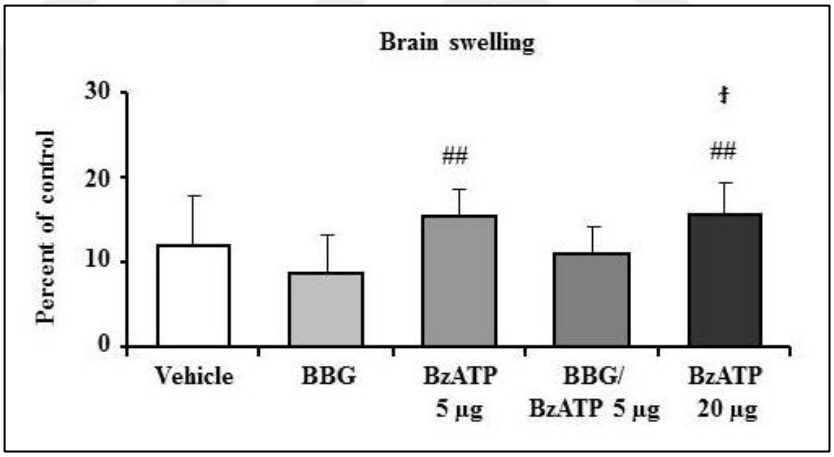


Figure 3.7. Brain swelling after 90 min of focal cerebral ischemia. Data are mean values ± S.D. (n= 7 animals/ group). ##p<0.01 compared with BBG treatment; ŧp<0.05 compared with BBG/BzATP 5 µg treatment.

Edema or brain swelling is one of the consequences of ischemia-reperfusion injury. As the severity of the edema increases, functional and behavioral consequences worsen [1]. Therefore, it is safe to state that decreasing the volume of edema is a desired outcome in the case of clinical cerebral ischemia treatment. Administration of BBG prior to MCAo resulted in a decrease in brain swelling (Fig 3.7). This decrease was not significant. On the other hand, in BzATP treated animals brain swelling was significantly higher than in BBG

treated animals. Percent of brain swelling did not increase with increased BzATP dose. However, when BBG and BzATP were administered together, percent of brain swelling was almost reduced to the level of BBG treatment alone.

Apart from the physiological parameters, functional outcomes should also be considered in the case of cerebral ischemia treatment. Therefore, neurological deficits of animals were scored in order to analyze the effects on motor function. According to the neurological scoring used, "0" indicates normal motor functions and "4" indicates the absence of any motor functions. In BBG treated animals, neurological deficit scores were significantly lower than the control group. Both BzATP groups showed slightly increased scores when compared with controls and significantly higher scores when compared with BBG group. When given together with BzATP, BBG significantly decreased the neurological deficit scores, indicating an improvement in motor neuron functions after 90 minutes of MCAo, followed by 24-hour reperfusion in mice (Fig 3.8).

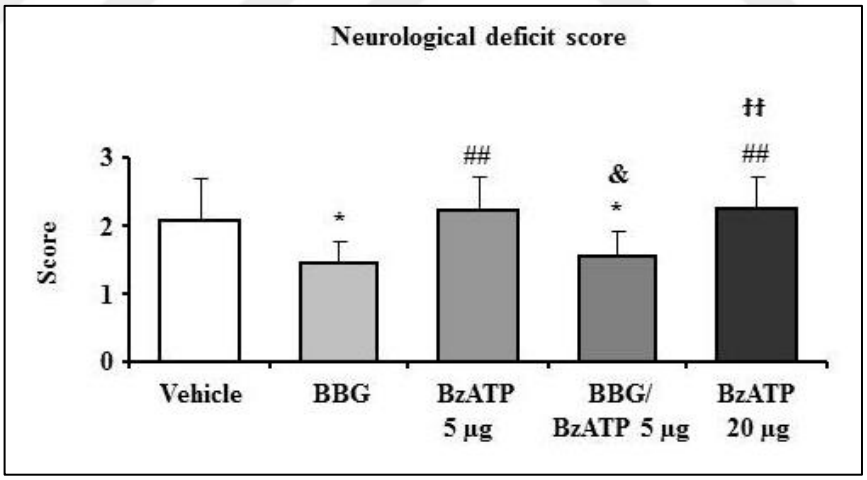


Figure 3.8. Neurological deficit score after 90 min of focal cerebral ischemia. Neurological deficit scores were evaluated 24 hr after 90 min of MCAo immediately before sacrification of animals. Data are mean values ± S.D. (n= 7 animals/ group). *p<0.05 compared with vehicle; ##p<0.01 compared with BBG treatment; &&p<0.01/ &p<0.05 compared with BzATP treatment; . ǂǂp<0.01/ ǂp<0.05 compared with BBG/BzATP 5 µg treatment.

## 3.4. P2X7R ANTAGONIST BBG PROTECTS AGAINST 30 MINUTES OF FOCAL CEREBRAL ISCHEMIA

In order to evaluate the role of $P2X_7R$ in neuronal survival, programmed cell death and related signaling mechanisms following an ischemic injury, 30 min of MCAo model was used. In 30-minute ischemic injury, due to the shorter time period of restricted blood flow in animals, the resulting infarct size is smaller than 90-minute ischemic injury. Therefore, this model is suitable for the study of neuronal survival and apoptosis mechanisms in the penumbra region. As with 90-minute MCAo model, changes in the blood flow of animals were closely monitored during the ischemia and reperfusion (Fig 3.9). As expected, blood flow was dropped to almost 15-20 per cent at the start of ischemia and increased slowly with the start of reperfusion. In the cases which the occlusion of the artery failed to decrease the blood flow or the removal of occlusion failed to increase the blood flow, animals were discarded from the experimental groups.

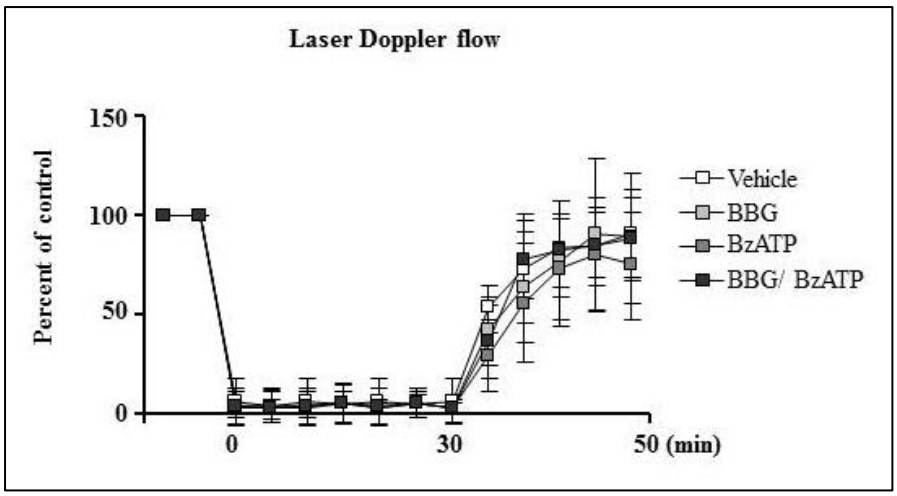


Figure 3.9. Blood flow changes after 30 min of focal cerebral ischemia. Percent decrease in blood flow during ischemia and percent increase during reperfusion was monitored for each of the operated animals.

