Astrocytic involvement in cortical inhibition and serotonin neuromodulation

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Abstract

A single astrocyte can ensheath more than 100,000 synapses within its domain. Thus, astrocytes are ideally positioned to integrate signals from a few synapses to have impact on all ensheathed synapses with high efficiency. As neuromodulators are released in a volume manner and are known to ellicit astrocyte calcium responses, we hypothesized that astrocytes may be effector cells, extending neuromodulator action to every synapse. Using live mouse brain slices, extracellular recordings of evoked excitatory postsynaptic potentials (eEPSPs), and select pharmacology, we assessed the astrocytic involvement in paired-pulse suppression and serotonin-mediated shaping of a simple sensory cortical network containing both excitatory and inhibitory activity.

Using a paired-pulse stimulus repeated every 20 seconds, we assessed the role of astrocytes in paired-pulse suppression by applying pharmacological agents in the bath perfusate to interfere with astrocyte function. We then applied them in the presence of the $GABA_A$ antagonist bicuculline to determine if effects were dependent on GABA. To assess the role of astrocytes in serotonin neuromodulation, serotonin was administered as a bolus to the bath perfusate upstream of the recording site to simulate transient effects on the network. Serotonin was applied both before and after bath application of pharmacological agents considered to affect astrocyte function or signaling mechanisms.

In the absence of neuromodulators or pharmacological agents, the first cortical eEPSP is much larger in amplitude than the second due to the recruitment of longer-lasting inhibitory activity resulting from the first stimulus. Pharmacological disruption of 1) astrocytic mGluR5 receptors, 2) astrocyte metabolism, 3) gap junctions/hemichannels, or 4) purinergic receptors resulted in a significant loss of this evoked inhibition in field recordings, suggesting that astrocytes may play a role in tonic aspects of network inhibition. Furthermore, all significance was lost when performed in the presence of bicuculline, suggesting that astrocytic involvement in paired-pulse suppression is $GABA_A$ dependent. In addition to effects seen on tonic cortical inhibition, serotonin effects on frequency transmission in the cortical network are significantly altered following

pharmacological astrocyte disruption. Lastly, serotonin-mediated frequency transmission could also be disrupted using P2 antagonists suggesting that ATP signaling (astrocyte currency) may be involved.

These data highlight a potential role for astrocytes in cortical inhibitory activity seen in this sensory cortical network and that serotonin acts on astrocytes to partially exert its modulatory influence.

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List of Abbreviations

5-HIAA - ***5***-hydroxyindoleacetic acid

5-HT - serotonin

5-HTP - *5*-hydroxytryptophan

Ab129 – acid blue 129

aCSF – artificial cerebral spinal fluid

ADP – adenosine diphosphate

AMP – adenosine monophosphate

APB – 2 – aminoethoxydiphenyl borate

ATP – adenosine triphosphate

BAPTA - 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid

BIC - bicuculline

cAMP – cyclic adenosine monophosphate

CBX - carbenoxolone

CNS – central nervous system

COX - cyclooxygenase

DAG - diacylglycerol

DPCPX - 8-Cyclopentyl-1,3-dipropylxanthine

eEPSP – evoked excitatory postsynaptic potential

E-NPPs - ecto-nucleotide pyrophosphatase/phosphodiesterases

E-NTPDs - ectonucleoside triphosphate diphosphohydrolases

EPSC - excitatory postsynaptic current

FFA – flufenamic acid

fPSP – field postsynaptic potential

GABA - gamma-Aminobutyric acid

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

IDA – iodoacetate

IP_3 – inositol triphosphate

IPSCs – inhibitory postsynaptic currents

MAO – monoamine oxidase

mGluR – metabotropic glutamate receptor

MPEP - 2-Methyl-6-(phenylethynyl) pyridine hydrochloride

MRS2179 - 2'-Deoxy-N^6-methyladenosine 3',5'-bisphosphate tetrasodium salt

NE – norepinephrine

NMDA - N-methyl-D-aspartate receptor

P1 – pulse 1

P2 – pulse 2

P2X – purinergic type 2X receptor

P2Y – purinergic type 2Y receptor

PAR-1 – protease-activated receptor type 1

PKC – phosphokinase type C

PLC – phospholipase type C

PPADS - Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt

SCH 582621 - 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine

SUR - suramin

TFLLR - L-Threonyl-L-phenylalanyl-L-leucyl-L-leucyl- L-argininamide

TPH - tryptophan hydroxylase

TRP – transient receptor potential

VMAT - vesicular monoamine transporter

VNUT - vesicular nucleotide transporter

1.0 Introduction

Astrocytes are the most abundant cell type in the brain and play important roles in ion homeostasis, metabolism, neurotransmitter clearance, synapse formation/removal, neurovascular coupling, and modulating neurotransmission. They are instrumental in metabolic processes as they respond accordingly to the activity dependent needs of neurons through the astrocyte-neuron lactate shuttle (Pellerin et al., 2007). Lactate is the oxidative substrate preferred by neurons. During neuronal activation, glutamate increases lactate consumption by neurons. Glutamate also increases glycolysis in astrocytes to provide neurons with lactate in order to maintain neurotransmission (Pellerin et al., 2007). Astrocytes also maintain a role in modulation of cerebral blood flow (Gordon et al., 2008).

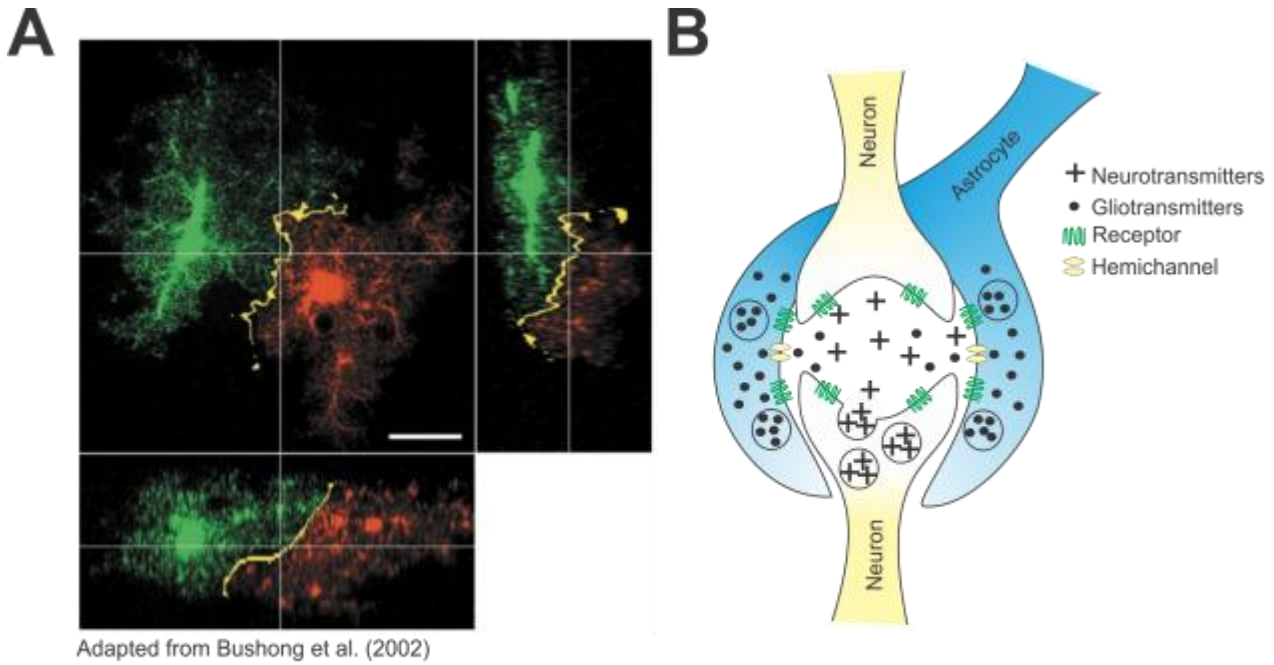
Blood vessels are covered by astrocyte end feet which regulate vasoconstriction and vasodilation of arterioles. Calcium signaling in astrocytes is coupled to changes in neural activity, which produces changes in blood flow. As oxygen availability is typically low in the brain (near the hypoxic threshold), astrocyte calcium increases lead to astrocyte lactate release and resulting vasodilation (Gordon et al., 2008).

Astrocytes are responsible for rapidly clearing the extracellular space. Of particular importance is the clearance of glutamate to prevent excitotoxicity during neurotransmission. This is required in order for neurons to survive and function normally (Anderson and Swanson, 2000). In addition to clearing glutamate, astrocytes also release glutamate as a gliotransmitter to contribute to the regulation of neural activity at the synapse (Anderson and Swanson, 2000). Furthermore, astrocytes play an important role in potassium homeostasis. During neuronal activity, potassium re-uptake by neurons is too slow to prevent potassium accumulation. A buildup of extracellular potassium would affect proper transmission and channel kinetics. Astrocytes compensate for this via three mechanisms; carrier operated potassium-chloride uptake, channel operated potassium chloride uptake, and they act as an interconnected (gap junctions) spatial buffer that effectively sequesters excess potassium away from neurons (Walz, 2000).

Astrocytes are thought to form a tripartite synapse with pre- and post-synaptic neurons. They envelope the synapse and can respond to activity at excitatory (glutamate) and inhibitory (GABA) synapses as well as possess cholinergic, adrenergic, and peptidergic receptors (Hosli and Hosli, 2000). Upon substrate-receptor activation, alterations in astrocyte calcium propagate from astrocyte to astrocyte in a syncytium that can widely affect brain function. This leads to the release of gliotransmitters, such as ATP, D-serine, and/or glutamate (Bezzi and Volterra, 2001). Therefore, it is possible that astrocytes modulate neuronal activity at many synapses across great distance.

1.1 Astrocytes are well positioned to modulate neurotransmission

20-40% of the total number of cells in the brain are astrocytes (Herculano-Houzel, 2014). They establish distinct territories that do not overlap except at narrow bands of profuse interdigitation between fine processes at their domain boundaries (Fig 1.1A)



Adapted from Bushong et al. (2002)

Figure 1.1: Astrocytes occupy distinct boundaries and form tripartite synapses with pre- and post-synaptic neurons. *A*, Image from Bushong et al. (2002) showing that astrocytes inhabit distinct domains and only interact with each other at their domain boundaries. *B*, schematic showing the tripartite synapse formed between an astrocyte and the pre- and post-synpatic neurons. Astrocytes respond to neurotransmitter release and can release gliotransmitters to modulate synaptic activity.

