The P2X Receptor Mediated Regulation of Inhibitory Synaptic Transmission

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Declaration

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Abstract

In the central nervous system ATP can be released by neurons and glial cells through similar pathways as other neurotransmitters. Extracellular ATP targets P2X purinoreceptors, causing an influx of Ca^{2+} ions which transmit very important messages for neurons and glial cells. Ionic signals modulated by P2X receptors can be transformed into the modulation of $GABA_A$ receptors. This work describes the universal interaction of P2X receptors down-regulating $GABA_A$ mediated currents via a Ca^{2+}-dependent mechanism.

I have shown that the postsynaptic modulation of GABA currents by P2X receptors is present in both the peripheral (DRG neurons) and central nervous system (cortical neurons). This effect is strongly regulated by an intracellular signalling cascade involving Protein Kinase C. Furthermore, tonically-activated $GABA_A$ receptors expressed on central neurons, containing alpha5 and delta subunits are also affected by this P2X and $GABA_A$ receptor interaction.

The purinergic modulation of $GABA_A$ receptors has significant implications for synaptic plasticity, an important mechanism of learning and memory in the central nervous system. The down-regulation of $GABA_A$ receptors on the postsynaptic membrane enhances the activity of NMDA receptors and thus increases synaptic efficacy. The study of the purinergic involvement in the induction of Long Term Potention is far from being understood. Ionotrophic purinoceptors represent a novel pathway of $GABA_A$ receptor modulation: release of ATP from neurons and astrocytes activates Ca^{2+}- signalling via purinergic P2X receptors, which can regulate $GABA_A$ receptors activity in both peripheral and central nervous system compartments. The physiological implications of this regulatory pathway are yet to be investigated.

Chapter 1
Introduction

For decades the nucleotide adenosine triphosphate (ATP) was merely considered a form of molecular currency for the transfer of energy between cells (Knowles, 1980). However, in 1972, experimental analysis revealed the potential of ATP to function as a neurotransmitter (Burnstock, 1972). This new extracellular role for an intracellular entity was widely disputed; engendering a flurry of research there-on-after. Now, there is a plethora of strong evidence indicative of the extracellular signalling properties of ATP, leading to the discovery of the purinergic receptors.

The role of extracellular ATP in fast excitatory synaptic transmission in the peripheral nervous system is well established (Silinksy et al., 1992). However, data from the last decade has demonstrated that ATP can also mediate fast excitatory transmission in the central nervous system (Jahr and Jessell, 1983; Edwards et al., 1992). Extracellular ATP can be released vesicularly from central neurons at both inhibitory and excitatory nerve terminals alone or along with GABA and glutamate (Burnstock, 2009). The precise demonstration of the fast excitatory purinergic transmission has been reported in many brain regions including, the spinal cord, hippocampus and the neocortex (Sawynok et al., 1993; Salgado et al., 1996; Pankratov et al., 2003b) where the action of extracellular ATP is mediated by the ionotrophic P2X receptor family.

Multiple purinergic receptors have been found to be expressed on another type of brain cell called glial cells. Despite being referred to as "brain glue," non-excitable cells which are

responsible for maintaining the surrounding environment of neurons, glial cells can also vesicularly release ATP, via a mechanism termed gliotransmission. Consequently, many important neuron-glial cell communications in the central nervous system have been described, introducing the concept of the tripartite synapse.

The high permeability of purinergic receptors to calcium at low membrane potentials creates a strong calcium dependence on the regulatory actions of extracellular ATP. P2X receptors ability to transmit robust calcium signals is a principal property which allows them to modulate the activity of other receptors and thus synaptic transmission. Intracellular calcium levels can affect the function of a plethora of membrane proteins including GABA receptors. To date research which focuses on the direct influence of ATP on inhibitory nerve terminals in the tripartite synapse is extremely limited, hence this current body of work concentrates on this aspect of purinergic signalling and predominantly focuses on GABA homeostasis in the central nervous system.

1.0 Physiology of purinergic receptors

One of the primary functions of extracellular ATP is to act as a ligand to activate the P2 purinergic receptors, which are non-selective cation channels permeable to Na^+, K^+ and Ca^{2+} ions (Ralevic and Burnstock, 1998). The secondary effects of purinergic neuromodulation are governed by the rapid breakdown of ATP in the synaptic cleft by resident ectonucleotidases. One of the breakdown products of ATP, adenosine diphosphate (ADP), can activate some P2 receptor subtypes. Another compound produced via the breakdown of ATP is adenosine, which acts as a ligand to the P1 receptors, a subclass of purinoreceptors that are expressed on all neuronal cells (North and Verkhratsky, 2006). The properties of adenosine in neurotransmission

are well studied, however due to its rapid degradation upon release; research exploring the effects of ATP on postsynaptic receptors still remains rather fragmented.

The P2 receptor family are further sub-categorised into the ionotrophic P2X receptors and the metabotropic P2Y receptors, distinctly coupled to G-proteins (Abbracchio and Burnstock, 1994; Illes and Ribeiro, 2004). Multiple purinergic receptors have been found to be expressed on another type of brain cell called glial cells, which can also release ATP via a process called gliotransmission. Consequently, important purinergic mechanisms involving neuron and glial cell interactions have been described in recent years (James and Butt, 2002; Lalo et al., 2008; Palygin et al., 2010; Koles et al., 2011).

It is acknowledged that the P2X receptors are comprised of seven different receptor isoforms, namely P2X1 to P2X7, which are widely distributed in both the peripheral and central nervous systems. Each subunit measures approximately between 379 and 595 amino acids in length (Khakh et al., 2001). The P2X receptor subunits exhibit both homomeric and heteromeric stoichiometry, evoking a wide range of kinetically distinct receptor functions. Torres et al. (1999) suggested the existence of several hetero-oligomers for P2X receptors: subunits 1/5, 1/6, 2/3, 2/5, 2/6, 3/5, 4/5, 4/6, 5/6. Research has demonstrated that P2X receptors assemble with a general ratio of at least three monomers to one functional receptor (North and Verkhratsky, 2006). For example, previous data has demonstrated that the molecular assembly of receptor subunits P2X1 and P2X3 consists of stable trimers on the cell membrane (Nicke, 1998). Additionally, it is established that P2X2 to P2X5 can form stable and functional homomeric receptors (Torres et al., 1999). Immunohistochemical studies and dark-field photomicrographs have documented the abundant distribution of P2X2 and P2X3 mRNA in adult DRG neurons (Vulchanova et al., 1997). The protein subunits may assemble as homomeric or heteromeric

receptors (P2X2/3) (Grubb and Evans, 1999). However, in the central nervous system the most predominant functional P2X receptor expressed on neurons is P2X4 (Norenberg and Illes, 2000; Lalo et al., 2007).

The characterisation of the currents elicited by the different subunit combinations of P2X receptors on DRG neurons have suggested the presence of "fast," "slow" and "mixed" type currents (Grubb and Evans, 1999; Sokolova et al., 2001). A comparison of the pharmacological profiles of the different receptor subunits combined with the tissue distribution patterns of P2X receptor mRNA, can determine which specific P2X receptor subtype corresponds to a particular characteristic response. The variety of P2X subunit combinations can yield a range of P2X receptor characteristics, including kinetically distinct phenotypes and various biophysical properties such as different sensitivities to receptor agonists and antagonists between the purinergic receptor subtypes. Therefore, the distinctive nature of the variety of P2X receptors expressed in the peripheral and central nervous systems provides a significant platform for a wide range of ATP signalling with a dynamic array of modulatory functions.

In contrast to the ionotrophic P2X receptors the metabotropic P2Y receptors are G-Protein coupled receptors, composed of the hallmark seven transmembrane domains. To date, ten subtypes have been identified and functionally defined (Abbracchio et al., 2003; Illes and Ribeiro, 2004) with the receptor binding sites residing on the sixth and seventh transmembrane domains (Burnstock, 1997). The receptor subtypes localised in the central nervous system include P2Y1, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14 (Moore et al., 2001). Despite the possible roles of P2Y receptors in neuromodulation and synaptic integration our research group is currently interested in the impact of P2X receptors on synaptic transmission in the central nervous system.

	P2X1	P2X2	P2X3	P2X4	P2X5	P2X6	P2X7
Molecular Properties							
Membrane Expression	Good	Good	Good	Good	Poor	No expression	Good
Dessensitisation[1]	< 1 s	> 20 s	< 1 s	> 20 s	> 20 s	N/A	> 20 s
Pore Dilation	No	Yes	No	Yes	N/A	N/A	Yes
PCa/P4Na	4.8	2.8	1.2	4.2	1.5	N/A	N/A
Fractional Ca2+ Conductance (%)[3]	12.4	5.7	2.7	11	4.5	N/A	4.6
Pharmacology							
Agonist EC50 values (μM)							
ATP	0.07	1.2	0.5	10	10	12	100
2meSATP	0.007	1.2	0.3	10	10	9	100
α,β-meATP	0.3	>300	0.8	>300	>300	>100	>300
BzATP	0.003	0.75	0.08	7	>500		20
Antagonist EC50 values (μM)							
Suramin	1	10	3	>500	4	>100	500
PPADS	1	1	1	>500	3	>100	50
TNP-ATP	0.006	1	0.001	15	N/A	N/A	>30
KN-62	N/A	N/A	N/A	N/A	N/A	N/A	0.3
RO-3	>10	>100	0.1	>100	>100	N/A	>100
NF279	9	30	50	>100	N/A	N/A	20
NF449	0.7	>100	>100	>100	N/A	N/A	>100
Physiology and Pathophysiology							
Major cellular expression	Smooth muscle	Neurons	Pain-sensing neurons	Microglia	Skeletal muscle	Broad Expression	Immune cells
Major role	Neuroeffector transmission	Taste, pre- and post-synaptic responses	Bladder reflexes, chronic pain, taste	Vascular remodelling, neuropathic pain	N/A	N/A	Bone reabsorption, chronic pain

Figure 1.0:1. A summary of the molecular, pharmacological and physiological properties of P2X receptors in the central nervous system. Table adapted from Jarvis and Khakh 2009**.**

1.1 ATP release from neurons and astrocytes in the central nervous systems

Due to the size of ATP molecules they are unable to simply diffuse across the plasma membrane (Chaudry, 1982), suggesting more specialised pathways are required for ATP release. A variety of release mechanisms can operate in the peripheral nervous system and in the CNS, exocytotosis being the prime release method. Previously, compelling evidence has been reported in support of the neuronal vesicular release of ATP in the neocortex (Pankratov et al., 2007). ATP is accrued and stored in synaptic vesicles, alone (Pankratov et al., 2007) or with other neurotransmitters, such as inhibitory GABA and excitatory glutamate (Mori et al., 2001; Jo and Role, 2002). The co-release of ATP occurs frequently at nerve terminals in the brain. Calcium dependent release of ATP was first recognised in 1977 by a group working on whole cell synaptosomes (White, 1977). Since this revolutionary study an abundance of research in support of the vesicular release of ATP has been published (Jo and Role, 2002; Pankratov et al., 2006; Lazaroski, 2012).

Understanding the mechanisms underlying the storage and release of ATP remain fragmented. It is not entirely sure whether exocytosis is wholly responsible for ATP secretion in the central nervous system, evidently there is much data to suggest otherwise (Wang et al., 2000; Halassa et al., 2009). Hence, other feasible non-vesicular approaches have been advocated, such as gap junction hemichannels and ATP binding cassettes (North and Verkhratsky, 2006).

The potential of astrocytes to release ATP by utilising similar methods employed by neurons has recently been revealed (Illes and Ribeiro, 2004) and studied in cultured expression systems (Queiroz et al., 1997; Jeremic et al., 2001). One study supports the hypothesis of ATP release from astrocytes by exocytosis, which may involve lysosomes (Zhang et al., 2003). As well as ATP, astrocytes are able to release many other gliotransmitters such as D-serine and

glutamate. The release of glutamate from astrocytes has many features which resembles synaptic vesicular release, as it is also dependent on the SNARE proteins (soluble *N*-ethylmaleimid sensitive factor attachment protein receptor) (Araque et al., 2000), a formation of a multi-complex of vesicular and membrane proteins (Halassa et al., 2009) and an elevation in the intracellular calcium concentration (Malarkey and Parpura, 2008). ATP release was also inhibited in mice which expressed a genetically modified dominant-negative SNARE complex (Pascual et al., 2005). The expression of the core machinery in glial cells required for vesicular release reinforces the idea of exocytotic release of ATP from astrocytes. Furthermore, vesicle imaging studies have associated the secretion of ATP from astrocytes evoking glial cell derived calcium waves (Pangrsic et al., 2007; Bowser and Khakh. 2007a; Bowser and Khakh, 2007b).

Most of the studies on the exocytotic release of ATP from astrocytes have been conducted *in vitro* on cultured cells, which have raised many questions about the functional significance of this neurotransmitter release method *in vivo* (Hamilton and Attwell, 2010). However, vesicular release of ATP from acutely isolated cortical astrocytes has been demonstrated in recent years (Pankratov el al., 2006), highlighting the physiological relevance of this ATP release mechanism from astrocytes.

A role for gap-junction hemichannels that assemble in astrocytes as hexamers of connexion 43 (Volterra and Meldolesi, 2005) has also been implicated as a mechanism for the astroglial release of ATP. However, the opening probability of hemichannels inside astrocytes is very low, but is increased marginally by reducing the intracellular Ca^{2+} concentration. This results in the release of glutamate and ATP from astroglia (Stout et al., 2002; Ye et al., 2003). Inhibition of the release of gliotransmitters is caused by the implementation of gap junction blockers, emphasising the role of hemichannels in astroglial ATP release (Volterra and Meldolesi, 2005).

Additionally, ATP binding cassettes (e.g. multi-drug resistance protein) have been implicated to evoke the release of ATP from astrocytes (Ballerini et al., 2002). Moreover, Darby et al. (2003), postulated that on binding to P2Y receptors, ATP activates swelling-induced chloride currents (*I*Cl, swell). This in turn releases ATP from the astrocytes (Darby et al., 2003). Hence, current evidence indicates that ATP secretion may occur through channels and transporters as well as via synaptic vesicles, but numerous aspects of these potential release pathways are yet to be explained.

1.2 P2X receptors and the importance of calcium signalling

Typically the amplitude of an ATP current evoked from activated P2X receptors expressed on central neurons ranges from 50-100 pA (Pankratov et al., 2008). Purinergic receptors are widely expressed in the central nervous system and despite the small amplitude of their response they represent an important modulatory pathway of synaptic function especially in consideration of their calcium signalling capabilities on both neurons and glial cells. All P2X receptors are permeable to calcium ions and the ratio of calcium permeability to other cations such as Na^+ and K^+ ranges between 1 to 10, depending on the P2X subunits present (Egan et al., 2006; Abbracchio et al., 2009). P2X receptors expressed in the somatosensory cortex have an extremely high permeability to calcium which is similar to the permeability of NMDA receptors to calcium (Abbracchio et al., 2009). Therefore, P2X receptors in the central nervous system represent an important source of calcium influx into the postsynaptic cell at resting membrane potentials when synaptic NMDA receptor channels are blocked by Mg^{2+} (Pankratov et al., 2003a).

A population of highly Ca^{2+} permeable P2X and P2Y receptors are expressed on astrocytes. The binding of ATP or its analogues evokes a conformational change within the P2X receptor,

which allows the passage of cations such as Ca^{2+}. The transient increase in the intracellular Ca^{2+} concentration can produce long-term changes, for example, cell proliferation and apoptosis (Neary et al., 1996; Ciccarelli et al., 2001). Bennett et al. (2005), describe the sustained response of calcium signalling in astrocytes after the application of ATP. As such, they discovered that this response was due to the perpetual influx of Ca^{2+} ions through P2X7, and is mediated by P2Y1 and P2Y2 receptors (Bennett et al., 2005).

An important property of P2X receptors is their ability to transmit robust Ca^{2+} signals even at resting membrane potentials (North, 2002), unlike NMDA receptors which require depolarisation to remove the Mg^{2+} ion block (Pankratov et al., 2003a). The ratio of neuronal P2X receptors permeability to calcium ions over other monovalent cations such as Na^{+} and K^{+} ranges from 2 to 12 (Pankratov et al., 2009). Similar results were obtained when testing for the permeability of NMDA glutamate receptors to calcium ions. Therefore, functional P2X receptors represent an important source of calcium influx to postsynaptic neuronal cells at low membrane potentials.

Calcium signalling in the CNS is crucial for neurotransmission and recent emphasis has been applied to its physiological role in purinergic mediated transmission on neurons and glial cells in the central nervous system (James and Butt, 2002). The receptors permeation to cations may be attributed to structural components, TM1 and TM2. The permeation capability of the channel pore is principally assigned to TM2, which also regulates conductance and Ca^{2+} influx into the cell (Egan et al., 2006). The role of TM1 in monovalent cation permeability has yet to be defined (Samways et al., 2008). The increase in intracellular Ca^{2+} levels is governed by P2X receptors via two specific mechanisms. Firstly, their ability to mediate a depolarizing current results in the opening of voltage-dependent Ca^{2+} channels. Secondly, P2X receptors are

permeable to Ca^{2+} subsequent to receptor activation by ATP producing a vast Ca^{2+} influx. Many research groups have measured the Ca^{2+} permeability's of P2X receptors – in most cases the permeability was close to more profuse cations present in the extracellular space, such as Na^{+} ions (Egan et al., 2006).

Cytosolic calcium plays a fundamental role for non-excitable cells in inter- and intracellular communications (Braet et al., 2004). Bennett et al. (2005) postulate that inositol-1,4,5-triphosphate ($IP_{3)}$ plays a central role to the junctional transmission of Ca^{2+} between astrocytes, initiated by a G-protein cascade on the activation of P2Y receptors. The groups model is based on IP_3 proceeding to diffuse through gap junctions to act on the internal Ca^{2+} stores, residing within the endoplasmic reticulum. Consequently, Ca^{2+} is released into the cytosol and activates phospholipase C to create more IP_3. This cycle is regenerative as the IP_3 produced diffuses and acts on neighbouring cells. Furthermore, scraping away astrocytes does not prevent Ca^{2+} signals propagating from one astrocyte to the other (Hassinger et al, 1996). Hence, this data suggests that astrocytes perhaps release a chemical transmitter alongside the diffusible Ca^{2+} signal. Imaging of ATP and Ca^{2+} were documented after experimental stimulation of an astrocyte (Wang et al., 2000).

Recently, the extent of the contribution of astroglial derived calcium signalling via P2Y activation has been disputed. Studies have revealed that a reduced or elevated level of P2Y-mediated calcium signalling failed to have an effect on synaptic transmission, in the hippocampus of mice with a genetically modified IP3 calcium activation pathway (Petravicz et al., 2008; Agulhon et al., 2010). However, the primary focus of this current study is the role of the ionotrophic P2X purinoreceptors in the bi-directional signalling between neuron and glial cells, which has largely been ignored.

Accumulating evidence has suggested that ATP arbitrates Ca^{2+} transmission, thus increasing the levels of cytosolic calcium in glial cells and neurons. Neuronal stimulation is able to induce Ca^{2+} signalling in astrocytes, which in turn is able to propagate back to neuronal synapses to modulate synaptic transmission. This neuron-glial cell signalling pathway highlights the potential of purinergic driven regulation of synaptic transmission and brain function mediated by Ca^{2+} ion conductance.

1.3 The role of glial cells in neurotransmission and the concept of the tripartite synapse

Although neurons are an essential component for the general functioning of the nervous system, studies have previously highlighted the importance of another type of brain cell called glial cells in synaptic transmission. Glial cells are non-neuronal cells residing within the nervous system, providing nutrition and support and maintaining homeostasis. They are widely dispersed in both the central and peripheral nervous systems. Amongst the glia in the CNS, the specialised functions of oligodendrocytes and microglia have long been established: axon myelination and immune defence in the CNS, respectively. However, the role of astrocytes has proven to be much more elusive.

Initially it was perceived that astroglia simply operated as "brain glue," essential for the distribution and interaction of neurons (Volterra and Meldolesi, 2005). Later studies have demonstrated that astrocytes express ion channels and receptors which are involved in neurotransmission (Volterra and Meldolesi, 2005), thus assigning a possible role to astrocytes as a component of neuronal signalling. Nevertheless, new insights suggest precise roles have been assigned to astroglial cells including, vasomodulation, regulation of synapse formation and facilitating myelination (Parri and Crunelli, 2003; Volterra and Meldolesi, 2005; Ishibashi et al.,

2006). The slow pace of these discoveries emphasises that many aspects of the principal functions of astrocytes are yet to be clarified.

Our static notions about astroglial cells perhaps derive from their inability to generate action potentials and therefore were deemed unexcitable. Upon finding that astrocytes can in fact be stimulated non-electrically our knowledge of the communication systems in the brain has been extended immensely (Volterra and Meldolesi, 2005). Glial cells play a dynamic role in neurotransmission as they are endowed with a generous number of receptors; permitting them to identify neuronal activity (North and Verkhratsky, 2006). All glial cells are highly sensitive to ATP as they abundantly express purinergic ATP receptors and accumulating evidence suggests that astroglial ATP may arbitrate Ca^{2+} transmission in the central nervous system (Kirischuk et al., 1995; Hassinger et al., 1996; Cotrina et al., 2000; Fields and Stevens, 2000; Verkhratsky and Steinhäuser, 2000; Wang et al., 2000). ATP release from astrocytes can also be generated by the P2Y receptors, mediating propagating Ca^{2+} waves via the actions on inositol-1,4,5-triphosphate (IP_3) (Verkhratsky and Steinhäuser, 2000; Bennett et al., 2005). The role of the P2X1-6 receptor subunits in astroglial processes remains enigmatic, however currents governed by P2X receptors in astrocytes have been reported in culture (Walz et al., 1994) and have been described recently in cortical astrocytes *in situ* (Lalo et al., 2008).

In the CNS astroglial cells are intimately associated with synaptic junctions where they help to maintain the integrity of the extracellular environment (Fields and Stevens, 2000). ATP can function as a powerful signalling molecule between neurons and glial cells as illustrated by experimental analysis of Schwann cells at synaptic and non-synaptic regions (Robitaille. 1995; Stevens and Fields, 2000). Therefore, the abundance of purinergic release mechanisms and the extensive distribution of purinergic receptors in the CNS suggest that ATP could mediate

communication between cells on a much larger scale. This hypothesis associates ATP's neuron-glial signalling potential with many dynamic functions outside maintaining the extracellular environment of surrounding neurons.

P2X receptors expressed on astroglial cells are activated by the diffusion of neurotransmitter released from the presynaptic membrane for synaptic transmission, arbitrating fast localised signalling as a result of enhanced intracellular calcium concentrations (Lalo et al., 2011). The ensuing ionic signals produced can be transformed into a plethora of physiological responses, including activation of intracellular signalling cascades, initiating the release of gliotransmitters, neuronal homeostasis and the calcium dependent modulation of receptors located on the postsynaptic membrane such as NMDA and GABA receptors. For instance, highly sensitive astroglial purinoreceptor subtype P2X1/5 (EC_{50} 50 nM) are able to recognise small changes in the concentration of extracellular ATP in the synaptic cleft and therefore able to detect neuronal activity. This suggests that astroglial P2X1/5 receptors may alter the neuronal support provided by astrocytes accordingly (Lalo et al., 2011). Thus, the activation of ionotrophic P2X receptors represent an innovative mechanism for the modulation of synaptic transmission mediated by glial cells, which involves the release of ATP from neurons and astrocytes in the central nervous system.

The role of neuron-glial communication is currently not fully understood (Fields and Stevens, 2000), however it is established that it allows the bi-directional interaction between neurons and glial cells (Volterra and Meldolesi, 2005). The release of ATP from astroglial cells makes them a major source of adenosine, a metabolite of the degradation of ATP in the synaptic cleft which can inhibit synaptic transmission upon the activation of neuronal P1 receptors. Thus, emphasising the significant role of astroglial derived purinergic transmission in modulating

synaptic signalling in the CNS. Although the modulatory role of adenosine in the brain is well studied a considerable amount of work is still required to ascertain the specific role of neuron and glial cell derived ATP on brain function. Understanding the purpose of this specialised tripartite complex (Fig.1.3:2) and its impact in controlling neuronal excitability and neurotransmission is perhaps vital in comprehending the pathophysiology of many neurological and psychiatric disorders.

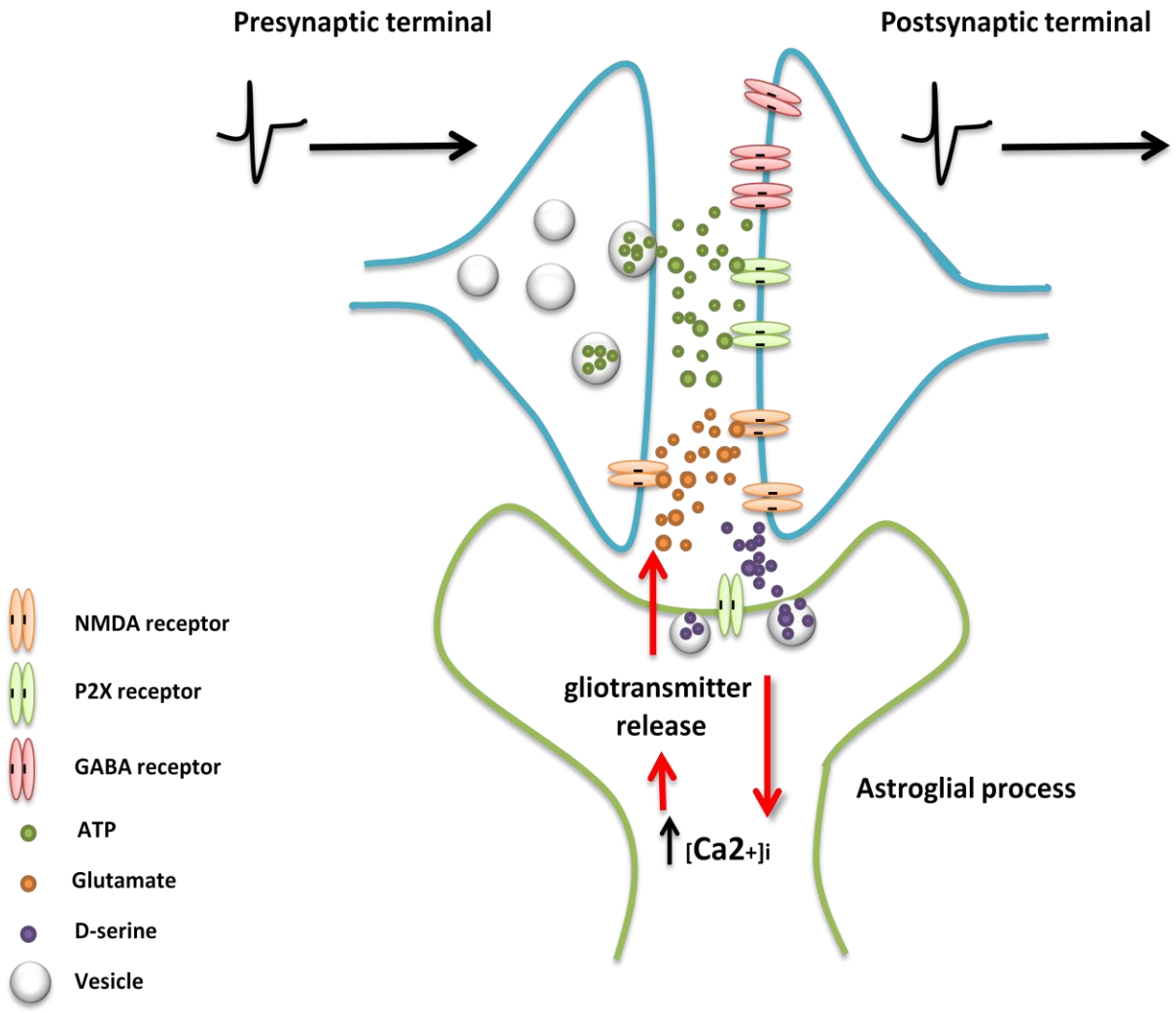


Figure 1.3:2. A schematic diagram of the tripartite synapse

Astroglial cells express many of the same receptors as neurons in the central nervous system. Upon the release of neurotransmitters from the presynaptic terminal, receptors expressed on astrocytes are also thought to be activated by the diffusion of neurotransmitter in the synaptic cleft. This causes oscillations in the intracellular calcium levels in the astrocytes which can then propagate to neighbouring astrocytes. The rise in intracellular calcium leads to the release of gliotransmitters such as ATP, glutamate and D-serine. These gliotransmitters act on the postsynaptic membrane to modulate synaptic transmission.

1.4 Roles for P2X receptors in the modulation of synaptic transmission and plasticity

1.4.1 Modulation of neurotransmitter release by presynaptic P2X receptors

There are a multitude of different receptors widely expressed on the presynaptic nerve terminal in the central nervous system, which include the ionotrophic P2X and metabotropic P2Y receptors (Illes and Ribeiro, 2004). Purinergic receptors operate as regulatory "hotspots" for ATP release and other neurotransmitters, ensuing neuronal stimulation of postsynaptic cells and cell signalling. The local positioning of these receptors permits efficient and controlled transmitter release accordingly. Moreover, this appropriate positioning of the highly Ca^{2+} permeable purinergic receptors emphasises their modulatory role, as they could directly yield transmitter release initiated by the Ca^{2+} influx through the receptors ion channel (MacDermott et al., 1999).

The modulation of neurotransmitter release by the P2 receptor family can either be facilitatory or inhibitory (primarily mediated by P2Y) (Sperlagh et al., 2007). The facilitatory action of presynaptic P2X receptors on acetylcholine release has been readily studied and confirmed by electrophysiological methods from motor nerve terminals of mice (Hong and Chang, 1998). The secretion of excitatory neurotransmitters such as glutamate is also regulated by presynaptic P2X receptors, and has been reported in the hippocampus and brain stem (Khakh and Henderson, 1998; Pankratov et al., 2002; Khakh et al., 2003). P2X receptors also promote the release of inhibitory neurotransmitters, specifically the major inhibitory neurotransmitter, γ-aminobutyric acid (GABA). Ubiquitously expressed ligand ATP was reported to facilitate GABAergic transmission between dorsal horn neurons (Hugel and Schlichter, 2000). Also, ATP produces an elevation in the intracellular Ca^{2+} levels in synaptosomes, resulting in the sequential release of GABA due to P2X3 receptor excitation (Gómez-Villafuertes et al., 2001). Modulation

of GABA release by P2X receptors has also been identified in the hippocampus, brainstem and spinal cord (Sperlagh et al., 2007). These findings encompass and appreciate the dynamic roles that P2X receptors play in neuromodulation and neurotransmission in the brain.

1.4.2 The purinergic driven regulation of receptors expressed on the postsynaptic membrane

Calcium is able to regulate the function of many intracellular and membrane proteins. One of the principle pathways of extracellular ATP action as a neurotransmitter is the modulation of postsynaptic receptors via a calcium dependent process as a result of their high calcium permeability. A crucial functional aspect of P2X receptors is their capacity to interact with other receptors involved in neurotransmission. In 1994, their ability to cross-talk with nicotinic acetylcholine receptors was demonstrated by Nakazawa et al. Application of both ATP and acetylcholine led to the reduction in one of the currents activated by either agonist, therefore indicative of reciprocal inhibition in rat sympathetic neurons. This novel study reported the dependence that activated acetylcholine and ATP receptor- mediated currents in neurons have on each other. The reciprocal inhibition appeared to be significantly influenced by the degree of channel opening as the suppression of the current of one agonist was alleviated once the excitatory current evoked by the other agonist had been desensitised (Nakazawa, 1994). Consequently, intense research on the negative interaction between P2 receptors and other receptors may provide new insights into the purinergic driven neuromodulation on the postsynaptic membrane in the peripheral and central nervous systems.

NMDA glutamate receptors are inactivated as a result of Ca^{2+} signalling (Legendre et al., 1993). The mechanism underlying NMDA inactivation is highly complex, but it is understood that calmodulin and phosphatase B are involved (Krupp et al., 1999). In this context, since Ca^{2+}

signalling through P2X receptors can influence the activity of NMDA receptors, perhaps they modulate the function of NMDA receptors in areas of the CNS where both glutamate and ATP are co-released (Burnstock, 2004). In hippocampal pyramidal neurons activation of P2X receptors by ATP binding produced a Ca^{2+}-dependent inactivation of the residing NMDA receptors (Pankratov et al., 2002a), which also involved the activation of an intracellular signalling cascade. This was demonstrated after short applications of ATP generated an acute decline in the amplitude of the NMDA-mediated excitatory postsynaptic currents (EPSCs). Moreover, NMDA evoked EPSCs in hippocampal CA1 neurons were hindered in the presence of P2X mediated currents. The study demonstrated that the introduction of a Ca^{2+} chelator called EGTA to the recording pipette eliminates the purinergic driven modulation of NMDA receptors (Pankratov et al., 2002a).

1.5 P2X and $GABA_A$ receptor interactions in the peripheral and central nervous systems

1.5.1 Molecular biology of $GABA_A$ receptors

GABA (γ-aminobutyric acid) is the predominant inhibitory neurotransmitter present in the central nervous system (CNS). These receptors assemble as heteropentameric glycoprotein complexes that can be formed from six different subunits with varying members present to produce the receptor: α (1-6), β (1-3) γ (1-3), δ, ε, and θ subunits (Persohn et al., 1992; Brandon et al., 2000). Oligonucleotide labelling studies have located GABA receptor units expressed in the cerebellum, hippocampus, cerebral cortex, thalamus, substantia nigra and spinal cord (Persohn et al., 1992). The composition of the receptor subunits has significant effects on the pharmacology and physiological functions of $GABA_A$ receptors in the CNS.

Research suggests that there are considerable variations in the pharmacology and subunit expression of the $GABA_A$ receptors expressed in embryonic and adult DRG neurons (Maddox et

al., 2004). Activation of $GABA_A$ receptors by agonists pentobarbital and diazepam suggests the incidence of α,β,γ-containing receptors, as such both embryonic and adult DRG neurons elicited $GABA_A$ currents after the application of 50μM of pentobarbital (Valeyev et al., 2000). However, adult DRG neurons were not sensitive to $GABA_A$ receptor antagonists picrotoxin or bicuculline, suggesting age related pharmacological differences of GABA receptors expressed on DRG neurons. Ionotrophic $GABA_C$ receptors cannot be suppressed by bicuculline due to their ρ subunit composition. The inability of bicuculline and picrotoxin to inhibit $GABA_A$ receptors on adult DRG neurons signifies the presence of receptors entirely assembled of ρ subunits, yet currents were still potentiated by pentobarbital which favours α,β,γ-containing receptors (Maddox et al., 2004). Dissimilarly, embryonic DRG neurons were sensitive to picrotoxin and bicuculline, thus suggesting the presence of a novel $GABA_A$ subunit composition residing on adult DRG neurons. Interestingly, $GABA_A$ subunits γ2 and γ3 are expressed on adult DRG neurons (Maddox et al., 2004), which previously highlighted the importance of GABA subunit composition in another study (Boue-Grabot et al., 2004).