In this model of ischemia, neurons in the affected penumbra undergo programmed cell death mechanisms and preventing apoptotic cell death while maintaining the normal physiological functions is one of the main goals of an ischemia treatment. To this end,

DNA fragmentation was assessed using terminal transferase biotinylated-dUTP nick end labeling (TUNEL) assay. TUNEL assay is a commonly used method for labeling the nicks in the DNA using terminal deoxynucleotidyl transferase enzyme to incorporate fluorescein tagged deoxy UTPs into these DNA nicks. It is used to identify the cells that are in late apoptosis period [200].

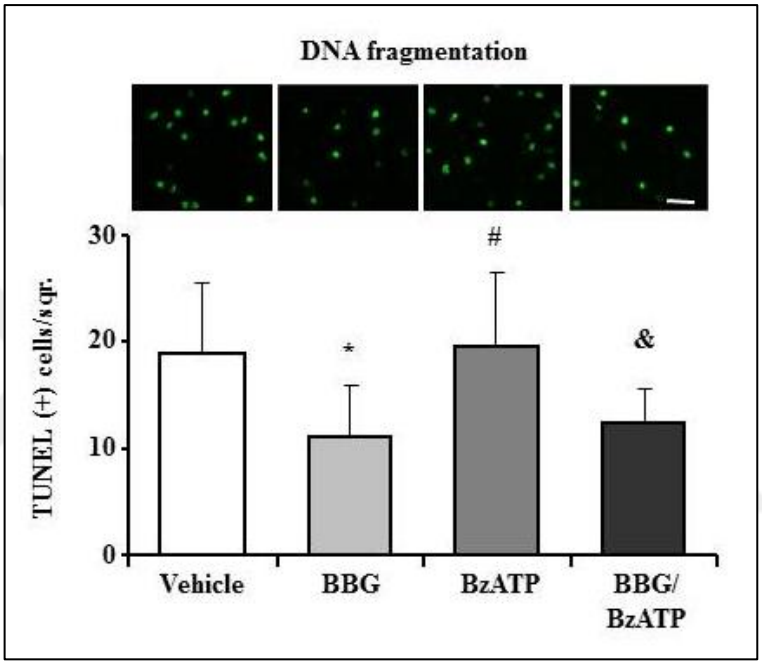


Figure 3.10. DNA fragmentation after 30 min of focal cerebral ischemia. Data are mean values ± S.D. (n= 7 animals/ group). *p<0.05 compared with vehicle; #p<0.05 compared with BBG treatment; &p<0.05 compared with BzATP treatment.

The results demonstrated that antagonism of $P2X_7$ receptor with BBG reduced the number of TUNEL(+) positive cells when compared with vehicle control. BzATP treatment slightly increased the number of TUNEL(+) positive cells when compared with the control group, but significatly increase the number of TUNEL(+) positive cells when compared with BBG-treated group. When BBG was given together with BzATP, it reduced the number of TUNEL(+) positive cells when compared with BzATP alone group. These results indicated that BBG alone decreased the number of apoptotic cells after 30-minute MCAo and that when applied together, BBG cancelled the impact of BzATP on apoptotic cell death (Fig 3.10).

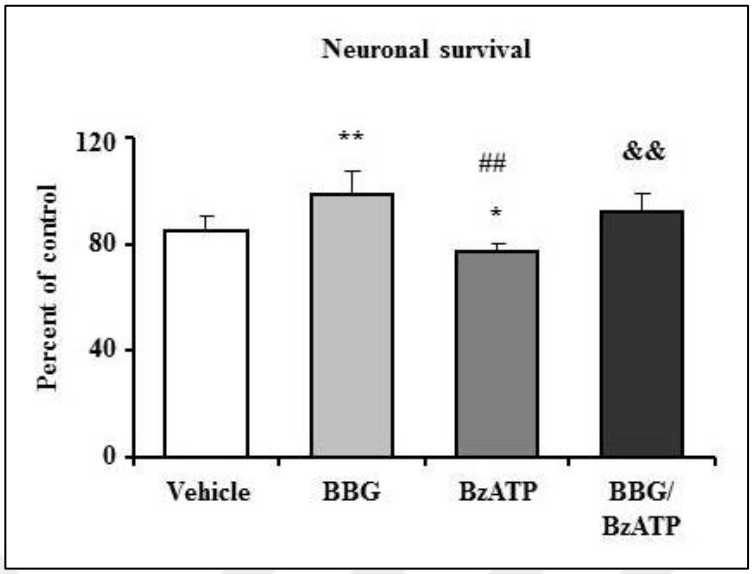


Figure 3.11. Neuronal survival after 30 min of focal cerebral ischemia. Data are mean values ± S.D. (n= 7 animals/ group). **p<0.01/ *p<0.05 compared with vehicle; ##p<0.01 compared with BBG treatment; &&p<0.01 compared with BzATP treatment.

Moreover, neuronal survival was also investigated. The results obtained correlated with DNA fragmentation assay results. BBG treatment increased the number of surviving neurons when compared with vehicle-given control group. Notably, BzATP resulted in a significantly less amount of surviving neurons when compared with vehicle control or BBG groups. Administration of BBG together with BzATP resulted in an increase in the amount of surviving neurons when compared with BzATP alone (Fig 3.11).

## 3.5. EFFECTS OF BBG AND BZATP ON SIGNALING PATHWAYS

The analyses so far indicated a favorable outcome from the inhibition of $P2X_7$ receptor in cerebral ischemia in mice. Therefore, it was decided to investigate the downstream signaling pathways responsible for this outcome. An extensive analysis of protein expression levels of survival kinases as well as their downstream targets were analyzed. First, whole protein lysates were prepared for vehicle-treated control group, BBG-treated group, BzATP-treated group and BBG+BzATP-treated group in the 30 minutes of MCAo model of focal cerebral ischemia. Equal amounts of total proteins were loaded into SDS-PAGE gels and the gels were transferred to polyvinylidene fluoride (PVDF) membranes

for Western blot analysis of protein levels. Each PVDF membrane was stripped at least once for the removal of bound antibodies and reprobed for beta actin protein in order to normalize the levels of protein expression. For the analyses of phospho proteins, membranes were first stripped of phospho protein antibodies and reprobed with an antibody which recognized the total of the same protein. Second stripping was carried out to remove the bound total protein antibody and lastly, the membrane was reprobed with beta actin antibody for the normalization of protein levels.