(Bushong et al., 2002). Astrocytes do not intermingle with each other but they profusely interweave their processes with other glial cell processes (Bushong et al., 2002). In addition, it is thought that astrocytes form a tripartite synapse with neurons in which the

astrocyte wraps around the synaptic connection between pre- and post-synaptic neurons (Fig 1.1B) (Araque et al., 1999a, Grosche et al., 1999, Ventura and Harris, 1999). As a single astrocyte can make connections with up to 140,000 synapses in its domain (Bushong et al., 2002), this puts them in an ideal position to integrate neuronal inputs and modulate synaptic activity.

1.2 Astrocytes in neurotransmission

Astrocytes were originally thought to play merely a structural role in the brain. However, recent evidence now supports that they play a role in modulating neuronal activity (Araque et al., 1999a, Allen and Barres, 2005, Panatier et al., 2011). As it is thought that astrocytes wrap around every synapse to form the tripartite synapse (Fig 1.1B) (Araque et al., 1999a, Grosche et al., 1999, Ventura and Harris, 1999), this would enable them to respond to neurotransmitter release (such as glutamate) at the synapse and respond accordingly to modulate wide brain function (Araque et al., 1998). They have been shown to possess receptors for various neurotransmitters such as acetylcholine, norepinephrine, serotonin, and glutamate among others (Hosli and Hosli, 2000). In particular the mGluR5 glutamate receptor can be activated to induce intracellular calcium elevations (Nakahara et al., 1997, Panatier and Robitaille, 2016), which is a crucial function of astrocyte signaling.

Metabotropic glutamate receptors are present on both glial cells and neurons. There are eight metabotropic glutamate receptors, which are classified into three groups. Group I includes mGluR1 and mGluR5 and are coupled to G_q G-proteins that activate phospholipase C (PLC) and generally modulate excitation. Group II consists of mGluR2 and mGluR3, while Group III includes mGluR4, mGluR6, mGluR7, and mGluR8. Group II and III are negatively coupled to adenylate cyclase through $G_{i/o}$ and are predominantly located pre-synaptically to generally decrease excitability (Gereau and Swanson, 2008, Sherman, 2014).

It has been shown that in response to elevated calcium, astrocytes release gliotransmitters, such as ATP (Bowser and Khakh, 2004). The tripartite synapse marks astrocytes as ideal candidates to modulate neuronal activity as they can respond to

synaptic activity accordingly, via calcium waves and gliotransmitter release, to regulate neurotransmission.

1.2.1 Astrocyte Calcium Signaling

Communication among astrocytes is achieved through calcium waves which are induced by transmitters released from neurons at the synapse (Dani et al., 1992). For example, glutamate has been shown to induce calcium oscillations in astrocytes (Cornell-Bell et al., 1990, Cornell-Bell and Finkbeiner, 1991, Porter and McCarthy, 1996). These glutamate-stimulated calcium elevations spread from one astrocyte to another which forms a long-range signaling pathway to affect extensive brain function (Cornell-Bell et al., 1990). It has been shown that astrocytes are connected at the border between domains where their fine processes interact through gap junctions (Finkbeiner, 1992). Calcium levels are thought to be increased via the second messenger, inositol triphosphate (IP_3), which is spread through these gap junctions. This results in a subsequent calcium increase in neighboring astrocytes (Charles et al., 1992, Leybaert et al., 1998). However, this passive diffusion is not sufficient to propagate the spread of calcium waves great distances. In addition, studies have shown that the intracellular calcium increase can jump between two groups of astrocytes that are not connected by gap junctions (Sneyd et al., 1994). Thus, there must be an extracellular mechanism by which calcium waves are regenerated and propagated. A likely mechanism is the release of gliotransmitters, particularly ATP (Stout et al., 2002, Guthrie et al., 1999), which can act on adjacent astrocyte to regenerate the calcium wave (Anderson et al., 2004). Therefore, astrocytes propagate calcium signals across short distances via diffusion of IP_3 through gap junctions to elevate intracellular calcium in connected astrocytes and across longer distances via gliotransmitter release that would act on neighboring astrocytes not connected through gap junctions (Fig 1.2).

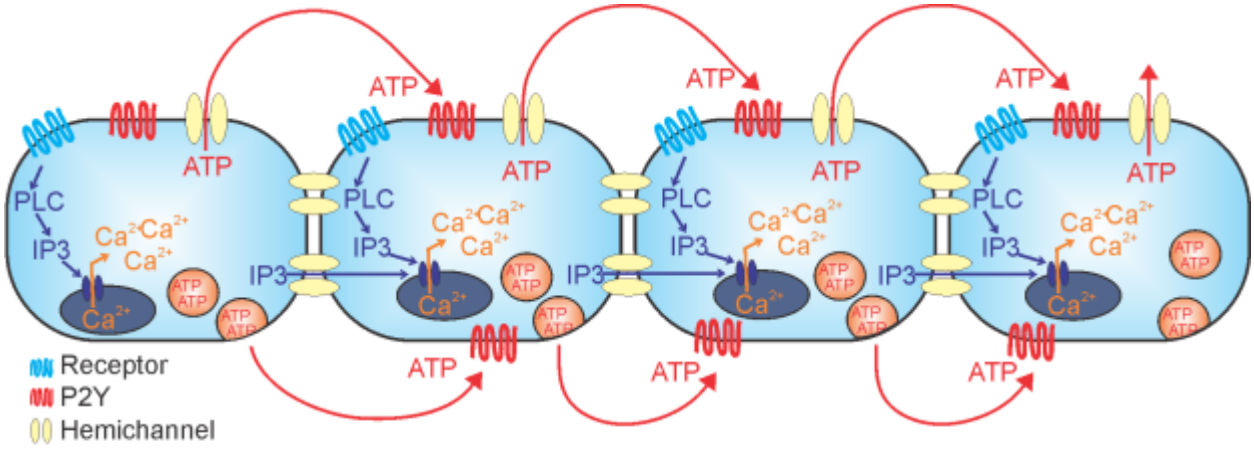


Figure 1.2: Astrocytes can propagate calcium waves throughout the astrocyte syncytium. Receptors that activate the PLC pathway leads to the formation of Ip_3. Ip_3 opens calcium channels in the ER causing intracellular calcium to rise. Ip_3 diffuses into neighboring astrocytes to propagate the calcium wave. However, this passive diffusion is not sufficient to propagate calcium waves great distances. ATP overcomes this by acting on astrocytic P2Y receptors to strengthen the calcium response and increase ATP release. ATP also propagates calcium waves to neighboring astrocytes that are not connected via gap junctions.

1.2.2 ATP is astrocyte currency

Increases in astrocyte intracellular calcium can trigger the release of the gliotransmitters, ATP, D-serine, and glutamate (Bezzi and Volterra, 2001). These gliotransmitters have been shown to act on neurons to regulate transmission (Parpura et al., 1994, Pasti et al., 1997, Araque et al., 1999b, Perea et al., 2009, Panatier et al., 2011). Moreover, evidence has been provided to support that astrocytes release ATP in response to elevated calcium. Astrocytes express PAR-1 receptors that can be activated by TFLLR to induce rises in calcium and gliotransmitter release (Debeir et al., 1997, Junge et al., 2004, Lalo et al., 2014). The ATP released can then act to induce further ATP release, which maintains and propagates calcium waves throughout the syncytium (Coco et al., 2002, Anderson et al., 2004, Guthrie et al., 1999). In addition to propagating calcium waves, ATP released from astrocytes can act to modulate synaptic activity though its actions on purinergic receptors on, neurons, interneurons, and neighboring astrocytes (Khakh et al., 2003, Koizumi et al., 2003, Zhang et al., 2003, Anderson et al., 2004, Lalo et al., 2014). These actions are excitatory or inhibitory and ATP can either be the sole transmitter or a co-transmitter to modulate synaptic activity (Abbracchio et al., 2009). Various purinergic receptor subtypes have been indicated to be widely distributed throughout the brain (Guthrie et al., 1999). This is significant as studies have demonstrated that high

concentrations of nucleotides are present in pathological states of the CNS (Abbracchio et al., 2009). For example, immediately after a traumatic insult, astrocytes respond by releasing ATP to activate microglia to form a protective barrier (Abbracchio et al., 2009). ATP can act directly or it can induce effects through the action of its metabolites when it is degraded.

1.2.3 ATP uptake and release

ATP is taken up by astrocytes via the chloride dependent vesicular nucleotide transporter (VNUT). This transporter is highly expressed in chromaffin granules and astrocytes (Sawada et al., 2008). However, the mechanism of ATP release is highly debated and there is evidence to support multiple methods including; vesicular release, ATP-binding cassette transporters, connexon or pannexin hemichannel gap junctions, and voltage-dependent anion channels which include P2X receptors (Abbracchio et al., 2009). The two mechanisms most highly debated between these are ATP release by exocytosis and ATP release through hemichannel gap junctions. For example, Haydon and colleagues have provided evidence to support the release of ATP by vesicular release from astrocytes. They used the transgenic dn-SNARE mouse model in addition to various manipulations of exocytosis (Lalo et al., 2014). However, other studies have shown that ATP can be released through hemichannels (Stout et al., 2002, Garré et al., 2010, Wei et al., 2014). In addition, one study suggested that ATP is initially released via exocytosis, which then modulates further release of ATP through hemichannels (Garré et al., 2010). ATP release is thought to be calcium dependent. However, some studies have shown ATP release in the absence of calcium demonstrating that there can be multiple ATP release pathways with varying dependence on calcium (Queiroz et al., 1999, Coco et al., 2002).