1.5.2 $GABA_A$ receptors and calcium modulation

Studies confirm that the release of neurotransmitter from GABAergic synapses is profoundly reliant on Ca^{2+} signalling in the CNS (Stefani et al., 1994). Whilst conducting our experiments variations in the control GABA responses were identified, which were more profound in high Ca^{2+} concentrations (**ref. Chapter 3, Fig. 3.2.1:12a)** Mozrymas' group documented that changes in the concentration of intracellular calcium in hippocampal neurons affects the desensitisation kinetics of the $GABA_A$ receptor and observed a run-down of the GABA receptor-mediated current (Mozrymas and Cherubini, 1998). The run-down was

markedly greater at higher Ca^{2+} concentrations. Also, it was noted that the run-down was accompanied by alterations in the desensitisation kinetics of the $GABA_A$ receptor. Analysis revealed that the diminishing current was attributable to intracellular Ca^{2+} and not due to an influx through voltage-dependent channels. These results suggest that the run-down in the GABA receptor-mediated currents is due to the activation of calcium dependent enzymes, which regulate the phosphorylation of the receptor and thus signalling potential of $GABA_A$ receptors (Mozrzymas and Cherubini, 1998). Currently, the agonist interactions of $GABA_A$ are well defined but the intracellular modulation of this ionotrophic receptor is yet to be fully understood.

Amounting evidence suggests that $GABA_A$ receptor mediated responses can be influenced by phosphorylation, kinases and intracellular Ca^{2+} (Stelzer, 1992). It has been reported that activation of Ca^{2+}/calmodulin-dependent phosphatase, calcineurin, suppresses $GABA_A$ receptor-mediated currents (Chen et al. 1990) and kinases such as, Ca^{2+}/calmodulin dependent protein kinase II (CaMK II), potentiates $GABA_A$ receptor responses (Wang et al., 1995). The importance of phosphorylation in the maintenance of receptor function was manifested when phosphorylating factor, Mg-ATP, was absent from the intracellular solution and dephosphorylation governed the $GABA_A$ receptor run down (Chen et al., 1990). Nevertheless, under the conditions of high intracellular Ca^{2+} the suppression was more profound even in the presence of Mg-ATP. In fact, perfusion of low Ca^{2+} solutions always occluded the diminution of the response generated by high Ca^{2+} solutions, indicative of the reversibility of the run down. In this context, the $GABA_A$ receptors can seemingly exist in two different states: phosphorylated (functional) or dephosphorylated (non-functional).

The dephosphorylation/phosphorylation cycle that occurs as part of the intracellular modulation of $GABA_A$ receptors can be regulated by the activation of specific receptors. The

influx of Ca^{2+} through ligand gated receptors such as NMDA causes an increase in the level of intracellular Ca^{2+}, suppressing the GABA current. Interestingly, this action is eradicated via the introduction of calcineurin inhibitors (Chen et al., 1990). The activation of calcineurin results in the loss of the phosphorylated functional receptor, however the identification of the specific kinase that mediates the phosphorylation of $GABA_A$ is not known. In contrast, influx through Ca^{2+} permeable 5-HT_3 channels facilitates GABA release in the hippocampus (Turner et al., 2004), but still emphasises the influence of intracellular calcium in this innovative mechanism.

In this context the Ca^{2+} plays a direct role in the modulation of $GABA_A$ receptors by promoting the activation of intracellular messenger cascades to which the receptors act as substrates. As yet, the functional relationship between a reduction in Ca^{2+} and the subsequent reduction in synaptic current arbitrated by $GABA_A$ receptors has not been identified. However, it could potentially have an impact several physiological and neuropathological mechanisms. These prospective processes range from synaptic plasticity, seizure activity associated with epilepsy and the efficacy of certain drugs (Mozrymas and Cherubini, 1998).

1.5.3 P2X receptor driven down-regulation of $GABA_A$ receptors

A potential role of P2X receptors as a consequence of their high affinity for Ca^{2+} ions could be the purinergic driven modulation of $GABA_A$ receptors. Their capacity to sequester high concentrations of calcium inside a cell could activate Ca^{2+}/calmodulin dependent phosphatases or kinases to modulate the $GABA_A$ receptor function, expanding the repertoire of P2X receptors modulatory roles in the central nervous system.

Previous results have established that the reciprocal inhibition between P2X and $GABA_A$ receptors can transpire on cultured rat dorsal root ganglion (DRG) neurons of the peripheral

nervous system (Sokolova et al., 2001). This pioneering study highlighted the ability of excitatory P2X-mediated currents to occlude propagating inhibitory $GABA_A$ receptor currents. Occlusion was elevated in the presence of intracellular Ca^{2+}. Therefore, the results suggest a novel Ca^{2+} dependent interaction between P2X and $GABA_A$ receptors on DRG neurons (Sokolova et al., 2001).

Boué-Grabot et al. (2004), demonstrated the concurrent activation of both P2X2 and multiple $GABA_A$ receptors expressed in *Xenopus* oocytes, results in cross-inhibition. This inhibition was heavily reliant on the composition of the $GABA_A$ receptor subunits, as P2X2 receptors failed to suppress currents mediated by $GABA_A$ receptors composed of $\gamma 2$ or $\gamma 3$ subunits (Boue-Grabot et al., 2004). This particular study disputes the concept that P2X3 receptors can occlude $GABA_A$ receptor mediated currents as proposed by Sokolova et al. (2001). Moreover, a later study by the same research group suggests that the mechanism underlying the reciprocal inhibition between the receptors is independent of Ca^{2+}, but occurs via direct interactions between the intracellular domains of P2X2, P2X3 or P2X2/3 receptors in the peripheral nervous system and P2X4 in the central nervous system with GABA (Boue-Grabot et al., 2004; Toulme et al., 2007; Jo et al., 2011). A similar mechanism has been described for the interactions between the dopamine D1 receptor and the glutamate receptor NMDA (Salter, 2003; Toulme et al., 2007). Hence, research into the $GABA_A$ and P2X receptor interaction is ever expanding and the underlying mechanism to this receptor communication remains controversial.

The balance between inhibitory neurotransmission which is maintained primarily by $GABA_A$ receptors and excitatory neurotransmission for which AMPA receptors are predominantly responsible is crucial for the functioning of the central nervous system. Dysfunctional GABA receptors have been implicated in the pathophysiology of many

neurological disorders such as anxiety, insomnia, epilepsy and schizophrenia (Spigelman et al., 2002; Mohler et al., 2012; Mohler et al., 2005). As targets of purinergic modulation they represent a novel pharmacological tool which could hold immense therapeutic potential. Therefore, one of the main objectives of this project was to understand the underlying mechanisms involved in this cationic and anionic receptor communication, which have yet to be explored.

1.5.4. Modulation of GABA-ergic inhibition by glial cells

Once a neurotransmitter is released into the synaptic cleft it is rapidly removed by uptake mechanisms or can be removed by enzymatic processes, which are involved in the synthesis and degradation of the neurotransmitter as in the case of acetylcholine which is rapidly degraded in the synapse by acetylcholinesterase. Residual neurotransmitter in the synaptic cleft must be terminated quickly to prevent the occurrence of excitoxicity and neuronal cell death. GABAergic neurotransmission is ceased by uptake mechanisms into neuronal or astroglial cell compartments via GABA transporters. A similar method of neurotransmitter recycling has also been demonstrated for glutamatergic neurotransmission. Astroglial glutamatergic transport predominates over neuronal uptake, whereas GABA uptake is primarily via neuronal mechanisms. However, the metabolism of neurotransmitters by astroglial cells is an important concept to the fundamental functioning of neurotransmission as dysfunctional neurotransmitter transport can have damaging results.

The transport of glutamate and GABA is far more complex than the general degradation of other neurotransmitters by enzymes and re-uptake into the presynaptic terminal as astrocytes are significantly involved. Astrocytes express the biological machinery to control the availability of the precursors to produce GABA and glutamate (Schousboe and Waagspetersen, 2003). In the

central nervous system excitatory neurons responsible for the release of glutamate are strongly dependent on the release of glutamate precursors from astrocytes, which are used to synthesise glutamate after neuronal uptake. Any interruption in the astroglial release of these precursors, specifically glutamate synthetase and pyruvate carboxylase which are only expressed in glial cells, results in the elimination of glutamatergic neurotransmission (Schousboe and Waagspetersen, 2003).

GABA homeostasis is regulated by GABA transporters which are expressed on neurons and astrocytes (Shigetomi et al, 2012). To date four transporters have been cloned GAT1-4 (Schousboe and Kanner, 2002). The role of the GABA transporters which are responsible for keeping extracellular GABA concentrations low has been demonstrated when GABA transporter inhibitors caused enhanced levels of GABA in the synaptic cleft. Glial cells express GAT1, GAT2 and GAT4 and thus are prime candidates for the removal of GABA from the synapse. Residual GABA that isn't recycled by neurons is taken up by astrocytes where it is then oxidised or transported back to neurons via the GABA-glutamine-glutamate shuttle. Glutamine is a metabolite of glutamate and is exclusively expressed in astrocytes (Broer and Brookes, 2001). Astrocytes are unable to convert glutamate into GABA by the modulation of enzymes; alternatively glutamine plays the role of a precursor of GABA from the breakdown of glutamate. Glutamine is transported to GABAergic nerve terminals where it is metabolised to produce glutamate and then undergoes decarboxylation to form GABA, which is regulated by enzyme glutamate decarboxylase.

The role of astroglial cells on the modulation of GABA homeostasis is intensively studied. Homeostatic mechanisms to ensure that the levels of extracellular GABA remain low are significant for the survival of neurons and the maintenance synaptic transmission. Another

purpose for the regulation of GABA receptor uptake by neurons and astrocytes is for the preservation of the fine balance that exists between GABAergic and glutamatergic neurotransmission. Any alterations in this balance between inhibitory and excitatory transmission can lead to a host of neurological disorders, primarily linked to epilepsy. Therefore, much research has concentrated on the potential of inhibitors which block GABA uptake in glial cells as prospective therapeutic agents for epilepsy (Schousboe et al., 2003).

1.5.5 Tonically active $GABA_A$ receptors in the central nervous system

$GABA_A$ receptors are also involved in another important mechanism in the central nervous system called tonic inhibition. Not only do $GABA_A$ receptors respond to the local release of GABA from the presynaptic membrane, but they also respond to a persistent tonic current caused by a spill over of neurotransmitter from the synaptic cleft. Tonic $GABA_A$ receptors are kinetically and pharmacologically distinct from their phasic counter parts that give rise to the traditional miniature inhibitory post synaptic currents (mIPSCs). Initially documented in cerebellar granule cells (Brickley et al. 1996; Wall et al. 1997; Hamann et al 2002) a transient tonic conductance has also been observed in multiple brain regions: hippocampus, thalamus, dentate gyrus, striatum, spinal cord, hypothalamus and neocortex (Yamada et al., 2007; Krook-Maguson et al., 2008; Belelli et al., 2009). In parallel with the incidence of tonic inhibition is the expression of a special subset of $GABA_A$ receptor subunits, which are specific to tonic $GABA_A$ receptors. These receptor subunits particularly expressed on neurons in the cerebellum and dentate gyrus include α6 and δ $GABA_A$ receptor subunits and α1 in the neocortex (Yamada et al., 2007). The differences in $GABA_A$ receptor subunit composition and brain region specificity can give rise to significantly distinct phasic and tonic $GABA_A$ receptors in the central nervous system. Immunohistochemical studies have demonstrated the extrasynaptic nature of the δ

subunit, but not all tonic $GABA_A$ receptors necessarily express δ subunits (Yamada et al., 2007). However, the important characteristics of the δ-subunit such as its high GABA sensitivity, extrasynaptic location and slow desensitization kinetics make it the perfect candidate for its involvement in tonic inhibition.

The physiological role of tonic $GABA_A$ receptors in the central nervous system is far from understood. It has been implicated in the control of neuronal firing patterns and excitability (Hamann et al., 2002; Semyanov et al., 2003) and it has also been demonstrated that impairments in tonic inhibition is involved in many neurological disorders such as Fragile X Syndrome, stress, idiopathic and temporal lobe epilepsy (Belelli et al., 2009). Since tonically active $GABA_A$ receptors are prime targets for various reagents (Santhakumar et al., 2007) it is highly possible that they could act as novel therapeutic targets for a plethora of neurological pathophysiologies. Although it has been suggested that tonic inhibition occurs on neocortical pyramidal neurons (Yamada et al., 2007) the underlying mechanism and the physiological relevance of this phenomenon is far from being elucidated.

1.6 Modulation of synaptic plasticity by post-synaptic P2X receptors

Various methods of increasing synaptic strength exist; long-term potentiation (LTP) is currently at the forefront of brain and behaviour research due to their contribution to learning and memory (Malenka and Bear 2004; Hansel et al. 2008). These two mechanisms can be mediated presynaptically by modifying neurotransmitter release or postsynaptically by the trafficking of receptors to and from the cell membrane. Our understanding about the role of P2X receptors in synaptic plasticity is relatively poor. However, some experiments have demonstrated the participation of P2X in synaptic plasticity in the CNS. For example, Yamazaki et al. (2003)

showed that application of ATP onto hippocampal CA1 neurons elicited LTP in neurons showing small Ca^{2+} currents, whereas LTD was detected in neurons exhibiting larger Ca^{2+} currents. Thus, there was a considerable correlation between the enormity of the long-term changes in plasticity and the size of Ca^{2+} signals following ATP application (Yamazaki et al. 2003). Broadly, agonists of P2X may generate modifications in synaptic strength by themselves, which may occur via cell death or brain trauma when large amounts of ATP is released (Pankratov et al. 2008).

The effects that P2X purinoreceptors have on synaptic plasticity can be clearly subdivided into either positive or negative categories. For instance, the inhibition of P2X receptors induces LTP, however P2X failed to facilitate LTP when NMDA receptors were suppressed (Pankratov et al. 2002a). Legendre et al. (1993) postulated that NMDA receptors can be inactivated via a Ca^{2+} dependent mechanism. This suggestion represents a viable system as to how P2X receptors can aid LTP initiation. Whereby, activated P2X receptors inhibit adjacent NMDA receptors causing an elevation in the threshold value to initiate LTP (Pankratov et al. 2008). In contrast, some evidence is indicative that ATP is able to augment LTP in the CNS (i.e. a positive effect). A positive modulator of P2X4 receptors, ivermectin, potentiates ATP currents and enhances LTP in wild-type mice but not in P2X4 receptor knock-out mice (Sim et al. 2006). Additionally, it has recently been discovered that activation of P2X receptors on the postsynaptic terminal by astroglial ATP causes the insertion of postsynaptic AMPA receptors in hippocampal neurons (Gordon et al. 2005). It has been suggested that P2X7 receptors were responsible as it is closely linked to phosphatidylinositol 3-kinase (Jacques-Silva et al. 2004), which is essential for AMPA receptor insertion (Man et al. 2003). Therefore, this particular study demonstrated

the possibility that ATP can produce enduring changes in the efficiency of the postsynaptic membrane.

A crucial functional aspect of P2X receptors is their capacity to interact with other receptors involved on neurotransmission. In 1994, their ability to cross-talk with nicotinic acetylcholine receptors was demonstrated by Nakazawa et al. Application of both ATP and acetylcholine led to the reduction in one of the currents activated by either agonist, therefore indicative of reciprocal inhibition in rat sympathetic neurons. This novel study reported the co-dependence of activated acetylcholine and ATP receptors on neurons. The reciprocal inhibition appeared to be significantly influenced by the degree of channel opening as the suppression of the current of one agonist was alleviated once the excitatory current evoked by the other agonist had been desensitised (Nakazawa, 1994). Consequently, intense research of negative interaction between P2 receptors and other receptors may provide new insights into neuromodulation on the postsynaptic membrane in the peripheral and central nervous systems.

NMDA glutamate receptors are inactivated as a result of propagating Ca^{2+} currents (Legendre et al., 1993). The mechanism underlying NMDA receptor inactivation is highly complex, but it is understood that calmodulin and phosphatase B are involved (Krupp et al. 1999). In this context, since Ca^{2+} signalling through P2X receptors can influence the activity of NMDA receptors, perhaps it regulates the functioning of these receptors in areas of the CNS where both glutamate and ATP are co-released (Burnstock, 2004). In hippocampal pyramidal neurons activation of P2X receptors by ATP binding produced the Ca^{2+}-dependent inactivation of the NMDA receptors (Pankratov et al., 2002a). This was demonstrated after short applications of ATP generated an acute decline in the amplitude of the NMDA receptor-mediated excitatory currents. Moreover, NMDA receptor evoked EPSCs in hippocampal CA1

neurons were hindered in the presence of P2X receptor-mediated currents. A Ca^{2+} chelator and P2X specific inhibitor PPADS eliminates P2X-mediated inhibition of NMDA receptors (Pankratov et al., 2002a).

1.7 The physiological relevance of extracellular ATP

Many studies have demonstrated the modulatory action of the purinergic receptors on the presynaptic membrane. The action of extracellular ATP on postsynaptic receptors, especially in native cells remains relatively unexplored. Current research suggests that the P2X purinoreceptors are able to influence synaptic plasticity, synaptic transmission and brain function; however the underlying mechanisms of such modulatory processes are far from being defined.

P2 purinoreceptors are widely expressed on glial cells in the central nervous system. A glial cell subtype, astrocytes are able to integrate in to the synapse and provide functional support to neighbouring neurons. Research has primarily focused on the role of astroglial metabotropic P2Y receptor derived calcium signalling and its impact on synaptic transmission. However, there is evidence in support of calcium signals mediated by the ionotrophic P2X1/5 receptors (Palygin et al., 2010) which are specific to astroglial cells. Therefore, activated P2X receptors expressed on astrocytes could be mediators of localised bi-directional communication between neurons and glial cells.

The generation of calcium signals by the release of extracellular ATP from astrocytes and neurons upon activating P2X receptors provides the foundations for a novel form of modulation of synaptic activity. Currently, other putative pathways of glial regulation, like the modulation of the activity of postsynaptic GABA receptors by gliotransmitters remain almost unexplored. Since it is well known that the pathophysiologies of many neurological diseases stem from

dysfunctional synaptic transmission and that gliotransmitters can regulate synaptic signalling, it is important to study of the role of purinergic modulation on postsynaptic receptors. Otherwise, the role of ATP as part of the tripartite synapse and its potential pharmacological consequences for the treatment of neurological diseases cannot be fully understood.

Chapter 2

Materials and Methods

2.0 Peripheral neurons

The dorsal root ganglion (DRG) neurons of the peripheral nervous system were prepared using 8-20 day old CBL57 mice, which were decapitated and then dissected to allow precise extraction of the DRG ganglia. The ganglia were then placed in a solution consisting of (mM): 150 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2 and 10 HEPES, pH adjusted to 7.3 with NaOH. Enzymatic treatment of the ganglia was carried out using the same solution with a lower calcium concentration (0.5 mM) containing 1.2 mg/ml of protease type XIV and incubated at 34^{O}C for 15 minutes. Once removed from the enzymatic treatment the ganglia are subsequently transferred back to the solution consisting of a normal calcium concentration (Lalo et al., 2001) and then kept for up to 8 hours for experiments. DRG neurons were isolated using the conventional trituration method using fire polished pipettes with tip diameters that ranged from 200-500 µm (Lalo et al., 2001).

2.1 Central neurons

Whole-cell recordings were made from acutely isolated pyramidal cells of the somatosensory cortex. Slices were prepared from 15-30 day-old CBL57 mice that were anesthetised using haloethane and immediately decapitated in agreement with the UK Home Office legislation. Once the brain was removed it was divided into two hemispheres in the coronal plane. The brain was placed in the slicing chamber and gassed with a 95% O2/5% CO2

mix. Slices were cut to a thickness of 300-350 µm in physiological saline at a temperature of 4°C to prevent tissue degradation. The preparation of the slices is similar to a previously described procedure (Lalo et al., 2006).

After the slices were prepared they were incubated in a chamber at room temperature for a minimum of two to four hours in physiological saline (mM) 130 NaCl, 2.7 KCl, 1 MgCl2, 2.5 CaCl2, 18 NaHCO3, 1 NaH2PO4 and 15 glucose, pH 7.3 when gassed with a 95% O2/ 5% CO2 mix until they were ready for isolation.

2.2 Cell isolation

Cells were isolated at room temperature from layer 2/3 of the somatosensory cortex. We used a non-enzymatic acute isolation procedure called vibrodissociation (Vorobjev, 1991; Lalo et al., 2006; Jun et al., 2011). This procedure involves suspending a pre-incubated slice in artificial saline inside the recording chamber which acts as a suitable culture dish for visualising the slice under a dissecting microscope. The slice is stabilised and held in place by a modified platinum wire approximately 0.5 mm in diameter, which has been moulded to specifically fit mouse brain slices.

A vibrating glass ball (diameter of 100-200 µm) attached to a flame sealed micropipette is moved slowly (20-50 µm) over the slice surface using a micromanipulator, vibrating at 100 Hz. (Fig. 2.2:2). The vibrating glass ball is moved laterally, deeper through the entire brain tissue in the desired area. This step is repeated to maximise the number of cells isolated from the specific brain region for experimental use. The external solution used for cell isolation included (mM): 140 NaCl, 2.7 KCl, 1 MgCl2, 2.5 CaCl2, 1 NaH2PO4, 18 NaHCO3 and 15 glucose, pH-adjusted to 7.3 with NaOH. After cell isolation the platinum wire and remaining brain slice are

omitted and the cells are left suspended in the solution until they sink to the bottom of the recording chamber and then they are ready for experimental use.

This innovative technique is useful as it preserves the cell dendrites and enables specific isolation of neurons even from older animals; it also prevents the possible impact of an enzyme on membrane proteins (Fig. 2.2:3). The mechanical isolation of native cells has many advantages over the use of cultured cells and cell lines as the "natural" receptor composition and physical arrangement of receptors is retained on the neurons. Also, there is the absence of adverse effects such as excitotoxicity, related to the excessive density of receptors, which can be typical for cell lines. Most studies which focus on the impact of P2X receptors on synaptic transmission and neuromodulation employ expression systems and cultured cells, however the series of experiments outlined in this current project uses native cells, emphasising the novelty and originality of our work.

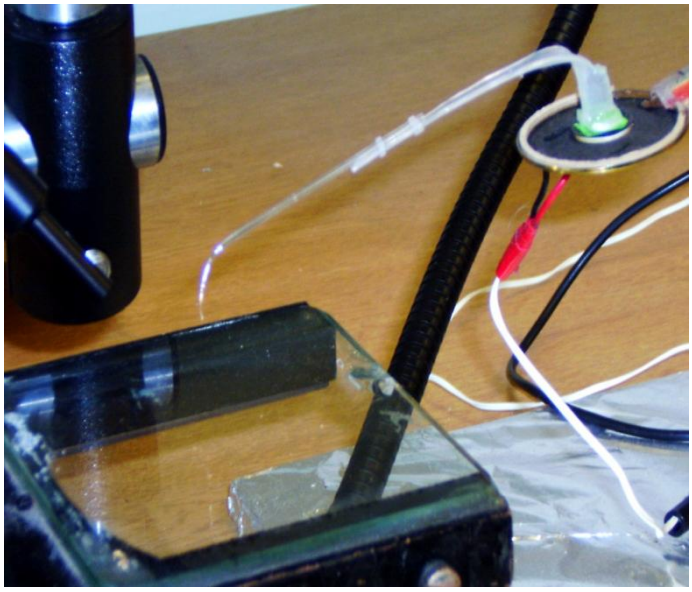


Figure 2.2:3. Photographs taken of the vibrodissociation setup used throughout the experiments conducted on acutely isolated cortical pyramidal neurons.

A vibrating glass ball is passed over the dissected coronal brain slice in regions L2/3 of the somatosensory cortex enabling the dissociation of neocortical neurons from the brain tissue for whole-cell patch clamp recording.

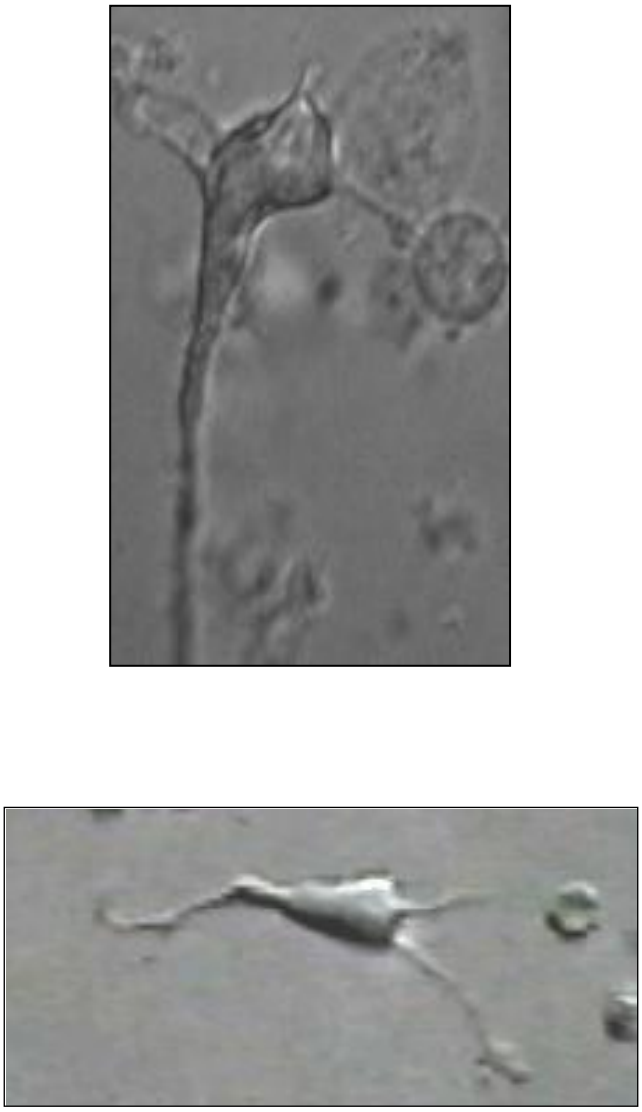


Figure 2.2:4. Examples of acutely isolated cortical L2/3 pyramidal neurons using the vibrodisscocation technique.

The acute isolation procedure preserves the dendrites and synaptic terminals of the pyramidal neurons and prevents any possible impact to membrane proteins by other enzymatic isolation methods.

2.3 Electrophysiological recordings

Whole-cell patch clamp recordings from isolated cortical pyramidal neurons in cortical layers II/III of brain slices were measured using an **Axopatch 200B (**Axon Instruments, USA) patch clamp amplifier. The membrane potential was voltage clamped at -80 mV and continuously perfused (1.5-2 ml/min) with an extracellular solution containing in mM: 140 NaCl, 2.7 KCl, 1 MgCl2, 2.5 CaCl2, 1 NaH2PO4, 18 NaHCO3 and 15 glucose, pH-adjusted to 7.3 with NaOH. Recordings were made using patch pipettes which were pulled using boroscillicate glass electrodes complete with filaments (World Precision Instruments), with resistances between 3-5 MΩ when filled with an intracellular solution containing (mM): 120 CsCl, 20 HEPES, 1 MgCl2, 3 Mg2ATP3, and 10 EGTA, pH-adjusted to 7.3 with CsOH. The intracellular calcium concentration of the cells was adjusted using high (10 mM) and low (0.2 mM) levels of calcium chelator, Ethylene Glycol Tetra-acetic Acid (EGTA).

The membrane potential was clamped at -80 mV unless otherwise stated and currents were filtered at 3 Hz. Liquid junction potentials were compensated using the patch-clamp amplifier. We did not use series resistance compensation because the cells were not of rounded shape and had dendrites typically, so compensation would not have been adequate. Also, the recorded current was not of a large amplitude and the series resistance was not very large so it did not bring significant error in the membrane potential. To monitor the cells viability and to ensure that it was still useful for experimental purposes a voltage step from -80 to -20 mV was routinely observed between fast applications of GABA in central neurons (Fig. 2.4:5). The response to the voltage-step is comprised of currents generated by voltage gated Na^+- and Ca^{2+} channels. The absence of a Na^+ current in response to the voltage-step would suggest that the cell is no longer suitable for voltage clamp experiments.

Field Excitatory Postsynaptic Currents (fEPSPs) were recorded in neocortical layer III with glass microelectrodes filled with artificial CSF (1-2 MΩ) (Pankratov et al. 2002b). Approximately 15 minutes before the delivery of the stimulus was allowed to ensure that the baseline had stabilised. The induction of LTP was caused by delivering trains of high frequency Theta Burst Stimulation (TBS). The intensity of TBS ranged from minimal, 20-30% below the threshold required to induce LTP to maximal which produced potentiation in nearly all cells tested.

2.4 Whole-cell patch clamp

During patch clamp recording an electrode made from a glass filament is sealed to an area of the cell membrane, which contains a small population of ion channels. The electrode used consists of a small tip often with an opening less than 1 μm. During our experiments the tip of the glass electrode was heated in a microforge to ensure that an even and smooth surface is produced which facilitates the formation of a membrane giga seal of a high resistance.

An intracellular solution is placed inside the glass filament of the pipette and makes contact with the silver wire that is attached to the head stage and amplifier, which records changes in ion flow across the membrane. The intracellular solution is of a similar composition to the extracellular solution that is perfused to the recording chamber, but can be modified to include specific drugs to change the recording conditions, for example when examining intracellular calcium signalling in cortical astrocytes (Palygin et al., 2010).

The recording electrode is lowered towards the cell and placed upon the cell membrane. To help form a giga seal between the electrode and the cell membrane negative pressure is applied which forces a patch of the membrane to make contact with the tip of the electrode. As a

result of the high resistance of the seal, ionic currents can be isolated and measured with little noise and mechanical disturbance to the recording.

Whole-cell patch clamp recordings consist of recording multiple ionic currents within the area of the patched membrane from a small population of membrane receptors. Once a giga seal is formed the position of the electrode on the cell membrane is maintained throughout the entire duration of the recording. More negative pressure is applied in order to rupture the patched membrane. When this occurs the intracellular solution moves into the cells cytoplasm and dialyzes the cell's intracellular contents. A reliable indicator which suggests that the cell membrane has been ruptured successfully and suitable for recording is the observation of a transmembrane current caused by the activation of voltage-gated Na^+ channels. In some cases the transmembrane current may also include responses mediated by K^+ and Ca^{2+} voltage-gated channels which was documented in our experiments conducted on DRG neurons (Fig. 2.4:5).

There are different forms of patch clamp recording methods. The variation used throughout this project is called whole cell voltage-clamp (Lalo et al., 2009). A holding voltage is set and uses a negative feedback mechanism contained inside the amplifier to clamp the cell at the desired voltage e.g. -80mV. This allows the changes in the ionic conductance across the membrane to be measured which can be interpreted as a receptor response when specific receptor agonists are applied (Grubb and Evans, 1999; Lalo et al., 2004; Lalo et al., 2009).

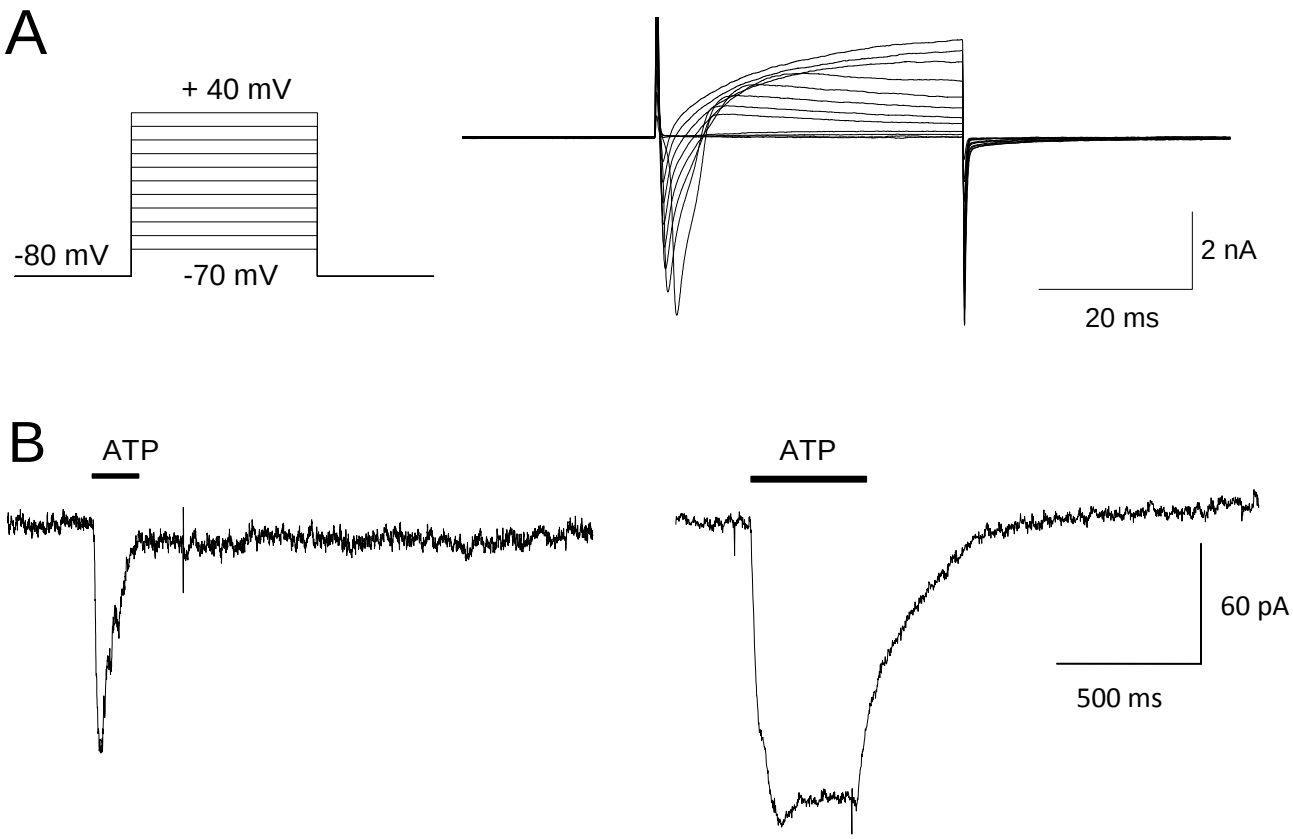


Figure 2.4:5. Voltage-clamp recordings in isolated DRG neurons of the peripheral nervous system.