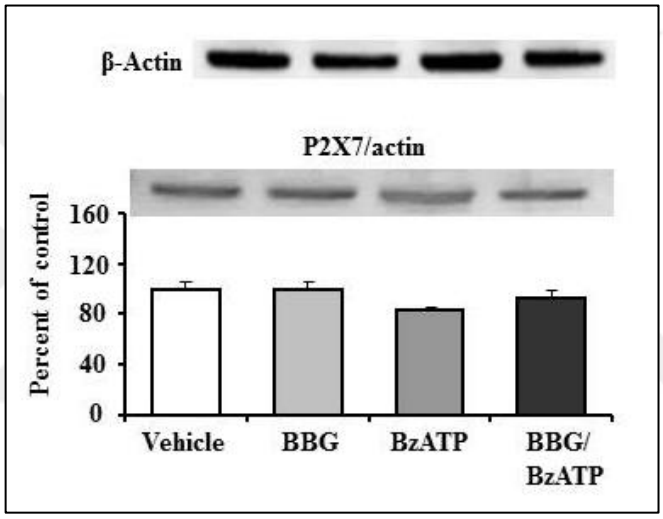


Figure 3.12. $P2X_7$ receptor protein levels after 30 min of focal cerebral ischemia.

It was suggested that the expression of $P2X_7$ receptor increased following an injury to the CNS [154], however the effects of the activator or the inhibitor on the expression of the protein was not documented. In this study, protein levels of the receptor was evaluated in each treatment group. The results showed that the treatments used in this study did not result in any significant change in the protein level of the receptor. Although in the BzATP administered group the protein level showed a trend towards decreasing and although such a decrease would suggest a negative feedback loop, it was not significant (Fig 3.12).

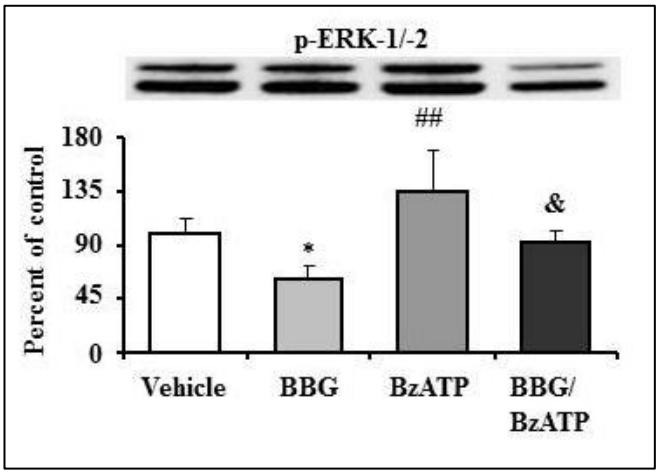


Figure 3.13. Phospho-ERK1/2 protein levels after 30 min of focal cerebral ischemia. *p<0.05 compared with vehicle; ##p<0.01 compared with BBG treatment; &p<0.05 compared with BzATP treatment.

Next, expression of the main survival kinase proteins was also evaluated. Phospho-ERK1/2 levels that were normalized against beta-actin protein level indicated that BBG treatment resulted in a significant decrease, whereas BzATP significantly increased the level of the proteins. When compared with BzATP alone group, BBG administration in addition to BzATP resulted in a significant decrease in the phospho-ERK1/2 levels (Fig 3.13).

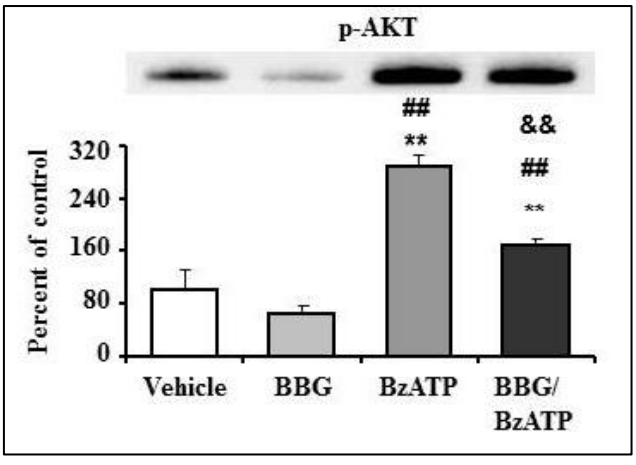


Figure 3.14. Phospho-AKT protein levels after 30 min of focal cerebral ischemia. **p<0.01 compared with vehicle; ##p<0.01 compared with BBG treatment; &&p<0.01 compared with BzATP treatment.

Protein levels of phospho-AKT was also evaluated for each experimental group. Interestingly, BBG treatment did not result in any significant change. On the other hand, BzATP group showed an almost 3-fold increase in phospho-AKT levels compared to vehicle control group. Even when BBG was administered together with BzATP, levels of phospho-AKT was partially reversed but was still statistically significant, suggesting that binding of BBG to the receptor might not be inducing the same pathways as BzATP did (Fig 3.14).

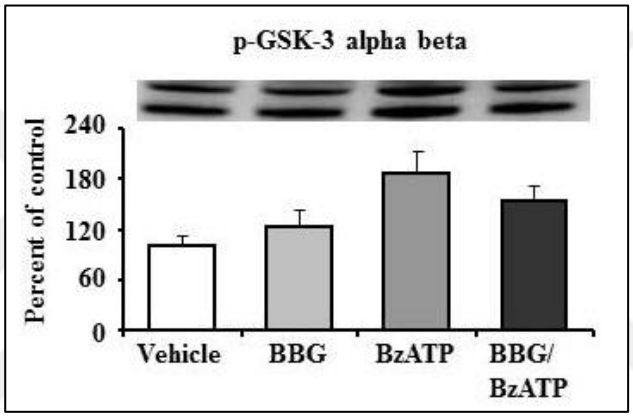


Figure 3.15. Phospho-GSK3 alpha and beta protein levels after 30 min of focal cerebral ischemia.

Phospho-GSK3 alpha and beta proteins were evaluated in the same graph. According to their normalization to beta-actin bands, neither of the treatments resulted in any significant change in these proteins. However, although not significant, a trend towards increasing could be observed with BzATP treatment and this increase was partially prevented when administered in the presence of the antagonist, BBG (Fig 3.15).

When total GSK3-alpha and total GSK3-beta proteins were normalized against beta-actin protein and compared among the experimental groups, it was observed that these two proteins were regulated at a somewhat similar extent. BBG treatment did not alter the levels of these proteins significantly. In BzATP group, there were statistically significant increases in both proteins when compared with vehicle control as well as BBG group. In BBG+BzATP group, total GSK3-alpha and total GSK3-beta proteins were significantly increased when compared with vehicle control or BBG-treatment groups (Fig 3.16).