1.2.4 ATP metabolism

After ATP is released, ectonucleotidase enzymes rapidly degrade it (Fig 1.3). ATP is metabolized into ADP, AMP, and finally adenosine which can also act as ligands for purinergic receptors (Abbracchio et al., 2009). The ectonucleotidases that breakdown ATP include the ectonucleoside triphosphate diphosphohydrolases (E-NTPDs), the ectonucleotide pyrophosphatase/phosphodiesterases (E-NPPs), alkaline phosphatases, and

ecto-5'-nucleotidases. E-NTPDs and E-NPPs hydrolyze ATP and ADP to AMP which is then hydrolyzed by ecto-5'-ectonucleotidase into adenosine. In addition, alkaline phosphatase equally hydrolyzes ATP, ADP, and AMP (Fig 1.3) (Zimmermann et al., 1998, Zimmermann, 2006). All products of ATP metabolism, including ADP, AMP and adenosine further act on their respective purinergic receptors to produce various effects. ATP therefore has the capability to mediate effects at multiple levels.

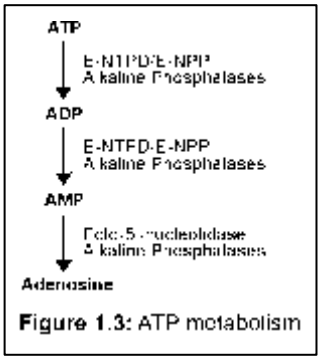


Figure 1.3: ATP metabolism

1.2.5 ATP receptors

Purinergic receptors are widely distributed throughout the brain. ATP plays a unique role in glial-neuron and glial-glial interactions as all types of functional glia express purinergic receptors. There are two groups of purinergic receptors, P1 and P2. Adenosine acts on P1 purinergic receptors, while ATP acts on the P2 purinergic receptors (Abbracchio et al., 2009).

P1 receptors for adenosine include the A1, A2a, A2b and A3 receptors which are coupled to G-proteins. A1 and A3 are coupled to $G_{i/o}$, while A2 receptors are coupled to G_s (Abbracchio et al., 2009). The actions of adenosine can often be either antagonistic or synergistic to those of ATP (Abbracchio et al., 2009), and have frequently been shown to be involved in many neuropathologies ranging from epilepsy and neurodegenerative disorders to psychiatric conditions (Boison, 2008).

P2 purinergic receptors are further divided into two subgroups, metabotropic P2Y receptors and ionotropic P2X receptors (Abbracchio et al., 2009). There are seven P2X receptor subtypes, $P2X_{1-7}$, which are cationic ligand operated channels that become permeable to sodium, potassium, and calcium upon binding of ATP (Khakh, 2001). Postsynaptic P2X receptors interact with various ionotropic receptors to have a reciprocal inhibitory effect, which is mediated through intracellular calcium to cause phosphorylation. The receptors that postsynaptic P2X receptors interact with include nicotinic acetylcholine receptors, A type gamma-Aminobutyric acid ($GABA_A$), and N-methyl-D-aspartate (NMDA) receptors (Khakh et al., 2000). There are eight P2Y receptor subtypes, $P2Y_1$, $P2Y_2$, $P2Y_4$,

$P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$, which are coupled to either $G_{q/11}$ or $G_{i/o}$ to activate the PLC pathway or inhibit cAMP respectively (Abbracchio et al., 2006). Extracellular ATP has been shown to induce intracellular ATP release from astrocytes through its action on both astrocytic P2X and P2Y receptors (Anderson et al., 2004).

1.2.6 ATP acts on interneurons

It is possible that ATP released from astrocytes acts on interneurons to contribute to the modulation of GABA release and in turn, inhibition. Bowser and Khakh showed that stratum radiatum interneurons are excited by ATP and consequently release GABA onto their postsynaptic targets. ATP increased inhibition of output pyramidal neurons by increasing the frequency of IPSCs and triggering IPSCs of larger conductance (Bowser and Khakh, 2004). These effects are in part mediated through P2Y receptors on interneurons as opposed to P2X or P1 receptors. No staining for purinergic receptors was seen in CA1 pyramidal neurons which was consistent with the lack of ATP evoked currents that was observed in these cells. However, interneurons were immunopositive for P2Y receptors further implicating them in modulation of inhibition. In addition, they found that interneurons expressing P2Y receptors were profusely surrounded by astrocytes with highly branched morphologies suggesting that they might be the source of ATP (Bowser and Khakh, 2004).

1.2.7 Glutamate can elicit calcium waves in astrocytes and ATP release

Astrocytes express receptors for glutamate, acetylcholine, norepinephrine, serotonin, and ATP (Haydon, 2001). The triggering stimulus for astrocytes involves vesicular release of glutamate from presynaptic nerve terminals (Bowser and Khakh, 2004). It is thought that glutamate acts on astrocytes at the synaptic nerve terminal and astrocytes release ATP at more distant sites to propagate the signal through the syncytium (Bowser and Khakh, 2004). It is possible that other transmitters, through their alteration of calcium in astrocytes, can alter this mechanism. Serotonin may be one such transmitter.

1.3 Serotonin

1.3.1 Serotonin neuromodulatory projection and anatomy

Serotonin neurons originate in the midbrain raphe nuclei and project extensively throughout the cortex (Fig 1.4). Serotonergic axons are present in all areas of the cortex and all cortical layers receive some serotonin afferents. Each area has a characteristic pattern of serotonin innervation (Wilson and Molliver, 1991). Bundles of large diameter axons are observed in successive sections as they ascend through the midbrain tegmentum, medial forebrain bundle, diagonal band, and supracallosal stria on their way to the cortex. The large diameter of these axons suggests that they are fibers of passage and once they reach the cortex, the serotonergic axons develop a distinct morphology of fine, densely branched plexuses that are extremely convoluted (Lidov et al., 1980). The density of serotonergic axons tends to be higher in sensory areas than in motor areas, but is substantially higher than noradrenergic and dopaminergic fibers throughout the cortex (Lidov et al., 1980).

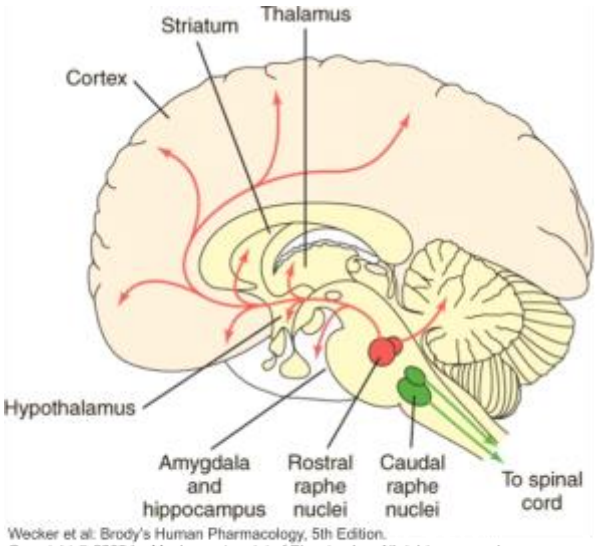


Figure 1.4: 5-HT projections from the raphe nuclei. Adapted from Brody's Human Pharmacology, 5th Edition. 5-HT projects from the raphe nuclei throughout the cortex

1.3.2 Serotonin receptors, release, and metabolism

All cells in the brain are directly or indirectly influenced by serotonin. There is a growing body of evidence to support that there are several subsystems of serotonin that serve different functions. However, the ability of all subsystems to produce, take up, store, and release serotonin is similar and they all require the same biosynthetic and transport mechanisms (Gaspar and Lillesaar, 2012).

Serotonin is synthesized from tryptophan. Tryptophan is first converted to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (TPH). A decarboxylase enzyme then converts 5-HTP into the active serotonin molecule, 5-hydroxytrypamine (5-HT) (Fig 1.5) (Gutknecht et al., 2009). After biosynthesis 5-HT is concentrated in the synaptic vesicles in neurons by the transporter, vesicular monoamine transporter (VMAT) (Abbracchio et al., 2006). To maintain homeostasis, 5-HT it is degraded by monoamine oxidase enzymes (MAOs) after its release. MAO-A is the main variant responsible for 5-HT metabolism in the CNS. MAO-A metabolizes 5-HT into 5-hydroxyindole acetaldehyde which is then further degraded to 5-hydroxyindole acetic acid (5-HIAA) (Fig 1.5) (Shih et al., 2011).

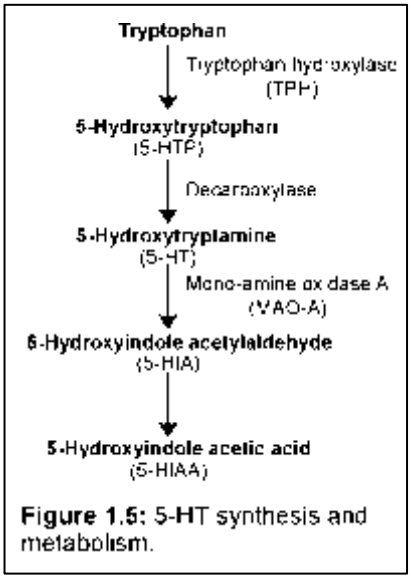


Figure 1.5: 5-HT synthesis and metabolism.

There are numerous types of serotonin receptors that have various mechanisms and effects. For example, some serotonin receptors act via adenylate cyclase and have inhibitory effects while others act via the phosphatidyl inositol second messenger system and have excitatory effects (Wilson and Molliver, 1991).There are thought to be as many as 13 different G-protein coupled receptors and one family of ligand gated ion channels. Serotonin receptors are subdivided into seven different groups (5-HT_1 to 5-HT_7) based on their structure and function. The 5-HT_1 receptor group consists of 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F} and are primarily autoreceptors that preferentially couple to $G_{i/o}$ to inhibit cAMP formation to regulate the release of neurotransmitters. The 5-HT_2 receptor group includes 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors that preferentially couple to $G_{q/11}$ to increase intracellular calcium via the IP_3 pathway. 5-HT_3 receptors are ligand gated ion channels similar to those for acetylcholine and GABA. 5-$HT_{4,6,7}$ receptors couple to G_s to promote cAMP. One study by Carson and coworkers suggested that 5-HT_5 receptors might be predominantly expressed in astrocytes and negatively coupled to adenylate cyclase (Carson et al., 1996). However, their function and mechanism remains unclear and there is little evidence to confirm that they are endogenously expressed (Hoyer et al., 2002). Many

serotonin receptor subtypes are expressed on both astrocytes and neurons throughout the brain (Shimizu et al., 1997, Hirst et al., 1998, Zhang et al., 2010). This wide variety and distribution of receptor types allows for a vast array of signaling actions.