A, example of transmembrane currents activated by series of voltage steps from -70 to 40 mV. Responses mediated by voltage-gated Na- and K- currents demonstrate good voltage-clamp conditions and cell viability. **B,** transmembrane currents activated by application of ATP (10 µM). The ATP currents coincide with observations made by Grubb and Evans (1999), a fast and rapidly inactivating current reminiscent of the P2X3 phenotype (left) and a slow sustained response, predominantly mediated by P2X2/3 receptors (right). Currents were recorded in different cells at a holding potential of -80 mV.

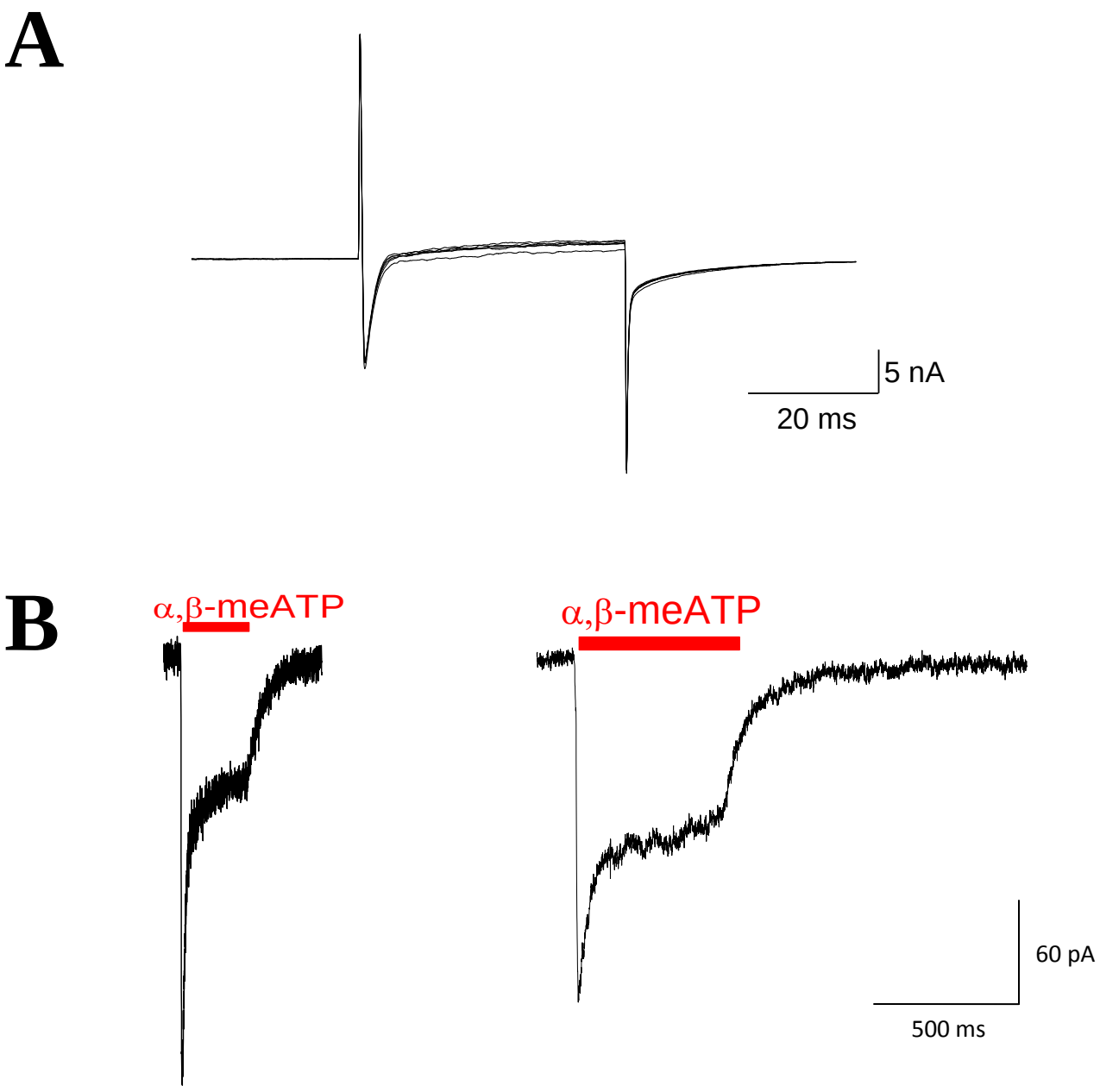


Figure 2.4:6. Voltage-clamp recordings taken from acutely isolated cortical pyramidal neurons of the central nervous system.

Examples of transmembrane currents activated by the application of P2X specific ATP analog α,βme-ATP (10 μM) to cortical pyramidal neurons. Again as observed in DRG neurons both receptor responses demonstrate different kinetically distinct phenotypes of the various homomeric and heteromeric P2X receptor complexes expressed in the central nervous system. Currents were recorded in different cells at a holding potential of -80 mV.

2.5 Field Excitatory Postsynaptic Potential (fEPSP) Recordings

Prepared slices were placed in the recording chamber and perfused with aerated artificial saline as the same composition as the incubating solution used to store slices before experiments commenced. The extracellular solution was gassed with a 95% O2 and 5% CO2 mix. The stimulating bipolar electrode (50μm thick Ni/Cr) was positioned in layer IV-V of the neocortex whereas the recording microelectrode was filled with identical ACSF, which was superfused over the slice. The recording microelectrode was positioned in layer II/III of the neocortex juxtaposed to the stimulating electrode, once a suitable fEPSP was found. The amplitude of stimulation to layer IV-V ranged between 0.5 and 3 Amps which consequently produced an EPSP of approximately 60% of the amplitude of the maximal response to ensure that any possible potentiation in the EPSP amplitude would be observed. Layer IV-V of the neocortex participates in callosal and ipsilateral corticocortical circuits (Hefti and Smith, 2000). The placement of the recording electrode in layer II/III was for the consistency with previous experiments on pyramidal neurons, which were acutely isolated from the same region of the neocortex. Layer II/III neurones receive vertical projections sent from neurones in layer IV/V (Feldman, 2000). Additionally, recording from this layer produced a clear EPSP which can be reproduced for more than two hours whilst recording, without any run-down. A stable amplitude was recorded in approximately 80% of all cortical slices using this particular recording configuration.

The induction of LTP was generated by utilising a standard Theta Burst Stimulation (TBS) protocol consisting of a 0.5 second train at 100Hz. In total 5 trains were delivered with 10 second intervals. Earlier experiments demonstrated that depending on the age of the animal shorter trains consisting of 2 or 3 were not sufficient to elicit LTP in the neocortex. Once the

baseline had stabilised after approximately 15 minutes a TBS of 5 pulses was applied to layer IV-V of the neocortex, producing an EPSP that reached a plateau lasting between 40-60 minutes each time.

To study the role of astroglial derived ATP on LTP in the neocortex the standard TBS protocol was used repeatedly in the presence of various receptor agonists and glial cell toxins. After the baseline had stabilised the drugs were delivered 10 to 20 minutes before TBS was administered and washed out 0 to 60 minutes afterwards.

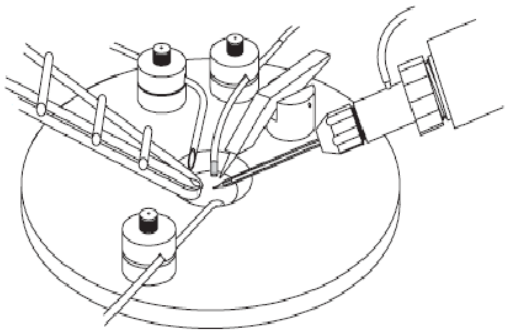
2.6 Drug Delivery

Agonists of P2X and $GABA_A$ receptors were applied at room temperature to acutely isolated single cells in the recording chamber by local perfusion via a rapid application system (5 ms) controlled by an electromagnetic valve (Fig. 2.6:7). Application of the receptor agonists to the patched neuron took approximately 2 seconds and applied at 3 minute intervals to prevent cumulative receptor desensitisation. Protein kinase inhibitors and receptor antagonists were also applied by bath perfusion along with the extracellular solution at a rate of 2 ml/min. In the case of allosteric modulator of δ-subunit GABA containing receptors, DS2, it was coapplied in the U-tube along with GABA.

Recording of receptor activated currents did not begin until the $GABA_A$ response had stabilised (**ref. Chapter 3, Fig. 3.2.1:12**). Consecutive applications of GABA were carried out before the experiments began to ensure that the inevitable calcium-dependent run-down of GABA responses was taken into account, particularly in 0.2 mM EGTA concentrations. The pharmacological changes observed during the experiments are all relative to the control conditions (before the application of the P2X agonists). The average of the GABA responses

recorded after the application of ATP were normalised to the control GABA responses. This was done for every experiment and thus prevents an underestimation of the impact of the P2X receptor agonists on $GABA_A$ receptor-mediated currents that could be caused by the rundown in the GABA receptor responses.

A



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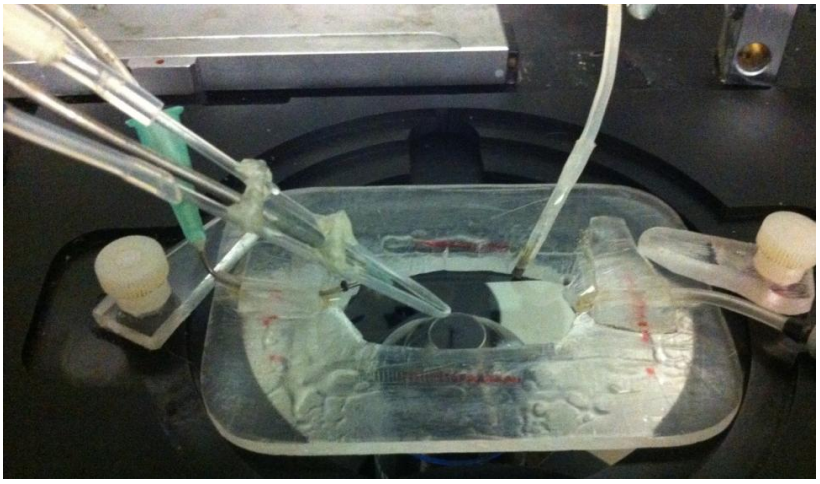

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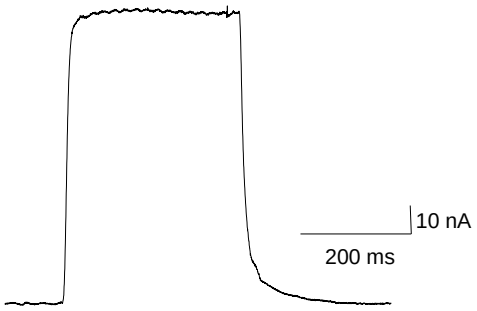

Figure 2.6:7. A schematic diagram showing an example of the U-tube set up that was implemented during experiments for drug applications to isolated pyramidal neurons of the neocortex.

A, the rapid perfusion of drugs on to isolated cells is performed by a U-tube (**B, left in the picture**) which is controlled by an electromagnetic valve. The advantages of using a rapid application system is that it allows a fast solution exchange time in the range of 5 ms and minimal drug consumption of < 50-100 mL/application. **C,** shows an application test recording taken from a patched isolated neuron using distilled water to examine the performance of the U-tube. Image **A** was adapted from Mortensen and Smart (2007).

2.7 Drugs

All drugs were purchased from Tocris Bioscience or Sigma Aldrich if otherwise stated.

2.8 Data analysis

All data is representative as the mean ± s.d. (n = number of cells) and statistical significance of the results was determined using the Student's t-test where necessary. The figures used to present the experimental results were created using Origin 6.0. A concentration response curve for the different $GABA_A$ receptor sensitivities to varying concentrations of GABA receptor agonist ranging from 0.1-200 µM were fitted to:

$$I/I_s=1/(1+(EC_{50}/[C]^n)$$

I = the current produced in GABA concentration $[C]$

I_s = concentration of agonist which causes saturation of the receptors (in our case 200 µM GABA)

EC_{50} = the concentration of the agonist that generates a half maximal response

n= Hill coefficient

When trying to fit a single sigmoidal curve to the concentration response curve an unsatisfactory fit was produced due to the biphasic nature of the GABA concentration response curve (**ref. Chapter 4, Fig. 4.1:19**). A range of GABA concentrations were used to activate the two different populations of $GABA_A$ receptors that are expressed in the central nervous system. They exhibit distinct levels of sensitivity to GABA, which is dependent on their location in the synapse. As a result, the formation of two separate sigmoidal curves which amalgamated both the tonic and phasic components of the concentration response curve produced a much better fit (Fig. 2.8:8).

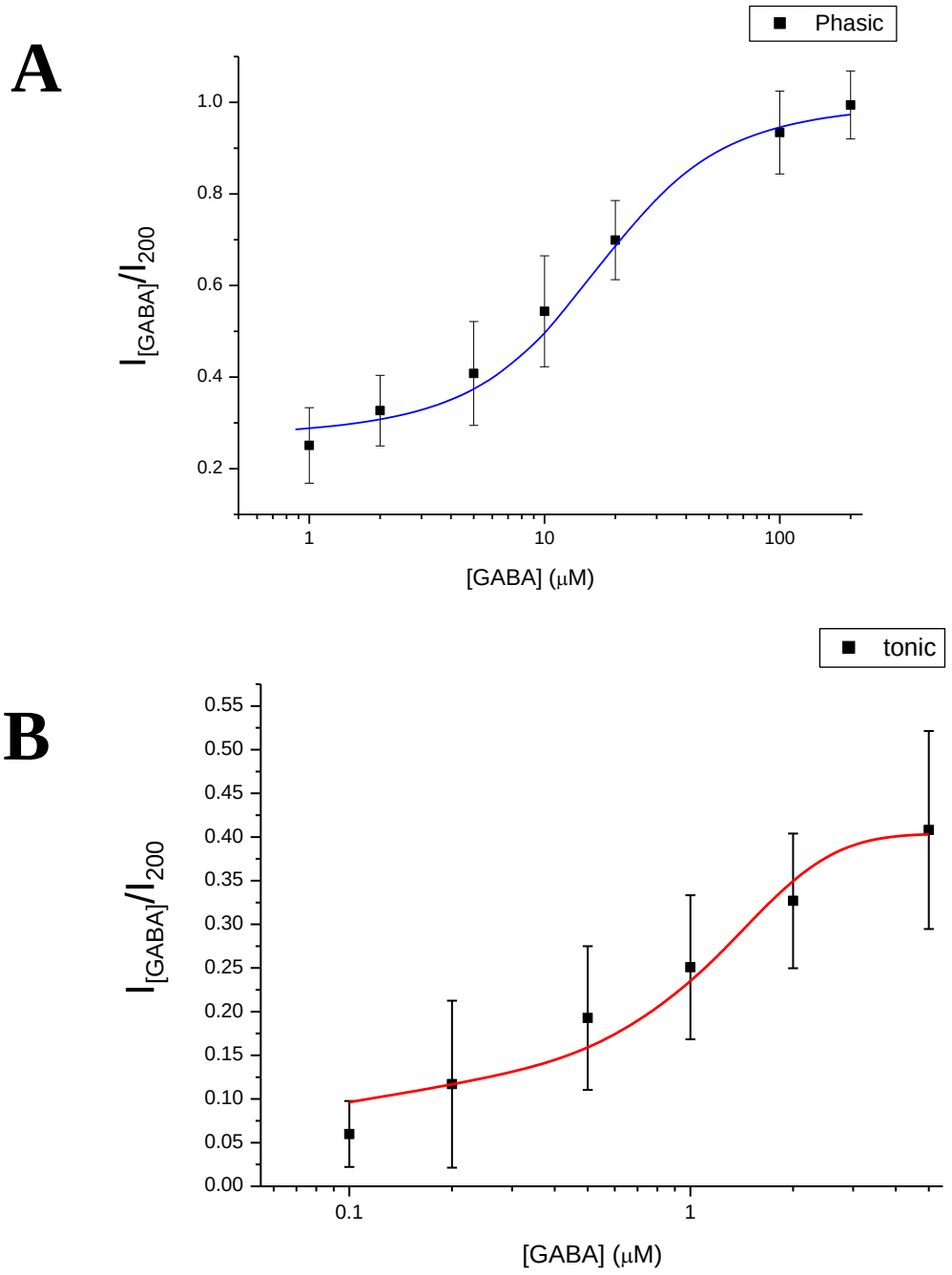

Figure 2.8:8. Concentration response curves for two different population of $GABA_A$ receptors expressed on cortical pyramidal neurons in the central nervous system.

A, phasic $GABA_A$ receptors respond to the local release of GABA from the presynaptic membrane. This subtype of $GABA_A$ receptor has a lower affinity for GABA and is activated by higher agonist concentrations. **B,** the activation of a different subset on GABA receptors, which is mediated by a persistent tonic current via a spill-over from the synaptic cleft. They have a significantly higher affinity for GABA than the phasic receptor subtype and thus require a lower concentration of agonist for activation. The responses represent the mean ± amplitude of response of GABA at different concentrations.

To monitor any changes in the amplitude of the excitatory postsynaptic potentials the slope of the fEPSPs was analysed using an orthodox method used previously in other studies (Fig. 2.8:9) (Fujii et al., 1995). The effects of the drugs on LTP were analysed by changes in the slope of the field EPSP (fEPSP) before and after the drug application. A linear approximation and mean square root analysis was used to calculate the slope of the rising phase of the EPSP. For each experimental protocol time courses were produced, an average of 6 consecutive individual EPSPs was recorded per minute. The data was normalised at a control value which was defined as the period before LTP induction and when the baseline was stable, referred to as 100%. Approximately 6-12 experiments of the normalised time courses were averaged. The data represents the mean +/-SEM.

The slope of the fEPSPs was considered statistically significant for each experiment by comparing the level of potentiation to the 100% control, verified by the one-population t-test. Differences between the levels of LTP to control conditions were determined by the two-population student t-test.

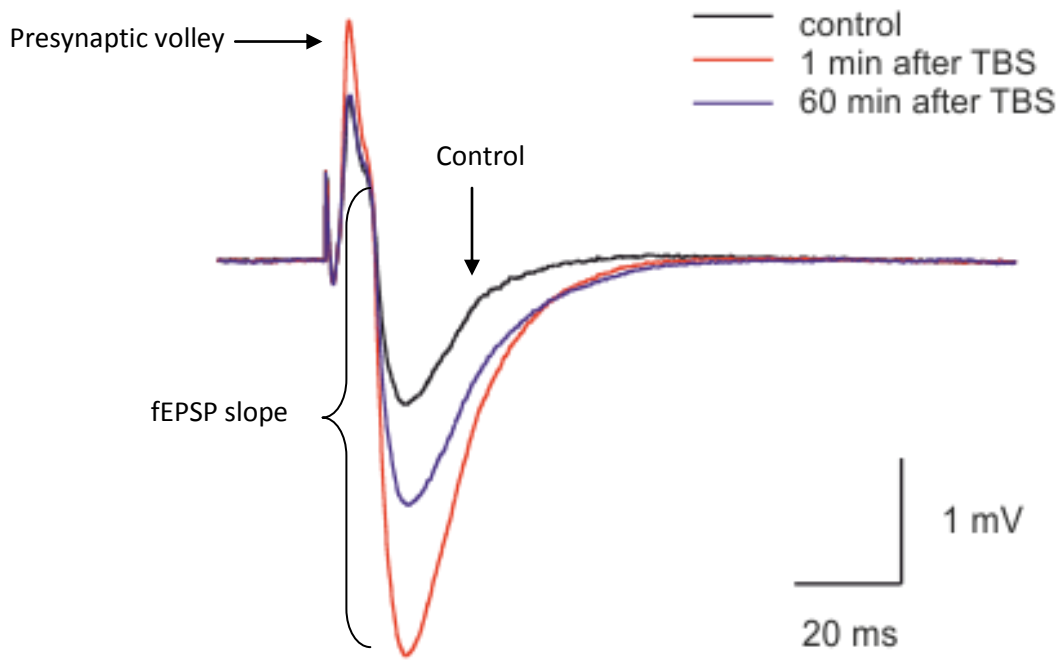

Figure 2.8:9. fEPSPs recorded under control conditions and after administering a standard Theta Burst Stimulation protocol used throughout all of the experiments conducted in the neocortex.

The initial spike of the fEPSP trace is the stimulus artefact produced by the stimulating electrode placed in layer IV-V. The rising phase of the excitatory postsynaptic potential is representative of the presynaptic volley which is caused by activity at the presynaptic nerve terminals; however the downward phase is indicative of postsynaptic potentials which gradually return back to baseline. One minute after TBS is applied (red) to the slice there is a dramatic increase in the amplitude of the EPSP with also a notable change to the slope which becomes significantly steeper and the EPSP wider. The EPSP eventually stabilises and 60 minutes after TBS (blue) application the amplitude of the EPSP decreases, but is still two-fold larger in amplitude than the control, before TBS (black). Therefore, the standard TBS protocol is able to generate long term changes in the amplitude of the EPSPs, suggesting that long term potentiation has been induced.

Chapter 3

P2X receptors down-regulate $GABA_A$ mediated currents in the peripheral and central nervous systems

3.0 Introduction

P2X receptors are ligand-gated ionotrophic receptors activated by ATP, which can transmit robust Ca^{2+} signals to neurons and glial cells in the central nervous system. The P2X receptors high affinity for calcium is principal to their capability to modulate other receptors involved in neurotransmission on the postsynaptic membrane. In 1994, their ability to cross-talk with nicotinic acetylcholine receptors was demonstrated by Nakazawa (1994). Application of both ATP and acetylcholine led to the reduction in one of the receptor-mediated currents activated by either agonist, indicative of reciprocal inhibition in rat sympathetic neurons. This novel study reported the dependence of activated acetylcholine and ATP receptors have on each other in neurons (Nakazawa, 1994). Consequently, intense research in to the negative interaction between P2 receptors and other receptors may provide new insights into neuromodulation on the postsynaptic membrane in both the peripheral and central nervous systems.

Preliminary data proposes that reciprocal inhibition between cationic P2X and anionic $GABA_A$ receptors occurs on cultured rat dorsal root ganglion (DRG) neurons (Sokolova et al., 2001). Indeed, this pioneering study revealed the potential of P2X receptor-mediated currents to

occlude propagating $GABA_A$ receptor currents. Occlusion was significantly elevated in the presence of intracellular Ca^{2+}. Therefore, this study suggests a novel Ca^{2+} -dependent interaction between P2X and $GABA_A$ receptors transpiring on DRG neurons (Sokolova et al., 2001).

Boué-Grabot et al. (2004) demonstrated that the concurrent activation of both P2X2 and multiple $GABA_A$ receptors expressed in *Xenopus* oocytes results in the inhibition of $GABA_A$ receptor-mediated responses. This inhibition was strongly reliant on the composition of the $GABA_A$ receptor subunits, as P2X2 receptors failed to suppress currents mediated by $GABA_A$ receptors composed of α, β, and γ2 or γ3 subunits (Boue-Grabot et al., 2004). This particular study disputes the concept that P2X3 receptors can occlude $GABA_A$ receptor evoked currents as proposed by Sokolova et al. (2001). Moreover, a later study suggests that the mechanism underlying the purinergic modulation of $GABA_A$ receptors is independent of Ca^{2+}, but interactions between the intracellular motifs of P2X2, P2X3, P2X2/3 or P2X4 receptors with $GABA_A$, (Boue-Grabot et al., 2004; Toulme et al., 2007; Jo et al., 2011) as with D1 and NMDA receptors (Salter et al., 2003). Hence, research into $GABA_A$ and P2X receptor interactions is ever expanding and the apparatus inducing receptor cross-talk is far from being clarified.

3.1 Inhibition of $GABA_A$ receptor currents by ATP and α,βme-ATP expressed on DRG neurons

The application of either ATP and P2X receptor specific agonist α,β-meATP significantly reduced the GABA receptor-mediated responses. ATP analogue α,β-meATP is described as a non-anhydrous compound and is not broken down into adenosine in the synaptic cleft like ATP. Therefore, we can assume that the down-regulation of the GABA receptor is caused by the activation of the P2X receptors rather than the A1 adenosine receptors. The effect of the purinergic modulation of the GABA receptors was greater under conditions where the

intracellular calcium concentration was not clamped (0.2 mM EGTA), parallel to observations previously reported by Sokolova et al. (2001). When 0.2 mM EGTA was used in the intracellular solution, the response to GABA (100 μM) recorded immediately after the application of 10 μM ATP was reduced to 67 ± 20% (n=7) of the control; this effect was statistically significant with a confidence level of P=0.02 (one-population t-test). In similar conditions, application of 10 μM α,β-meATP inhibited GABA-activated currents to 69 ± 27% (n=5) of control (Fig.3.1:10). The significance level for this effect was P=0.02 (one-population t-test). Interestingly, there is no statistically significant difference between α,β-meATP and ATP in 0.2 mM EGTA concentrations (verified by two-population t-test) despite their effects being significantly different separately.

On the contrary, when high EGTA concentrations (10 mM) were used in the intracellular medium, activation of the P2X receptors did not cause inhibition of the GABA receptors. The GABA response recorded after the application of ATP was slightly potentiated to 110 ± 69% (n=11) of control and application of α,β-meATP prevented the P2X mediated inhibition of $GABA_A$-mediated currents, 100 ± 5% (n=6). Comparison of the action of α,β-meATP in the presence of 0.2 mM EGTA and 10 mM EGTA (Fig.3.1:11) revealed that difference in the effects were statistically significant (P=0.005, two-population t-test).

The two predominant P2X receptor subtypes expressed on DRG neurons are homomeric P2X3 and heteromeric P2X2/3 receptors (Cook et al., 1997; Vulchanova et al., 1997) which exhibit both fast and slow kinetically distinct responses. Our results highlight the role of both receptor subtypes in the P2X receptor driven down-regulation of $GABA_A$ receptors expressed on DRG neurons, but this effect is limited to 0.2 mM EGTA concentrations only. Hence, our results

underline the crucial role that Ca^{2+} signalling plays in this interaction between P2X and $GABA_A$ receptors in DRG neurons.

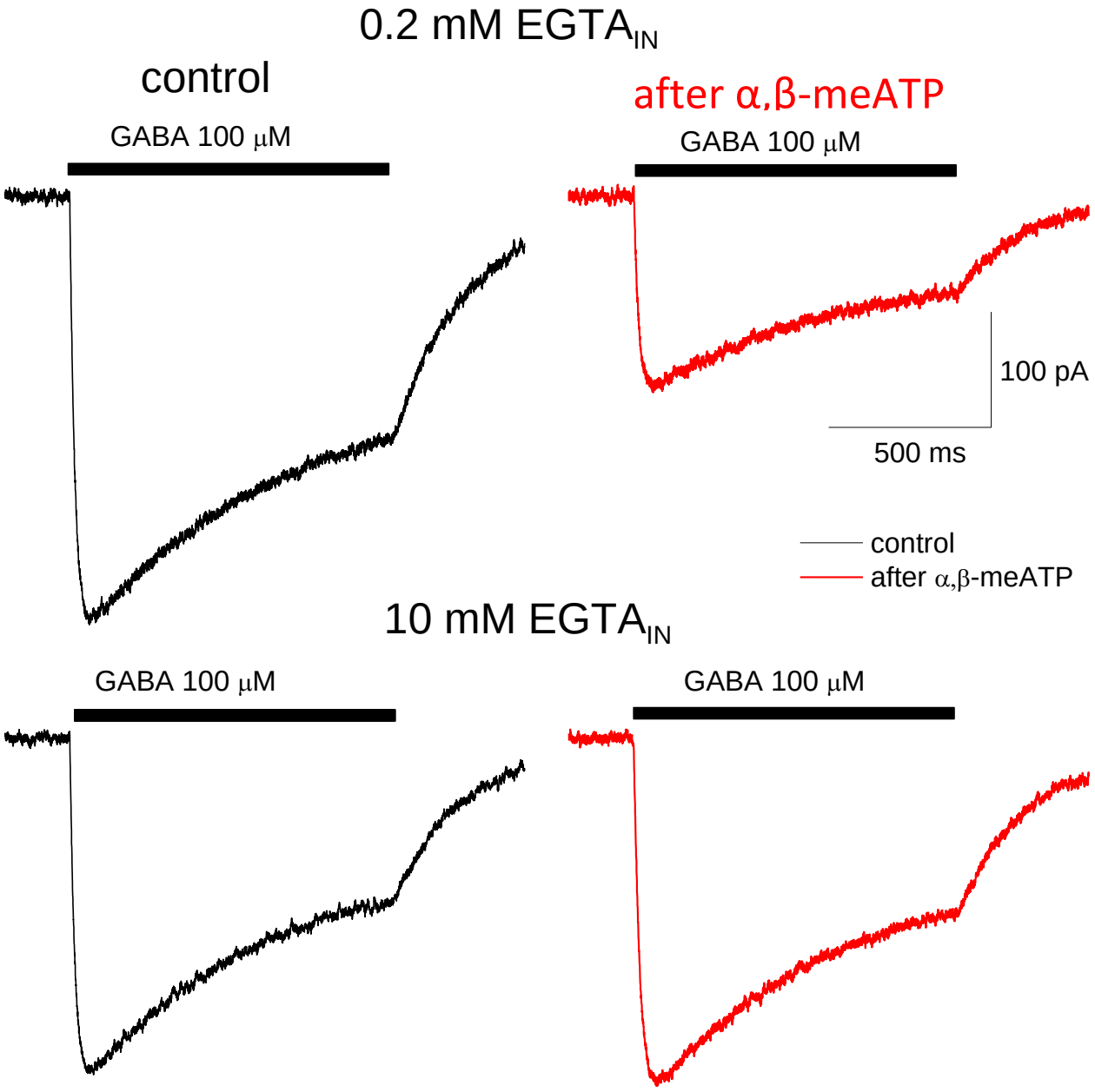

Figure 3.1:10. The effect α,β-meATP on $GABA_A$ receptor-mediated responses elicited from DRG neurons in low and high Ca^{2+} concentrations.

Recorded $GABA_A$ receptor mediated currents before (left) and after (right) P2X receptor activation in both 0.2 mM and 10 mM EGTA concentrations on dorsal root ganglion neurons. P2X receptors are able to down-regulate $GABA_A$ receptor-mediated currents in low EGTA concentrations only, highlighting the Ca^{2+} dependence of this receptor interaction. Currents were recorded in different cells at a holding potential of -80 mV.

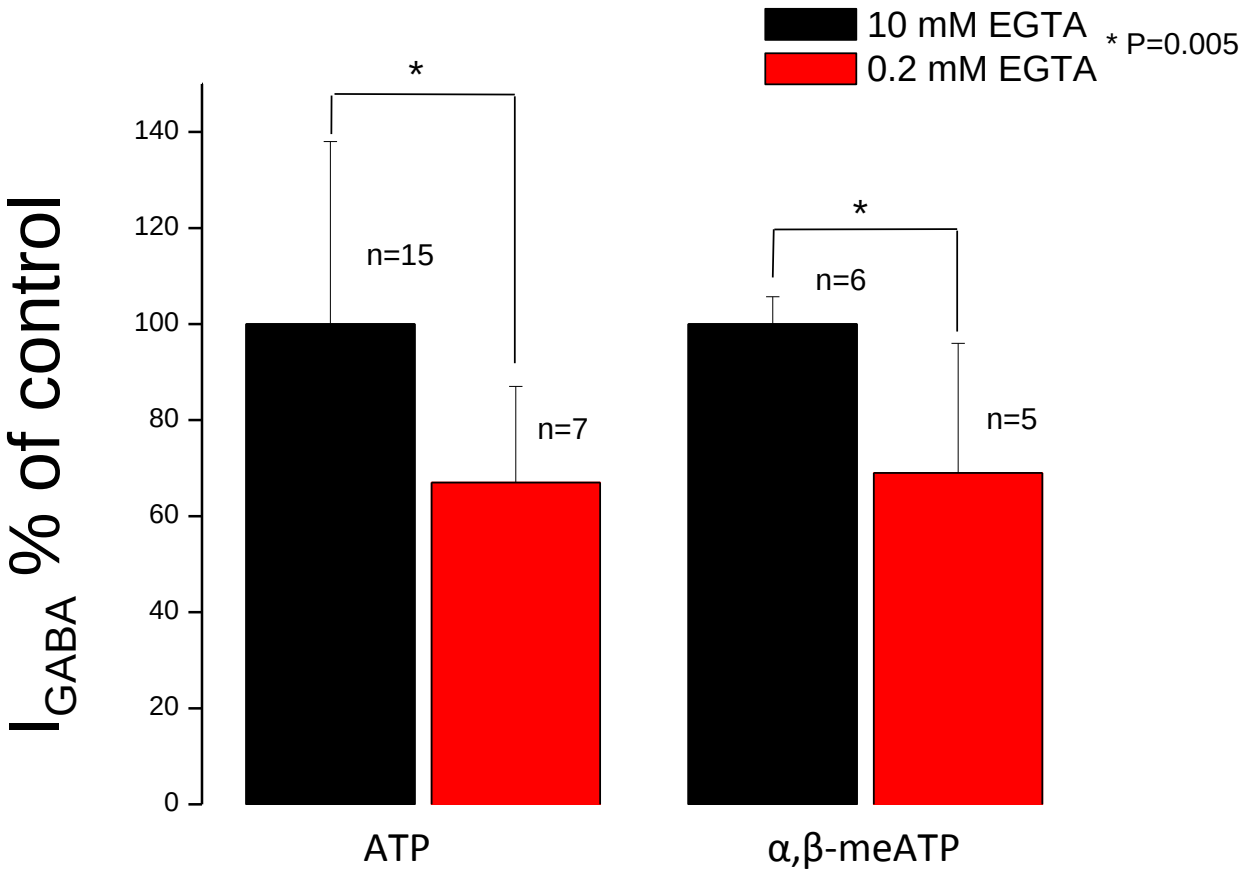

Figure 3.1:11. A comparison of the significance of the effect of ATP and α,β-meATP in 0.2 mM and 10 mM EGTA concentrations on $GABA_A$ receptor-mediated currents expressed on DRG neurons in the peripheral nervous system.

In 0.2 mM EGTA concentrations the evoked GABA current immediately after the application of both ATP and α,β-meATP were inhibited. However, in 10 mM EGTA concentrations there was no down-regulation of the $GABA_A$ receptor-mediated currents, indicating the significance of calcium in this receptor interaction. The impact of α,β-meATP and ATP of GABA on currents was statistically significant with a confidence level of P=0.005 (one population t-test). Despite both affects of the P2X agonists being statistically significant separately they failed to show any significance when their affects were compared together in 0.2 mM EGTA concentrations (two population t-test).