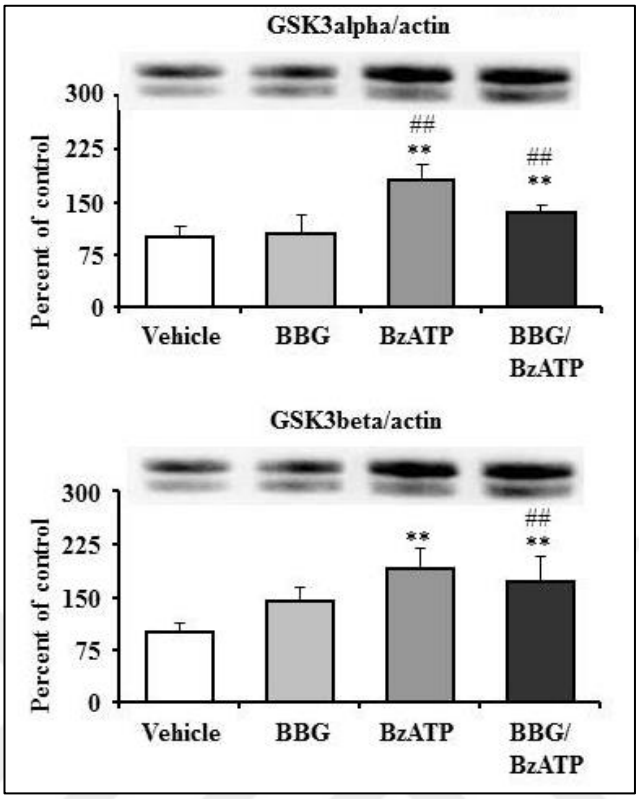


Figure 3.16. GSK3 alpha and beta protein levels after 30 min of focal cerebral ischemia. **p<0.01 compared with vehicle; ##p<0.01 compared with BBG treatment.

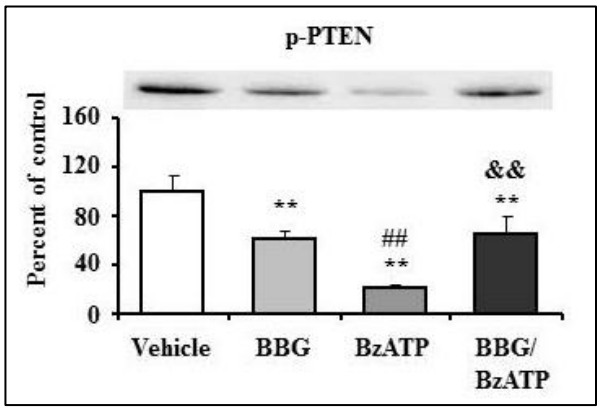


Figure 3.17. Phospho-PTEN protein levels after 30 min of focal cerebral ischemia. **p<0.01 compared with vehicle; ##p<0.01 compared with BBG treatment; &&p<0.01 compared with BzATP treatment.

In BBG-treated group, phospho-PTEN, a negative regulator of phospho-AKT, was significantly lower than the vehicle control group. Interestingly, phospho-PTEN was

further decreased in BzATP group when compared with BBG-treatment. In BBG+BzATP together treatment group, phospho-PTEN level was significantly lower than vehicle control and was significantly higher than BzATP alone group (Fig 3.17).

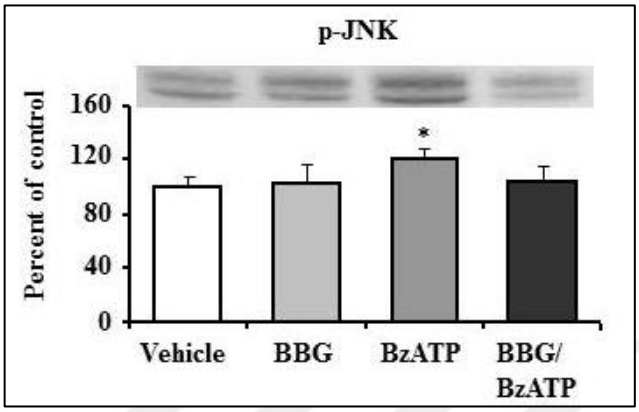


Figure 3.18. Phospho-JNK protein levels after 30 min of focal cerebral ischemia. *p<0.05 compared with vehicle.

Phospho-JNK protein levels were also evaluated for each experimental group following focal cerebral ischemia. The level of this protein was somewhat stable in the groups evaluated, except in the BzATP alone group in which the protein was significantly higher than the vehicle control group. Administration of BBG with BzATP, however, decreased the level of the protein to a degree that was slightly and insignificantly higher than the vehicle control (Fig 3.18).

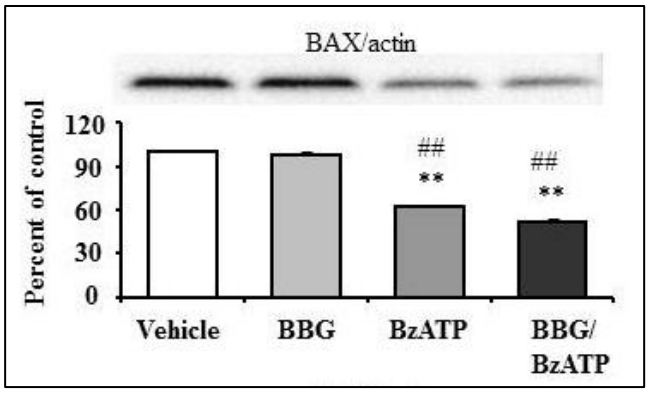


Figure 3.19. Bax protein levels after 30 min of focal cerebral ischemia. **p<0.01 compared with vehicle; ##p<0.01 compared with BBG treatment.

Pro- and pre-apoptotic protein levels were also investigated in this study. One such proapoptotic protein was Bax, whose levels were not affected from a treatment with BBG. Interestingly, BzATP alone as well as BBG+BzATP resulted in a statistically significant decrease in the level of Bax when normalized against the level of beta-actin protein (Fig 3.19).

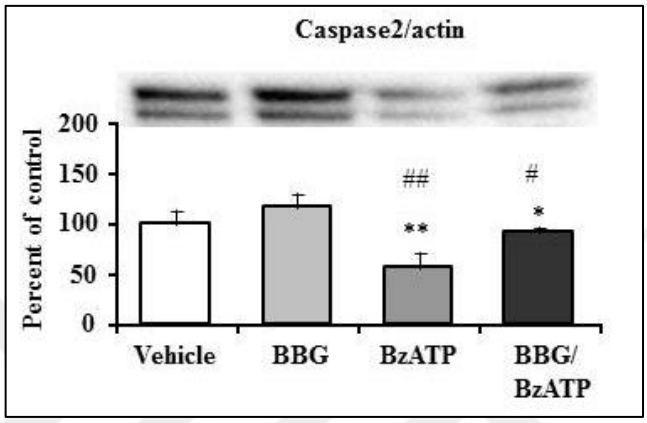


Figure 3.20. Caspase-2 protein levels after 30 min of focal cerebral ischemia. **p<0.01 compared with vehicle; ##p<0.01 compared with BBG treatment; &&p<0.01 compared with BzATP treatment.