1.3.3 Serotonin effects on spontaneous and evoked EPSP/IPSP amplitudes and frequency in the cortex

Serotonin neurotransmission is implicated in a vast array of behavioral and physiological states and functions including mood, sleep, anxiety, among many others (Jacobs and Azmitia, 1992). It can induce a facilitation effect in response to excitatory amino acids glutamate, NMDA, and quisqualate (Nedergaard et al., 1987), and it also has possible effects on GABAergic interneurons (Sheldon and Aghajanian, 1990). It can facilitate GABA release via activation of 5-HT_{2A} receptors, which increases interneuron excitability via inhibition of TASK-3 potassium channels. This leads to membrane depolarization and resulting increase in action potential firing (Deng and Lei, 2008). Serotonin effects can be postsynaptic as its receptors are distributed over large areas of the cell membrane (Nedergaard et al., 1987). Therefore, depending on the receptor type and location serotonin can exert wide range of inhibitory or excitatory effects in different neural networks.

1.3.4 Astrocytic recruitment of interneurons

It has been suggested that neuronal activity and glutamate release can trigger calcium elevations in astrocyte networks (Dani et al., 1992, Porter and McCarthy, 1996). Glutamate levels reach excess during neuronal release, which results in some of it escaping the synaptic cleft. This excess has been shown to reach sufficient concentrations to bind and activate receptors on astrocytes (Dzubay and Jahr, 1999). The glutamate receptor mGluR5 is found on astrocytes and is coupled to G_q and G_{11} G-protein variants that, when activated, result in stimulation of the IP_3 pathway, leading to mobilization of intracellular astrocyte calcium (Nakahara et al., 1997, Pasti et al., 1997, Panatier and Robitaille, 2016). In response to elevated calcium, astrocytes release ATP, which acts on interneurons to modulate inhibition (Khakh et al., 2003, Koizumi et al., 2003, Lalo et al., 2014). P2Y receptors have been found on astrocytes and interneurons. P2Y receptors on astrocytes

induce further ATP release (Anderson et al., 2004), while P2Y receptors on hippocampal interneurons increase GABA release (Bowser and Khakh, 2004). It has also been shown that astrocyte-derived adenosine can regulate basal synaptic transmission in the hippocampus (Panatier and Robitaille, 2016). However, whether this is first released as ATP and then broken down to adenosine or just released as adenosine is not yet known. Thus, through mGluR5 activation, astrocytes regulate basal synaptic transmission and neuronal synchrony (Panatier and Robitaille, 2016).

1.3.5 Rationale and hypotheses

As astrocytes can ensheathe up to 140,000 synapses, astrocytes are in a prime position to regulate synaptic activity and amplify neuromodulatory actions. It has now been established in the hippocampus that astrocytes can respond to a single synaptic event through mGluR5 activation and subsequent calcium transients that enable gliotransmitter release for modulation of synaptic strength (Nakahara et al., 1997). This makes for an incredibly powerful and efficient neuromodulatory network. In addition to this, widely distributed serotonin afferents and receptors can exert a vast array of functions on synaptic activity that may involve alterations of astrocytic gliotransmitter release in order to affect every synapse. Given that P1 and P2 receptors can be activated by astrocytic release of ATP and, if necessary, breakdown to adenosine by ectonucleotidases, astrocytes may have influence on cortical inhibitory interneuron activity similarly to what has been observed in hippocampal circuits (Bowser and Khakh, 2004). Thus, given the unique astroglial ability to respond to single events and influence a vast number of surrounding synapses, as well as able to extend neuromodulator action to every synapse, we hypothesize that:

1. **Astrocytes are involved in the somatosensory cortical paired-pulse suppression** and

2. **Serotonin acts on astrocytes to affect large scale cortical inhibition**

2.0 Materials and Methods

2.1 Slice Preparation

Six to ten week old male C57BL/6 mice were anesthetized with isoflurane and then decapitated. The brain was rapidly removed and immediately submersed in ice-cold artificial cerebrospinal fluid (aCSF). A vibrating slice cutter (Leica VT 1200) was used to make 350 um thick coronal sections that were taken from the hindlimb somatosensory cortex. Brains were sliced in ice cold aCSF containing the following (in mM): 123 NaCl, 3.5 KCl, 1.2 NaH_2PO_4, 2 $CaCl_2$-$2H_2O$, 2 $MgSO_4$-$7H_2O$, 0.4 L-ascorbic acid, 26 $NaHCO_3$, 10 dextrose, and 2 lactate (pH 7.4 when saturated with 95% O_2 and 5% CO_2). Slices were cut into halves and immediately transferred to a recovery chamber and incubated in aCSF saturated with 95% O_2 and 5% CO_2 for one hour at 30°C and then kept at room temperature for an additional hour prior to experimentation. Slices were kept at room temperature for up to 6 hours for experimentation. Experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by the University of Saskatchewan Committee on Animal Care and Supply.

2.2 Electrophysiology

Slices were placed in a perfusion chamber (~2 ml volume) under nylon strings attached to a U-shaped platinum frame submerged in and continually perfused with aCSF saturated with 95% O_2 and 5% CO_2 at a rate of ~4 ml/min at room temperature. Slices were imaged using a Nikon SMZ1000 stereozoom microscope for placement of recording and stimulating electrodes. fEPSP recordings were obtained using a differential amplifier (DP311; Warner Instruments) connected to a Digidata 1440A (Molecular Devices) using pClamp 10.2 software (Molecular Devices). Signals were captured at 2kHz, high-pass filtered at 1 Hz and low pass filtered at 300 Hz. Layer IV/V was stimulated using a paired-pulse paradigm with paired stimulations 50 ms apart every 20 seconds for the duration of each experiment. Recording electrodes were pulled on a vertical Narshige PC-10 two-step puller using borosilicate capillaries. Recording electrodes were filled with 0.9% saline and positioned in layer II/III of the hindlimb somatosensory cortex using the MP-285

manipulator (Sutter, Novata, CA). Stimulation (10-30 uA; 0.6 ms; 0.33 Hz) was applied to layer IV/V using a concentric bipolar stimulating electrode (TM88CCINA, WPI, Sarasota, FL, USA) via a constant current stimulator (Grass S48 Stimulator) controlled by pClamp through the digital output on the Digidata 1440A (Molecular Devices). Only recordings of maximal postsynaptic potentials (PSP) greater than 1.2 mV were used in this study (conducted at ~60% of max). Serotonin (5-HT; 2mM) was dissolved in normal aCSF and delivered to the chamber via bolus application (100 uL) through a syringe connected to PE-10 tubing (Harvard Apparatus) positioned directly upstream of the recording site through a 16 gauge needle. Given the flow rate, volume of the recording chamber (2 ml), and the fact that 5-HT had to diffuse into the tissue (~150 um deep), it was estimated that 5-HT reached the recording site at less than 50 uM concentration. The effects observed were found to be consistent with bath perfusion of 20 uM 5-HT (data not included). Bolus injection was used to simulate more closely the phasic physiological release as opposed to the slow stable increase in concentration seen with perfusion in the bath, which more closely resembles tonic release. Bolus application relates to the rapid release of neuromodulators associated with environmental stimuli essential for the modulation of executive functions.

2.3 Drugs

Drugs purchased from Sigma Aldrich include iodoacetate (IDA), 2-aminoethoxydiphenylborane (APB), carbenoxolone (CBX), flufenamic acid (FFA), acid blue 129 (Ab129), bicuculline (BIC), and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). Suramin was purchased from Tocris. Serotonin (5-HT) was purchased from Sigma Aldrich or Abcam and ATP was purchased from Sigma Aldrich. IDA, APB, CBX, FFA, and 5-HT were dissolved in normal aCSF before experiments. 5-HT was kept on ice during experiments. MPEP, and BIC were dissolved in normal aCSF and kept frozen as stock solutions. Drugs were perfused through the slice in the bath perfusate.

2.4 Bicuculline experiments

Baseline recordings were obtained prior to the application of BIC. BIC was then perfused in the bath and the resulting effects on fEPSP amplitude were allowed to stabilize. BIC in addition to MPEP, IDA, CBX, or Ab129 were then perfused and allowed to stabilize.

2.5 5-HT experiments

A baseline recording was acquired prior to the application of 5-HT. 5-HT was then applied via bolus injection in the absence of pharmacological agents and its effects were allowed to recover to baseline. The slice was then perfused with one of the drugs and allowed to stabilize. 5-HT was applied in the presence of the drug and the effect was compared to its effects in the absence of the drug. This was repeated for each pharmacological agent used in this study.

2.6 Data Analysis

The recorded fEPSPs were analyzed using the Clampex program to determine the maximum amplitude of pulse 1 and pulse 2. Data were normalized to the average of 10 baseline recordings. Drug effects were compared in the presence and absence of BIC. Data for the drug effects in the absence of BIC were obtained from experiments before the application of 5-HT. 5-HT experiments were analyzed by comparing the amplitudes of both pulses to pulse 1 during the application of 5-HT in the presence and absence of pharmacological agents.

2.7 Statistics

Data were expressed as the mean ± standard errors. Statistical significance was assessed using paired students t-tests. Significance was determined when $p < 0.05$. All statistical comparisons were performed using Microsoft Excel.

3.0 Results

In order to assess the astrocytic role in cortical inhibition and neuromodulator effects we used a paired-pulse paradigm to study evoked inhibition. It has previously been established that paired-pulse suppression in the visual (Rozas et al., 2001) and somatosensory (Ming et al., 2015) cortex is dependent on $GABA_A$ receptors. The first evoked field postsynaptic potential (fPSP) represents a summation of both evoked excitatory and spontaneous inhibitory/excitatory PSPs (IPSPs/EPSPs). Most of the evoked inhibitory PSPs (IPSPs) come at a delay due to the extra synapse involved in excitatory recruitment of local interneurons. Thus, due to this delay and the much longer duration of IPSPs, the second evoked fPSP represents the summation of evoked EPSPs, evoked long-lasting IPSPs (from first stimulation) and spontaneous IPSPs/EPSPs. The difference between the first and second stimulation is thus, evoked inhibition. By comparing the amplitudes of pulse 2 (P2) and pulse 1 (P1) in the paired-pulse ratio, evoked inhibition can be assessed (P2/P1). In the absence of pharmacological agents P1 is larger than P2 due to recruited inhibition (Fig. 3.1). Various pharmacological agents with and without neuromodulators are used to assess the role of astrocytes in cortical inhibition as well as neuromodulator-mediated alteration of inhibition.