3.2 P2X and $GABA_A$ receptor interactions are strongly dependent on calcium influx through the purinergic receptors

3.2.1. Variability in the control GABA responses

In the earlier stages of experiments we observed a marked variability (up-to 50%) of the amplitude of $GABA_A$-activated currents in the control phase of the protocol; often there was a significant run-down of subsequent responses to GABA (Fig. 3.2.1:12). When 0.2 mM EGTA was used in the intracellular solution, the average amplitude of the initial response to the application of 100 μM GABA was 135 ± 43 pA, whereas the average amplitude of the second control response was 76 ± 31 pA (n=34). Conversely, with 10 mM of intracellular EGTA the initial GABA receptor-mediated response was 258 ± 74 pA and the subsequent response reached 267 pA ± 79 (n=35). On the third or fourth GABA application the recorded responses appeared to stabilise. The time interval between the each of the responses was 3 minutes. It should be emphasized that no ATP or α,β-meATP had been applied to cause such an effect as these responses were recorded before the application of the P2X receptor agonist. This occlusion could not be attributable to the desensitisation kinetics of $GABA_A$ receptors as sufficient time was left between each application (3 minutes) to omit cumulative agonist-provoked desensitisation (Chen et al., 1990).

In all of our experiments it was noted that this initial GABA rundown happened only when the intracellular solution containing 0.2 mM of Ca^{2+}-chelator was used. The variability of control $GABA_A$ receptor mediated currents was much less (5-10%) when the concentration of intracellular Ca^{2+}-ions was kept at physiological baseline level of 50-100 nM by using 10 mM EGTA (Egelman and Montague, 1999). Furthermore, it was noted that the amplitude of $GABA_A$ responses usually underwent significant changes when we recorded the response of the cell to a

voltage step from -80 to -20 mV between applications of GABA. This voltage-step was routinely used for monitoring the cell viability and conditions of voltage-clamp. The response to the voltage-step comprised of currents mediated by voltage gated Na^+- and Ca^{2+}-channels. Research suggests that $GABA_A$ receptors can be dephosphorylated via a Ca^{2+}/calmodulin dependent phosphatase (Chen et al., 1990; Chen et al., 1995) and phosphorylated by CaMKII and PKC (Houston et al., 2008;Houston et al., 2009; Brandon et al., 2000) rendering the receptor non-functional. However, the mechanisms underlying this phenomenon are not completely understood. Hence, to provide more stable conditions for control responses to GABA, another voltage step protocol (-80 to -70 mV) was introduced to monitor the cells between drug applications. This protocol did not activate the Ca^{2+}-channels and consequently the GABA control response variability was reduced to 10-20%.

The run-down of GABA-activated currents in the presence of low concentrations of intracellular Ca^{2+}-chelator could not be avoided completely. There was a significant drop in the amplitude of response to the second application of GABA (Fig. 3.2.1:12a) followed by a slow decline in consecutive responses. To minimize the influence of this run-down on our results, we recorded at least three to four control responses to GABA before applying P2X receptor agonists. To estimate the effect of the P2X receptors, the amplitude of GABA receptor-mediated currents was normalized to the average amplitude of several control GABA-currents recorded before (2 responses minimum) and after (1-3 responses) the application of the P2X receptor agonist. This protocol allowed for the compensation of the variability in GABA-activated currents, which could potentially give a lower estimate for the effect produced by the P2X agonists. Therefore, our results produce a more conservative estimate on the interaction between P2X and GABA receptors.

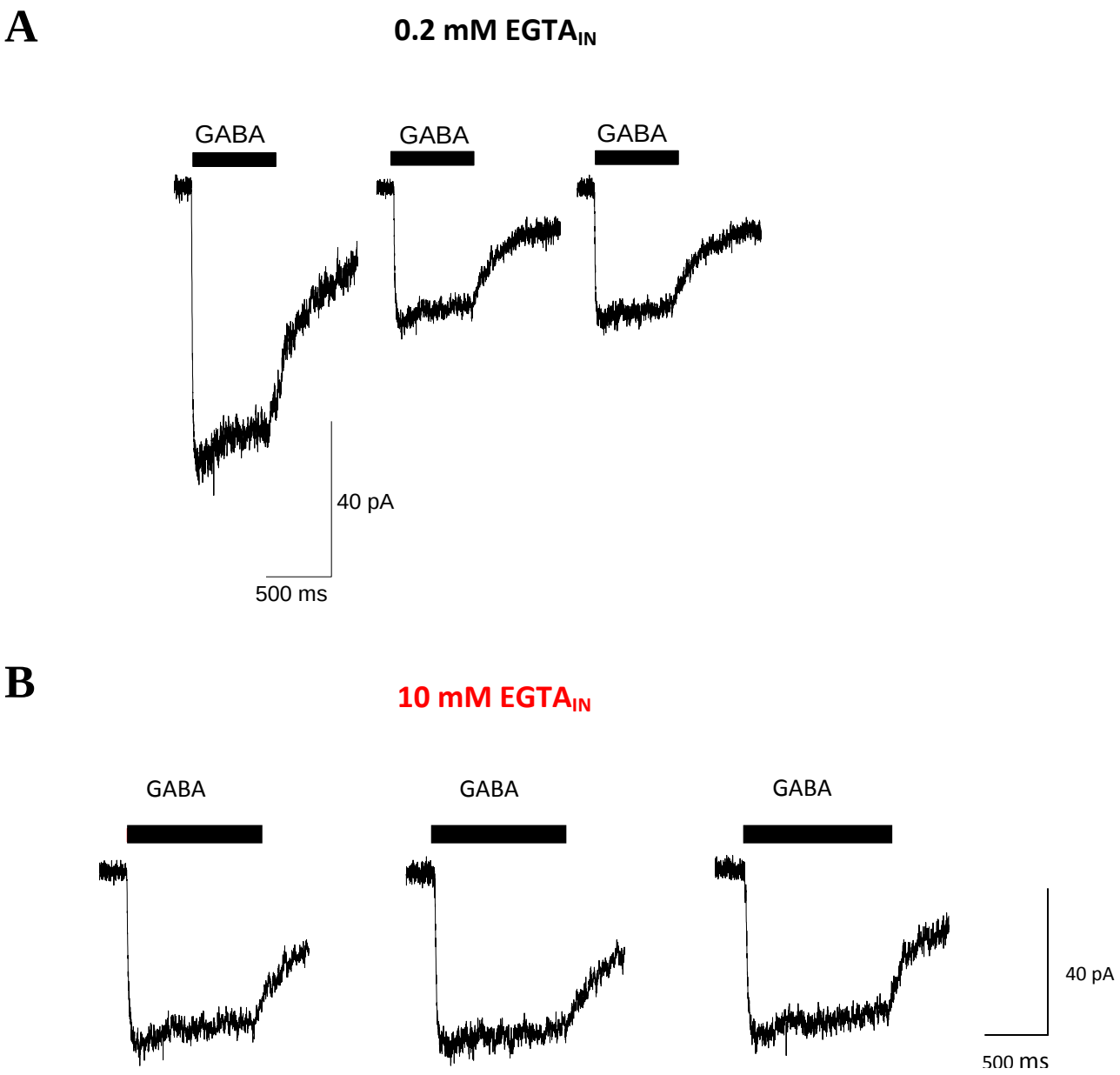

Figure 3.2.1:12. Variability in the initial control $GABA_A$ mediated currents in low and high EGTA concentrations.

A, shows the initial receptor mediated responses on the application of GABA (100 µM) before P2X agonist application in low Ca^{2+} chelator concentrations (0.2 mM EGTA). There is a significant level of rundown between the first and second responses, but the second and third responses are relatively of the same amplitude. There was virtually no variability in the initial GABA receptor responses in 10 mM EGTA (**B**).

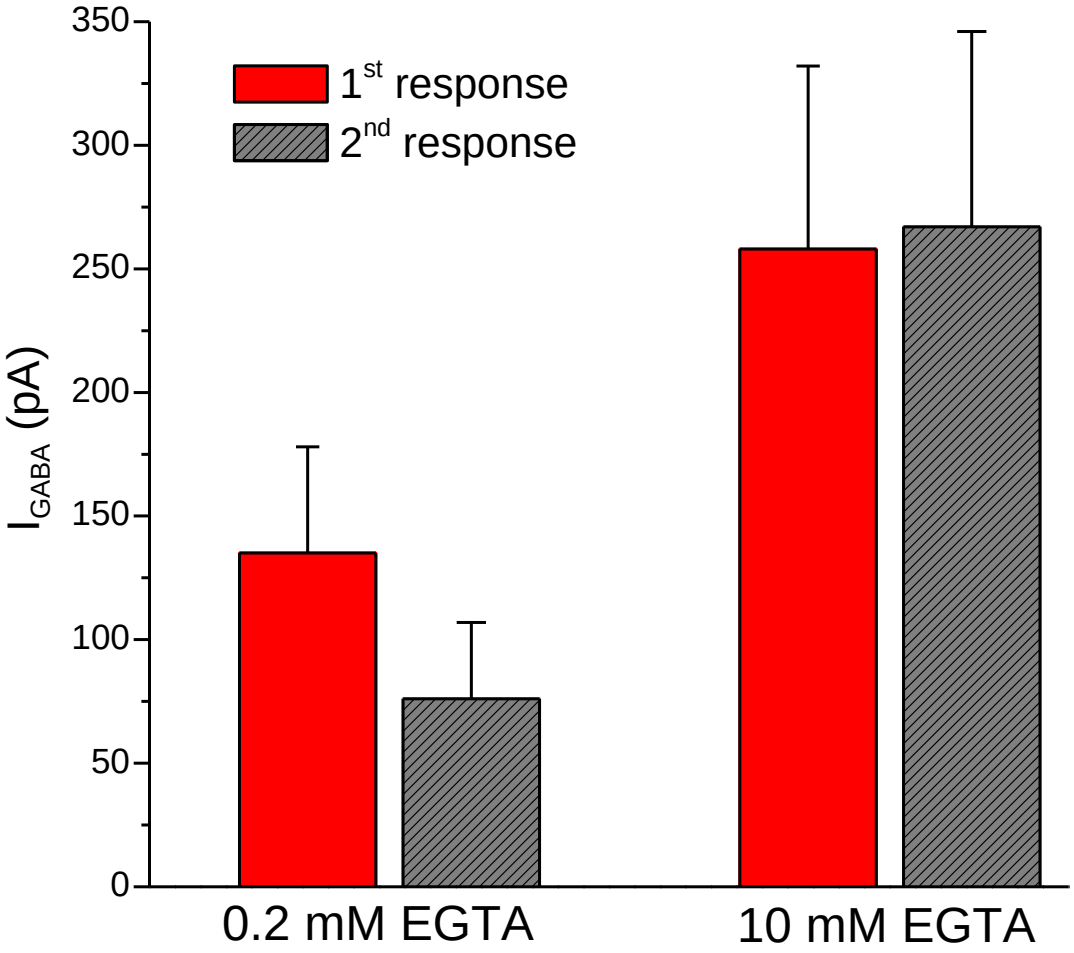

Figure 3.2.1:13. A comparison of the average amplitude of the first and second control GABA responses in 10 mM and 0.2 mM EGTA concentrations.

The stability of the GABA mediated control currents is greater in 10 mM EGTA when compared to the 0.2 mM EGTA intracellular concentrations. Currents were recorded in different cells at a holding potential of -80 mV.

3.2.2 Examining the role of voltage-gated calcium channels in the receptor interaction

To test the hypothesis that it is strictly Ca^{2+}-influx through P2X receptors which down-regulates $GABA_A$ receptors, we used an experimental protocol where by Ca^{2+}-entry was provided by voltage-gated calcium channels (Fig. 3.2.2:14) instead of activating the P2X receptors using ATP. Rather unexpectedly, this protocol did not reproduce the effect of the P2X receptor agonists. We observed not only the inhibition but also the rebound potentiation of $GABA_A$ mediated currents in 30% of cells tested. This large potentiation indicates a prolonged increase in the sensitivity of the activated $GABA_A$ receptors to it's agonist. The extent of the potentiation varied from 20 to 200% when compared to the control responses.

Inhibition of the $GABA_A$ receptor mediated responses was much smaller than one could expect from such a large Ca^{2+}-influx through the voltage-gated Ca^{2+}-channels. The GABA response recorded after the stimulation of the Ca^{2+}-channels was inhibited just to 80-90% of control; in the fraction of cells tested there was no inhibition at all. The magnitude of the inhibition caused by the activation of the voltage-gated Ca^{2+}-channels (Fig. 3.2.2:14) is comparitively smaller than the magnitude of the $GABA_A$ receptor inhibition caused by Ca^{2+} influx through activated P2X receptors (Fig. 3.1:10).

These observations suggest that not only Ca^{2+}-influx, but also activation of the P2X receptors themselves is essential for the inhibition of $GABA_A$ receptors. We observed neither potentiation nor inhibition of the $GABA_A$ response by Ca^{2+}-channels with 10 mM EGTA in the intracellular medium. This implies that the level of intracellular calcium is still important for both the up- and down-regulation of the $GABA_A$ receptors.

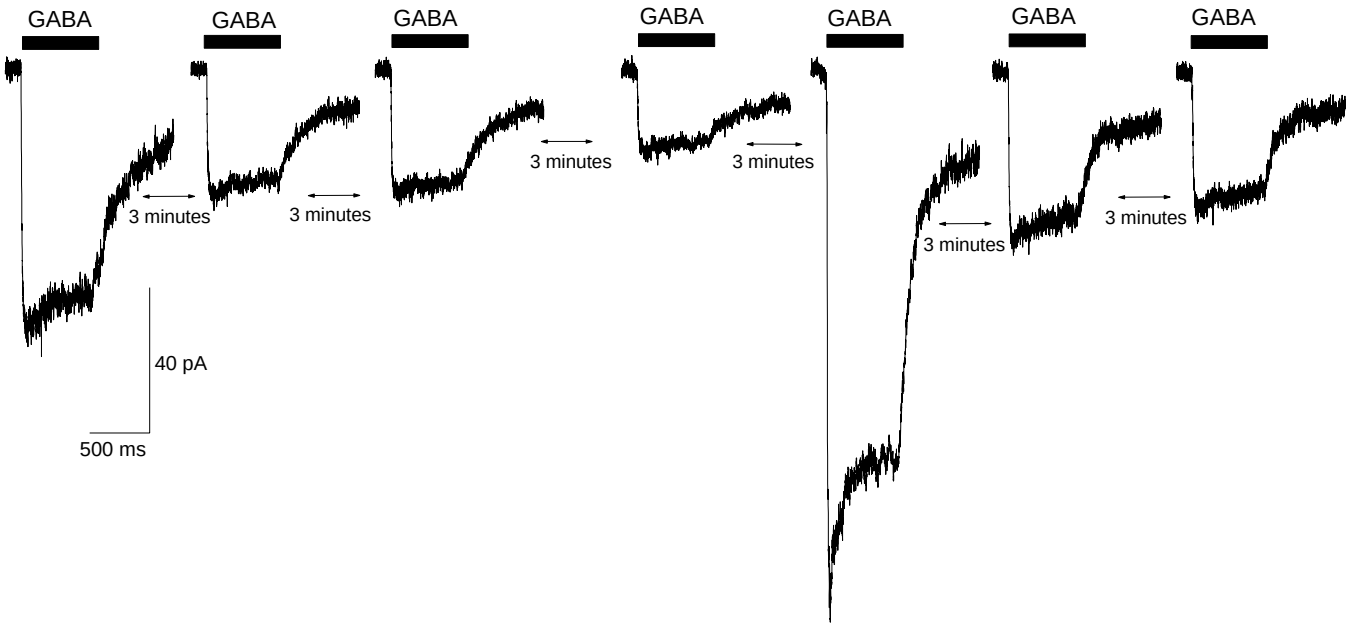

Figure 3.2.2:14. The effect of voltage-gated Ca^{2+} channels activated by a -80 to -20 mV voltage-step.

Note that inhibition of the GABA-current was followed by rebound potentiation in 30% of cells tested. The level of inhibition observed during the experiments was much smaller than one could expect from such a large calcium influx through the voltage-gated calcium channels. In fact some of the cells tested showed no inhibition of the GABA evoked currents. This suggests that activation of the P2X receptors as well as calcium influx through the purinergic receptor channel is vital to the receptor interaction. Currents were recorded with 0.2 mM EGTA in the intracellular medium.

3.3 The effect of the intracellular modulation of $GABA_A$ receptors on the P2X driven inhibition of GABA currents

Since there is no effect on $GABA_A$ receptor mediated currents upon the activation of the P2X receptors in 10 mM EGTA concentrations this suggests that Ca^{2+} influx via P2X receptors is essential to this receptor interaction. It is widely acknowledged that $GABA_A$ receptors can undergo Ca^{2+}-dependent modulation by phosphorylation (Kittler and Moss, 2003). This modulation could provide a basis for the interaction between P2X and $GABA_A$ receptors.

To study the effects of phosphorylation of the $GABA_A$ receptor on this mechanism we applied both Cyclosporine A and Staurosporine to the cells. Blocking Phosphatase II did not alter the inhibition of the $GABA_A$ current 62 ± 17% (n=7), but blocking PKC (98 ± 13% n=7) and CamKII (97 ± 13% (n=5) almost completely abolished the P2X driven inhibition of $GABA_A$ evoked responses (Fig. 3.3:15). The action of Staurosporine and KN93 when compared to the action of ATP in control conditions was statistically significant with a confidence level of P=0.02 (two population t-test). Thus, the results suggest that the functional linkage between the P2X and GABA receptor responses is via modulation by a Ca^{2+}-dependent phosphorylation process, rather than direct coupling between the receptors as previously reported (Toulme et al., 2007; Boue-Grabot et al., 2004 ; Jo et al., 2011).

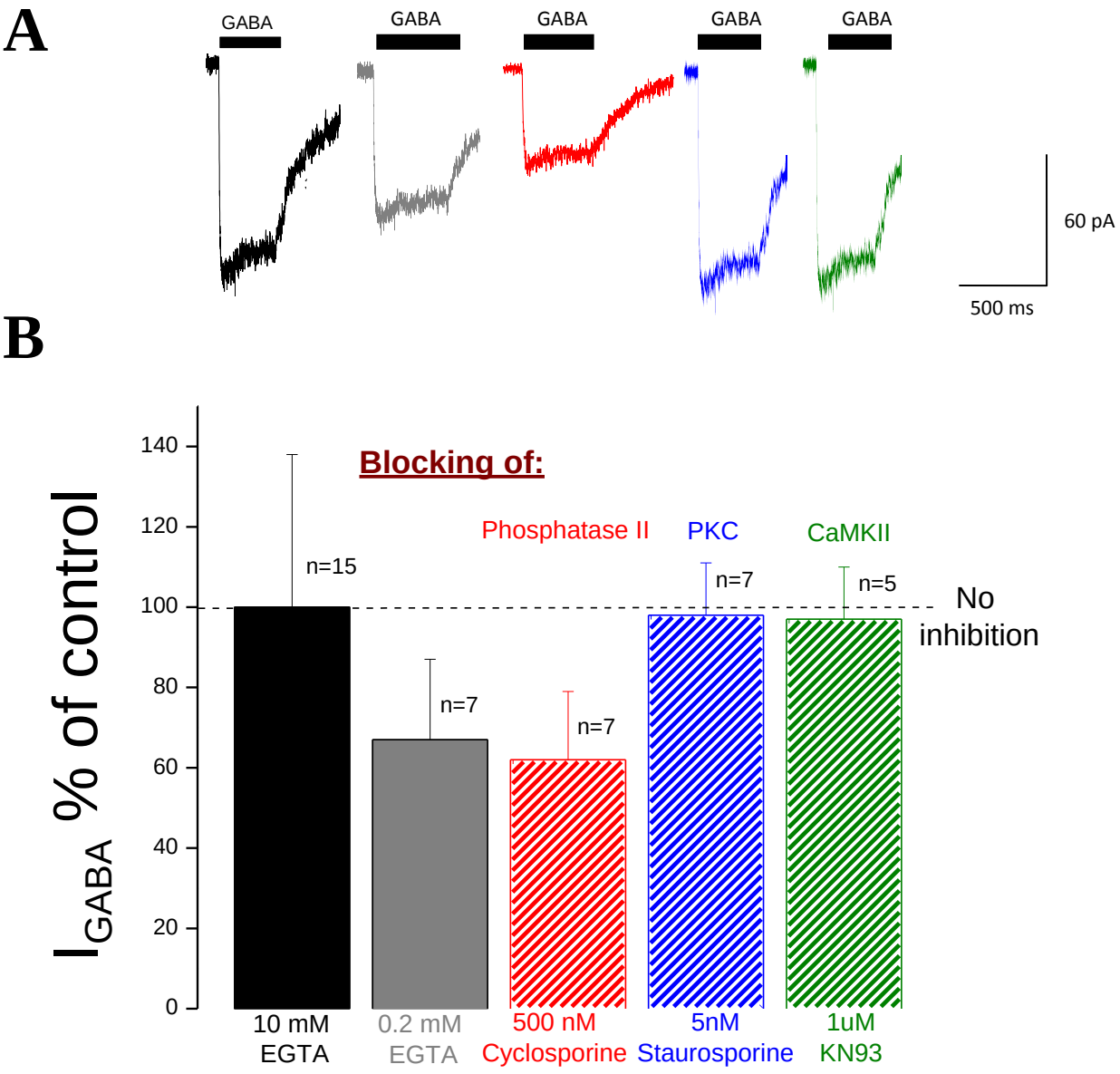

Figure 3.3:15. The effect of Staurosporine and Cylosporine A on P2X receptor mediated inhibition of $GABA_A$ evoked currents in DRG neurons.

A, GABA receptor-mediated responses recorded in high and low EGTA concentrations and under the influence of phosphatase and kinase blockers**. B,** Blocking Phosphatase II using Cyclosporine A had no effect on the impact of P2X receptors on $GABA_A$ mediated currents as a similar level of inhibition was observed in control conditions. Blocking PKC and CaMKII almost completely eliminated this effect, suggesting that phosphorylation is vital to this mechanism. The action of Staurosporine and KN93 was statistically significant with a confidence level of P=0.02 (two population t-test). All experiments involving phosphatase and kinase inhibitors were carried out using 0.2 mM EGTA in the intracellular medium.

One of the most principal pathways of ATP action as a neurotransmitter can be the direct calcium dependent modulation of neighbouring receptors expressed on the post synaptic membrane, such as NMDA (Pankratov et al., 2002a) and GABA receptors. Our results show that ATP is able to down-regulate $GABA_A$ receptor function in the peripheral nervous system; however excitatory ATP fast neurotransmission has also been demonstrated in the CNS (Pankratov et al., 1998). Its exact role in modulating synaptic transmission is not yet clarified.

Research has shown that P2X receptors can localise at inhibitory GABAergic synapses in the CNS (Lalo et al., 2006). The co-localisation of P2X with other receptors could be crucial to their modulatory actions on other membrane proteins expressed on the postsynaptic membrane. The influx of Ca^{2+} through P2X receptors may cause the activation of secondary messenger signalling cascades to cause the up or down-regulation of the co-localised receptors. Since GABA dysfunction in the central nervous system has been implicated in many neurological disorders such as schizophrenia and epilepsy it is important to also configure the impact of purinergic modulation on $GABA_A$ receptors expressed in the CNS as the next series of results will highlight.

3.4 The P2X receptor driven down-regulation $GABA_A$ receptors on acutely isolated L2/3 neo-cortical pyramidal neurons

Activated P2X receptors were also able to occlude $GABA_A$ receptor mediated currents in acutely isolated L2/3 neocortical pyramidal neurons via a similar mechanism that we previously observed in DRG neurons of the peripheral nervous system (**ref. Fig. 3.1:10**). In low calcium chelator concentrations (0.2 mM EGTA) GABA receptor responses recorded after the application of the P2X receptor agonist were reduced to 58 ± 11% (n=23) and 60 ± 18% (n=16) of control after ATP and α,β-meATP application respectively (Fig. 3.5:16). Both effects were statistically significant with confidence level P=0.005 (one-population t-test). However, comparison of the action of ATP and α,β-meATP on GABA receptor-mediated responses revealed that their effects are not significantly different. No effect of P2X receptors on GABA receptor evoked currents was observed in 10 mM EGTA concentrations on the application of ATP or α,β-meATP (Fig. 3.5:17).

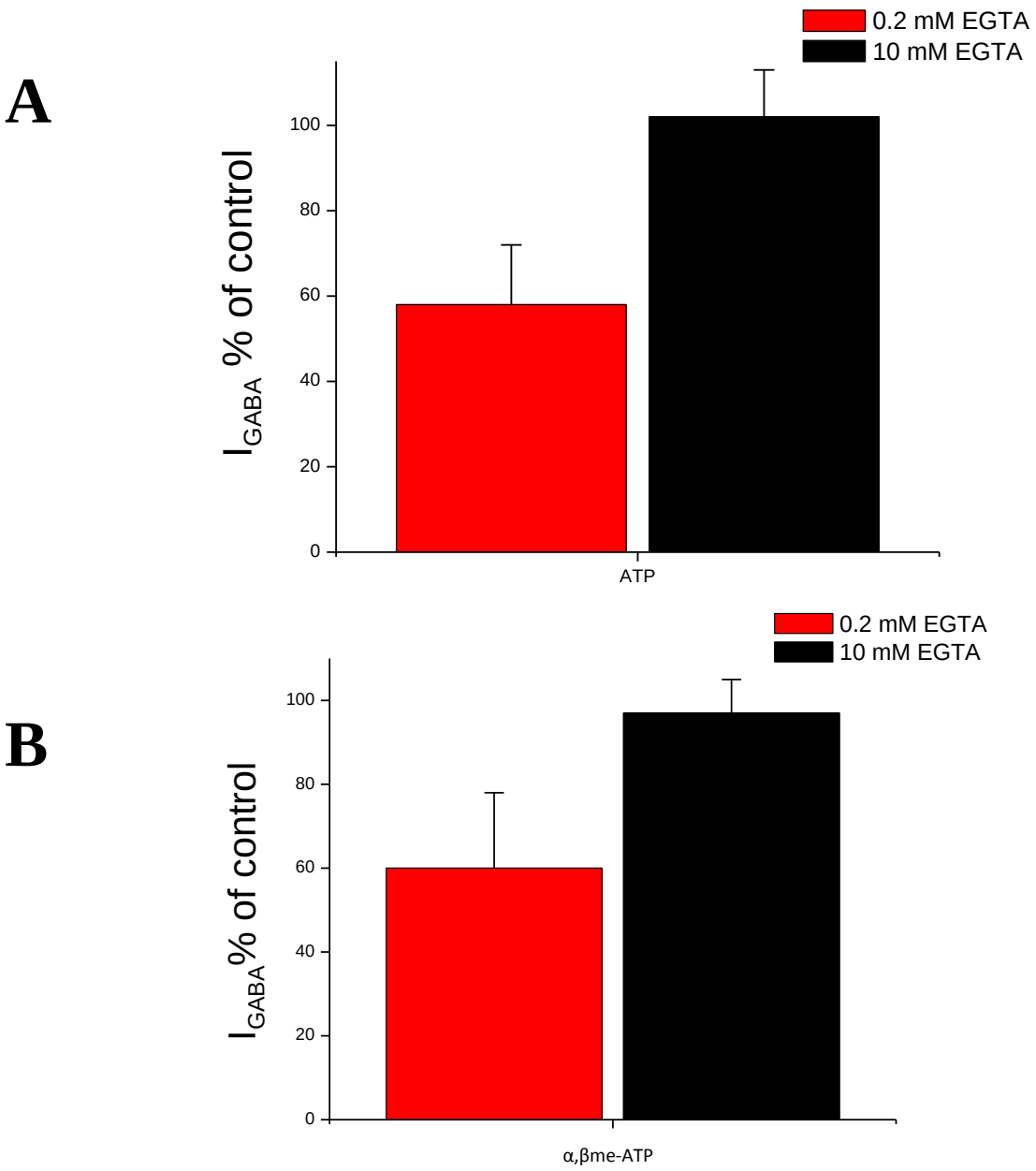

Figure 3.4:16. A comparison of the effect of ATP and P2X receptor specific agonist α,β-meATP on $GABA_A$ receptor evoked responses elicited from acutely isolated L2/3 pyramidal neurons in 10 mM and 0.2 mM EGTA concentrations.

A, in 0.2 mM EGTA concentrations the application of ATP inhibited the receptor mediated currents of $GABA_A$ receptors expressed on neocortical pyramidal neurons. However, P2X receptor activation with 10 mM EGTA concentrations in the intracellular medium failed to suppress the GABA currents. **B,** Similarly, the application of α,β-meATP caused the down regulation of GABA mediated currents in 0.2 mM EGTA concentrations but not in 10 mM EGTA. The down regulation of GABA evoked currents were statistically significant with a level of confidence of P=0.005 when both P2X receptor agonists were applied in separate experiments. The results highlight the calcium dependence of the receptor interaction in the central nervous system, parallel to previous results obtained in the peripheral nervous system.

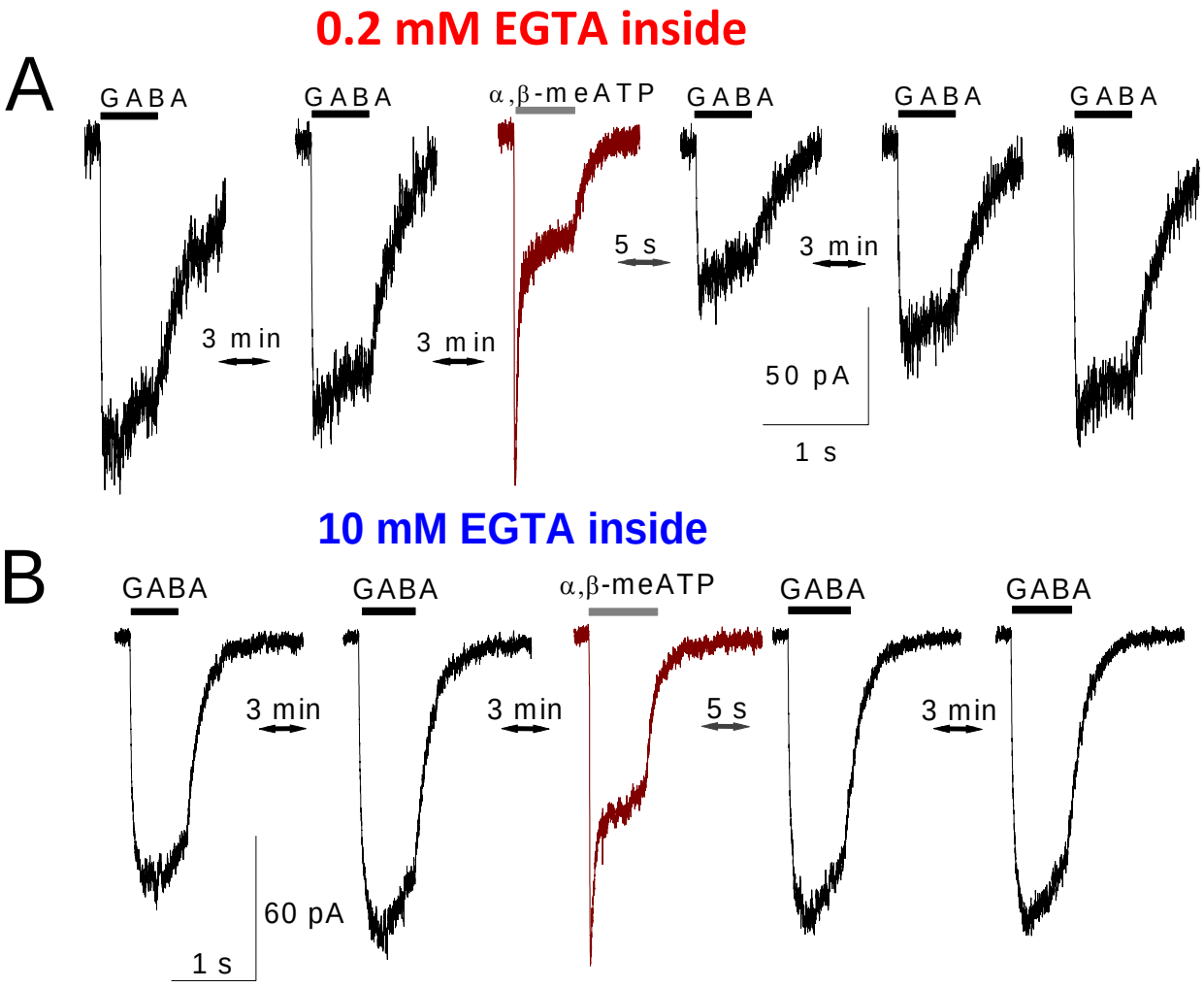

Figure 3.4:17. The effect of α,β-meATP on $GABA_A$ evoked responses elicited from acutely isolated L2/3 pyramidal neurons is mediated by Ca^{2+}.

A, shows receptor mediated responses activated by the application of GABA (100 μM) before and after the application of α,β-meATP (10 μM) in 0.2 mM EGTA concentrations. Activated P2X receptors are able to inhibit $GABA_A$ receptor mediated currents in low EGTA concentrations expressed on central neurons. **B**, the amplitude of GABA responses before and after the introduction of ATP in high calcium chelator concentrations (10 mM EGTA) reveals the failure of α,β-meATP to inhibit $GABA_A$ responses. Currents were recorded in different cells at a holding potential of -80 mV. The figure was taken from (Lalo et al., 2009).

3.5 PKC plays a significant role in the P2X driven modulation of $GABA_A$ receptors in the central nervous system

Previously we have shown that $GABA_A$ receptors are targets of modulation by intracellular signalling cascades involving Ca^{2+}-dependent phosphorylation in the peripheral nervous system (Fig. 3.3:15). Since P2X receptors are also able to down-regulate $GABA_A$ receptors expressed on cortical neurons via a Ca^{2+}-dependent mechanism (Lalo et al., 2009), we then went on to look at the impact of $GABA_A$ receptor phosphorylation on this mechanism in the central nervous system.

Application of blockers of cAMP-dependent kinase, PKA, failed to eliminate the P2X receptor-mediated inhibition of currents elicited from $GABA_A$ receptors on cortical neurons. GABA responses recorded after the application of ATP under the influence of H-89 and KT5720 were still inhibited 67 ± 23% (n=26) of control and 49 ± 16% (n=5) accordingly. Our results indicate that PKA is not involved in the down regulation of GABA evoked currents by P2X receptors.