Protein level of Caspase-2 was also evaluated for each experimental group. In the BBG group, the protein did not represent any significant change. On the other hand, BzATP group showed a statistically significant decrease in Caspase-2 levels when compared with the vehicle control group or when compared with BBG-treated group. Even when BBG was administered together with BzATP, protein level of Caspase-2 was only partially increased but the decrease that was primarily caused by BzATP was still statistically significant when compared with the vehicle control or BBG groups (Fig 3.20).

In the BBG group, Caspase-9 level was not significantly altered. On the other hand, BzATP group showed a statistically significant decrease in Caspase-9 levels when compared with the vehicle control group or when compared with BBG-treated group. Even when BBG was administered together with BzATP, decreased levels of Caspase-9 was not affected by the addition of BBG. In BBG+BzATP together group, Caspase-9 was significantly lower than both vehicle control and BBG-treated group (Fig 3.21).

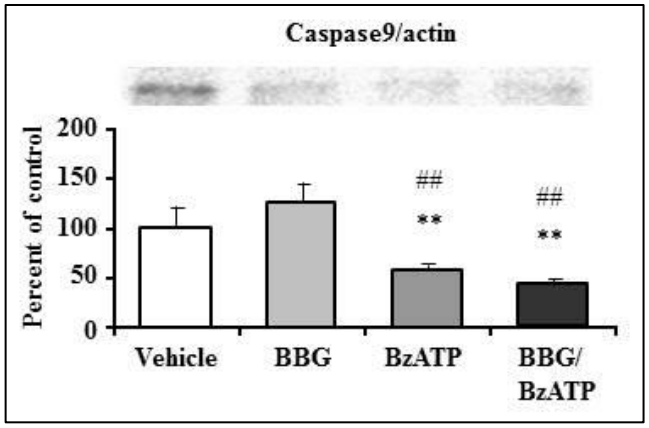


Figure 3.21. Caspase-9 protein levels after 30 min of focal cerebral ischemia. **p<0.01 compared with vehicle; ##p<0.01 compared with BBG treatment.

### 3.6. P2X7 RECEPTOR EXPRESSION IN PRIMARY NEURON CULTURE

For further analyses of the function of $P2X_7$ receptors in neuronal survival and apoptotic cell death *in vitro*, distribution of the receptor was investigated in primary neuron culture. To this end, cortices from the brains of newborn mouse were removed, dissociated and cultured. Isolated cells were kept in culture for 7 days, until they were considered as "mature" neurons and formed synapses and networks with each other. At the $7^{th}$ day in culture, cells were fixed and stained with anti-$P2X_7R$ antibody together with either neuronal nuclei marker, NeuN or astroglial cell marker, GFAP. The results of double immunostaining procedures showed that $P2X_7$ receptors only colocalized with NeuN positive cells, indicating a neuron-specific expression (Fig 3.22).

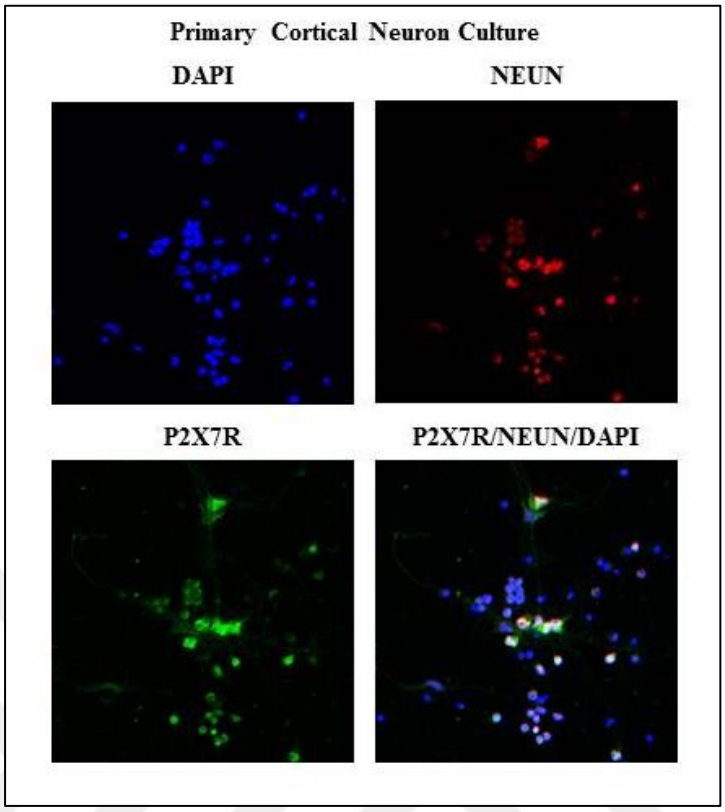


Figure 3.22. Immunoreactivity of $P2X_7R$ in primary cortical neuron culture. In primary neurons, $P2X_7$ receptor expression was demonstrated in NeuN+ cells. DAPI was used as a nuclear counter stain.

GFAP positive cells had a characteristic morphology that was consistent with astrocytes *in vitro* and they were negative for $P2X_7R$ immunoreactivity (Fig 3.23).

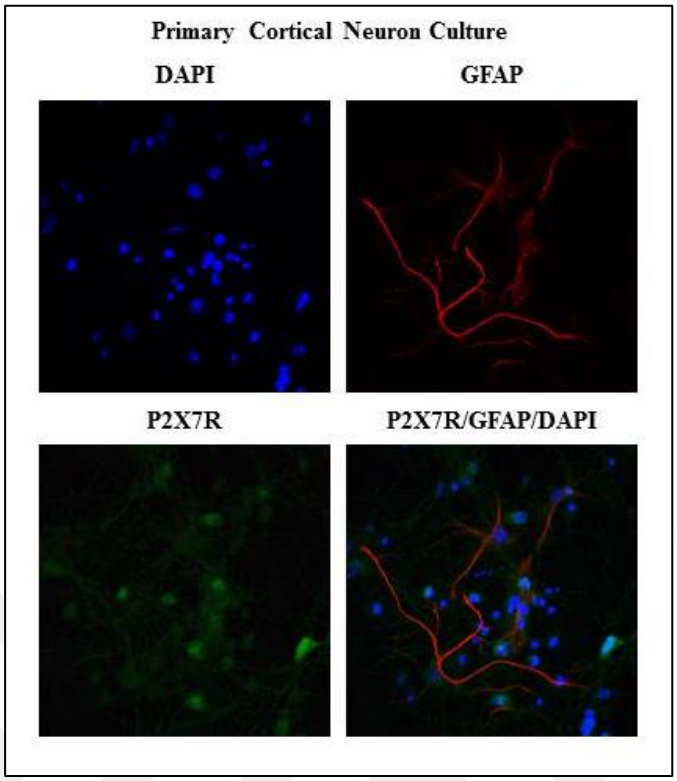


Figure 3.23. $P2X_7R$ and GFAP double staining in primary cortical neuron culture. In primary neurons, $P2X_7$ receptor expression did not colocalize with GFAP+ cells. DAPI was used as a nuclear counter stain.