3.1 Astrocytic ATP signaling is involved in cortical evoked inhibition

First, drugs that alter astrocytic function were applied to acutely isolated slices to assess the role of astrocytes and ATP on cortical evoked inhibition. MPEP was used to block mGluR5 receptors on astrocytes, IDA to inhibit glycolysis to reduce ATP, 2-APB to inhibit intracellular rise in astrocytic calcium, CBX and FFA to block ATP release through hemichannels, and Ab129 to block P2Y receptors on interneurons (Fig. 3.2). The amplitudes of P1, P2 and the P2/P1 were analyzed before and after the application of the drugs to assess their effects.

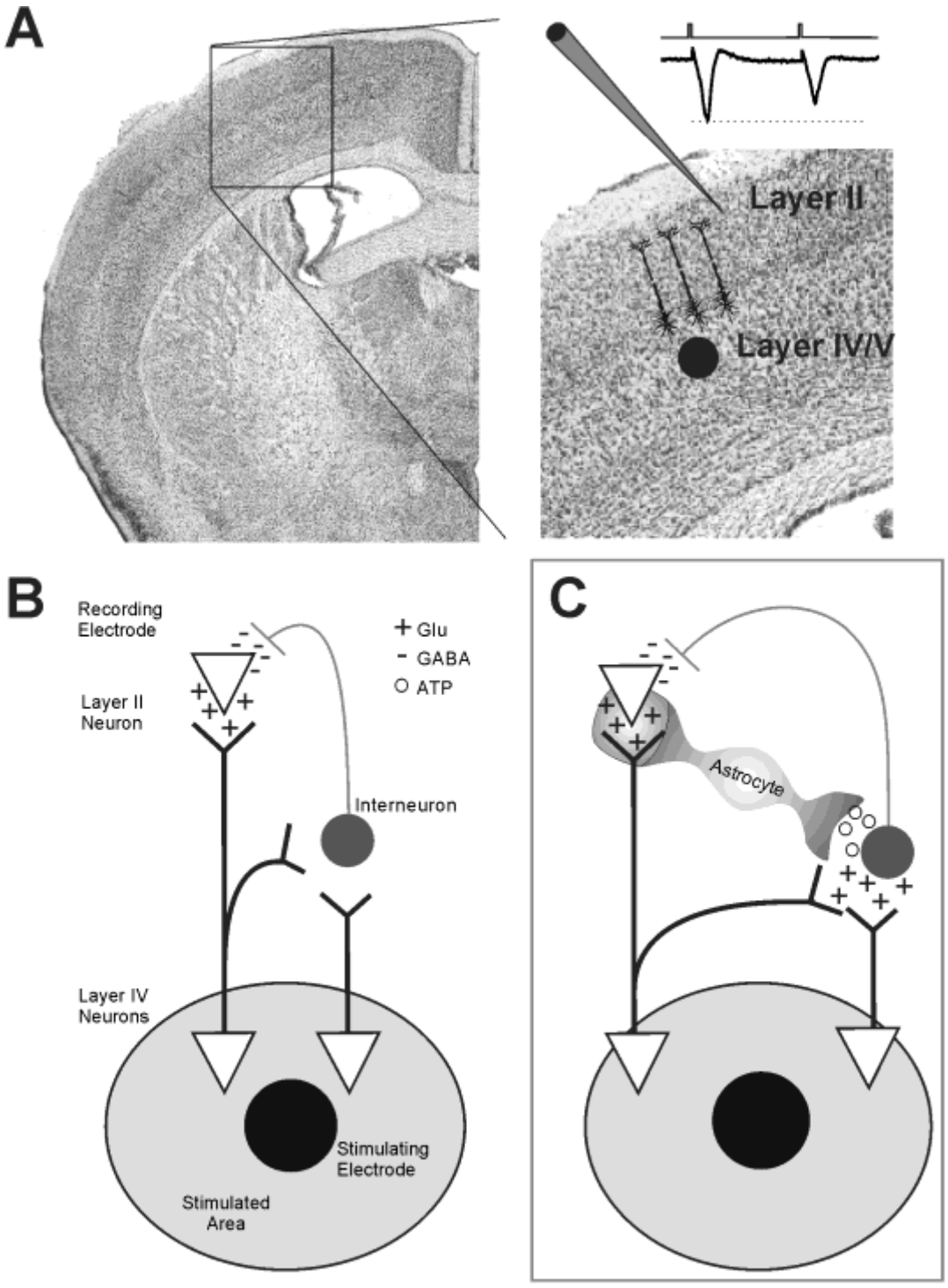


Figure 3.1: Cortical inhibition was assessed in the somatosensory cortex using extracellular recordings of a paired-pulse paradigm. *A*, The stimulating electrode (black dot) was placed in layer IV/V while the recording glass electrode was placed in layer II. Evoked inhibition is represented in the second pulse as illustrated by the decrease in amplitude. *B*, Stimulation of collateral/adjacent neurons that synapse on inhibitory interneurons recruit longer duration inhibitory post-synaptic currents that arrive at a delay to the direct excitatory post-synaptic currents. *C*, It is hypothesized that astrocytes are also involved in recruiting inhibitory interneuron activity via purinergic signaling.

Given that mGluR5 glutamate receptors are mainly expressed on astrocytes and have been shown to induce astrocyte calcium waves (Nakahara et al., 1997, Panatier et al., 2011), the glutamate antagonist MPEP (25uM) was applied to block astrocyte activation by glutamate. MPEP caused a decrease in the amplitude of P1, but no change in the amplitude of P2. The resulting effect on the P2/P1 represents a possible decrease in evoked inhibition (Fig. 3.3A).

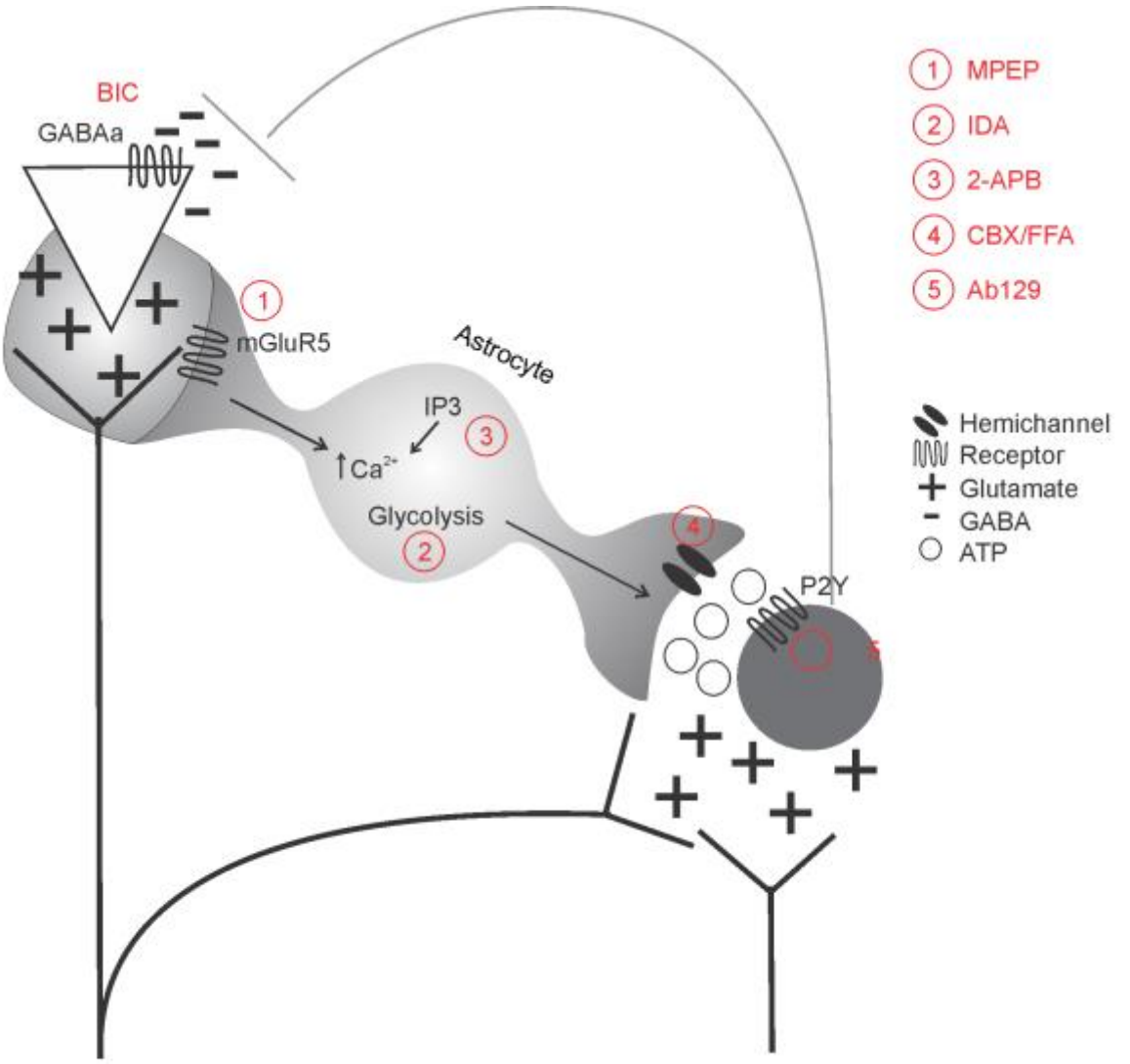


Figure 3.2: Sites of action of the pharmacological agent used. *1*, MPEP is an mGluR5 antagonist. *2*, IDA blocks GAPDH to inhibit glycolysis which would reduce ATP available for release. *3*, 2-APB is an Ip3 antagonist which inhibits release of calcium from intracellular stores. *4*, CBX and FFA block hemichannels from which ATP can be released. *5*, Ab129 is a P2Y receptor antagonist which would inhibit the effects of ATP.