GABA receptors have a conserved Protein Kinase C (PKC) binding domain located on their β subunit (Brandon et al., 2000; Brandon et al., 2002; Kittler et al., 2003) and it is established that PKC is able to modulate the function of $GABA_A$ receptor mediated responses in cultured hippocampal and cortical neurons (Connolly et al., 1999). To study the involvement of PKC in this mechanism we applied 5 nM of Staurosporine to the cells and recorded responses to $GABA_A$ before and after the application of P2X receptor agonist, ATP (Fig. 3.6:18). In this instance Staurosporine was able to completely block the purinergic modulation of $GABA_A$ receptors by 101 ± 10% (n=4).

Staurosporine is marketed as a broad spectrum kinase inhibitor with an IC_{50} of ~7 nM for PKA and an IC_{50} of ~3 nM for PKC (Tamaoki et al., 1986). At a concentration of 5 nM it is highly probable that we were partially blocking both kinases with preference to PKC. As such we then went to reconfirm our results by applying a more specific blocker of PKC, GF109203X (Fig. 3.2.1:18). The $GABA_A$ receptor-mediated response recorded immediately after the activation of the P2X receptors resulted in the almost complete abolishment of $GABA_A$ inhibition by 92 ± 13% (n=6). To further establish the involvement of PKC, sub-threshold concentrations of both Staurosporine and GF109203x were applied simultaneously. Consequently, introducing both kinase inhibitors to the cells produced a slight potentiation of the $GABA_A$ mediated responses and no inhibition (105 ± 9% n=7). A comparison of the action of ATP in control conditions and under the influence of inhibitors of PKC revealed that the difference in the effects were statistically significant with a confidence level of P=0.02 (two population t-test). Therefore, our results strongly suggest a potential role for PKC in the interaction between these two ionotrophic receptors.

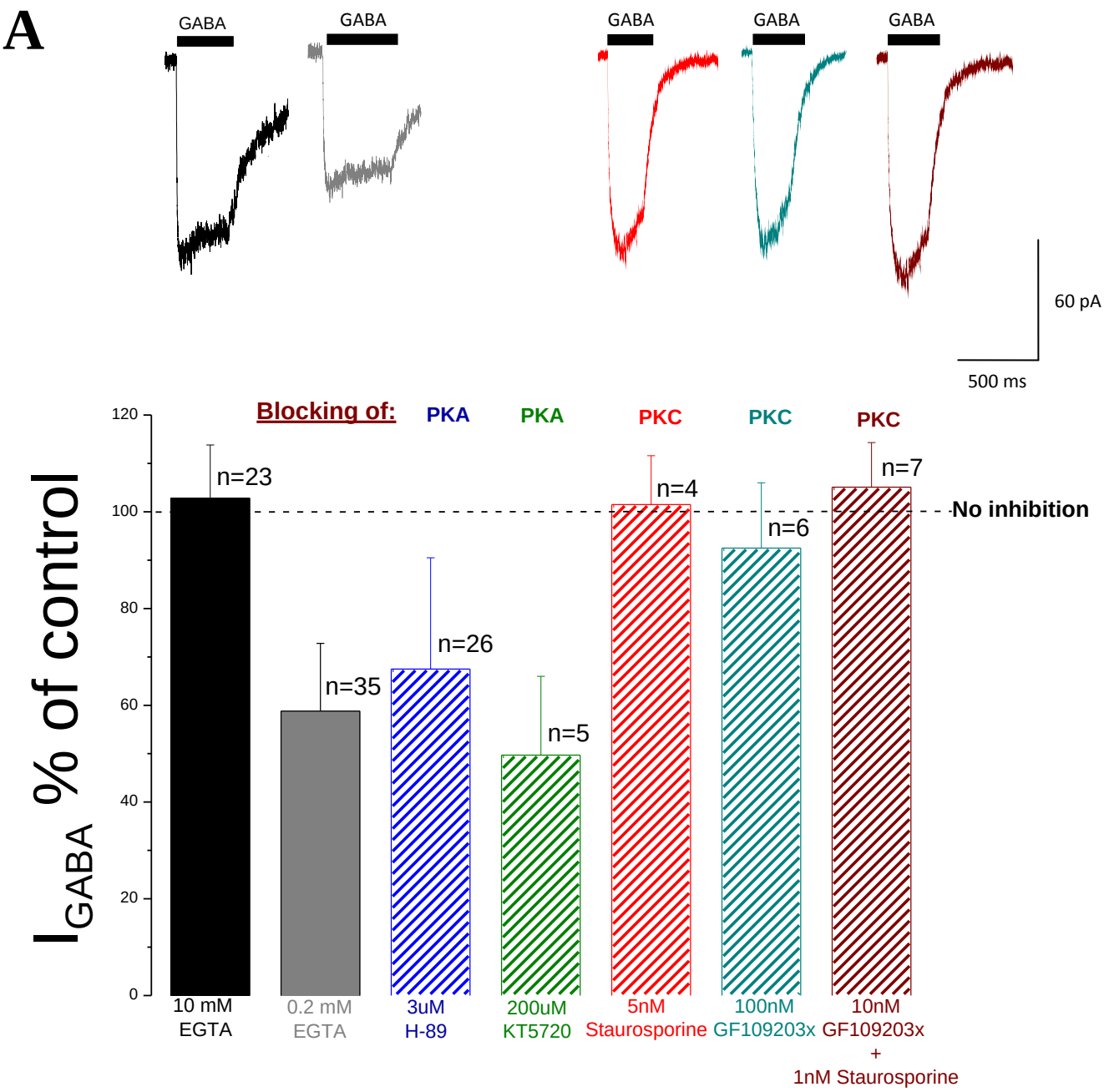

Figure 3.5:18: PKC is able to completely abolish the P2X receptor-mediated down-regulation of $GABA_A$ receptor currents in isolated cortical neurons.

A, the blocking of PKA has no effect on the ability of activated P2X receptors to inhibit $GABA_A$ receptor evoked currents. However, blocking PKC with Staurosporine, GF109203x and then a combined application of both kinase inhibitors almost or completely blocked the purinergic modulation of $GABA_A$ receptors. **B,** comparison of the action of ATP in control conditions and under Staurosporine and GF109203x revealed that the difference in the effects were statistically significant with a confidence level of P=0.02 (two population t-test). All experiments in which kinase inhibitors were washed on to the cells 0.2 mM EGTA concentrations were used unless otherwise stated.

3.6 Discussion

The presence of fast excitatory purinergic synaptic transmission has only been demonstrated in a number of regions in the central nervous system: the spinal cord (Jo and Schilchter, 19993), locus coeruleus (Nieber et al., 1997), hippocampus (Pankratov et al., 1998) and many more (Hugel et al., 2000). Research has shown that P2X subunit expression occurs on both the soma and dendrites of pre- and postsynaptic cells in the CNS (Vulchanova et al., 1997; Kanjhan et al., 1999), which highlights the potential of P2X receptors to modulate the function of other receptors at synapses in the brain via pre- and postsynaptic processes. P2X receptors are major contributors to nociceptive pain signalling in the spinal cord (Burnstock, 2000) and thus have become the focus of intense pharmacological research. Therefore, it is important to elucidate the function of ATP signalling in the central nervous system and the impact that the action of ATP can have on brain function.

To examine this paradigm we observed the postsynaptic actions of P2X on $GABA_A$ receptor mediated currents as it has been previously reported that P2X receptors can modulate inhibitory $GABA_A$ currents on DRG neurons and *Xenopus oocytes* (Sokolova et al., 2001; Toulme et al., 2007). We studied the P2X driven down-regulation of $GABA_A$ receptor mediated transmembrane currents in both the peripheral and central nervous system, where the underlying mechanism is strongly dependent on intracellular Ca^{2+} concentrations. This is demonstrated continuously throughout our experiments as ATP and it's non-hydrolysable analogue α,β-meATP only had an effect on $GABA_A$ mediated responses in 0.2 mM EGTA and not 10 mM EGTA where the intracellular Ca^{2+} concentration is kept at physiological baseline levels. Additionally, the control GABA responses are significantly variable in 0.2 mM EGTA solutions, emphasising the potential calcium dependence of this interaction. Therefore, our findings

contradict previous experimental data which disputes the necessity of Ca^{2+} in this receptor interaction (Toulme et al., 2007).

There are many P2X receptor isoforms expressed in the peripheral and central nervous systems, evoking a wide range of kinetically distinct P2X receptor phenotypes. Whilst conducting our experiments we observed different phenotypes of currents elicited from activated P2X receptors (fast, slow and mixed responses), suggesting the possibility that the amount of calcium influx through the receptor channel is variable and dependent on the kinetics of the P2X receptor subtype. Since we observed the occlusion of GABA mediated currents regardless of the type of P2X receptor activated in both the peripheral and central nervous systems reinforces the idea that purinergic modulation of $GABA_A$ receptors is a universal phenomenon.

The purinergic modulation of $GABA_A$ receptors is not solely attributable to Ca^{2+} influx through the P2X receptors as the state of the $GABA_A$ receptors themselves may play a significant role in respect of their subunit composition and phosphorylation state. Observations have demonstrated that $GABA_A$ receptors harbour a series of highly conserved phosphorylation sites specifically on their β- and γ-subunits (Brandon et al., 2000; Vithlani et al., 2011). As phosoproteins $GABA_A$ receptors are prone to intracellular modulation via a Ca^{2+}-dependent phosphorylation process (Brandon et al., 2002; Kittler et al., 2003; Kumar et al., 2005; Houston et al., 2009). We have studied the impact of $GABA_A$ phosphorylation on the receptor interaction. Our results show that by inhibiting PKC the action of P2X receptors on $GABA_A$ mediated currents is completely abolished and these experiments were performed in low concentrations of Ca^{2+} chelator (0.2 mM EGTA).

Emphasis is put on the role of Ca^{2+} in the inhibition of $GABA_A$ receptors as we observed a significant difference between the average of the first and second $GABA_A$ receptor control responses. This effect is restricted to 0.2 mM EGTA concentrations only (Fig. 3.2.1:11a), which further implicates Ca^{2+} in the down-regulation of $GABA_A$ evoked responses in the peripheral and central nervous systems. Research has demonstrated that the stability of $GABA_A$ receptors is mediated by clatharin-dependent endocytosis and involves PKC (Connolly et al., 1999; Kittler et al., 2003; Arancibia-Cárcamo et al., 2009). Therefore, the P2X receptor driven inhibition of $GABA_A$ receptor function may also be attributable to GABA receptor trafficking via a Ca^{2+}-dependent and an intracellular signalling cascade.

By activating voltage gated Na^+- and Ca^{2+} channels using a voltage step of -80 to -20 mV between several GABA applications we have established that Ca^{2+} channels have a small effect on GABA responses (Fig. 3.2.2:14). For instance, the GABA response recorded directly after the voltage-step stimulation is smaller when compared to the two control responses. However, their mechanism of action is not similar to the rundown in $GABA_A$ mediated currents. In fact what we observed is the rebound potentiation of the GABA response.

Rebound potentiation has been documented in cerebellar Purkinje neurons where excitatory currents were able to prolong inhibitory GABA receptor activation (Kano et al., 1992). This mechanism is stimulated by a transient increase in intracellular Ca^{2+} levels, caused by the activation of voltage-gated channels (Kano et al., 1996) which activate protein kinases. Since we have demonstrated the involvement of PKC in the functional modulation of $GABA_A$ receptors it may be possible that it also plays a crucial role in the rebound potentiation at inhibitory synapses. This experimental data concerning the effect of voltage-gated Ca^{2+} channels is preliminary and requires more vigorous experimental analysis to specifically determine this mechanism.

Nevertheless, this extended GABA receptor signalling, which enhances the efficacy of synaptic inhibition may have important implications for learning and memory (Brandon et al., 2002). .

In conclusion, so far we have demonstrated that P2X receptors are able to modulate $GABA_A$ receptors expressed on the post synaptic membrane via a Ca^{2+} dependent mechanism. The phosphorylation and function of $GABA_A$ receptors can be regulated by PKC in cortical pyramidal neurons. Therefore, P2X receptors may dynamically modulate other postsynaptic receptors by directly activating Ca^{2+} dependent signalling cascades in the central nervous system.

Chapter 4

The inhibition of $GABA_A$ receptors which mediate tonic inhibition by P2X receptors in the central nervous system

4.0 Introduction

The fast inhibitory synaptic transmission in the central nervous system is principally derived from the synaptic release of γ-aminobutyric acid (GABA), which activates postsynaptic $GABA_A$ receptors. $GABA_A$ receptors modulate excitatory synaptic transmission by either hyperpolarizing the cell membrane or by shunting excitatory inputs (Yamada et al., 2007).

It is widely acknowledged that there are two types of $GABA_A$ current which exists in the central nervous system: type 1 and type 2. The type 1 current is produced by GABA receptors that are located in the synaptic cleft and activated by high transient concentrations of GABA released from the presynaptic terminal. The type 2 receptor-mediated currents are produced by $GABA_A$ receptors which are highly sensitive and require a much lower concentration of GABA for receptor activation. In subsequent chapters we will be referring to the type 1 receptor as phasic $GABA_A$ receptors and type 2 receptors as tonic $GABA_A$ receptors.

Only recently has the function of phasic $GABA_A$ receptors and tonic $GABA_A$ receptors which reside outside of the synaptic cleft been integrated (Wall and Usowicz, 1997; Nusser et al., 1998; Semyanov et al., 2004). The spill over of GABA from the synapse innervates tonic

$GABA_A$ receptors to produce a prolonged GABA tone termed, tonic inhibition. Conventionally, functional phasic receptors consist of α, β and γ subunits, however tonically activated GABA receptors are composed of a distinct set of highly sensitive subunits, including α5- and δ-subunits in the central nervous system.

Whilst studying the impact of P2X receptors on neurotransmission in both the peripheral and central nervous systems we have demonstrated the down regulation of phasic $GABA_A$ receptor-mediated currents as a result of being targets of purinergic modulation. P2X receptors are able to inhibit GABA responses this way as a result of their high Ca^{2+} permeability which engenders the activation of an intracellular signalling cascade. The application of kinase inhibitors to the recording chamber resulted in the removal of the inhibition of GABA receptor-mediated responses, particularly in the presence of blockers of Protein Kinase C. Therefore, we surmise that this receptor interaction is Ca^{2+}-dependent and also involves the regulation of $GABA_A$ receptors by PKC phosphorylation.

Dysfunctional $GABA_A$ receptors that mediate tonic inhibition in the central nervous system have been implicated in many neurological disorders (Jacob et al., 2008). Extrasynaptic tonic $GABA_A$ receptors are prime targets for various reagents (Cheng et al., 2006; Santhakumar et al., 2007) it is highly possible that they could act as novel therapeutic targets for a plethora of neurological pathophysiologies. Although it has been suggested that tonic inhibition occurs on neocortical pyramidal neurons (Salin et al., 1996) the underlying mechanism and the physiological relevance of this phenomenon is far from being elucidated, and thus they became a key focus in this project.

4.1 Pharmacologically isolating subunit specific $GABA_A$ receptors mediating tonic inhibition

Tonic conducting $GABA_A$ receptors are localised outside the boundaries of the synaptic cleft and they are transiently activated via a spill over of neurotransmitter from the synapse. The tonically active current is caused by a specific subset of $GABA_A$ receptor subunits that are highly sensitive and have slower desensitizing kinetics, consisting primarily α1, α5, α4 and δ subunits (Nusser et al., 1998; Mortensen and Smart, 2006; Glykys and Mody, 2008; Olsen and Sieghart, 2009). δ-$GABA_A$ receptor subunits are restricted to extrasynaptic locations (Nusser et al., 1998) and studies have demonstrated their expression in the somatosensory neocortex (Yamada et al., 2007).

To confirm that we were selectively activating tonic rather than phasic $GABA_A$ receptors, receptor mediated responses were measured from acutely isolated pyramidal neurons. Due to the differentiation of GABA sensitivities conferred by the different subunit compositions of the receptors we applied various concentrations of GABA to the cells, ranging from 0.1 µM to 200 µM to produce a dose response curve.

The broad spectrum of GABA concentrations applied were (in µM) 0.1, 0.2, 0.5, 1, 5, 10, 20, 100 and 200 (Fig 4.1:19). From the dose-response curve it can be deduced that tonic $GABA_A$ receptors have an EC_{50} of 0.89 µM which is in accordance with previous data (Brown et al., 2002; Borghese et al., 2006). Even at concentrations below this value we still observed activation of the tonically active receptors, which emphasises the high sensitivity of extrasynaptic $GABA_A$ receptors to ambient GABA concentrations.

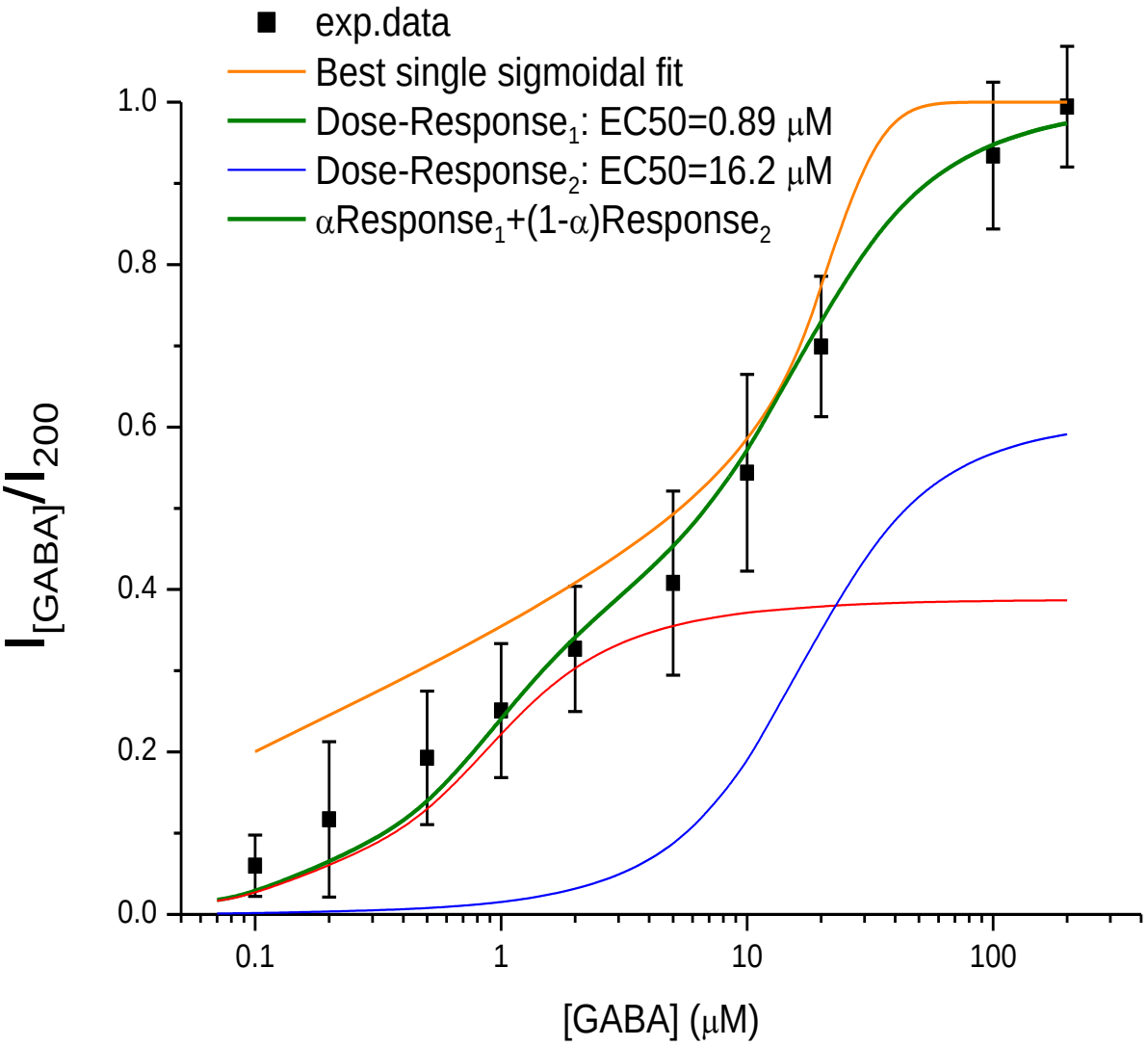

Figure 4.1:19. $GABA_A$ receptor sensitivity of neocortical pyramidal neurons

A dose-response curve showing the relationship between varying concentrations of GABA and the peak activated receptor currents. From the GABA concentration-dose response curve it is evident that two populations of GABA receptors are present. We observed responses mediated by the high affinity GABA receptors at the lower agonist concentrations (0.1-5 μM) and responses mediated by receptors with a lower affinity at the higher concentrations of GABA (10-200 μM). All of the responses were normalised on the GABA current which was generated by a saturating concentration of agonist during the experiments. We observed receptor saturation at 200 μM of GABA. The data was accumulated from individual acutely isolated pyramidal neurons of the neocortex. All recordings were conducted using 0.2 mM EGTA in the intracellular solution at a holding potential of -80 mV.

4.2 δ-subunit $GABA_A$ containing receptors mediate tonic inhibition in the somatosensory cortex

The expression of the GABA δ-subunit has been previously recognised in the somatosensory cortex (Yamada et al., 2007) and they are major contributors to the tonic conductance in this brain region. To study the subunit specificity for phasic and tonic $GABA_A$ receptors we introduced DS2, a novel positive allosteric modulator of the δ-subunit containing receptors (Wafford et al., 2009) to the extracellular solution whilst conducting experiments.

Under control conditions we observed a mean amplitude in the range of 50-250 pA (n=12) in response to the application of 0.2 μM of GABA. After introducing DS2 (300 nM) into the extracellular medium concurrently with the fast application of 0.2 μM of GABA the mean activated receptor response was considerably potentiated ranging from 100-400 pA (n=7) (Fig. 4.2:20). The average increase of DS2 influenced $GABA_A$ receptor mediated responses was 95% ± 67% n=7 when compared to control conditions. The effect of DS2 on activated $GABA_A$ receptor currents was statistically significant at a confidence level of P=0.01 (one-population t-test). Our results confirm the expression of δ-subunits on neocortical pyramidal cells and thus this specific extrasynaptic GABA receptor subtype is major contributor and accountable for mediating tonic inhibition in the somatosensory cortex.

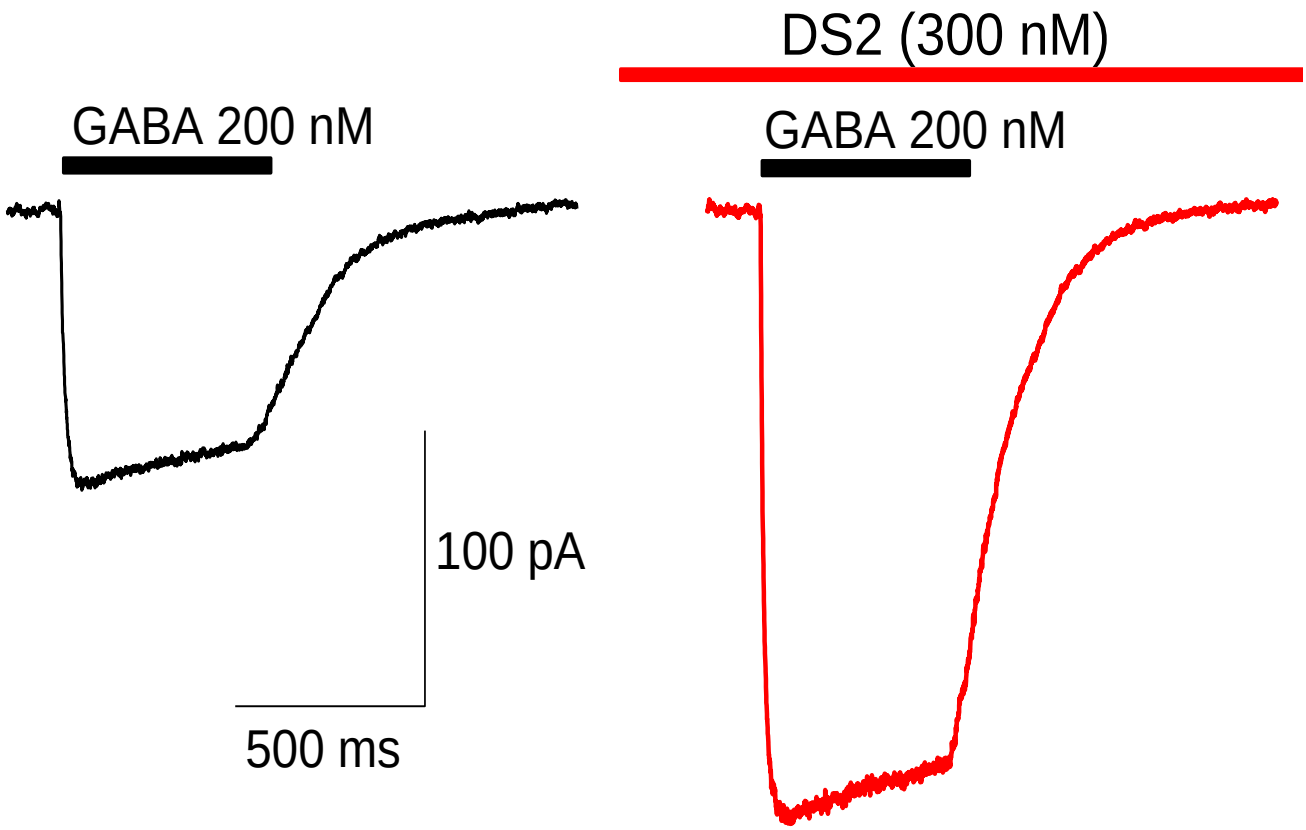

Figure 4.2:20. The pharmacological isolation of δ-subunit containing $GABA_A$ receptors that mediate tonic inhibition in cortical pyramidal neurons.

In control conditions (left) 200 nM of GABA applied to the recording chamber produced a mean response in the range of 50-250 pA (n=12). However, this response was significantly potentiated under the influence of a novel allosteric modulator of the δ-$GABA_A$ receptor subunits, DS2 (right), ranging from 100-400 pA (n=7). δ-subunit containing $GABA_A$ receptors are located extrasynaptically and have been demonstrated to have a major role in conducting tonic currents in the somatosensory cortex (Yamada et al., 2005). All experiments were carried out in 0.2 mM EGTA**.** Currents were recorded at a holding potential of -80 mV.

4.3 $GABA_A$ receptors mediating tonic inhibition are targets of purinergic modulation

Due to our previous results obtained when studying the sensitivity of extrasynaptic $GABA_A$ receptors to their agonist (Fig. 4.1:19) we applied 1 μM of GABA to acutely isolated pyramidal neurons before and after the introduction of the P2X specific agonist, α,β-meATP (10 μM). As previously observed with phasic $GABA_A$ receptors, our results demonstrate that extrasynaptic $GABA_A$ receptors are also inhibited by P2X receptor activation. Tonic $GABA_A$ receptor responses were reduced to 66 ± 16% n=19 of control in low EGTA concentrations (Fig. 4.3:21). However, in 10 mM EGTA, no inhibition mediated by α,β-meATP application was observed, 96 ± 8% n=11. We used non-anhydrous ATP analogue α,β-meATP to eliminate the possible effects of adenosine when ATP is broken down in the synaptic cleft. Individually, both of these effects were statistically significant with a confidence level of P=0.005 (one-population t-test).

We also studied the action of ATP on the P2X receptor driven down-regulation of tonic $GABA_A$ receptors (Fig. 4.3:22). Fast application of ATP to the recording chamber caused the inhibition of the tonic receptors recorded immediately after the activation of the ATP receptor in low EGTA concentrations, 62 ± 19% n=16. In 10 mM concentrations of calcium chelator the tonic $GABA_A$ response was inhibited to a similar level as we observed on application of α,β-meATP, 92 ± 7% n=11 of control. Both effects were significant with a confidence level P=0.005 (one population t-test). Hence, tonically activated $GABA_A$ receptors are also prime targets of the modulatory actions of purinergic receptors.

We observed inhibition of both phasic and tonic $GABA_A$ receptors in the central nervous system and of phasic receptors on DRG neurons in the peripheral nervous system. Inhibition was caused by the activation of P2X receptors with both ATP and α,β-meATP, which have specificity for different P2X isoforms. Therefore, our results highlight the universal nature of

this mechanism and the possible expanse of the modulatory repertoire of purinergic receptors on the postsynaptic membrane in the central nervous system.

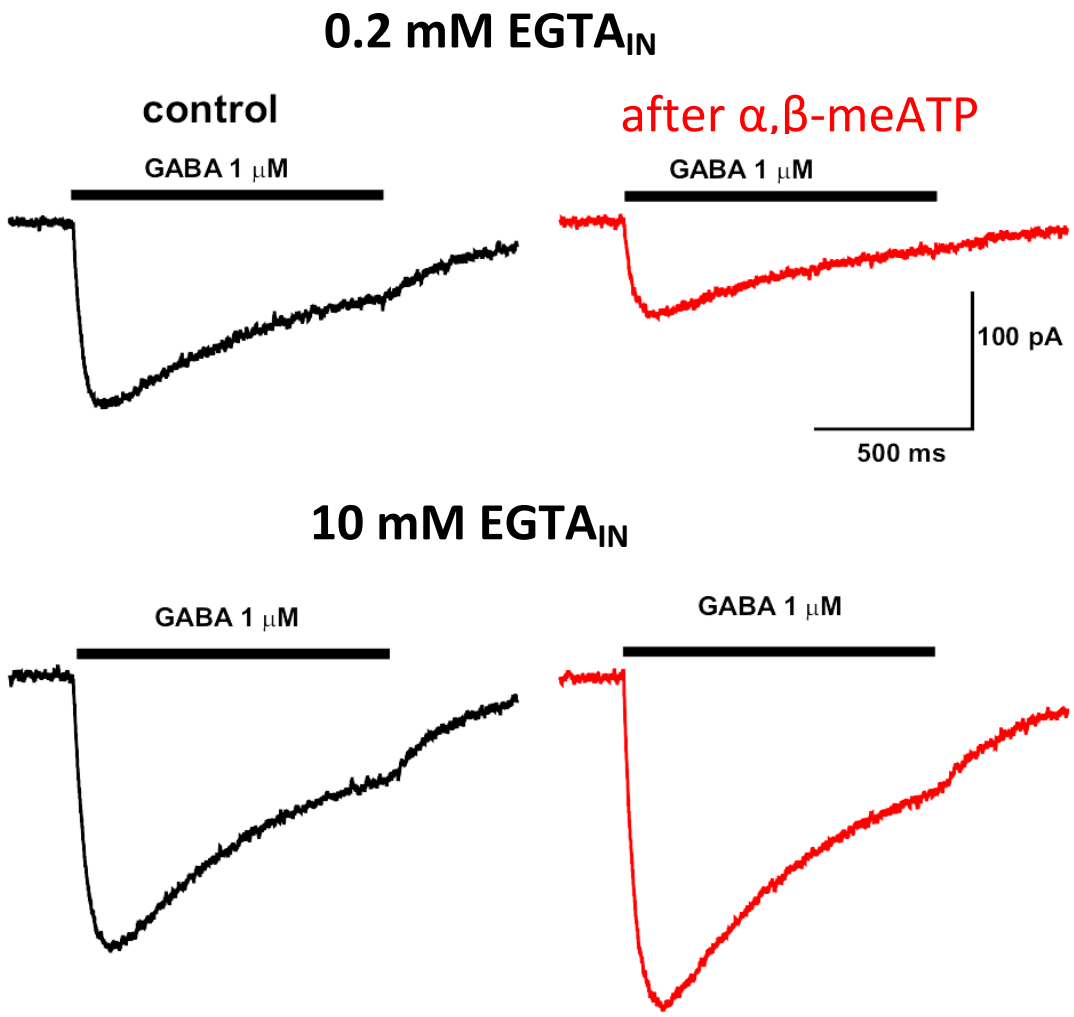

Figure 4.3:21. Tonically active $GABA_A$ receptors are also targets of purinergic modulation

Activated P2X receptors are able to inhibit $GABA_A$ receptors that reside beyond the boundaries of the synaptic cleft expressed on isolated cortical pyramidal neurons in 0.2 mM EGTA concentrations. As seen previously with phasic $GABA_A$ receptors there was a significant level of inhibition of the tonic GABA receptor mediated currents after the activation of the P2X receptor. Since no effect of the P2X receptor agonist application was observed in 10 mM EGTA concentrations, indicating a definite role of calcium in this mechanism. All recordings were conducted with a holding potential of -80mV

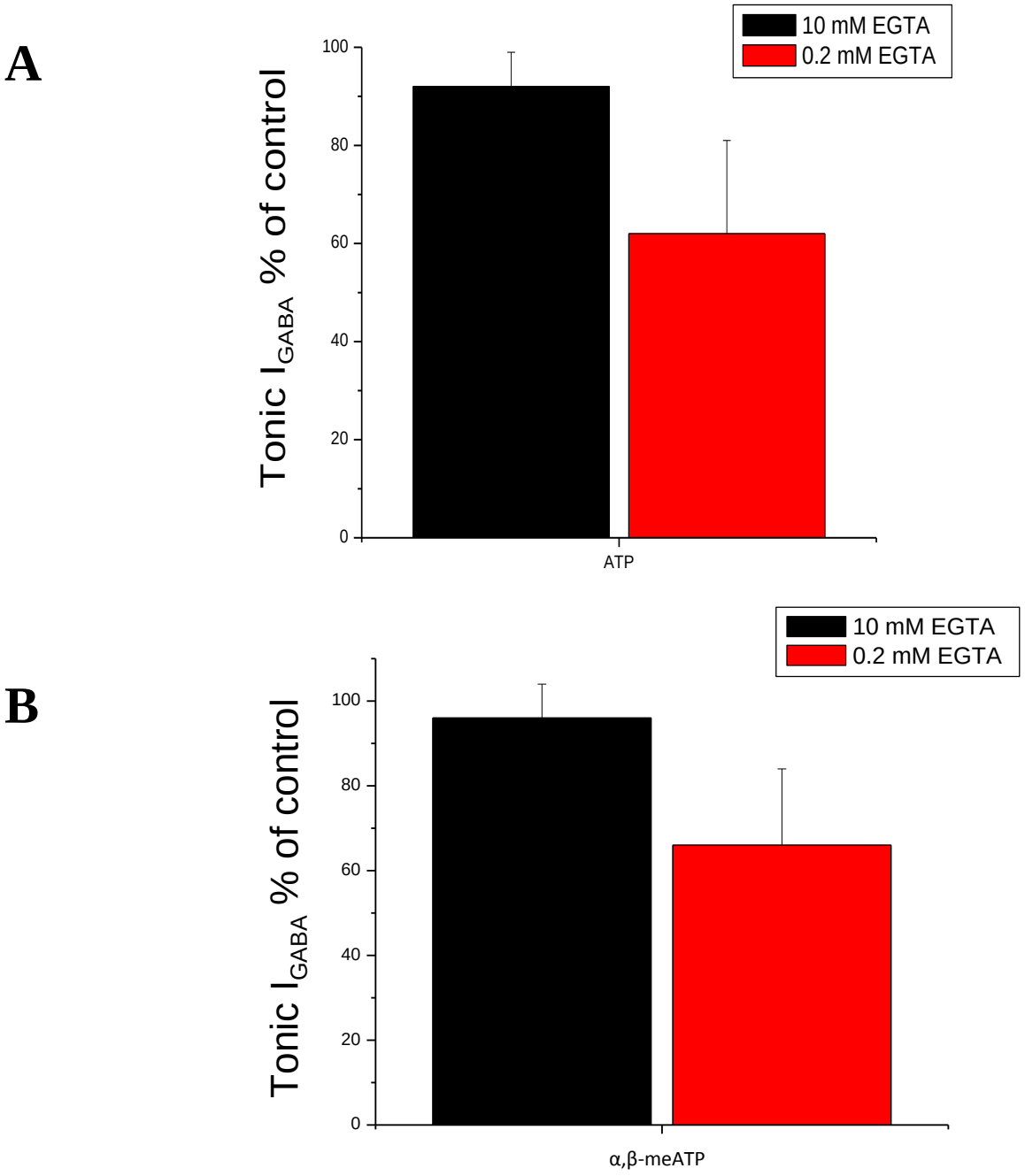

Figure 4.3:22. A comparison if the impact of the application of P2X receptor agonists ATP and α,β-meATP on tonically activated $GABA_A$ receptors.