## 3.7. INTRACELLULAR CALCIUM CONCENTRATION IN PRIMARY NEURON CULTURE

In this study, it was observed that $P2X_7$ receptor antagonist, BBG, increased neuronal survival in both optic nerve transection and focal cerebral ischemia models. Based on that, it was initially assumed an opposite effect for $P2X_7$ receptor agonist, BzATP. However, BzATP did not result in any further toxic effect in neuronal survival. On the other hand, when the signaling pathways responsible for those effects were evaluated, evidence supporting the protective role of BBG was not obtained. In fact, protein levels of survival-promoting pathways (such as, pAKT, pERK) were higher in the BzATP-treated group. This raised the question whether BzATP and BBG exerted their effects through secondary messengers, such as intracellular $Ca^{2+}$. It is a well-known feature of P2X receptors that,

once activated, they increase mobility of intracellular $Ca^{2+}$ levels. Therefore, it was decided to track the changes in $Ca^{2+}$ levels in response to agonist or antagonist administration.

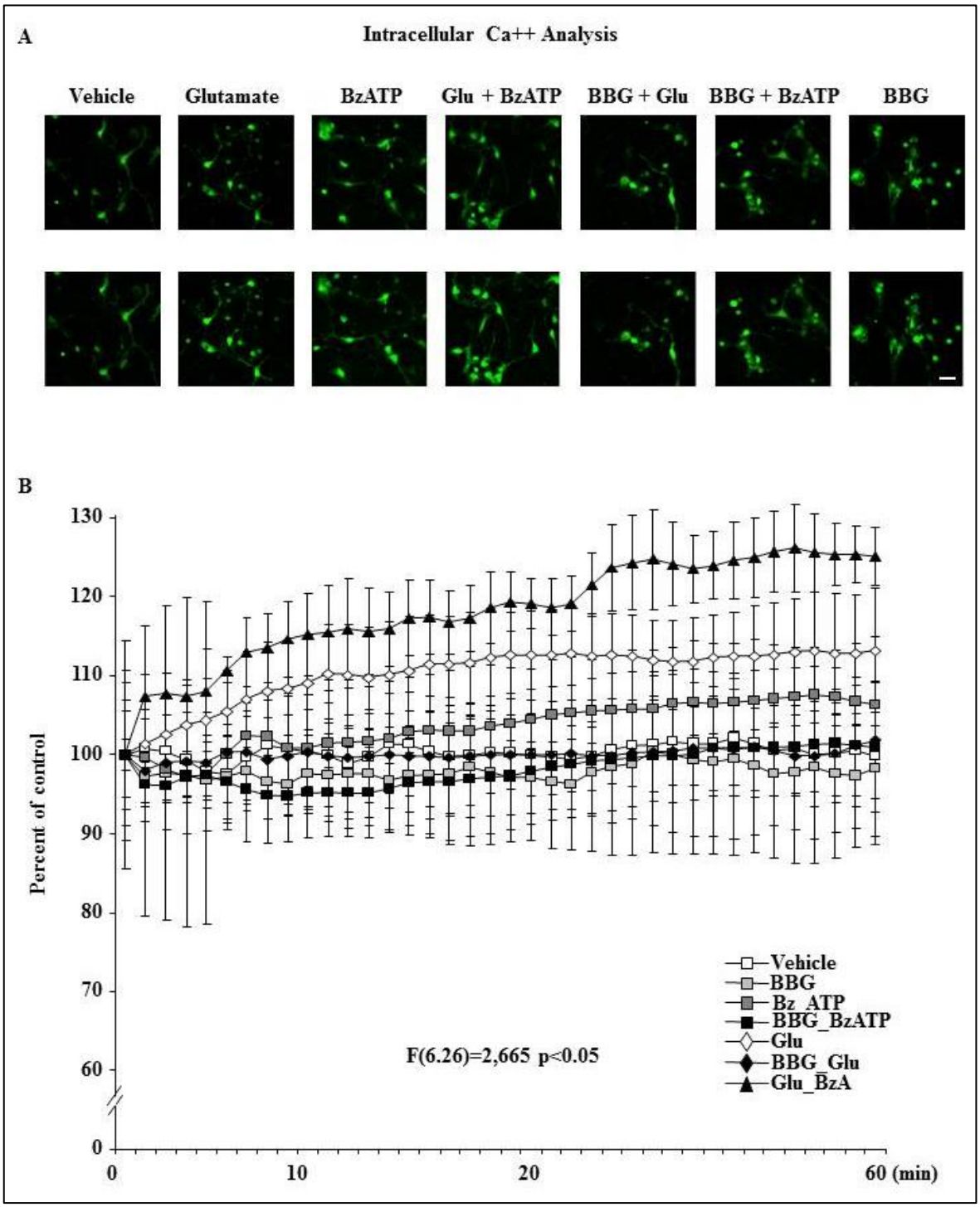


Figure 3.24. Intracellular calcium levels in primary cortical neuron culture.

To be able to track these changes in real time, an *in vitro* model in primary cortical neuron culture was used. Fura-2-Acetoxymethyl ester (Fura-2AM) is a cell permeable and high affinity intracellular calcium indicator and is frequently used to image the changes in intracellular $Ca^{2+}$ levels. When Fura-2AM is applied to culture medium, it is readily taken up by the cells, de-esterified by cellular esterases and the subsequent FURA-2 free acid binds to intracellular free $Ca^{2+}$, and upon binding, the dye changes its excitation peak from 240 to 380 nm. The effects of receptor antagonist and agonist on intracellular $Ca^{2+}$ levels were demonstrated in Figure 3.24.