Additional studies provide evidence that astrocytic ATP is involved in synaptic inhibition. It has been shown that ATP released from astrocytes in the hippocampus (Khakh et al., 2000, Khakh et al., 2003, Bowser and Khakh, 2004, Lalo et al., 2014) as well as in the cortex (Lalo et al., 2014) modulates synaptic inhibition. To investigate whether the same is true in the somatosensory cortex we used iodoacetate (200uM; IDA) to deplete astrocytic ATP levels. Iodoacetate is an inhibitor of the glycolysis enzyme GAPDH and hinders astrocyte function. Interfering with glycolysis leads to a drop in ATP levels in astrocytes, in turn, weakening astrocyte calcium wave propagation and signaling via gliotransmitters (Anderson et al., 2004). As lactate is the main energy source for neurons, and astrocytes shuttle lactate to neurons (Tarczyluk et al., 2013), 2mM of lactate was added to the aCSF to ensure an energy source for neurons. In a similar trend to MPEP application, the application of IDA showed a decrease in the amplitude of P1 and an increase in the amplitude of P2. The resulting increase in the ratio suggests a decrease in evoked inhibition (Fig. 3.3A). By interfering with astrocyte glycolysis we were able to alter cortical inhibition.

If astrocytes are the source of ATP, its release could be causing the effects seen on evoked inhibition. We used pharmacological agents to interfere with possible mechanisms of ATP release. Gap junction hemichannels have been shown to be expressed in astrocytes (Dermietrel et al., 1991) and carbenoxolone (CBX), a hemichannel blocker, has been used to block astrocyte gliotransmitter release (Andersson et al., 2007, Brokamp et al., 2012). It is possible that ATP is released through hemichannels (Stout et al., 2002). Thus, we perfused our slices with CBX (50uM) to assess the effect on this simple inhibitory circuit. In the presence of CBX there was no change in the amplitude of P1. However, P2 showed an increase (Fig. 3.3A). The increase in P2 amplitude with no change seen in P1 amplitude suggests CBX affects evoked inhibition.

Astrocytic P2Y receptors have been shown to be involved in ATP-induced ATP release from astrocytes (Anderson et al., 2004) and the P2Y receptors on interneurons is a mechanism by which ATP could mediate inhibition. Therefore, we used the P2Y antagonist acid blue 129 (100uM; Ab129) to block ATP receptors on astrocytes and interneurons. In the presence of AB129, P1 amplitude decreased, P2 amplitude increased, and there was a resulting increase in the P2/P1 (Fig. 3.3A). Ab129 can act indirectly by reducing ATP

release from astrocytes thereby reducing the amount available to act on interneuron P2Y receptors or it can act directly on P2Y receptors on interneurons to inhibit ATP effects.

We hypothesized that astrocytes mediate paired-pulse suppression through the release of ATP onto interneurons. Interneurons release GABA, which then acts on neurons to inhibit synaptic activity. We perfused another set of slices with the $GABA_A$ receptor antagonist bicuculline (2uM; BIC) to determine if the effects seen with the MPEP, IDA, CBX and Ab129 were GABA dependent. There remained changes in P1 amplitude for MPEP, IDA and Ab129 as seen in the absence of BIC (Fig. 3.3). In addition, CBX P1 amplitude also remained the same, with no difference, as in the absence of BIC (Fig. 3.3). However, all significance was lost in the amplitudes of P2 and P2/P1, which are representative of evoked inhibition (Fig. 3.3B). All pharmacological agents used reduce ATP and its release from astrocytes. As BIC blocks $GABA_A$ receptors, and MPEP, IDA, CBX or Ab129 did not show any further effect on the P2 amplitude or the P2/P1, these results indicate that pharmacological effects on evoked inhibition are dependent on $GABA_A$.

In addition to the above mentioned pharmacology, we used 2-APB to inhibit the intracellular rise in astrocytic calcium, and FFA to block large anion channels including hemichannels. As calcium waves help activate and propagate astrocyte signals as well as stimulate the release of gliotransmitters. In this study we used the IP_3 receptor blocker, 2-APB (100 uM), to inhibit the release of intracellular calcium to weaken astrocytic calcium wave propagation. With the application of 2-APB, P1 and P2 showed a decrease in amplitude but the P2/P1 did not show a significant change (Supplemental Fig. 3.1). Given that the application of 2-APB showed no significant change in P2/P1, evoked inhibition was not affected. Furthermore, use of the large anion channel blocker flufenamic acid (50 uM; FFA) also supports an ion channel mechanism. Although FFA produced a decrease in the ratio similar to CBX, FFA only decreased P1 amplitude which is in contrast to CBX only increasing P2 amplitude (Supplemental Fig. 3.1).

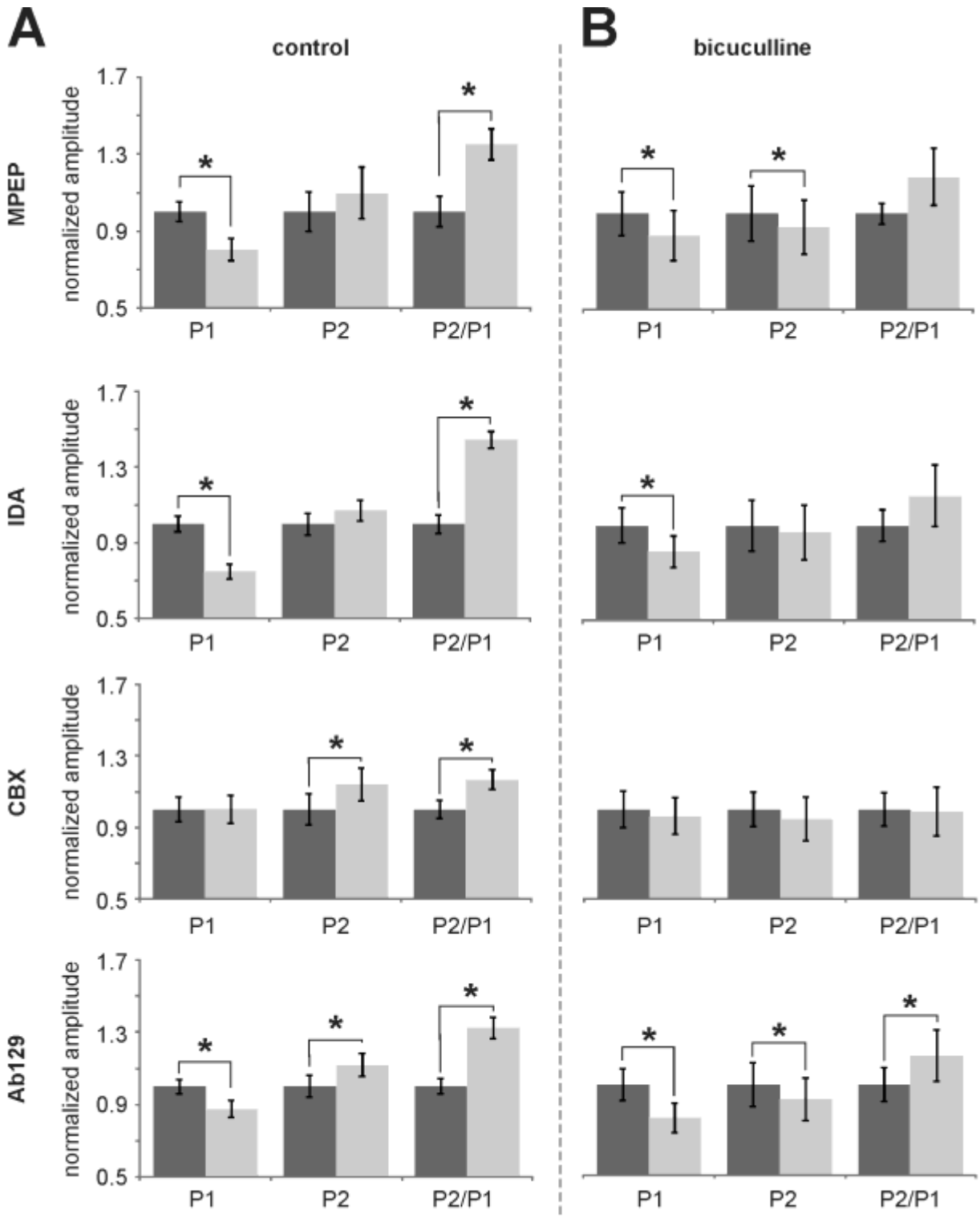
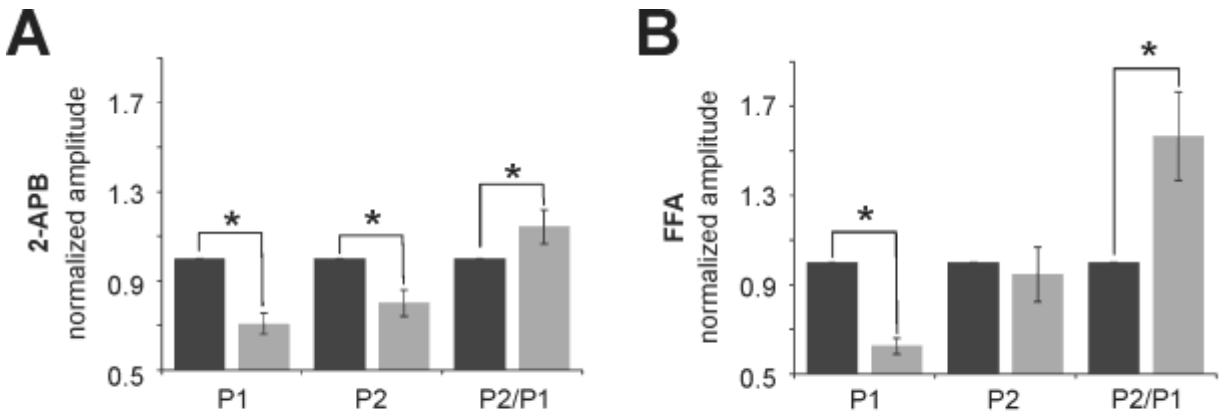
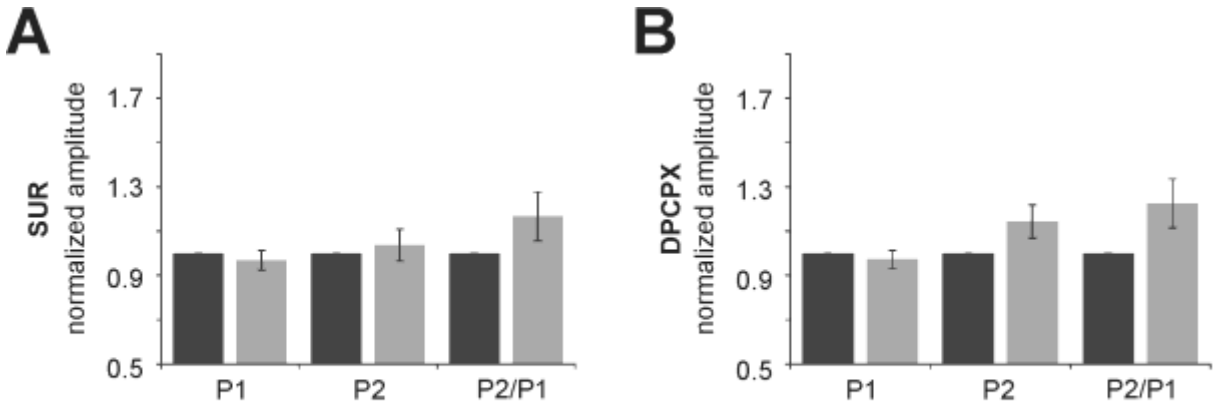