The down-regulation of tonically active $GABA_A$ receptors by purinergic modulation in the neocortex is a calcium dependent process. **A,** in 0.2 mM EGTA there is a considerable reduction in the average amplitude of the $GABA_A$ response recorded immediately after the application of the P2X agonist ATP, but this effect was absent in 10 mM EGTA concentrations **B,** when α,β-meATP was applied to the isolated neurons there was also a significant decrease in the GABA receptor response in 0.2 mM EGTA concentrations only.

4.4 Purinergic modulation of extrasynaptic $GABA_A$ receptors is mediated by protein kinases

Previously we have revealed that $GABA_A$ receptors of the peripheral nervous system are modulated by a Ca^{2+}-dependent intracellular signalling cascade as are the phasic $GABA_A$ receptor-mediated currents in the central nervous system. To examine the possible effects of intracellular modulation on tonically active receptors we followed the same protocol that we utilised when studying the impact of phosphorylation on synaptic $GABA_A$ receptors (**ref. Chapter 3, Fig. 3.6:18**).

Tonically active $GABA_A$ receptor responses recorded immediately after activation of the P2X receptors were inhibited in low EGTA concentrations (Fig. 4.3:21). However, this effect was absent when the same protocol was performed using a high concentration of EGTA (10 mM EGTA) in the intracellular medium as no inhibition was observed. When 0.2 mM EGTA was introduced to the patch pipette alongside the application of PKC blocker, GF109203x, the purinergic induced inhibition of tonic GABA receptor responses was hampered, 95 ± 14% n=9 of control (Fig. 4.4:23). Similarly, applying sub-threshold concentrations of GF109203x and Staurosporine completely eradicated the purinergic modulation directed at extrasynaptic GABA receptors, 102 ± 13% n=7 of control. Both effects were statistically significant with a confidence level of P=0.005 (one-population t-test). Therefore, the inhibition of tonic conducting $GABA_A$ receptors is similar to phasic $GABA_A$ responses, whereby, the interaction between both $GABA_A$ receptor subtypes and P2X receptors is Ca^{2+}-dependent and potentially modulated by PKC.

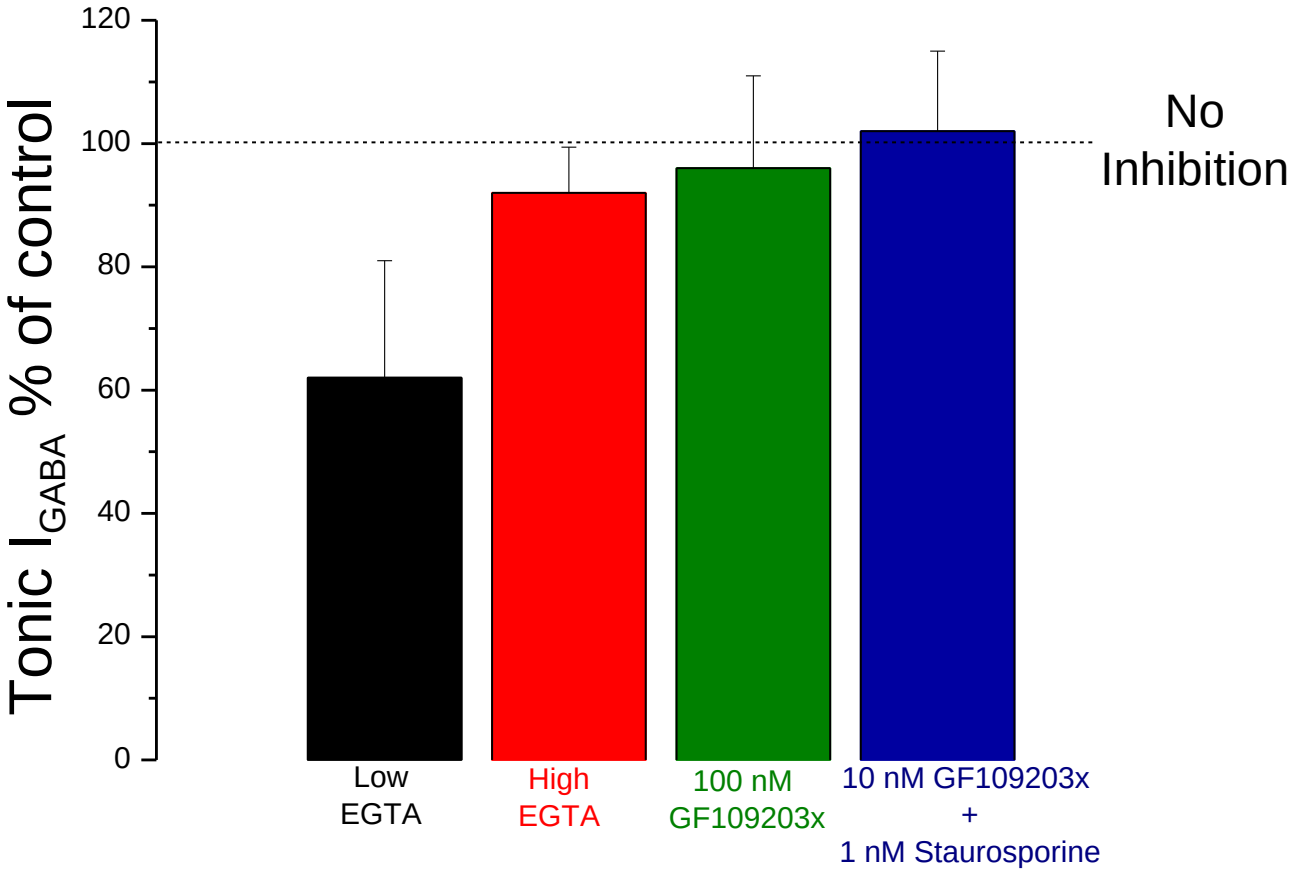

Figure 4.4:23. The impact of intracellular modulation of tonically active $GABA_A$ receptors and the interaction with P2X receptors on cortical pyramidal neurons

The PKC blocker GF109203x is able to prevent the P2X receptor driven down-regulation of tonically activated GABA receptors, parallel to observations made on phasic GABA receptors. The introduction of sub-threshold concentrations of GF109203X and Staurosporine (10nM and 1nM respectively) blocked the P2X mediated inhibition to a greater effect than GF109303X alone. Our results highlight the significant role of PKC in the interaction of P2X receptors with $GABA_A$ receptors which mediate tonic inhibition in the in the central nervous system.

4.5 Discussion

Tonically-activated $GABA_A$ receptors expressed on central neurons, containing α5- and δ-subunits are also affected by this P2X and $GABA_A$ receptor interaction. We observe when there is 0.2 mM EGTA in the intracellular medium of the recording pipette a pronounced decrease in the amplitude of the GABA receptor response when GABA is applied immediately after the application of the P2X receptor agonist. This effect of P2X receptor activation is absent in high concentrations of calcium chelator (10 mM EGTA). Therefore the functional linkage between the P2X and the tonic GABA receptor activated responses is an influx of calcium through the purinergic receptors, equivalent results were obtained with phasic $GABA_A$ receptors.

The influence of P2X receptors on tonically active $GABA_A$ receptor currents is also similar to their actions on phasic $GABA_A$ receptors as our results show that their effect is mediated by the activation of an intracellular signalling cascade involving PKC. Perfusion of sub-threshold concentrations of PKC blockers Staurosporine and GF109203x hampered the P2X receptor driven inhibition of tonic GABA responses. Since no effect of P2X receptors was demonstrated in high concentrations of calcium chelator and the addition of PKC inhibitors eradicated the purinergic modulation of tonic GABA receptors, the results indicate the strong dependence of calcium and PKC to this receptor interaction on isolated neocortical neurons.

Expression of δ-subunits in the somatosensory cortex has been demonstrated (Yamada et al., 2007) and our results confirm this finding with the potentiation of GABA mediated responses on the application of δ-subunit allosteric modulator DS2. The ability of P2X receptors to modulate phasic $GABA_A$ receptors expressed on peripheral and central neurons and their aptitude to block tonically active $GABA_A$ receptors enforces the idea that this receptor interaction is a

global mechanism. Hence, our findings highlight the significance of P2X receptor modulation and their potential impact on neuronal signalling in the central nervous system.

Chapter 5
The underlying mechanisms of P2X and $GABA_A$ receptor interactions

5.0 Introduction

Previous experimental evidence studying the reciprocal inhibition between $GABA_A$ and P2X receptors expressed on dorsal root ganglion neurons demonstrated that the purinergic modulation of $GABA_A$ receptors is driven by a calcium dependent mechanism in the peripheral nervous system (Sokolova et al., 2001). However, another experimental group suggested that this receptor interaction occurs independently of calcium. They describe an alternative mechanism for the receptor interaction that is attributed to the direct coupling of the intracellular motifs on both of the receptors (Boue-Grabot et al., 2004).

The inhibition of GABA-mediated currents upon the activation of the purinergic receptors was observed when agonists ATP and respective analogue α,βme-ATP were applied in low concentrations of calcium chelator EGTA (0.2 mM). On the contrary, no marked effect of the P2X receptors was measured on GABA mediated currents when a higher EGTA concentration was used in the intracellular medium. Therefore, our results strongly support the role of calcium in the ability of P2X receptors to down regulate GABA receptors in the somatosensory cortex. This is further reinforced by the observations of the long term changes in GABA receptor evoked responses that can be modified by Ca^{2+} chelators and protein kinases (**ref. Chapter 3, Figs. 3.3:13 and 3.6:16**). Nevertheless, the underlying mechanisms of this receptor interaction have yet to be elucidated.

Usage of protein kinase inhibitors could help to explore the possible effects of intracellular signalling pathways on the receptor interaction. However, this approach should be used with caution. Many kinase inhibitors are classed as broad spectrum inhibitors, whereby a single blocker can inhibit a diverse range of protein kinases depending on the specific concentration that is applied to the cells. For instance, Staurosporine can inhibit a variety of different kinases including both PKA with an IC_{50} of ~7 nM and PKC with an IC_{50} of ~3 nM (Tamaoki et al., 1986). Some blockers were reported to inhibit other protein kinases with a potency close to their assumed targets (Davies et al., 2000; Lochner and Moolman, 2006). Therefore, using just one protein kinase inhibitor to demonstrate the physiological relevance of a particular pathway can hardly be seen as reliable. To minimize the possible impact of non-specific action of protein kinase blockers, during our experiments we utilized multiple compounds that inhibited kinases. This helped discriminate between protein kinases which could possibly be involved in the calcium dependent modulation of GABA receptors (**ref. Chapter 3, Fig. 3.6:16).**

5.1 Activated P2X currents inhibit $GABA_A$ receptor-mediated currents on summation

Sokolova et al. (2001) previously studied the negative interaction between P2X and $GABA_A$ receptors expressed on peripheral dorsal root ganglion neurons. They demonstrated that the experimental linear summation during the co-application of both receptor agonists was much smaller than their predicted arithmetical response, in conditions where the intracellular calcium concentration was not clamped by intracellular calcium chelator. However, the occlusion was removed in low levels of intracellular calcium, highlighting the significant role of calcium in the receptor interaction.

To examine the effects of calcium on the summation of the GABA and P2X responses we used a modified experimental protocol (Fig. 5.1:23). We recorded a series of control responses to the application of 100 μM GABA to cortical neurons followed by the concurrent activation of both receptors. Subsequent responses to GABA application were also recorded to test a recovery of GABA-response after putative attenuation. Response to ATP alone was recorded at the end of the experiment, to avoid a possible long-term effect on the amplitude of GABA receptor-mediated currents.

When low EGTA (0.2 mM) was used in the intracellular solution the amplitude of the observed response when both ATP and GABA were co-applied was significantly reduced. The theoretical sum of the individual receptor mediated responses was much larger than the receptor mediated response recorded during our experiments. The effect of summation was 67 ± 8% (n=11) of control, which was statistically significant at a confidence level of P=0.02 (one-population t-test). Therefore, when implementing the protocol using high EGTA concentrations one would expect that the absence of summation demonstrated on the coapplication of GABA and ATP in low EGTA concentrations would be abolished.

Despite our expectations the addition of a higher concentration of calcium chelator (10 mM) in the intracellular medium failed to recover the deficit in summation. The response to the coapplication of agonists was much smaller than the predicted sum of both receptor mediated responses and summation was inhibited by 87 ± 14% (n=13) of the control. This effect was statistically significant with a confidence level of P = 0.05 (one-population t-test).

Similarly, when non-anhydrous ATP analogue α,β-meATP was coapplied with GABA the total amplitude of the response was comparable to the effect of ATP, 85 ± 9% n=7 of the

predicted sum of both agonists (Fig. 5.1:22b); this effect was statistically significant with a confidence level of P=0.02 (one-population t-test). In fact the observed summed response is of similar amplitude to the control P2X agonist response, suggesting that GABA did not contribute to the response as the receptors evoked current was occluded.

Introducing H-89 (inhibitor of PKA) to the extracellular solution whilst conducting the summation protocol, had no effect on the P2X summation of P2X and phasic GABA receptor-mediated currents (Fig. 5.1: 22c) (76 ± 13% n=16). This result was not unexpected as previously blockers of PKA were unable to prevent the down regulation of GABA responses by activated P2X receptors on DRG and central neurons. However, considering the potential role of PKC in this receptor interaction we would expect PKC blockers to compensate for the deficit in summation. When the PKC inhibitors were applied to the recording chamber they were unable to obstruct the impact of ATP on GABA during the summation protocol (69 ± 11% n=7) and summation could not be recovered under these conditions. Both of these effects were statistically significant with a confidence level of P=0.02 (verified by one-population t-test).

We have previously shown that cortical pyramidal neurons receive tonic inhibitory currents which are mediated by $GABA_A$ receptors located extra-synaptically and mediate tonic inhibition (Semyanov et al., 2004; Glykys and Mody, 2007) are also targets of purinergic modulation. The activity of specific subset of $GABA_A$ receptors is modulated by calcium dependent phosphorylation (Jacob et al. 2008). Therefore, one would expect to observe a deficit in summation on the co-application of ATP and GABA to tonically active receptors too. To validate this hypothesis all of the previous experiments conducted on phasic $GABA_A$ receptors were repeated on the $GABA_A$ receptors that mediate tonic inhibition in the central nervous system and identical results were observed (Fig. 5.1:23).

The experimental results conducted in the peripheral nervous system and central nervous system significantly highlight the role of calcium in the interaction between P2X and $GABA_A$ receptors, contrary to the involvement of direct coupling between the receptors (Boue-Grabot et al., 2004; Toulme et al., 2007; Jo et al., 2011). Nevertheless the fact that the GABA and ATP responses are not additive during the summation experiments suggests that direct interaction between the intracellular components of the receptors could also be partially accountable.

If direct coupling does not take a part in the underlying mechanism for this receptor interaction, then we would not expect a deficit in summation with a high concentration of intracellular Ca^{2+}-chelator. However, our results show otherwise. The significant deficit in summation was still present in 10 mM EGTA concentrations, suggesting that calcium is not entirely responsible. Nevertheless, the absence in summation is significantly greater in low EGTA concentrations as opposed to high EGTA concentrations. The reduced level of summation described under 10 mM EGTA could be caused by a reduction in P2X-mediated receptor signalling as a consequence of the direct coupling. Enhancement in the levels of the deficit in summation witnessed in low levels of calcium chelator suggests that calcium dependent phosphorylation is more predominant. Therefore, the most appropriate assumption is that the receptor interaction is mediated by two mechanisms: modifications by Ca^{2+} signalling and receptor phosphorylation and to some degree direct coupling between the receptors.

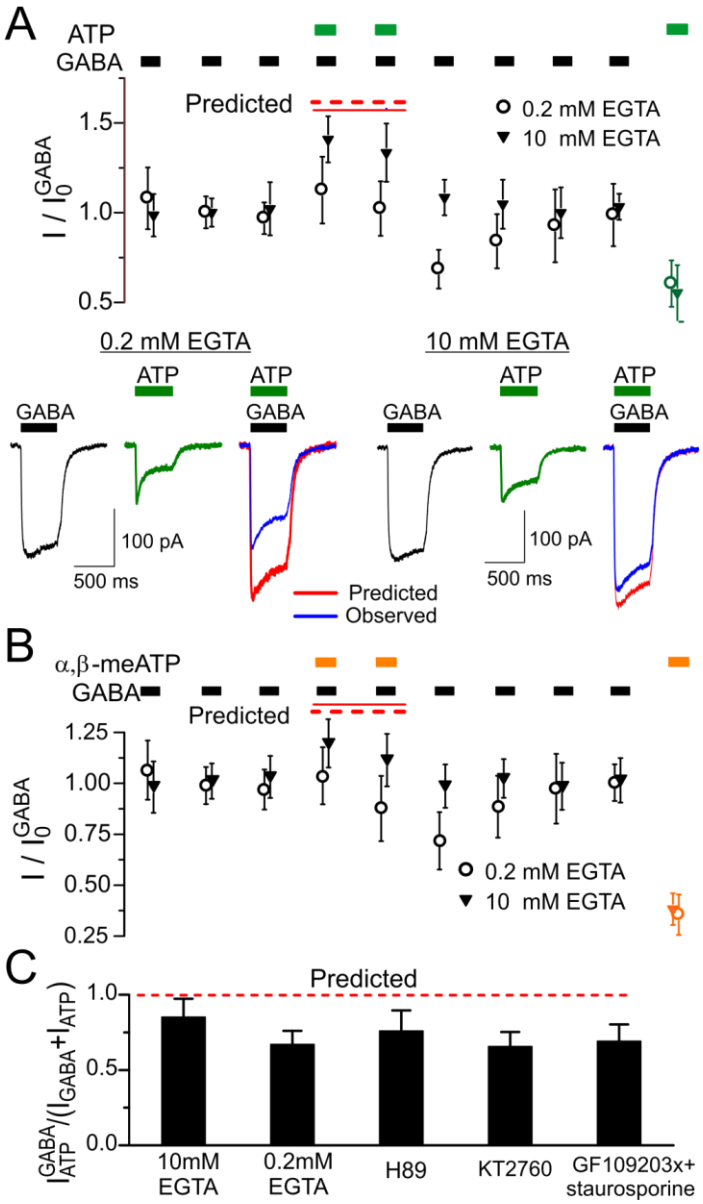

Figure 5.1:24. Summation experiments conducted on phasic $GABA_A$ receptors expressed on cortical pyramidal neurons in low and high concentrations of calcium chelator.

To selectively activate phasic $GABA_A$ receptors in the neocortex we applied 100 µM of GABA. **A,** In 0.2 mM EGTA the amplitude of algebraic predicted sum of GABA and ATP responses (red) when both agonists were applied together is larger than the amplitude of the observed experimental response (blue). The same effect was demonstrated in 10 mM EGTA concentrations, suggesting a role for direct coupling in this receptor interaction. **B,** both 0.2 mM and 10 mM EGTA concentrations were unable to recover the deficit in summation when 10 µM of α,βme-ATP and 100 µM GABA were co-applied, as observed previously with ATP under the same conditions. Note that the deficit in summation of the GABA mediated currents is greater in 0.2 mM of EGTA when compared to the absence of summation in 10 mM EGTA concentrations on the application of ATP and α,βme-ATP. Application of kinase inhibitors also failed to have an effect on the deficit in summation (**C**).

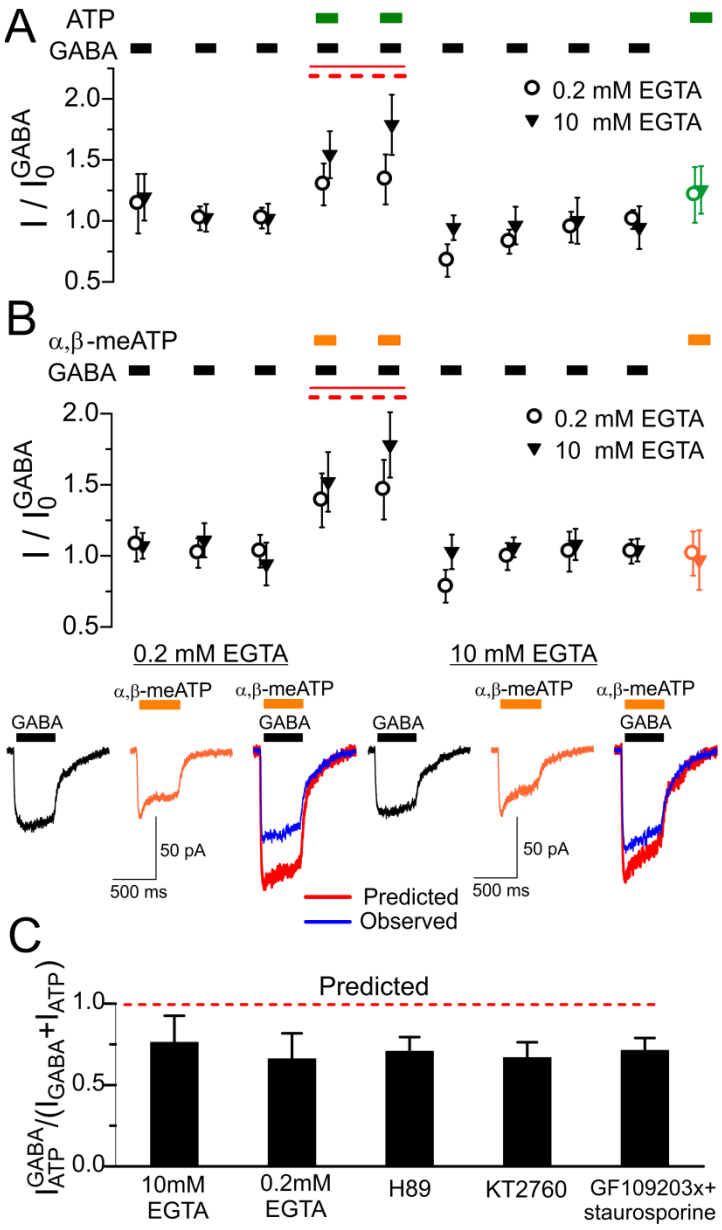

Figure 5.1:25. The involvement of direct coupling in the interaction between P2X and tonic $GABA_A$ receptors in the central nervous system.

A, demonstrates the deficit in summation when 1 µM GABA was applied to cortical neurons. **B,** the addition of 10 mM of EGTA or the action of kinase blockers also failed to recover the deficit in summation when both agonists were applied together when studying the effects of summation on tonically active GABA receptors. The results suggest tonically active $GABA_A$ receptors are also affected by the deficit in summation. Also, it is possible that calcium is not solely responsible for the deficit in summation and direct coupling between the receptors may also be involved. The application of kinase inhibitors also failed to have an effect on the deficit in summation (**C**). Recordings were taken from different cells at a holding potential of -80mV.

5.2 The absence of reciprocal inhibition of the purinergic receptors by $GABA_A$ receptors

In the central nervous system GABA is the major inhibitory neurotransmitter which regulates excitatory synaptic transmission. An excitatory neurotransmitter occluding the actions of inhibitory transmission in the central nervous system is a relatively novel paradigm. It has been reported that P2X receptors and $GABA_A$ receptors can cross-talk with one another on DRG neurons. Sokolova et al. (2001) also noted the non-additive effect when both ATP and GABA were applied together as their observed response was much smaller than their predicted algebraic sum of the individual responses, probably as one receptor response is inhibiting the other. They concluded that $GABA_A$ receptors can occlude P2X receptors and vice versa (Sokolova et al., 2001).

Sokolova et al. (2001) has previously suggested that the down-regulation of P2X-mediated currents by activated $GABA_A$ receptors is more predominant than the down-regulation of $GABA_A$ receptor function by P2X-mediated currents. To study this hypothesis we utilized our summation protocol to examine the effects of GABA receptor evoked currents on the summation of consecutive P2X receptor-mediated currents. We recorded a series of control responses to the application of ATP to cortical pyramidal neurons followed by the simultaneous activation of both receptors. In high concentrations of calcium chelator (10 mM EGTA) the calcium independent deficit in summation was still demonstrated as the amplitude of the observed response when ATP and GABA were applied together was still smaller than the theoretical response. The effect of summation was 76 ± 8% n=6, which was statistically significant with a confidence level of P=0.05 (verified by one population t-test).

In keeping with prior results, the ATP response recorded immediately after the application of both receptor agonists was not inhibited (Fig. 5.2:24). In fact the observed response was

slightly potentiated, 105 ± 19% n=6 and statistically significant with a confidence level of P=0.05 (one population t-test). Hence, P2X receptors of the somatosensory cortex were not inhibited immediately after the application of GABA, contrary to previous experiments conducted on DRG neurons (Sokolova et al., 2001), which suggests that this negative receptor interaction is not reciprocal. In our own experiments we witness a significantly higher level of the deficit in summation in low concentrations of calcium chelator (0.2 mM) compared to high levels of EGTA (10 mM). The small deficit in summation observed in high concentrations of EGTA could represent reciprocal inhibition as there may be a decrease in the ATP mediated fraction of the current. This assumption has yet to be studied experimentally; however it is consistent with the hypothesis suggested by Sokolova et al. 2001.

This difference in experimental evidence may perhaps be due to differences in the subunit composition of the P2X receptors. The most abundant P2X receptor subtype expressed on dorsal root ganglion neurons are P2X3 are P2X2/3. However, neurons of the somatosensory cortex show a significant level of expression of the P2X4 receptor subtype. Both receptor subtypes have different functional properties (Grubb and Evans, 1999; North and Suprenant, 2000; Jarvis and Khakh, 2009; Nicke et al., 2012) which could account for the diverse results.

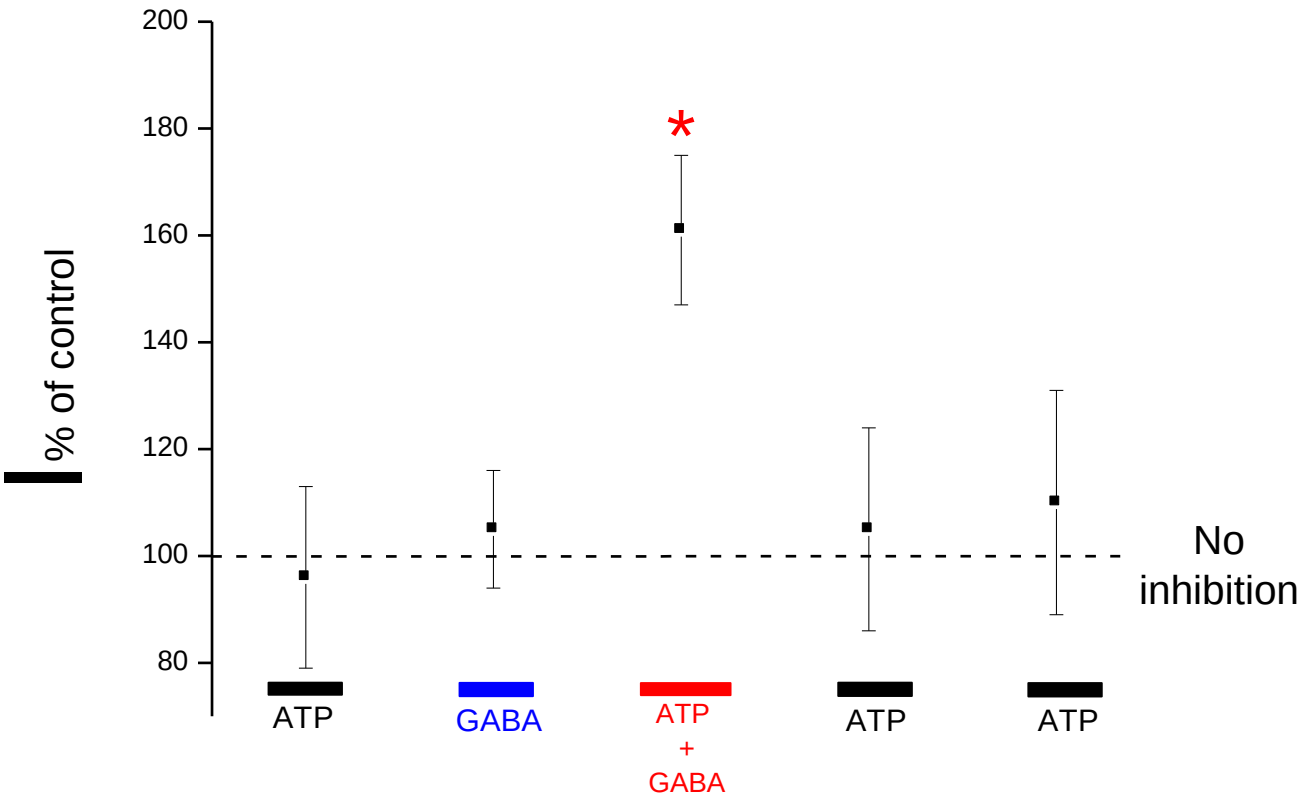

Figure 5.2:26. A time course demonstrating the consecutive application of ATP in order to observe the impact of activated $GABA_A$ receptors on P2X mediated currents on neocortical neurons.

A previous study suggested that the receptor interaction between ATP and GABA receptors was reciprocal on dorsal root ganglion neurons (Sokolova et al 2001). To study this theory P2X and GABA receptor agonists were applied to cortical pyramidal neurons in the central nervous system with 10 mM EGTA in the intracellular medium. The deficit in summation is still significant (*), but the relative lack of change in the amplitude of the ATP responses is also noteworthy. $GABA_A$ mediated currents were unable to inhibit evoked ATP responses in the somatosensory cortex as previously reported in the peripheral nervous system (Sokolova et al., 2001). Broadly, our results suggest that the functional occlusion between the two receptors is not reciprocal in the somatosensory cortex. The boxes represent the mean SEM ± for several recordings taken from different cortical neurons.

5.3 Discussion

ATP and GABA are two fast acting neurotransmitters that can be co-released at excitatory and inhibitory synapses. A previous study demonstrated that the functional cross talk between ionotrophic P2X and $GABA_A$ receptors and postulated that this was a calcium dependent mechanism in the peripheral nervous system (Sokolova et al., 2001). At high concentrations of intracellular calcium the sum of the observed response, where ATP and GABA receptors were coactivated, was much smaller than the predicted response, derived from the algebraic sum of the individual control ATP and GABA responses. This effect was not apparent in conditions where the intracellular calcium concentrations were low. However, an opposing study reports that this interaction occurs via a calcium independent process called direct coupling. This involves the direct interaction between the intracellular QST motif on the carboxyl terminus of the P2X receptor and the intracellular loop on the β-subunit of the $GABA_A$ receptor. However, the underlying mechanism for this negative receptor interaction has yet to be determined.

During our own experiments in the peripheral and central nervous systems we have shown that the receptor interaction is calcium dependent and mediated by the impact of PKC modulation on phasic and extrasynaptic $GABA_A$ receptors. In spite of this knowledge when both receptor agonists were coapplied to patched cortical neurons the recorded response was much smaller in magnitude when compared to the predicted sum of both responses in low and high EGTA concentrations. This deficit in summation was also observed when PKC inhibitors were introduced to the extracellular solution. Similar results were obtained when studying this effect on extrasynaptic $GABA_A$ receptors which mediate tonic inhibition. Since high EGTA concentrations and PKC inhibitors, GF109203x and Staurosporine, failed to recover the absence

summation our results are indicative that calcium is not solely responsible for this deficit in summation, but direct coupling may also play a role. Nevertheless, the deficit in summation is greater in lower EGTA concentrations when compared to the level of the deficit in higher EGTA concentrations. These results are in line with our previous studies documenting the reduction in the GABA receptor currents (**ref. Chapter 3**) in the peripheral and central nervous systems. Therefore, the data highlights that calcium dependent phosphorylation has a more prominent effect over direct coupling.

Studies have suggested that $GABA_A$ and P2X receptors are able to reciprocally interact with one another, whereby P2X receptors can occlude $GABA_A$ receptor mediated responses and the same can occur in reverse (Sokolova et al., 2001; Boue-Grabot et al., 2004). However, our own experiments conducted on cortical pyramidal neurons did not show the reported $GABA_A$ receptor mediated down- regulation of ATP currents. Generally the deficit in summation is marginal in the presence of 10 mM EGTA compared to 0.2 mM EGTA concentrations. One could presume when taking into account our earlier results that the deficit in summation would have been recovered in high levels of EGTA. Clearly the data demonstrates an opposite effect which may be interpreted as the reciprocal cross-talk; the ATP mediated component of the response is inhibited. This hypothesis requires further experimental exploration before a more definitive explanation can be described. The dissimilar results could also be due to physiological differences between dorsal root ganglion neurons and cortical neurons, which express different P2X receptor subtypes.

In summary, we have shown that the P2X down-regulation of $GABA_A$ receptor currents is a calcium dependent process involving the activation of and intracellular signalling pathway relating to PKC. However, using high concentrations of calcium chelator in the intracellular

solution and application of PKC blockers had no effect on the deficit of summation observed when both agonists were applied simultaneously. Hence, our results unify the two opposing theories as the underlying mechanism to this receptor interaction seems to involve predominantly a longer-lasting calcium dependent phosphorylation and to some extent direct coupling of the intracellular components of the two receptors.