Intracellular $Ca^{2+}$ levels were not altered by BBG and were almost indistinguishable from the levels obtained in vehicle control group. When compared with vehicle controls, BzATP resulted in a slight increase in the intracellular $Ca^{2+}$ levels. This increase was completely blocked by BBG when given together with BzATP. Glutamate excitotoxicity is one of the pathophysiological processes resulted from focal cerebral ischemia and causes extensive $Ca^{2+}$ influx and eventually extensive cell death [1]. Therefore, to mimic glutamate toxicity conditions in the ischemic brain, cultured primary cortical neurons were stimulated with glutamate. As expected, glutamate increased the intracellular $Ca^{2+}$ levels. BBG, when given together with glutamate, reversed the increase in calcium levels that were previously induced by glutamate. When BzATP was administered together with glutamate, a distinctive increase in the amount of intracellular calcium was observed.

# 4. DISCUSSION

The objective of the thesis was to investigate the effects of activation and inhibition of the receptor on neuronal survival following ischemic stroke and optic nerve (ON) transection in mice. Due to the conflicting data in the literature, it was important to provide solid evidence for the role of the receptor. There was even a debate in whether the receptor was expressed in neuronal or glial or both cell types in the CNS. Therefore, before starting further experiments, the expression profile of the receptor in the brain and in the retina was identified. It was demonstrated for the first time using immunofluorescence analyses that neuronal cells of the brain widely expressed the protein. In contrast to the literature stating a glial expression, GFAP(+) cells did not colocalize with $P2X_7R$ immunoreactive cells in striatal or cortical brain sections or in primary cortical neuron cultures. $P2X_7R$ immunoreactivity was also demonstrated in retinal ganglion cells and in bipolar cells of inner nuclear layer in frozen retinal sections prepared after optic nerve transection. In all the optic nerve transection experiments, only one eye was operated, whereas the other eye was used as a control. This allowed the direct comparison of the treatment administered to the eye with control tissue.

In this study, double immunofluorescence analyses of at least 3 independent animals were carried out. For all immunofluorescence analyses, two negative controls; one without primary antibody and the other without secondary antibody were also carried out in order to eliminate the risk of false positives. Furthermore, absence of $P2X_7R$ immunoreactivity in astroglial cells could raise the question whether the antibodies used were faulty. Therefore, the double immunofluorescence analyses were repeated with a different antibody from a different vendor. The data obtained with the second antibody was consistent with the ones obtained earlier. As a result, it can be safely suggested that $P2X_7$ receptor is expressed in the neuronal cells in the brain and in retinal ganglion cells and in bipolar cells of inner nuclear layer, whereas astroglial cells do not express this receptor in mice.

The ongoing debate whether $P2X_7Rs$ have protective or degenerative effects on the expansion of central nervous system injuries was inconclusive at the time of the conductance of this thesis. Following the identification of the expression profile of the

receptor, it was decided to investigate the outcomes of activation *vs*. inhibition following ischemic injury as well as optic nerve transection. To this end, a potent and selective antagonist, Brilliant Blue G (BBG) that was widely used in the literature was chosen. For the activation of the receptor, BzATP was chosen as it is the most potent agonist of the receptor. In the optic nerve injury model, antagonism of $P2X_7R$ resulted in the protection of neuronal cells. On the other hand, BzATP administration did not cause any significant effect on the survival of retinal ganglion cells when compared with vehicle control group, but BzATP caused a significantly decreased number of surviving RGCs than BBG group. In the ischemic injury models, BBG also provided protective effects on all the parameters studied. It was assumed that as BzATP activated the channel, it would result in worsened outcomes following an injury. Interestingly, BzATP did not significantly alter the viability of neurons, nor caused an extensive amount of cell death. To rule out the insufficiency of the dose used in exerting unfavorable effects, another group of 4-fold increased BzATP dose was included. That was the maximum concentration that can be given *in vivo* due to the solubility of the molecule. However, the results obtained from 4-fold increased BzATP dose did not differ from the previous results. It could be speculated that BzATP could not further activate the receptor that was initially activated by excess ATP in the extracellular space due to ischemic injury. This could be because the two pharmacological agents used are acting on the different regions of the receptor, hence activating different pathways. In addition, when BBG was used together with BzATP, BBG was able to reverse the unfavorable outcomes of BzATP administration, suggesting a more potent pathway involved upon BBG stimulation.

It should be noted that in this thesis two different ischemia models were used: 90 min of MCAo and 30 min of MCAo. 90 min ischemia model is a severe acut injury model and used to evaluate the effects of treatments on infarct volume, brain swelling and neurological deficit scores. In the 30 min ischemia model, disseminate neuronal injury in the striatum is induced and this injury mainly affects small and medium sized neurons [201, 202]. This shorter injury model is less severe and allows the analyses of the changes in the protein levels that are involved in survival and apoptotic pathways.

Based on the intriguing results obtained with BBG, BzATP and with their combination, it was decided to evaluate the expression of proteins that were involved in different cellular signaling pathways. First, expression of $P2X_7R$ was evaluated. Although the literature

suggested an increase in the expression of the receptor following an ischemic injury, a significant change in the level of this protein was not observed. This may be due to the use of different animal species or different animal strains or the use of different models to mimic focal cerebral ischemia. Especially with P2X receptors, it is quite common that the receptors exert inter-species differences and hence, an agonist or an antagonist that is effective in one animal species may not be effective in others [27]. Furthermore, in *in vitro* studies of these purinergic receptors, where they are ectopically expressed in cells that do not normally express them, homomer or heteromer structures that did not normally form *in vivo* were observed [27]. This may partly explain the discrepancy among the reports in the literature.

On the other hand, it was reported that activation of $P2X_7$ receptor by ATP or another agonist resulted in the dephosphorylation of the receptor which may be responsible for the feedback control of the receptor [203]. In addition, it was demonstrated that ATP binding to the receptor activated mechanisms that phosphorylated and eventually redistributed the receptor between plasma membrane and cytosol [204]. It was also suggested that this phosphorylation and redistribution was due to the activation of GRK3 protein [204]. Interestingly, in the same study, authors also reported a nuclear localization for $P2X_7$ receptor and postulated that this translocation may be resulted from phosphorylation of different residues and may be responsible for the activation of different pathways.

Regarding the modulation of signaling pathways related to survival, BzATP was found to activate survival kinases, AKT and ERK-1/2, both of which were known to take part in ameliorating the ischemic tissue loss [205]. The upregulated expression of these kinases in response to $P2X_7$ receptor stimulation is consistent with previous studies [206, 207]. Additionally, the enzymes GSK3 alpha and beta which affect numerous proteins ranging from transcription factors to cell cycle regulators, were reported to have increased level of expression in response to $P2X_7$ receptor activation through a mechanism different than AKT [208]. Based on that, an increase in GSK3 alpha and beta expressions in response to BzATP treatment can be considered consistent with the previous reports. Interestingly, JNK, a kinase having both survival related and apoptosis inducing properties, was increased in response to BzATP. Considering the role of JNK in secondary cell damage following increased intracellular $Ca^{2+}$ levels [209, 210], it can be suggested that BzATP-mediated activation of JNK pathway leads to cell death. Intriguingly, BzATP increased the

protein levels of both survival kinases and stress kinases. While the reason behind this is still not known, it can be postulated that this very fact may resulted in the effects of BzATP following ischemic injury or optic nerve transection, because BzATP did not further worsen the outcomes in terms of survival when compared with vehicle control. Even though BzATP did not have any negative effects, it did not have positive effects on cellular survival either and this could be attributed to the activation of both survival and stress kinases. It is tempting to speculate that the effects of activation of $P2X_7$ receptor was dependent upon the context of its being activated, i.e. dependent on the presence of other factors, such as extracellular ATP or intracellular $Ca^{2+}$ overload.