Figure 3.3. Pharmacology implicates astrocytes and purinergic signaling in cortical paired-pulse suppression. * means $P < 0.05$, paired *t*-test (n=9-45 from 6-15 animals, BIC; n=11-12 from 5-6 animals).



Supplemental Figure 3.1: Pharmacology implicates astrocytic ATP. *A*, 2-APB causes a decrease in P1, P2 and the P2/P1. *B*, FFA decreased P1 only resulting in an increase in the P2/P1. * means $P < 0.05$, *paired t-test*, (2-APB; n = 11 from 4 animals, FFA; n = 26 from 11 animals).

To further investigate the role of ATP in evoked inhibition, the non-selective P2 receptor antagonist suramin (50uM; SUR) was applied. There was no change in P1, P2 or the P2/P1 (Supplemental Fig. 3.2). However, the SUR concentration used may have been too low as most studies use a concentration of 100uM. In addition, as ATP is rapidly degraded extracellularly into adenosine, we used the selective adenosine A1 receptor antagonist DPCPX (300-600nM) to assess its impact on cortical evoked inhibition. DPCPX showed no change in P1, P2, or the P2/P1 (Supplemental Fig. 3.2) demonstrating that adenosine A1 receptors are not involved in mediating evoked inhibition under basal conditions.



Supplemental Figure 3.2: Pharmacology implicates ATP signalling. A, SUR showed no effect. B, DPCPX showed no effect. * means $P < 0.05$, *paired t-test*, (SUR; n = 13 from 6 animals, DPCPX; n = 16 from 7 animals).

3.2 5-HT mediated effects on paired-pulse suppression are GABA dependent

Serotonin (5-HT) and norepinephrine (NE) have been shown to alter inhibition in the entorhinal cortex (Deng and Lei, 2008, Xiao et al., 2009). As ATP and astrocytes play a role in mediating paired-pulse suppression, it is possible that they also mediate neuromodulator effects on paired-pulse suppression. We first assessed the response to 5-HT in the absence of pharmacological agents, using the paired-pulse paradigm. Then we applied MPEP, IDA, CBX, Ab129, 2-APB, FFA, SUR, and DPCPX to interfere with astrocytic ATP release and determine if ATP plays a role in mediating the response to 5-HT.

In order to assess if astrocytes and ATP are involved in mediating 5-HT signaling we first applied 5-HT in the absence of any drug. 5-HT was applied as a bolus application to simulate transient physiological release. This caused P1 amplitude to decrease and P2 amplitude to increase leading to an increase in the P2/P1 (Fig. 3.4A). These results suggest that 5-HT application decreases evoked inhibition.

If 5-HT decreases inhibition in the cortex, the changes should be sensitive to GABA blockade. Thus, we applied 5-HT in the presence of the $GABA_A$ antagonist BIC. BIC virtually abolishes the response to 5-HT compared to control experiments (Fig. 3.4B).

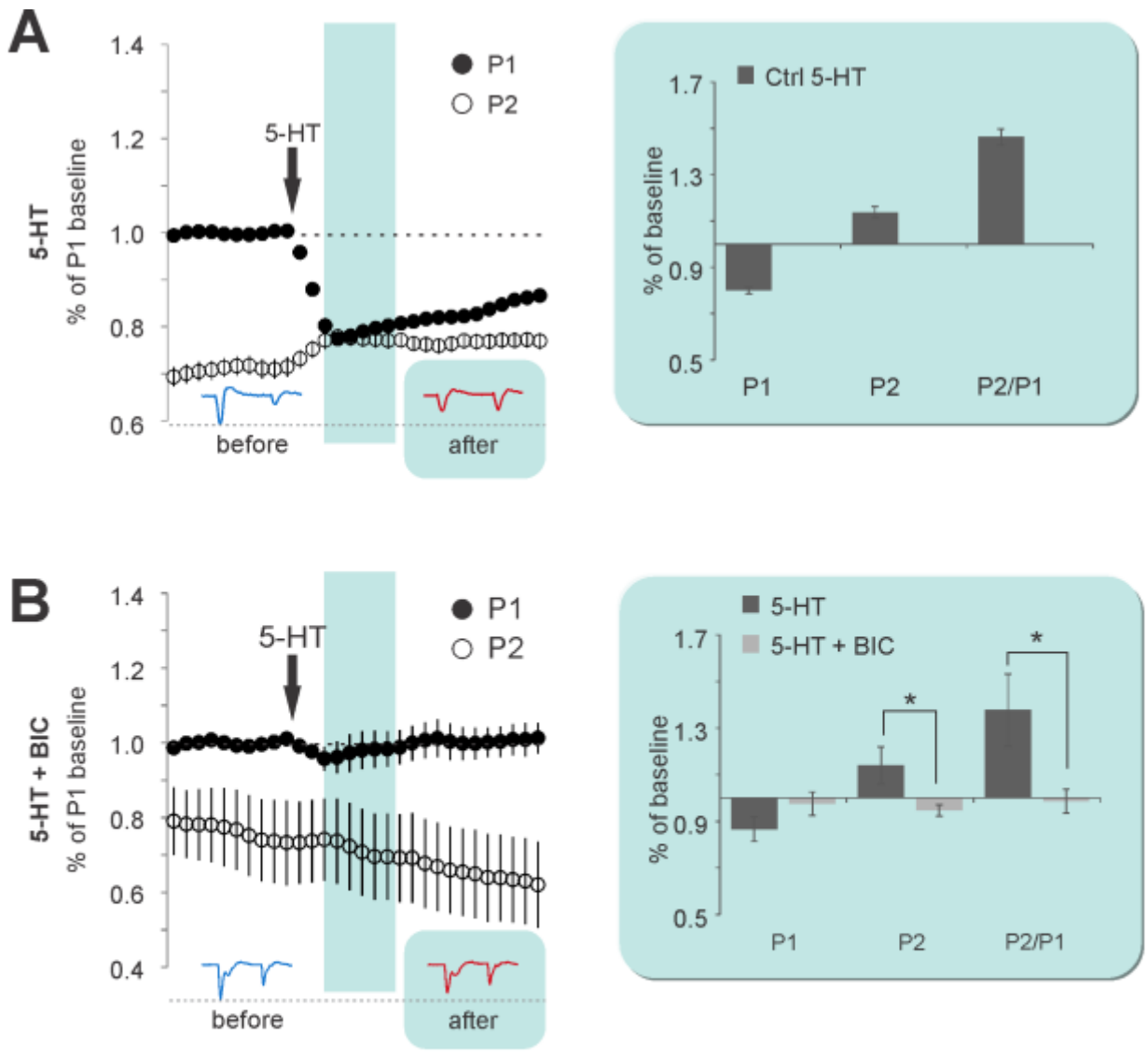


Figure 3.4: 5-HT decreases inhibition and is GABA dependent. *Left*, time series showing the effects of 5-HT on P1 and P2 amplitude in the presence and absence of BIC over the course of an experiment. *Insets*, histograms showing the change in amplitude of P1, P2, and P2/P1 compared to baseline recordings in response to application of 5-HT in the presence and absence of BIC. *A*, application of 5-HT decreases P1 and increases P2 resulting in an increase in P2/P1 indicating a reduction in inhibition. *B*, In the presence of BIC, 5-HT effects are virtually abolished, demonstrating that 5-HT effects involve GABA. * means $P < 0.05$, paired t-test, (5-HT control; n = 120 from 63 animals, BIC; n = 10 from 7 animals).

3.3 Astrocytes are involved in 5-HT effects on paired-pulse suppression

Could it be that 5-HT is altering the astrocytic involvement in inhibition? By interfering with ATP levels and its release from astrocytes, 5-HT should no longer decrease evoked inhibition to the same extent. To test whether 5-HT effects are mediated through

ATP and astrocytes, MPEP, IDA, CBX, Ab129, 2-APB, FFA, SUR, and DPCPX were perfused through the slice prior to the application of 5-HT. According to our hypothesis, these agents should hinder the 5-HT effects on evoked inhibition. In the presence of these pharmacological agents, 5-HT showed a decrease in both P1 and P2 compared to controls in which P1 amplitude decreased and P2 amplitude increased (Fig. 3.5). The decrease in P1 amplitude was the same as seen in controls. The inverse response in P2 resulted in a dampened increase in the P2/P1 demonstrating that MPEP, IDA, CBX, and Ab129 reduce the 5-HT effect on evoked inhibition.

In addition, 5-HT was applied in the presence of the IP_3 antagonist 2-APB (Supplemental Fig. 3.3), and another hemichannel gap junction blocker FFA (Supplemental Fig. 3.3). In the presence of these drugs, 5-HT produced similar effects as those shown in the presence of MPEP, IDA, CBX, and Ab129. P1 amplitude also decreased to the same extent as controls and P2 amplitude decreased compared to the increase seen in controls. This resulted in a dampened increase in the P2/P1 (Supplemental Fig. 3.3). The FFA results are consistent with the results previously shown using CBX.