Chapter 6

The physiological consequences of the P2X driven down-regulation of $GABA_A$ receptors in the central nervous system

6.0 Introduction

The expression of P2X receptors on neurons has been demonstrated in many brain areas (Burnstock, 1976; Buell et al., 1996; North, 1996; North, 2002; Illes and Ribeiro, 2004), but recent evidence suggests that they also reside on a second type of cell in the brain, glial cells (Verkhratsky et al., 2009). Astrocytes, a type of glial cell in the central nervous system, are non-excitable cells. Research examining the precise function of glial cells in the central nervous system has remained dormant as they have forever been observed as supporting cells to neurons. The glutamate induced oscillations in the intracellular calcium concentration in astrocytes (Cornell-Bell et al., 1990; Charles et al., 1991), causes the activation of an intracellular signalling cascade involving inositol trisphosphate (IP3) and the release of calcium from intracellular stores. This finding engendered a flurry of research into the potential role of astrocytes in modulating neurotransmission. Furthermore, evidence that astrocytes enwrap synapses, express a multitude of receptors and release gliotransmitters such as ATP, D-Serine and glutamate signified that they played a significant role in regulating neuronal function and excitability.

Cytosolic calcium plays a fundamental role for non-excitable cells in inter- and intracellular communications (Braet et al., 2004). Accumulating evidence suggests that ATP

may arbitrate Ca^{2+} transmission (Wang et al., 2000), thus increasing the levels of cytosolic calcium in glial cells and neurons as part of the tripartite synapse. Neuronal stimulation is able to induce Ca^{2+} signalling in astrocytes, which in turn is able to propagate back to neuronal synapses to modulate synaptic transmission, highlighting the potential of purinergic regulation of synaptic transmission mediated by Ca^{2+} ion conductance.

There has been a plethora of studies which describe the induction of an important mechanism associated with learning and memory termed long term potentiation (LTP), particularly in the CA1 region of the hippocampus (Weirazko, 1996; Fuji, 2004; Yamazaki et al., 2003; Henneberger and Rusakov, 2010; Ievglevskyi et al., 2012). However, research has also demonstrated that the neocortex is also linked to learning and memory (Castro-Alamancos et al., 1995) and neuroimaging studies revealed that patients suffering from amnesia showed activation of regions within the neocortex when asked to recall information (Purves, Neuroscience 2004). LTP is an important mechanism that involves an increase in synaptic efficacy associated with learning and memory. It has been demonstrated that silent synapses where there is an absence of AMPA receptors are transformed to functional synapses by the induction of LTP in thalamocortical slices (Isaac et al., 1997). LTP is state dependent (requires the coupling of both pre- and postsynaptic activity), exhibits input specificity (potentiation is restricted to active synapses) and also displays associativity (weak and strong stimulation occurs simultaneously at neighbouring synapses and both synapses are strengthened) (Bliss and Collingridge, 1993). These unique features of LTP indicate that it is a useful mechanism for memory development.

LTP can be induced in the central nervous system by two main mechanisms, which include: an elevation in the quantity of neurotransmitter released from the presynaptic cell; an increase in the functional expression of receptors on the postsynaptic membrane and disruptions

in the uptake and degradation methods of specific neurotransmitters (Bliss and Collingridge, 1993). The mechanism of LTP induction involves the activation of the receptors expressed on the postsynaptic membrane, predominantly glutamate NMDA receptors. An increase in intracellular calcium levels via influx through the NMDA receptor channel is vital for the induction of LTP. Lisman et al. (2002) proposed that the direction of the changes in synaptic plasticity whether it be potentiation or depression is dependent on the extent of the calcium influx into the cell (Malenka et al., 1988). A high magnitude of calcium influx causes LTP by activating secondary messenger CamKII and a low calcium influx activated by calcineurin causes Long Term Depression (LTD) (Lisman et al., 2002). Therefore, NMDA receptors are a primary source of calcium on the postsynaptic cell, which activates intracellular signalling cascades to produce LTP in the brain.

Many studies have demonstrated that ATP plays a significant role in the induction of long term potentiation (LTP) with particular emphasis on the CA1 region of the hippocampus (Fujii et al., 1995, 2002, 2003, 2004; Pankratov et al., 2009). Yamazaki et al. (2003) demonstrated that the application of ATP onto hippocampal CA1 neurons elicited LTP in neurons showing small Ca^{2+} currents, whereas (Long Term Depression) LTD was detected in neurons exhibiting larger Ca^{2+} currents. Thus, there is a considerable correlation between the enormity of the long-term changes in plasticity and the size of Ca^{2+} signals following ATP application (Yamazaki et al., 2003). The P2X receptors high permeability to Ca^{2+} even at resting membrane potentials makes them perfect candidates to mediate synaptic plasticity in the central nervous system.

Long term changes in synaptic efficacy are maintained by alterations in gene expression, which frequently occur in the neocortex and has Hebbian properties i.e. an increase in stimulation to the presynaptic neuron causes and increase in synaptic strength (Bliss and

Colligridge, 1993). LTP in the neocortex can be induced by signalling to the presynaptic terminal as a mechanism to increase the release probability. Glial cell derived signalling molecules coined gliotransmitters act on the postsynaptic membrane to modulate NMDA receptors and effecting the facilitation of LTP in the central nervous system. D-Serine is produced in neurons and glial cells as they harbour serine racemase, which catalyses the conversion of L-serine into D-Serine (Haydon and Carmignoto, 2006). Once released D-Serine diffuses across to the postsynaptic NMDA receptors, binding to the glycine site. Consequently, there is a significant rise in the field Excitatory Postsynaptic Potential (fEPSP) (**ref. Chapter 2, Fig. 2.9:7**) as the NMDA receptors are not saturated under resting conditions (Henneberger and Rusakov, 2010). Hence, gliotransmitters are also accountable for modulating NMDA receptors and thus inducing long term potentiation.

Growing evidence shows that glial derived ATP is involved in the induction of long term potentiation (LTP). Vesicularly released ATP is able to modulate the activity of postsynaptic NMDA receptors by activating intracellular secondary messengers such as PKA in a calcium dependent manner (Pankratov et al., 2002a). Furthermore, we have shown previously that astroglial derived ATP is able to inhibit both phasic and tonic $GABA_A$ receptors on the postsynaptic membrane in the somatosensory cortex (Lalo et al., 2009). Extracellular ATP released from glial cells also acts postsynaptically to enhance the expression of AMPA receptors on the postsynaptic membrane and thus increasing the level of synaptic efficacy. This effect can be blocked by glial cell toxin fluoroacetate (FAC) (Haydon and Caramignoto, 2006; Henneberger and Rusakov, 2010). Therefore, extracellular ATP's unique signalling properties and modulatory capabilities as part of the tripartite synapse could also contribute to the formation

of learning and memory in the neocortex. However, the specific role of astrocytes in synaptic plasticity in the neocortex has yet to be fully determined.

6.1 The induction of LTP in cortical slices

Under control conditions the induction of LTP was recorded to test the suitability of the designed stimulating protocol in inducing the potentiation of the postsynaptic response in cortical brain slices. The stimulating electrode was placed in layer IV-V of the neocortex (involved in in callosal and ipsilateral corticocortical circuits) and the recording electrode was positioned in layer II/III to record the postsynaptic response opposite the stimulating electrode. Before initiating any stimulation to the slice stabilisation of the baseline was essential before starting the experiments as this would act as a control, defined as the period before the induction of LTP and referred to as 100% during analysis of the fEPSP slope. Therefore, a delay of 10-20 minutes was required before the administration of any form of electrical stimulation to the brain slice.

To produce an enhancement in synaptic efficacy a standard Theta Burst Stimulation (TBS) protocol was used, which consisted of a 0.5 second train at 100Hz. There were a total of 5 trains which were delivered at 10 second intervals. Previously, experimental data demonstrated that depending on the age of the animal shorter trains consisting of 2 or 3 were not sufficient in inducing LTP in the neocortex (data not shown). Once the baseline had stabilised a TBS of 5 pulses was applied to layer IV-V of the neocortex with an amplitude that ranged between 0.5 and 3 Amps producing an EPSP that reached a plateau lasting between 40-60 minutes each time (Fig. 6.1: 25). Using this configuration, recording from layer IV-V using this standard TBS protocol produced a clear EPSP which can be reproduced for more than two hours whilst recording, without any run-down of the EPSP and a stable amplitude was recorded in approximately 80% of all cortical slices.

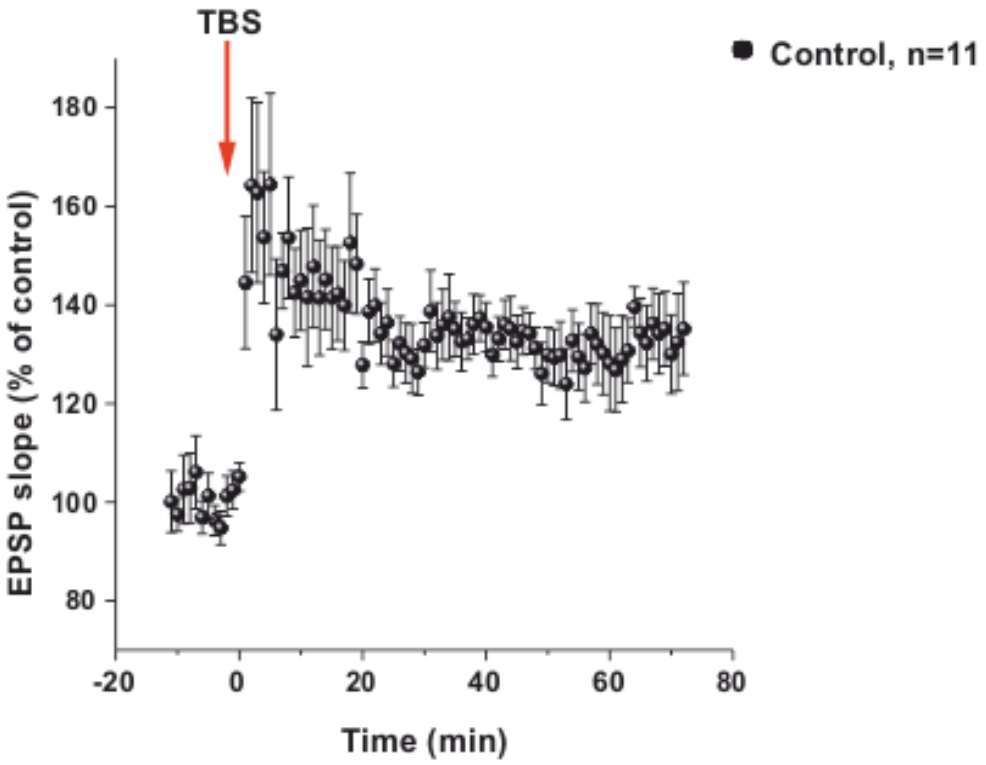

Figure 6.1:27. Long term potentiation induced electrically by TBS in cortical brain slices.

The standard Theta Burst Stimulation (TBS) used throughout the study of LTP is sufficient to produce enhancements in synaptic efficacy in cortical slices under control conditions. After the baseline has stabilised TBS is elicited and there is a significantly large increase in the fEPSP slope, which gradually decreases to a stable plateau 20 minutes after TBS. The control experiments confirm that LTP could be induced using a train of high frequency stimulation like the standard TBS protocol. Data points represent the mean ± SEM of recordings from slices. The results were statistically significant with a confidence level of P=0.001 (one population t-test).

6.2 The involvement of gliotransmitters in LTP induction

Once thought as dormant cells, which function as supporting components to neurons and fundamental to the maintenance of homeostasis in the brain, there is now a plethora of evidence that suggests astrocytes contribute and modulate synaptic transmission. Since they are also able to release gliotransmitters which can act on the pre- and postsynaptic membranes suggests that their potential as mediators of synaptic signalling as part of the tripartite synapse is of paramount importance.

Astroglial cells express multiple receptors, many of which are also expressed on neurons. The release of neurotransmitters from the presynaptic terminal causes the activation of the functional receptors expressed on astrocytes. Consequently, the activation of the astroglial receptors produces a calcium influx and causes oscillations in the intracellular calcium concentration in the astrocytes. The rise in intracellular calcium leads to the release of gliotransmitters such as ATP, glutamate and D-Serine (Zhang and Haydon, 2005).

It has been established that the external application of gliotransmitter D-Serine to brain slices produces an increase in LTP in the postsynaptic response at the Schaffer collateral pyramidal cell synapse of the CA1 region in the hippocampus (Henneberger and Rusakov, 2010). Due to the fact that D-Serine is produced in glial cells, which contain D-Serine racemase, responsible for the conversion of L-serine to D-Serine (Haydon and Carmignoto, 2006) makes it an ideal candidate out of the selection of gliotransmitters to induce LTP. The calcium dependent release of D-Serine results in its diffusion across to the postsynaptic membrane as an agonist to the glycine binding site on the NMDA glutamate receptors. Research has

demonstrated that D-Serine is the co-agonist to NMDA receptors in the hippocampus during LTP (Panatier et al., 2006).

During our own experiments conducted in the neocortex, D-Serine was applied 10 minutes before and 10 minutes after the TBS stimulation. It was discovered that rather than the initial increase and gradual sustained plateau which was observed in the control experiments, LTP under D-Serine appears to be inhibited as an increase in synaptic plasticity was not observed (Fig. 6.2:26). The effect of D-Serine was statistically significant with a confidence level of P=0.005 (one population t-test). The discrepancy between the data could be due to the concentration of D-Serine already being relatively high in the neocortex and therefore neuronal NMDA receptors are already saturated unlike the CA1 hippocampal pyramidal neurons (Henneberger and Rusakov, 2010). The failure of D-Serine to cause LTP is the neocortex overshadows the potential of gliotransmitters to induce LTP, however further study in required to determine the role of gliotransmitters in learning and memory, specifically in the neocortex.

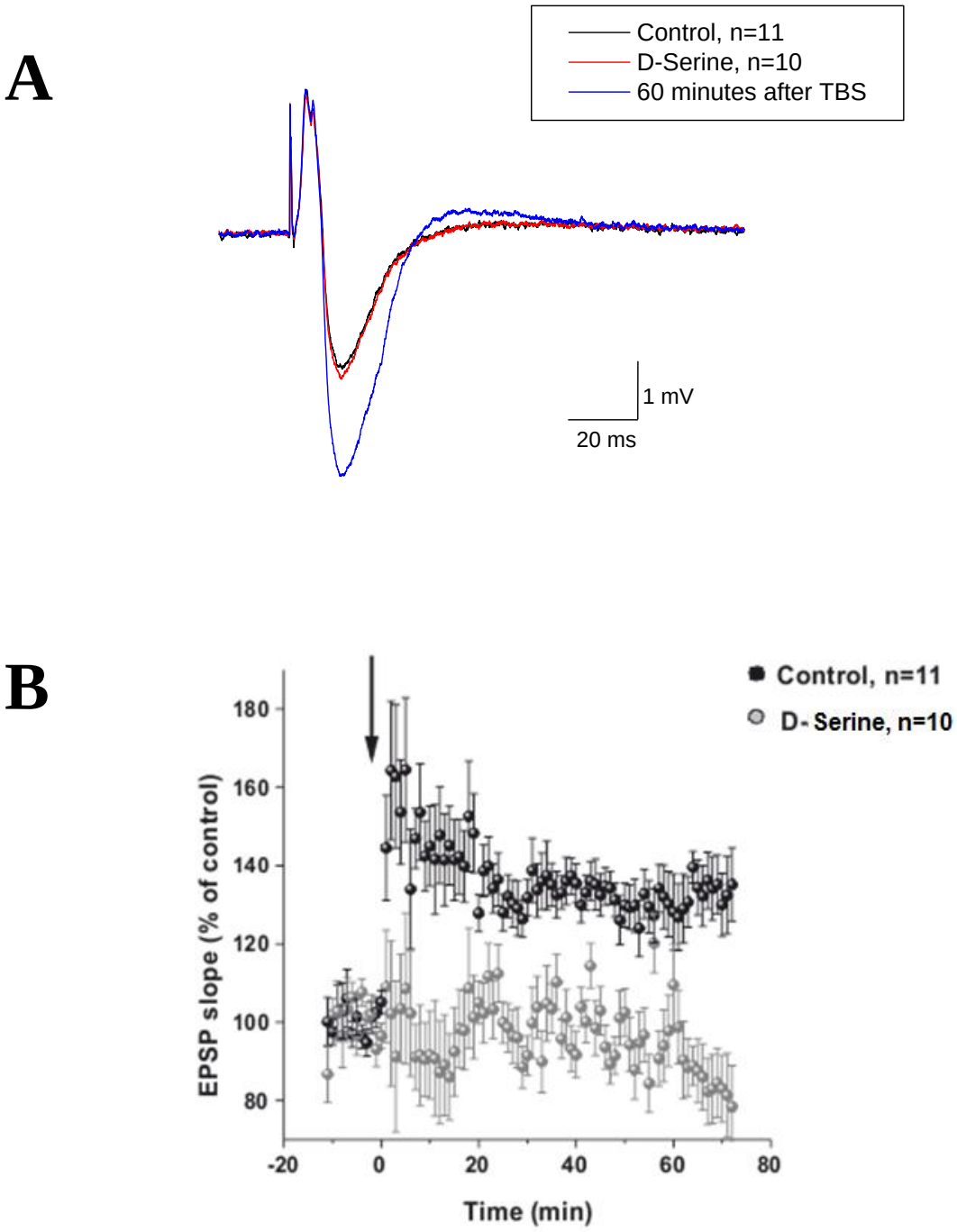

Figure 6.2:28. Gliotransmitter D-Serine does not induce LTP in the cortex.

D-Serine is released from astroglial cells and binds to the glycine binding site on the NMDA receptor. The application of 3 μM D-Serine 10 minutes before the implementation of TBS and 10 minutes afterwards causes a depression in LTP contrary to previous reports (Henneberger and Rusakov, 2010). This effect was statistically significant with a confidence level of $P<0.005$.

6.3 The role of glial cells in the induction of LTP in the neocortex

The gliotransmitter D-Serine surprisingly did not provide the expected potentiation of the postsynaptic response in the neocortex. Therefore, it was important that the role of glial cells in LTP the neocortex was established. Calcium signalling is essential to the induction of D-Serine activated NMDA receptor LTP as the vesicular release of D-Serine from astrocytes is calcium dependent. However, loading an astroglial cell with calcium chelators had no effect on the induction of synaptic plasticity in hippocampal CA1 pyramidal neurons (Diamond et al., 1998). However, another study demonstrates that by clamping the intracellular calcium concentration inside the astrocytes potentiation of the fEPSP was blocked as a result of a decreased level of NMDA co-agonist occupancy on the glycine binding site (Henneberger and Rusakov, 2010). Therefore the physiological relevance of astrocytes in learning and memory remains controversial. To study the possible involvement of glial cells in the enhancement of synaptic plasticity we applied Fluoroacetate (FAC) to cortical slices. FAC is a glial metabolic toxin and is selective for astrocytes because their membranes are more permeable to FAC than neurons (Hassel et al., 2002), due to the presence of acetate transporters on the astrocytes. FAC targets the TCA cycle specifically and has been shown to inhibit LTP in the hippocampus even with a high frequency stimulation protocol (Henneberger and Rusakov, 2010).

The application of 3 mM FAC in the extracellular solution to the slices occurred 20 minutes before the implementation of the standard TBS protocol and also continued 60 minutes afterwards. Unlike the control where there is a significant increase in the amplitude of the EPSP slope as a consequence to the induction of LTP, under FAC LTP induction is inhibited. In fact there were no considerable changes in the fEPSP slope and it remained fixed at approximately 100% for the entire duration of the experiment. The effect of FAC on long term potentiation in

the neocortex was statistically significant with a confidence level of P=0.01 (one population t-test). Therefore, the action of FAC which is known to target the metabolic cycle of glial cells and its effect on inhibiting the induction of LTP, suggests a role for glial cells in the development of synaptic plasticity in the neocortex.

The impairment of the induction of LTP by clamping the intracellular concentration inside individual astrocytes can be reversed by exogenous D-Serine (Henneberger and Rusakov, 2010). Hence, we used this hypothesis to rescue the occlusion of astroglial cell induced LTP by co-applying D-Serine to the slice with FAC. The combined application of FAC and D-Serine occurred 10 minutes before TBS and for 60 minutes after TBS (Fig. 6.3:27). The amplitude of the fEPSP slope declined by approximately 85% after TBS and eventually stabilised to control level (100%). No potentiation was observed 20 minutes later. This effect was statistically significant with a confidence level of P=0.02 (one population t-test). Therefore, our results indicate that although the data suggests that astrocytes are involved in the induction of LTP gliotransmitter D-Serine was not able to recover LTP under the inhibitory actions of FAC.

To further investigate the role of glial cells in the generation of LTP in the neocortex we employed genetically modified dominant negative SNARE mice (dnSNARE). dnSNARE mice are useful in studying the physiological consequences of astrocytes as they express a dominant negative SNARE complex in astrocytes (Pascual et al., 2005). The SNARE complex is an important functional feature of the vesicular release machinery in neurons and astrocytes for the exocytosis of transmitters. Astroglial cells in the hippocampus expressing the dnSNARE gene exhibited a decrease in the levels of LTP induced by TBS, implicating a role for glial cells in long term potentiation.

Administering the standard TBS protocol to layer IV-V of the neocortex of the dnSNARE brain slices failed to produce the effects of LTP (Fig. 7.3:28). There was only a minute increase in the fEPSP slope from the 100% control level in the dnSNARE mice, contrary to the results obtained in wild type mice. The effect of dnSNARE mice on LTP on glial cells in the neocortex is statistically significant with a confidence level of P=0.005 (one population t-test). Therefore, the dysfunctional vesicular release apparatus of dnSNARE mice means that gliotransmitters cannot be released to modulate the neuronal NMDA receptors on the postsynaptic membrane, inferring a role to gliotransmitters in the induction of LTP.

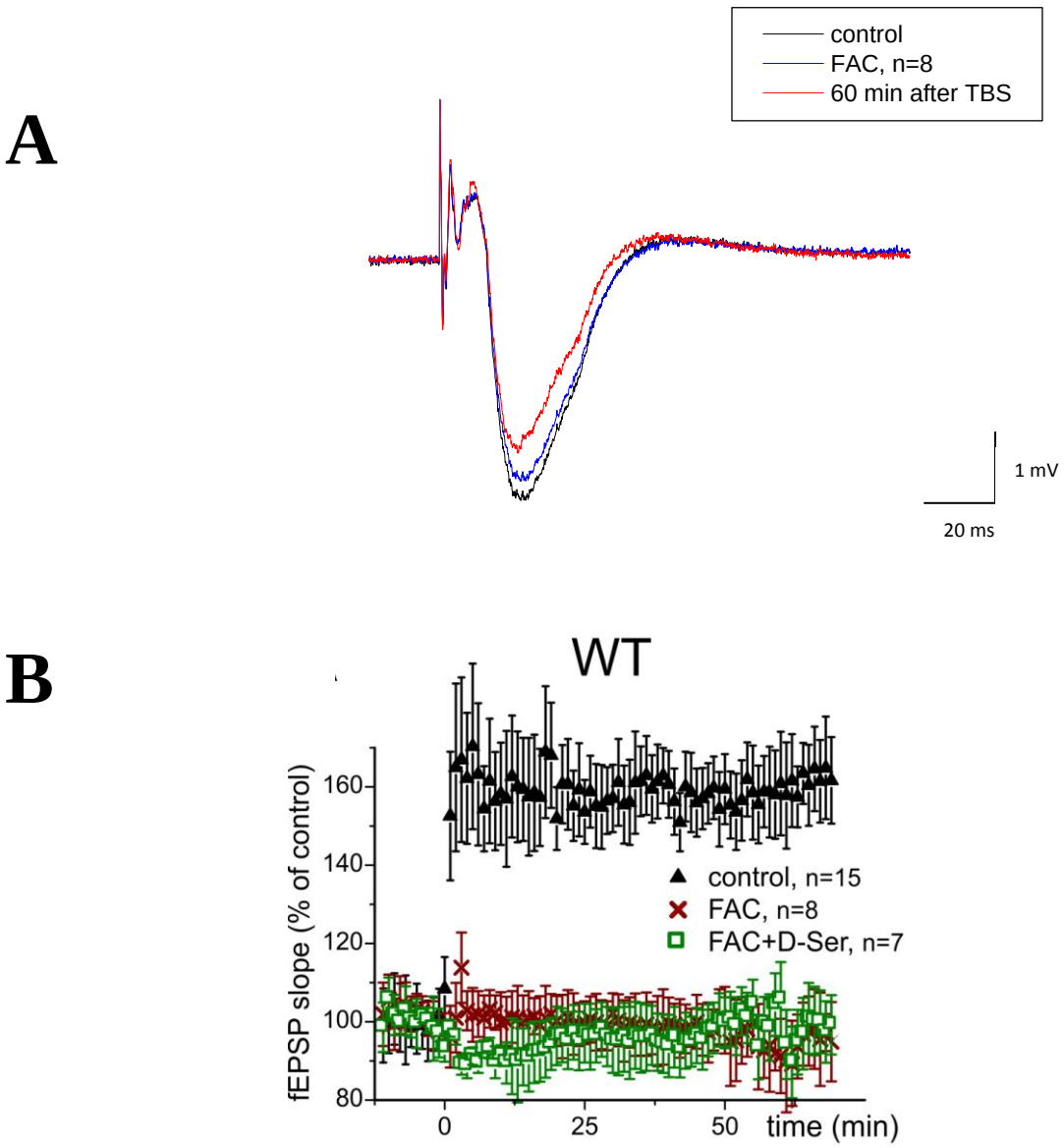

Figure 6.3:29. The inhibition of Long-term potention by glial cell toxin Fluroacetate (FAC).

A, the application of specific glial cell metabolic toxin 3 mM FAC blocked the induction of LTP in the neocortex, indicating a role for glial cells in modulating synaptic plasticity. **B,** D-Serine was unable to recover the abolishment of LTP mediated by FAC when co-applied with the metabolic poison. Therefore, the data suggests that glial cells play a significant role in LTP induction. Then data presents the mean+/- SEM, for n=8 and n=7 respectively, of FAC and FAC + D-Serine applications. Effects were statistically significant with confident levels of P=0.01 and P=0.02 respectively.

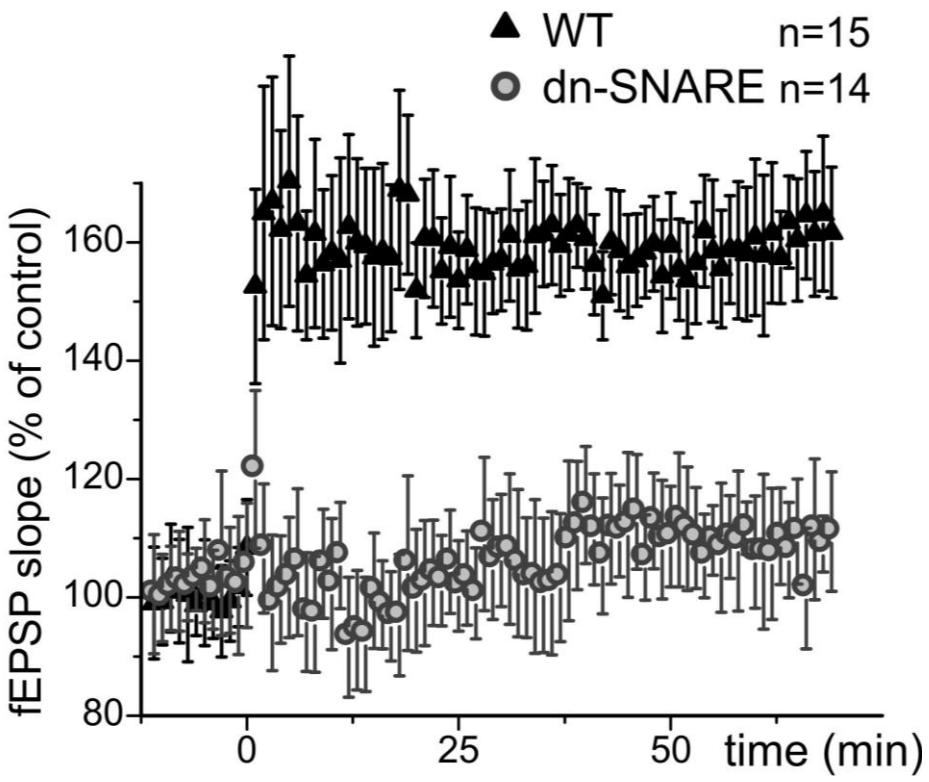

Figure 6.3:30. SNARE complex deficient transgenic dnSNARE mice inhibit LTP in the neocortex.

A comparison between control experiments conducted on both wild type mice and dnSNARE mice suggests a role for gliotransmitters in the induction of LTP. The data shows the mean+/-SEM, for n=12, of the dnSNARE control experiments which were statistically significant with a confidence level of P=0.005.

6.4 The role of glial derived ATP in the induction of LTP

Previously, observations have suggested that the functional linkage between NMDA receptor mediated LTP and glial cells is the release of gliotransmitters from astrocytes, with particular emphasis on D-Serine. When we apply specific astroglial cell metabolic toxin FAC to the cortical slices there is no potentiation of the fEPSP slope as observed in the control and D-Serine is not able to reverse this effect. Therefore, this suggests the involvement of glial cells as a facet in the mechanism for LTP induction, but questions the involvement of D-Serine in synaptic plasticity in the neocortex. The role of glial cells is also highlighted when dnSNARE mice also fail to elicit LTP as the there is a deficit in the release of gliotransmitters to act on the postsynaptic membrane.

Using the dnSNARE mouse model we applied 3 μM of D-Serine to cortical slices in the extracellular solution 10 minutes before TBS and for 10 minutes after TBS. When comparing the control dnSNARE experiments with the application of D-Serine, it is apparent that there is no significant difference between the two effects. There is no potentiation of the fEPSP in the dnSNARE control and the amplitude of the fEPSP slope remains at approximately 100% throughout the experiment. Similarly, there is no potentiation upon the application of D-Serine in dnSNARE mice (Figs. 7.3:28 and 7.4:29). The effects of dnSNARE mice and D-Serine were not statistically significant, despite resulting in similar effects on LTP induction.

It is widely acknowledged that extracellular ATP is a major component in the tripartite synapse and can be released from neurons to modulate pre- and postsynaptic receptors. The release of ATP from glial cells can also impact the function of postsynaptic receptors. We have previously studied the calcium dependent release of astroglial ATP in the neocortex using a "sniff cell" approach and cultured HEK-293 cells (Lalo et al., 2009). The HEK-293 cells were

transfected with P2X2 receptors and produced a burst of pulsatile currents when astroglial cell PAR-1 receptor agonist TFLLR was applied, which were later inhibited by P2X antagonist PPADS. Hence, the data suggests that ATP release from the adjacent astrocytes triggers the pulsatile currents in the HEK293 cells which are mediated by activated P2X receptors. Also, the acutely isolated cortical astrocytes which were loaded with calcium indicator Fluo-4 AM displayed elevated intracellular calcium levels upon PAR-1 receptor activation. Therefore it is natural to assume that astroglial derived ATP can effect postsynaptic $GABA_A$ receptors in a calcium dependent manner and modulate the induction of LTP.

We have shown that astroglial cell derived ATP is able to modulate $GABA_A$ receptors expressed on neocortical pyramidal neurons (Lalo et al., 2009). Thus, we studied the effects of extracellular ATP on LTP in the neocortex. An analogue of ATP called ATPγS has been demonstrated to induce LTP in the hippocampus (Weirazko, 1996) and it selectively activates P2X1 and P2X1/5 which are abundantly expressed on astrocytes (Rettinger et al., 2000; Palygin et al., 2010). ATPγS (10uM) was applied 10 minutes before and for 10 minutes after electrical stimulation. After the removal of ATPγS there is a gradual increase in the fEPSP slope which results in a stable plateau for more than 60 minutes. This effect is reminiscent of the induction of LTP in wild type mice (Fig. 6.2:28). Although the period of time for the potentiation to be exhibited is longer in dnSNARE mice, it is evident that ATPγS is competent in its abilities to rescue the neocortical LTP phenotype (Fig. 6.4:29). The effect of the ATPγS application was statistically significant when compared to the dnSNARE mice control experiments with a confidence level of P=0.02. Therefore the data suggests that the induction of LTP in cortical slices also includes a purinergic component, which influences the activation of NMDA receptors expressed on the postsynaptic membrane to enhance synaptic efficacy. Furthermore, the fact that

LTP can be generated even in dnSNARE mice indicates that glial cells themselves are not important, but it is the gliotransmitters that they release and the resulting signals that they exert on neuronal receptors.

The positive response subsequent to the application of ATPγS on inducing LTP in cortical slices after electrical stimulation was further established with the use of ionotrophic receptor blockers in a series of experiments. Research on NMDA receptors has predominantly focused on those expressed on neurons and the function of NMDA receptors expressed on glial cells was considered inconsequential. This is mainly due to the voltage dependent Mg2+ block, which many perceived would mean that glial NMDA receptors would be inactive at resting membrane potentials (Palygin et al., 2011). Despite previous thoughts it has been noted that astroglial cells (Lalo et al., 2006) in particular express NMDA receptors which are of a different subunit composition to neuronal NMDA receptors and are not subject to Mg^{2+} block of their channel pores at physiological concentrations (Karadotir et al., 2005; Lalo et al. 2006).

The most ubiquitous NMDA receptor subunit expressed on glial cells is NR2C.D. UBP141 is a potent inhibitor of NMDA receptors which contain NR2C,D subunits and thus is a selective blocker of glial cell signalling. It had a modest inhibitory action on NMDA receptors expressed on cortical pyramidal neurons (Palygin et al., 2011), which makes it a useful tool in studying glial cell induced LTP. The NMDA receptor blocker was washed in to the recording chamber on to cortical slices of wild type mice 10 minutes before TBS and removed immediately afterwards. Under the application of UBP141 there was no induction of LTP it the cortical slices instead we observed a small decrease in the fEPSP slope after the electrical stimulation which was maintained throughout the majority of the experiment and progressively increased back to

control level of 100%. The effect of UBP141 was statistically significant with a confidence level of P=0.02 (one population t-test).