On the other hand, BBG decreased the levels of pAKT and pERK-1/2. BzATP, even when administered together with BBG, triggered the otherwise inhibited survival and cellular proliferation signaling cascades via increasing the levels of pAKT, pERK-1/2, and reducing the level of pPTEN. The reducing effects of BBG on the levels of survival kinases were incompatible with its neuroprotective role as demonstrated in neuronal survival assays. These results suggested that BBG exerted its neuroprotective functions through other mechanisms that involve $Ca^{2+}$ influx-regulated signaling cascades (such as $Ca^{2+}$-dependent endoplasmic reticulum stress-activated unfolded protein response pathway), instead of the well-known AKT or ERK-1/2 pathways. Another mechanism involved in BBG-mediated neuroprotection may involve glutamate receptors. During ischemia, glutamate is released to the extracellular space. When released, it causes $Ca^{2+}$ influx through different mechanisms, and recent studies suggested that $Ca^{2+}$ overload may not be the absolute inducer of cell death. Instead, the new theory suggested that cellular death mechanisms were activated by a "source-dependent" $Ca^{2+}$ influx and the subsequent activation of specific pathways [210]. One other mechanism involved in BBG-mediated neuroprotection may involve pannexin channels. It was reported that pannexin channels contributed to the ischemic damage by helping $P2X_7$ receptors form pore structures in plasma membrane [51]. Pannexin channels also contributed to $Ca^{2+}$ conductance of $P2X_7$ receptors and NMDA receptors [51-55]. On the other hand, BBG also blocked pannexin channels mediated $P2X_7$ receptor pore formation and this phenomenon may be the reason behind the neuroprotective effects observed with BBG.

In conclusion, it was demonstrated that $P2X_7$ receptors were expressed in neurons of CNS as well as in retinal ganglion cells and bipolar neurons of retina. It was also demonstrated

that antagonism of the receptor increased the number of surviving neurons while decreasing the amount of apoptotic cells through mechanisms possibly involving intracellular $Ca^{2+}$ ion mobility as well as other $Ca^{2+}$ ion-regulated receptors, such as glutamate receptors. It was also demonstrated that BzATP stimulated the activation of both survival and apoptotic mechanisms following an injury that dual activation of these pathways may result in survival or death in a context-dependent manner. Future studies are required to elucidate the underlying signaling pathways activated by antagonism and agonism of $P2X_7$ receptor in order to provide solid insights into potential therapy strategies in stroke patients.

# 5. CONCLUSION

In this thesis, it was aimed to investigate the effects of activation and inhibition of the receptor on neuronal survival following ischemic stroke and optic nerve (ON) transection in mice. To this end, *in vivo* animal studies as well as *in vitro* primary culture assays were performed. Protein expression studies carried out in tissues of an in vivo ischemia model. This helped us identify the proteins involved in the neuronal survival and apoptotic pathways after the application of $P2X_7$ receptor agonist BzATP and antagonist BBG following an ischemic injury. Since the receptor antagonist BBG ameliorated the detrimental effects of ischemia and helped increase the number of surviving neurons, survival kinases and apoptosis-related proteins were analyzed. This is the first study which identified the downstream signaling pathways regulated by $P2X_7$ receptor activation or inhibition under injury conditions. To our knowledge, this is the first study demonstrating the BzATP's effect on signaling pathways in ischemic injury in mice. BzATP did not worsen the outcomes of ischemia, while BBG had ameliorating effects. Here, it was demonstrated that this effect was mainly due to the inability of BzATP-induced receptor activation did not increase the expression of pro-apoptotic proteins. Instead, BzATP induced expression of survival kinases ERK-1/2 and especially, AKT. This is the first study which demonstrated an increase in the expressions of AKT and ERK-1/2 following MCAo in mice. Nonetheless, it was also demonstrated that BzATP increased the expression of stress kinase, JNK. This may explain the effects of BzATP on the level of surviving and apoptotic neurons, since BBG resulted in favorable outcomes compared to BzATP following ischemic and optic nerve injuries. The findings related to BzATP's regulatory effects on the expression of significant survival pathways could be considered as one of the main contributions to the literature. However, future studies should be carried out in order to expand the findings in terms of expression of proteins involved in survival and $Ca^{2+}$ ion regulation.

It has been well known that $P2X_7$ receptor antagonist BBG could increase neuronal survival in ischemic injury. However, the pathways responsible for such increase was not known at the time of the start of this study. This is the first study investigating the expression of downstream signaling proteins following the activation of the receptor in ischemia. Surprisingly, it was demonstrated that BBG did not increase survival kinases.

Instead, it ameliorated the amount of intracellular $Ca^{2+}$ overload. Here, it was suggested for the first time that BBG's neuroprotective role could be associated with its effects on intracellular $Ca^{2+}$ levels and possibly, on $Ca^{2+}$-regulated proteins or plasma membrane channels, instead of the well-known survival proteins, such as AKT. Based on these findings, it may be possible in the future to design pharmacological small molecules which can both inactivate $P2X_7$ receptor and activate the survival pathways, so that the treatment used can be beneficial in clinical trials. This study may also encourage the use of combination treatments that can activate the survival mechanisms, while inhibiting $Ca^{2+}$-related cellular toxicity.

On the other hand, the results suggested another window of opportunity for the use of $P2X_7$ receptor antagonist, BBG, in cancer treatment. Considering the fact that BBG did not increase the expression of survival and proliferation-related proteins, its use for the inhibition of the receptor in different cancer types. It has been reported that $P2X_7$ receptor expression and subsequent intracellular $Ca^{2+}$ levels were altered in several cancer cell lines, including lung cancer [211], breast cancer [212], pancreatic cancer [213], glioma [214], prostate cancer [215], cervical cancer [216] or ovarian cancer [217]. It was also reported that this receptor was involved in cancer-related pain, especially in bone cancer [218]. Therefore, the results obtained in this study suggested that BBG could be used in cancer treatment and the use of BBG in cancer treatment could provide beneficial effects in terms of both inhibition of cancer proliferation and inhibition of cancer-related pain in patients. Based on these data, this study encourages future work in order to investigate the effects of inhibition of $P2X_7$ receptor in treatment of cancer growth and pain in clinical trials.

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