The application purinergic receptor inhibitor NF449 which selectively blocks P2X1 and P2X1/5 receptors also impaired the induction of LTP when it was applied 20 minutes before TBS and for 60 minutes afterwards. These P2X receptor subtypes were shown to be predominant in the purinergic signalling of cortical astrocytes (Lalo et al., 2008), contrary to neurons where P2X2, 3 and 4 receptors made a major contribution. Therefore, NF449 could be considered as a blocker of glial purinergic signalling in our experimental context. The response of the fEPSP to the NF449 application paralleled that of UBP141 as again the fEPSP slope decreased for a considerable amount of time and gradually returned back to 100% (Fig. 6.4:30). The effect of the preferential inhibition of glial P2X receptors by NF449 is statistically significant with a confidence level of P=0.01 (verified by the one population t-test). The inhibition LTP by blocking P2X1 and P2X1/5 purinoceptors suggests that these particular astroglial purinergic receptors are responsible in the mechanism of ATP driven LTP. Furthermore, the above data suggests that the functional relationship of LTP in the neocortex is between the NMDA and P2X receptors. The precise underlying mechanism of ATP induced LTP remains unclear and requires further investigation.

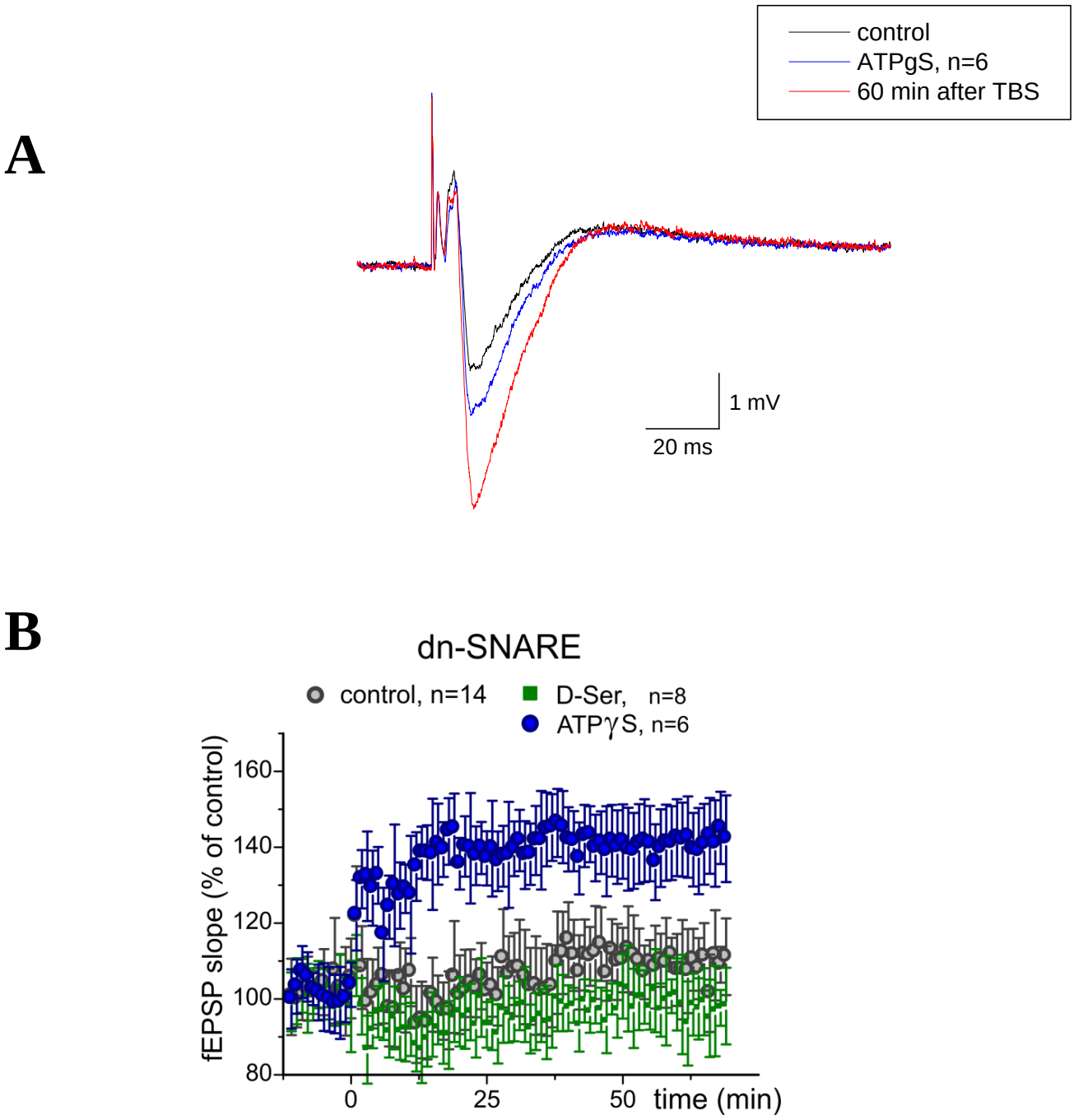

Figure 6.4:31. Different roles for glia-driven ATP and D-serine in induction of synaptic plasticity in the neocortex.

3 μM of D-Serine was applied to cortical slices 10 minutes before the electrical stimulation and for 10 minutes afterwards. D-Serine was unable to recover LTP in dnSNARE mice. **A and B,** the action of 10μM ATPγS which was applied for the same time frames as D-Serine induced LTP in dnSNARE mice, suggesting a purinergic role in the induction of LTP and confirming that D-Serine does not contribute to LTP in the neocortex. Both effects were statistically significant with confidence levels of P=0.02 and P=0.01 (one population t-test) respectively. However the effect of D-Serine was not statistically significant when compared to the control dnSNARE experiment.

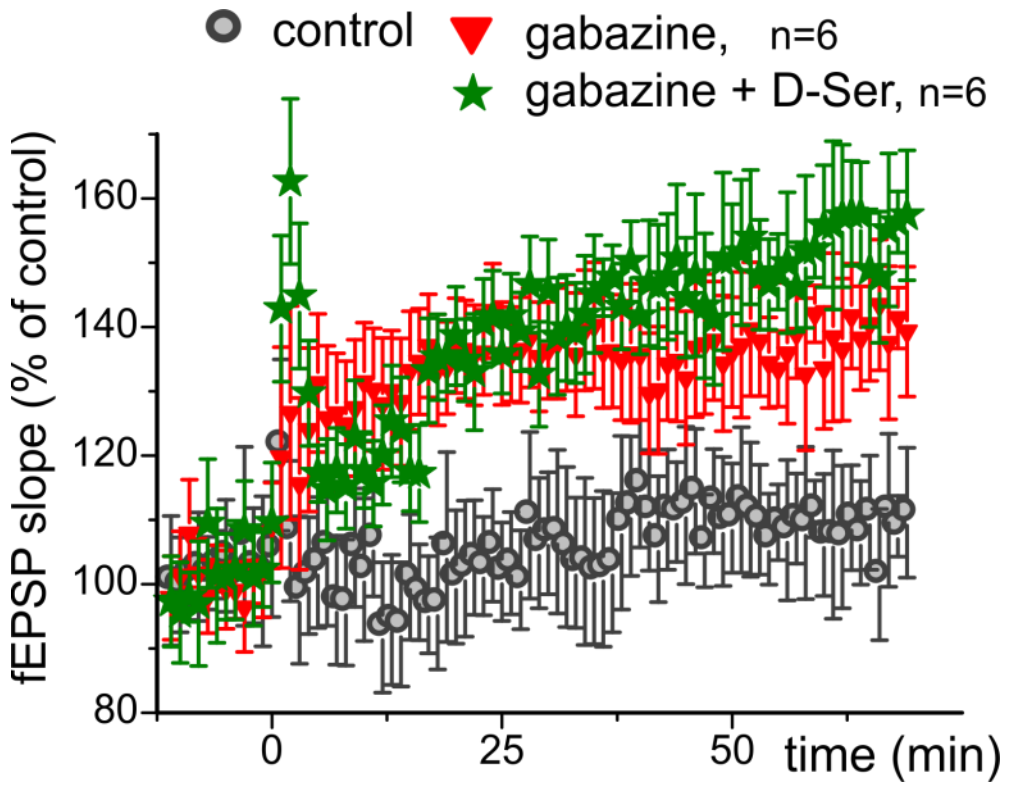

Figure 6.4:32. Impairment of exocytosis of gliotransmitters dramatically reduced the magnitude of LTP in the dn-SNARE mice.

Attenuation of GABA receptors by antagonist gabazine (150 nM) significantly increased the magnitude of LTP in dnSNARE mice. In the presence of gabazine, D-Serine became capable to increase the LTP magnitude in the neocortex of dnSNARE mice. These data suggest that down-regulation of inhibitory receptors by ATP released from astrocytes is essential for the induction of LTP in the neocortex.

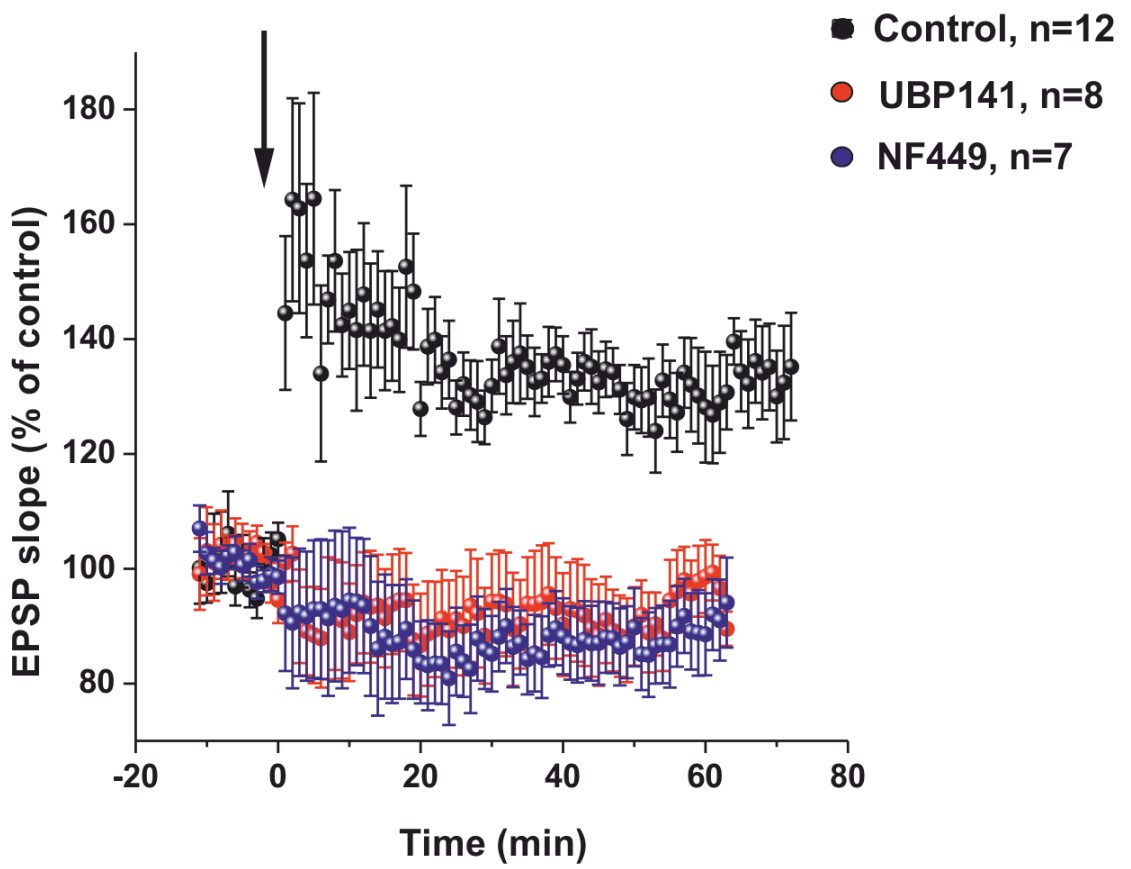

Figure 6.4:33. The inhibition of Long-term potentiation by glial specific ionotrophic receptor blockers in wild type mice.

The standard TBS protocol is able to elicit LTP in the neocortex of wild type mice. However, the application of 3mM UB141, a glial specific NMDA receptor blocker prevents the induction of LTP by electrical stimulation in the neocortex. Similarly, with application purinergic receptor blocker NF449 (3 µM), which specifically blocks P2X1 and P2X1/5 receptor subtypes which are abundantly expressed on glial cells no potentiation of the fEPSP slope is observed. Both of the effects were statistically significant with confidence levels of P=0.02 and P=0.01 respectively.

6.5 Discussion

In recent years a significant effort has been devoted to defining the role of ATP derived from glial cells in the central nervous system with a strong emphasis on the action of astrocytes in mediating synaptic transmission. It is wildly acknowledged that glial cells express receptors which are also expressed abundantly on neurons and also possess the cellular machinery to release chemical transmitters into the synaptic cleft such as D-Serine, ATP and glutamate. Consequently, astroglial cells are able to integrate themselves into the synapse and mediate neuronal signalling as part of the tripartite synapse. Numerous studies have shown that ATP plays a fundamental role in the induction of long term potentiation in the CA1 region of the hippocampus (Fujii et al., 2004) as well as the possibility of glial cells also being involved in the development of learning and memory (Pankratov et al., 2008).

Since most of the work on synaptic plasticity has been conducted in the hippocampus we focused our attention to the neocortex. Under control conditions LTP was induced by a 5 pulse Theta Burst Stimulation (TBS) protocol (Fig. 6.1:25). In nearly all of the slices LTP was elicited using the same electrical stimulation protocol which was applied to layer IV-V and detected in neurons in layers II/III of the neocortex. Consequently, there was a potentiation of the fEPSP slope of approximately 30%, which was prolonged for more than 60 minutes after TBS, signifying LTP induction. The high frequency stimulation to presynaptic neurons in layer IV-V produces potentiation via elevated neurotransmitter release, which in turn influences astrocytes and postsynaptic neurons to generate LTP.

The external application of gliotransmitter D-Serine to brain slices produces an increase in the potentiation in the postsynaptic response recorded at the Schaffer collateral pyramidal cell

synapse of the CA1 region in the hippocampus (Henneberger and Rusakov, 2010). This is a plausible hypothesis as D-Serine is stored and created in astrocytes which harbour serine racemase, which is accountable for converting L-serine into D-Serine. Once released it binds to the glycine site on the postsynaptic NMDA receptor to modulate the channels function. On the contrary to the reported results, the external application of D-Serine to the cortical slices had almost no effect, 4% potentiation was recorded and LTP was not induced. The divergence in the results could be a result of the higher concentration of D-Serine in the neocortex compared to the hippocampus, which means that the neuronal NMDA receptors are already saturated. Also, the expression of different NMDA subtypes may also be critical as the presence of non-conventional NMDA receptors can be desensitised to D-Serine when the membrane potential is too strong, causing an inhibitory effect. This would require further investigation to elucidate the expression levels of such NMDA receptors on cortical neurons and glial cells.

To deduce the role of astrocytes as mediators of enhancing synaptic efficacy in the neocortex we employed methods to impair the function of glial cells. Fluoroacetate (FAC) is a specific glial metabolic toxin which inhibits the tricarboxylic acid cycle (TCA cycle). Studies have reported that D-Serine is able to increase the amplitude of NMDA receptor EPSCs in CA1 pyramidal cells, suggesting that the NMDA receptors are not saturated in resting conditions (Henneberger and Rusakov, 2010). However, the application of FAC to the cortical brain slices resulted in the inhibition of LTP by electrical stimulation and could not be rescued by the application of D-Serine (Fig. 6.9:27). It was suggested that under FAC glial cell function is not fully impaired and as a result a resting level of D-Serine is released from the remaining functioning cells. Subsequently the tonic concentration of D-Serine is sufficient to activate the postsynaptic NMDA receptors. Since the NMDA receptors in the neocortex are already highly

saturated the tonic D-Serine current will have no resulting effect. When glial cell function is deficient in the neocortex LTP induction fails and cannot be recovered by D-Serine in the neocortex. Hence, the data assigns an important role to glial cells in LTP induction and suggests the involvement of gliotransmitters in learning and memory in the neocortex.

Glial cell signalling can also be abolished by specific glial cell ionotrophic receptor antagonists. The strong inhibitory action of UBP141 is restricted to NMDA receptors expressed on glial cells as they are composed of NR2C,D subunits which are not found in neuronal NMDA receptors. When NMDA blocker UBP141 was applied there was no induction LTP by TBS (Fig. 6.4:30). Glutamate NMDA receptors are a primary source of calcium influx into glial cells via activation of glutamate released from the presynaptic membrane. Therefore, the intracellular calcium concentration is not increased and gliotransmitters cannot be exocytosed from the astrocytes which is a calcium dependent process. The inhibition of this signalling pathway prevents LTP and highlights the essential role of glial cells in facilitating the induction of LTP.

The most predominant functional P2X receptor subtypes expressed on astrocytes are P2X1 and P2X1/5 receptors. Application of a specific blocker of these P2X receptor subtypes, NF449 obstructed the induction of LTP by electrical stimulation. Hence, extracellular ATP is unable to activate the glial cell specific P2X receptors and there is no influx of calcium, parallel to the results obtained under UBP141. The experimental data underlines the significance of glial NMDA and P2X receptors in LTP induction. The released gliotransmitters (ATP and D-Serine) as a result of receptor activation act on the neuronal receptors on the postsynaptic membrane and modulate their function, such as ATP down regulating $GABA_A$ receptors which can facilitate LTP caused by elevated excitatory neurotransmission.

The presence of both NMDA and ATP receptors has been described as being fundamental for the induction of LTP when the application of NMDA alone failed to facilitate synaptic plasticity in the same way that the co-application of ATP and NMDA did in the hippocampus (Fujii et al., 2003). The role of glial derived ATP is to enhance the action of NMDA by causing transient increase in the intracellular calcium concentration on the post synaptic membrane. ATP causes the depolarisation of the postsynaptic membrane, which in turn removes the magnesium block of the neuronal NMDA receptor channel producing a source of calcium influx into the cell. Lisman et al. (2002) postulated that the extent of the increase in the intracellular calcium concentration drives the direction of the plasticity (Lisman et al., 2002). A large influx of calcium facilitates LTP which can be provided by the activation of NMDA by glial derived ATP. A consequence of the ATP evoked calcium influx is the activation of intracellular signalling cascades to produce ATP induced LTP (Fujii et al., 2004).

The role of glial cells in synaptic plasticity was reconfirmed in cortical slices taken from dnSNARE mice where the vesicular release apparatus are impaired. Electrical stimulation of the slice in layer IV-V produced no LTP induction and the same occurred under the influence of D-Serine. The function of the TBS protocol is to depolarise the presynaptic cells for the release of neurotransmitters which then bind to receptors expressed on the postsynaptic membrane and glial cells. Although the glial cells will be able to respond to the binding of the corresponding agonist to its receptors producing oscillations in the intracellular calcium concentration, the astroglial cells are deficient in releasing any gliotransmitters in dnSNARE mice to act on the postsynaptic membrane. Thus, long term potentiation cannot be induced in this transgenic mouse model and highlights the importance of the release of gliotransmitters in this mechanism of learning and memory.

The application of non-hydrolysable ATP analogue ATPγS was able to facilitate LTP caused by electrical stimulation in dnSNARE mice. However, it is unclear whether ATPγS alone could produce LTP without the aid of the TBS protocol. One of the direct mechanisms of ATP action on synaptic plasticity could be reducing the threshold required to induce LTP by inhibiting $GABA_A$ receptor activity via activation of P2 on the postsynaptic membrane. The reduced activation of postsynaptic receptors in dnSNARE mice, particularly NMDA receptors, may be a cause for the absence of an effect by D-Serine on LTP in the neocortex. To conclude, the fact that LTP can be induced even in dnSNARE mice, it is not the glial cells themselves that is important but it is the release of gliotransmitters such as ATP and D-Serine and the signals that they exert on the postsynaptic membrane that contribute to the development of learning and memory.

Chapter 7
Discussion

The data obtained from this series of experiments show that the activity of the $GABA_A$ receptors were affected by the activation of the P2X receptors expressed in both the peripheral and central nervous system. Our results suggest that the entry of calcium through the purinergic ion channel served as a signalling molecule to facilitate the modulation of the inhibitory receptors and acted as the functional linkage between the P2X and $GABA_A$ receptor responses. Moreover, the presented results maintain the ideology that enhanced intracellular calcium concentrations leads to the activation of intracellular signalling cascades which are responsible for the phosphorylation of the $GABA_A$ receptor. The examination of the underlying mechanism of this receptor interaction is not very well explored, however endeavouring to do so has led to the discovery of a calcium dependent pathway which points to the involvement of an intracellular signalling kinase, Protein Kinase C that is fundamental to this novel receptor communication.

There are two main factors which emphasise the significant role of the calcium dependent component of the purinergic driven down regulation of $GABA_A$ receptors. Firstly, when the levels of calcium chelator was enhanced in the intracellular medium (10 mM EGTA) the action of the P2X receptors on $GABA_A$ receptor currents when activated with ATP or P2X specific agonist α,βme-ATP was abolished. On the contrary the opposite effect was observed in low concentrations of calcium chelator (0.2 mM EGTA), which promoted the occlusion of the GABA mediated currents recorded immediately after the activation of the ATP receptors on both

DRG and central neurons. These results are parallel with another group that have also shown the calcium dependent inhibition of $GABA_A$ receptor evoked currents by P2X receptors in DRG neurons (Sokolova et al., 2001), which is in disagreement with another group who postulate that the receptors communicate via direct coupling (Boue-Grabot et al., 2004; Toulme et al., 2007) Secondly, functional P2X receptors exhibit a high permeability to calcium at low membrane potentials, which means they are a prime source of calcium influx into the cell. Therefore, the inhibitory influence of P2X receptors on $GABA_A$ receptors is most likely to be dependent on the calcium influx through the purinergic ion channel.

Early on during our experiments conducted on DRG neurons we noted that the amplitude of the control GABA responses were significantly variable in 0.2 mM EGTA intracellular solutions, but gradually stabilized after consecutive applications of receptor agonist. The variability in GABA mediated responses was not observed in 10 mM EGTA concentrations, further emphasizing the magnitude of the calcium dependence to this receptor interaction. Other groups have established this receptor interaction by focusing on a comparison between the predicted algebraic sum of the co-application of ATP and GABA and the actual response to the simultaneous application of two agonists recorded during their experiments. This method is severely influenced by the rundown of $GABA_A$ currents, which as we have observed is inevitable when using a lower concentration of Ca^{2+} chelator inside the cell, and may underestimate the impact of activated P2X receptors on the GABA mediated responses. This could explain the controversy between the data of two different research groups (Sokolova et al., 2001; Boue-Grabot et al., 2004; Toulme et al., 2007; Jo et al., 2011). The method we used during our own experiments is significantly more robust and more sensitive towards the rundown of the GABA response.

Experimental evidence suggests that calcium may not be solely responsible for $GABA_A$ and P2X interactions as the functional state of the $GABA_A$ receptors themselves may play a significant role. NMDA receptors expressed on hippocampal neurons are also subjects of the P2X mediated down regulation via a calcium dependent mechanism involving phosphorylation (Pankratov et al., 2002). It is widely accepted that $GABA_A$ receptors undergo intracellular modulation via the activation of intracellular signalling cascades (Kittler and Moss, 2003) and we have focused on the impact of intracellular $GABA_A$ modulation on the interaction between the two receptors. The introduction of phosphatase an kinase inhibitors Cylcosporine A, Staurosporine and KN93 to the extracellular solution revealed that Phosphatase II has no effect on the down regulation of GABA mediated currents mediated by activated P2X receptors on DRG neurons. However, upon the blocking of PKA and CamKII the purinergic inhibition of $GABA_A$ receptors was eliminated. Furthermore we later provided evidence for the role of PKC in this mechanism when the coapplication of Staurosporine and GF103209x successfully occluded the effect of P2X receptors on $GABA_A$ receptors on central neurons. Observations have demonstrated that $GABA_A$ receptors possess a sequence of highly conserved phosphorylation sites on their β and γ subunits (Brandon et al., 2000; Vithlani et al., 2011). As phosphoproteins $GABA_A$ receptors are targets of intracellular modulation via a Ca^{2+}-dependent phosphorylation process (Brandon et al., 2002; Kittler et al., 2003; Kumar et al., 2005; Houston et al., 2009). Our results confirm that the modulatory impact of P2X receptors on GABA receptors expressed on central neurons is governed by the calcium dependent activation of PKC.

Despite the strong evidence which suggests that the receptor communication between the two ionotrophic receptors is calcium dependent (Sokolova et al., 2001; Lalo et al., 2009) the underlying mechanism of this receptor interaction still remains controversial. Another group

postulates that this receptor interaction occurs independently of calcium, but is a result of direct coupling of the intracellular components of the receptors via a QST motif located on the carboxyl terminus of the P2X receptor (Toulme et al., 2007). However, the role of calcium is questioned after observations of the summation experiments also provide a possible role for direct coupling between the receptors. When the functional linkage between the two receptors is removed via the application of PKC inhibitors and a high dose of calcium chelator the deficit in summation is not recovered, which would be expected if calcium was solely responsible for this receptor interaction (**ref. Chapter 3, Fig. 3.3:13**). Therefore the results imply that P2X receptors ability to modulate GABA receptor mediated responses is dependent on Ca^{2+} and phosphorylation, but to some extent may also involve direct coupling, marrying the two opposing paradigms of this receptor communication.

When making our observations of this receptor interaction in the central nervous system we used central neurons isolated from the somatosensory cortex. The study of extracellular ATP on functional receptors expressed postsynaptically is relatively unexplored, specifically in native cells. For cell lines it is typical to find an excessive density of receptors which can have adverse effects on neurotransmission, using native cells is beneficial as these effects are absent. Also, in native cells the receptors are positioned in their "natural" arrangement and receptor composition. Therefore when pursuing the idea of looking at the effect of extracellular ATP on postsynaptic receptors in the central nervous system we utilized native cells acutely isolated from the brains of mice.

In our voltage clamp experiments on acutely isolated pyramidal neurons the $GABA_A$ receptors exhibited inhibition of their response recorded immediately after the application of the purinergic receptor agonists in 0.2 mM EGTA concentrations. As previously observed in DRG

neurons this effect was not present under 10 mM EGTA concentrations, highlighting the role of calcium in this receptor mechanism. Furthermore, the level of inhibition was to a marginally larger degree in central neurons than in DRG neurons. Since our results support the theory that this interaction is predominantly calcium dependent the varying kinetically distinct P2X responses that we observe (both fast, slow and mixed responses) suggests the expression of a mixed population of P2X receptors on both DRG and central neurons which would give rise to different calcium permeability's. This observation coincides with the fact that one of the most predominant P2X receptor subtypes expressed on DRG neurons, P2X3, results in a rather limited calcium influx when activated (Virginio et al., 1998). On central neurons P2X4 is the most predominant P2X receptor expressed and has a slower dessentisation rate than P2X3 and therefore a greater calcium influx, which would explain the higher degree of inhibition documented in central neurons compared to dorsal root ganglion neurons.

Not only do $GABA_A$ receptors respond to the local vesicular release of receptor agonist from the presynaptic terminal but another highly sensitive subclass of $GABA_A$ receptors can also respond to the low concentrations of GABA as a result of a spill over from the synaptic cleft, in a principal mechanism termed tonic inhibition. Experimental observations suggest that this novel form of $GABA_A$ receptor signalling is also a target of purinergic modulation. Additionally, the fact that we observed different phenotypic currents elicited from activated P2X receptors, suggests the possibility that the amount of calcium influx is variable depending on which P2X receptor subunits are expressed. We have demonstrated the inhibition of GABA mediated currents by extracellular ATP in the peripheral and central nervous systems, which express different populations of purinergic receptor subtypes. Furthermore, the occlusion of both phasic

and tonically activated $GABA_A$ receptors is indicative that this mechanism is not P2X receptor subunit specific and is a universal phenomenon.

The physiological implications of this regulatory pathway are yet to be fully investigated; however our results in brain slices reveal the potential of this innovative receptor interaction in the central nervous system (Lalo et al., 2009). It has been established in recent years that purinoreceptors play a crucial role in synaptic plasticity, synaptic transmission and brain function. Much of the spotlight of purinergic research is currently focused on its potential role in synaptic plasticity due to its important association with learning and memory, most of this work has been conducted in the hippocampus with little emphasis on the somatosensory cortex. However our data significantly suggests that astroglial derived ATP is a strong contributor to long term potentiation in the neocortex. The extent of a purinergic role in inducing long term potentiation and other physiological processes is far from being fully understood, equally the underlying mechanisms to which also remain indeterminate.

The extracellular signalling properties of ATP provide the core foundations of inter- and intra-astroglial cell signalling in the central nervous system. The high permeability of P2X receptors to calcium is principal to their modulatory actions on the pre- and postsynaptic receptors at nerve terminals. The integration of astroglial cells in to the synapse by enwrapping synapses to form the tripartite synapse, provides a source of functional support for neurons and consequently synaptic transmission. Astrocytes are also able to transmit robust and regenerative calcium signals which target other astrocytes and neurons (Hassinger et al., 1996). Depolarisation of the presynaptic nerve terminal causes the release of neurotransmitters. This in turn causes an increase in cytosolic calcium concentration within astrocytes, promoting the

generation of propagating calcium signals and the release of gliotransmitters, which propagate back to neuronal synapses to modulate synaptic transmission.

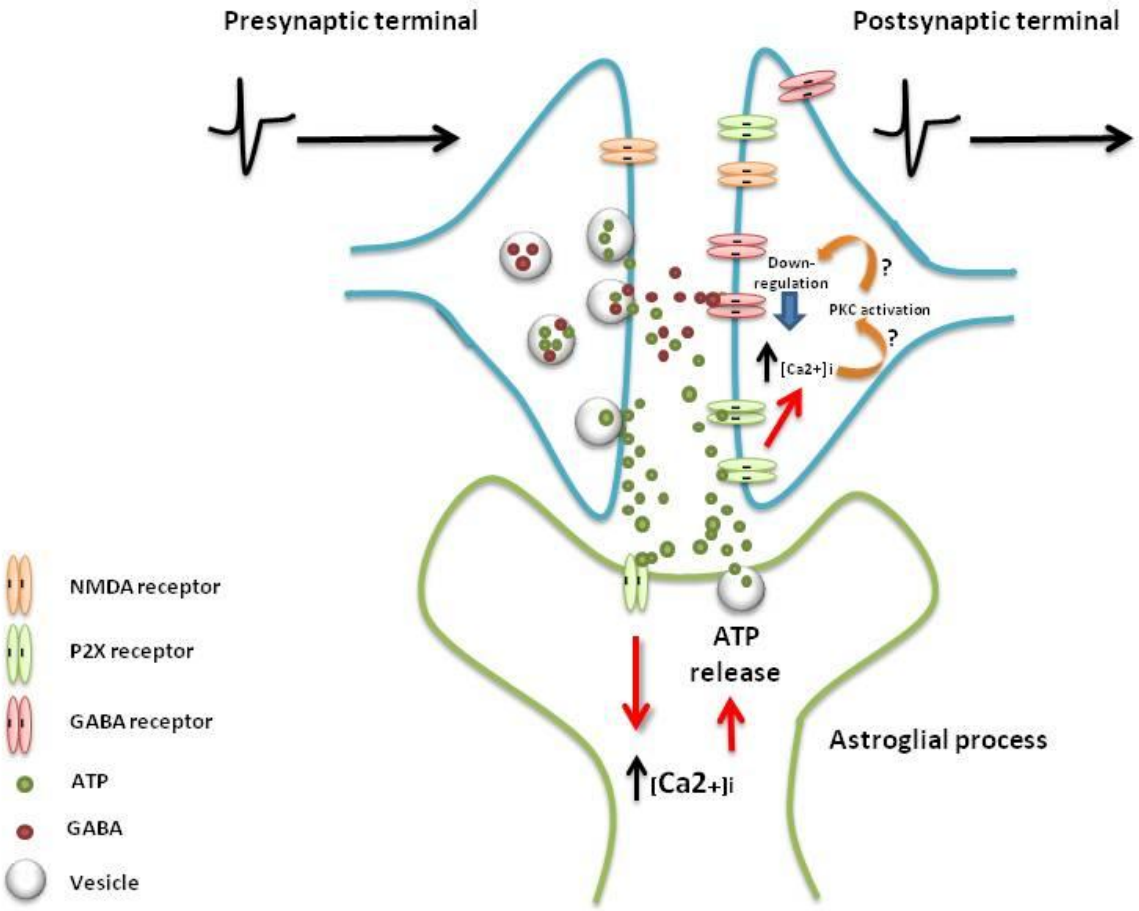

Figure 7:34. A summary of the action of the purinergic receptors on $GABA_A$ receptors expressed on the postsynaptic membrane.

ATP is co-released alongside GABA or alone in synaptic vesicles and diffuses across the synaptic cleft to activate postsynaptic P2X receptors. The high permeability of P2X receptors to calcium is fundamental to actions of purinergic modulation in the central nervous system. Our studies demonstrate the down-regulation of synaptic and extrasynaptic $GABA_A$ receptors in a calcium dependent manner, and also suggest the involvement of an intracellular signalling cascade. Astrocytes also express P2X receptors, once activated there is an increase in the intracellular calcium concentration which stimulates the release of gliotransmitters such as ATP, glutamate and D-serine. Glial cell derived ATP can also modulate postsynaptic $GABA_A$ receptors, representing a novel mechanism of GABA receptor regulation which could have important implications for GABA homeostasis and synaptic plasticity.

Since astrocytes abundantly express P2X purinoreceptors and can release ATP as a gliotransmitter, this suggests the presence of a unique modulatory network involving neuron-glial cell communications potentially of a purinergic origin. At a functional level the precise role of astroglial cell derived P2X receptors is currently unknown, however previous studies and our data indicates that astroglial derived ATP induces long term potentiation in the central nervous system. This is a relatively unexplored territory which requires more intense study, but essentially the paradigm of LTP induction by astroglial ATP extends the realms of the P2X receptors modulatory repertoire.

Once assumed to be just "brain glue" providing functional support to neurons, research into the role of astrocytes remained dormant for a long time. However, recently researchers have devoted much effort in pursuing astrocytes, attempting to define their precise regulatory impact on synaptic transmission and brain function. In their role as supporting cells, glial cells are responsible for the uptake of the residual neurotransmitter left in the synaptic cleft after release, such as glutamate and GABA as a method of maintaining the correct concentration of chemical transmitter at the synapse to prevent exocytotic cell death of neurons. The study of the maintenance of GABA homeostasis by astrocytes in the central nervous system is a relatively unexplored topic which is currently under intense study. P2X purinoreceptors embody a pioneering mechanism of $GABA_A$ receptor regulation. Neuronal and astroglial cell derived ATP elevates the concentration of intracellular calcium concentration via stimulating P2X receptors, which in turn mediates the modulation of $GABA_A$ receptors in the peripheral and central nervous system.

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