3.4 P2 receptors are involved in 5-HT effects on paired-pulse suppression

Given that astrocytic calcium waves can trigger the release of ATP and Ab129 hindered the 5-HT response, we further investigated the role of ATP in 5-HT signaling using the non-selective P2 blocker SUR. Using a non-selective P2 antagonist allowed us to assess if ATP was acting through either P2Y and P2X. 5-HT in the presence of SUR resulted in no change in the amplitudes of P1, P2 or P2/P1 (Fig. 3.6A). Again, this may have been a consequence of the SUR concentration of 50uM instead of 100uM. Future experiments will need to be done to verify this possibility.

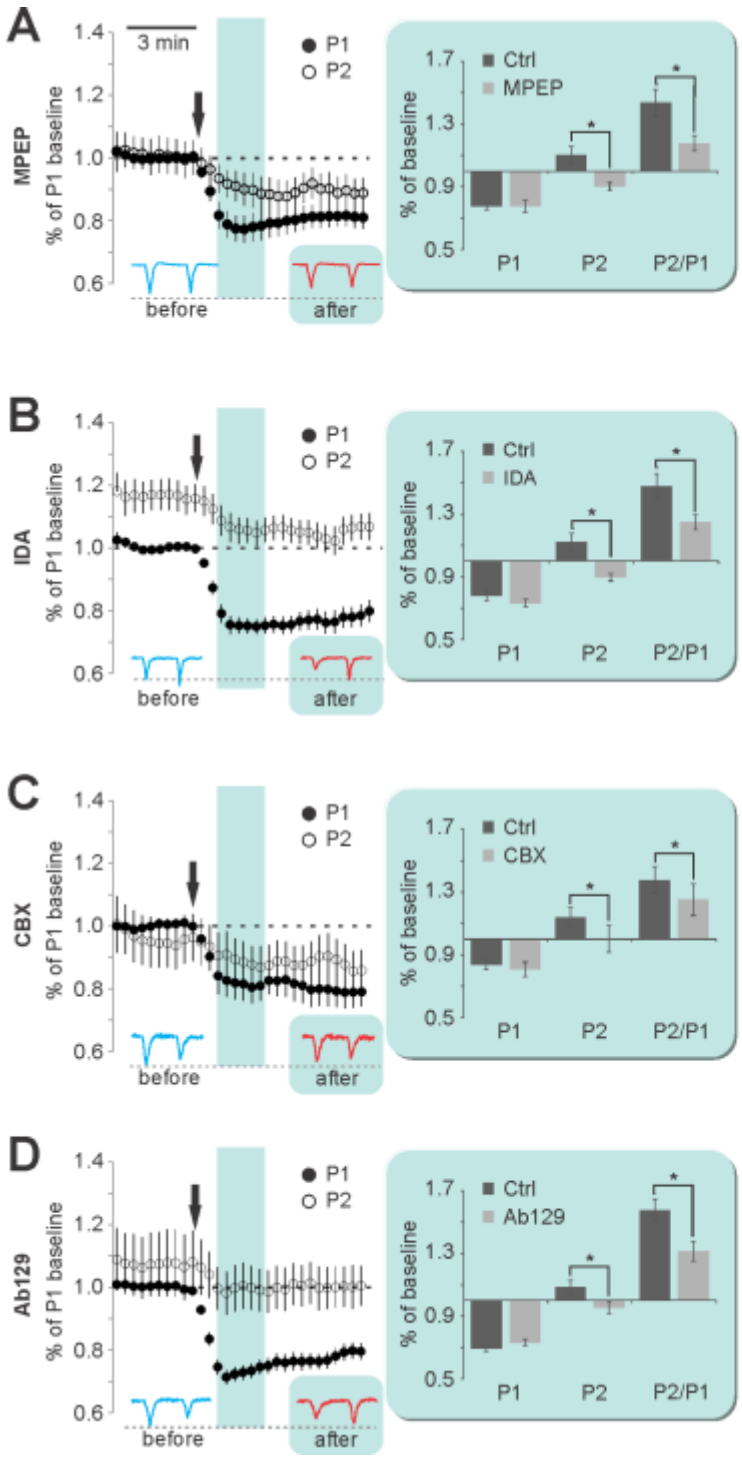
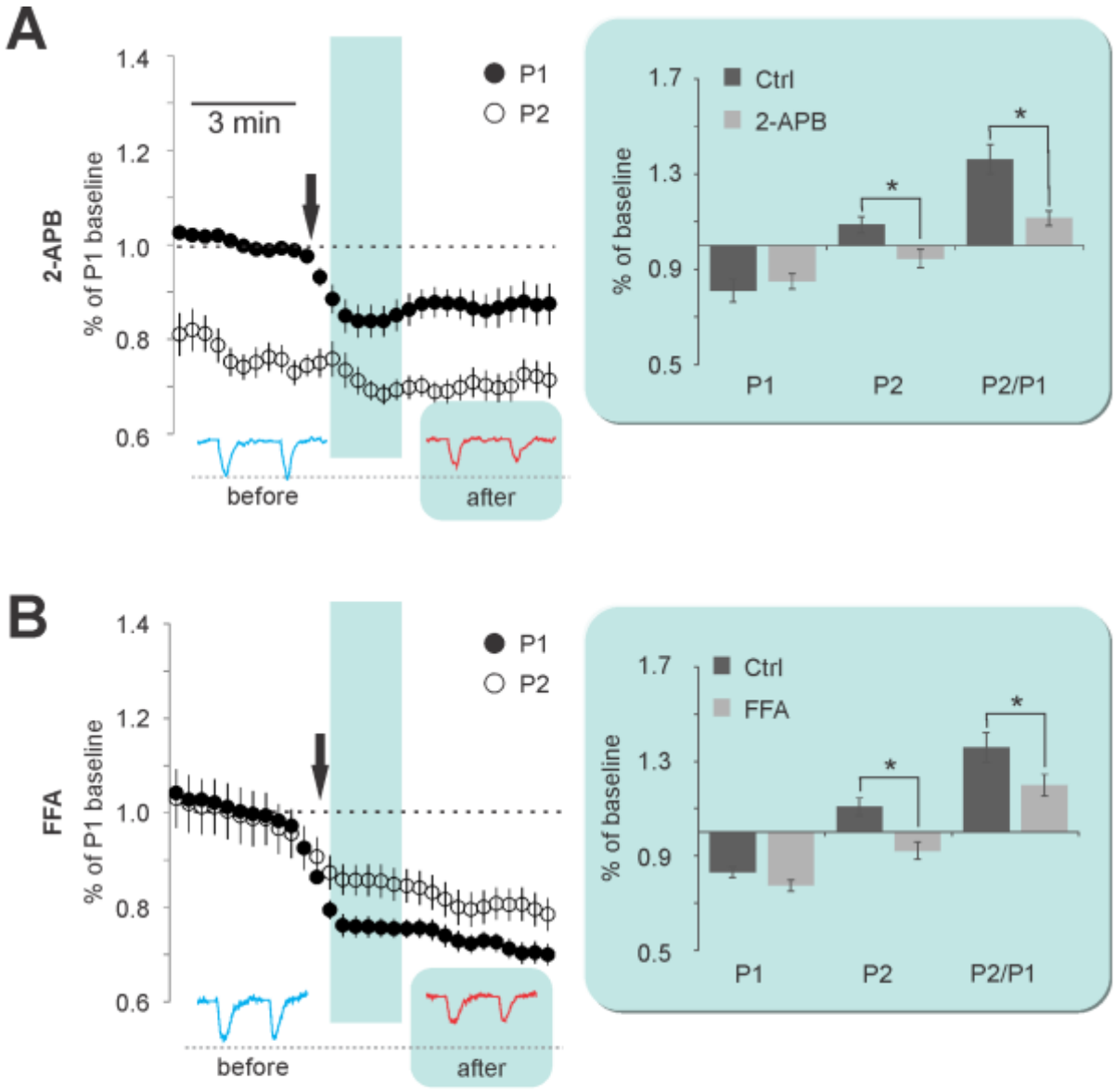


Figure 3.5: 5-HT effects involve astrocytes and ATP. *Left*, time series showing the amplitudes of P1 and P2 with the application of 5-HT in the presence of MPEP, IDA, CBX, and Ab129 over the course of an experiment. *Insets*, histograms showing the change in amplitude of P1, P2, and P2/P1 compared to baseline recordings in response to application of 5-HT in the presence of MPEP, IDA, CBX, or Ab129. In the presence of pharmacological agents there was a decrease in the amplitude of P2 compared to the paired effects of 5-HT before the application of pharmacological agents in which there was an increase in P2 amplitude. Thus, the pharmacological agents used resulted in a reduction of 5-HT effects on the P2/P1. * means $P < 0.05$, paired *t-test*, (n = 9-28 from 6-15 animals).



Supplemental Figure 3.3: 5-HT effects involve astrocytes and ATP. *Left*, time series showing the amplitudes of P1 and P2 with the application of 5-HT in the presence of 2-APB and FFA over the course of an experiment. *Insets*, histograms showing the change in amplitude of P1, P2, and P2/P1 in response to application of 5-HT compared to application of 5-HT in the presence of 2-APB and FFA. In the presence of 2-APB or FFA, there was a decrease in the amplitude of P2 compared to the paired effects of 5-HT before the application of pharmacological agents in which there was an increase. Thus, 2-APB and FFA resulted in a reduction of 5-HT effects on the P2/P1. * means $P < 0.05$, paired *t-test*, (2-APB;n = 11 from 4 animals, FFA; n = 26 from 11 animals).

In addition, as ATP is degraded rapidly to adenosine by ectonucleotidase enzymes, it is possible that adenosine is involved instead of ATP. To test whether ATP is mediating 5-HT effects and not adenosine, we applied 5-HT in the presence of the adenosine A1

receptor blocker DPCPX. This also did not change the 5-HT response effectively ruling out adenosine A1 receptors as being involved in 5-HT effects (Fig. 3.6B).

As interfering with astrocyte function and ATP leads to a change in the effects of 5-HT on paired-pulse suppression, astrocytes and ATP may be mediating 5-HT effects. In the presence of pharmacological agents that hinder astrocyte function and ATP levels, P2 had a

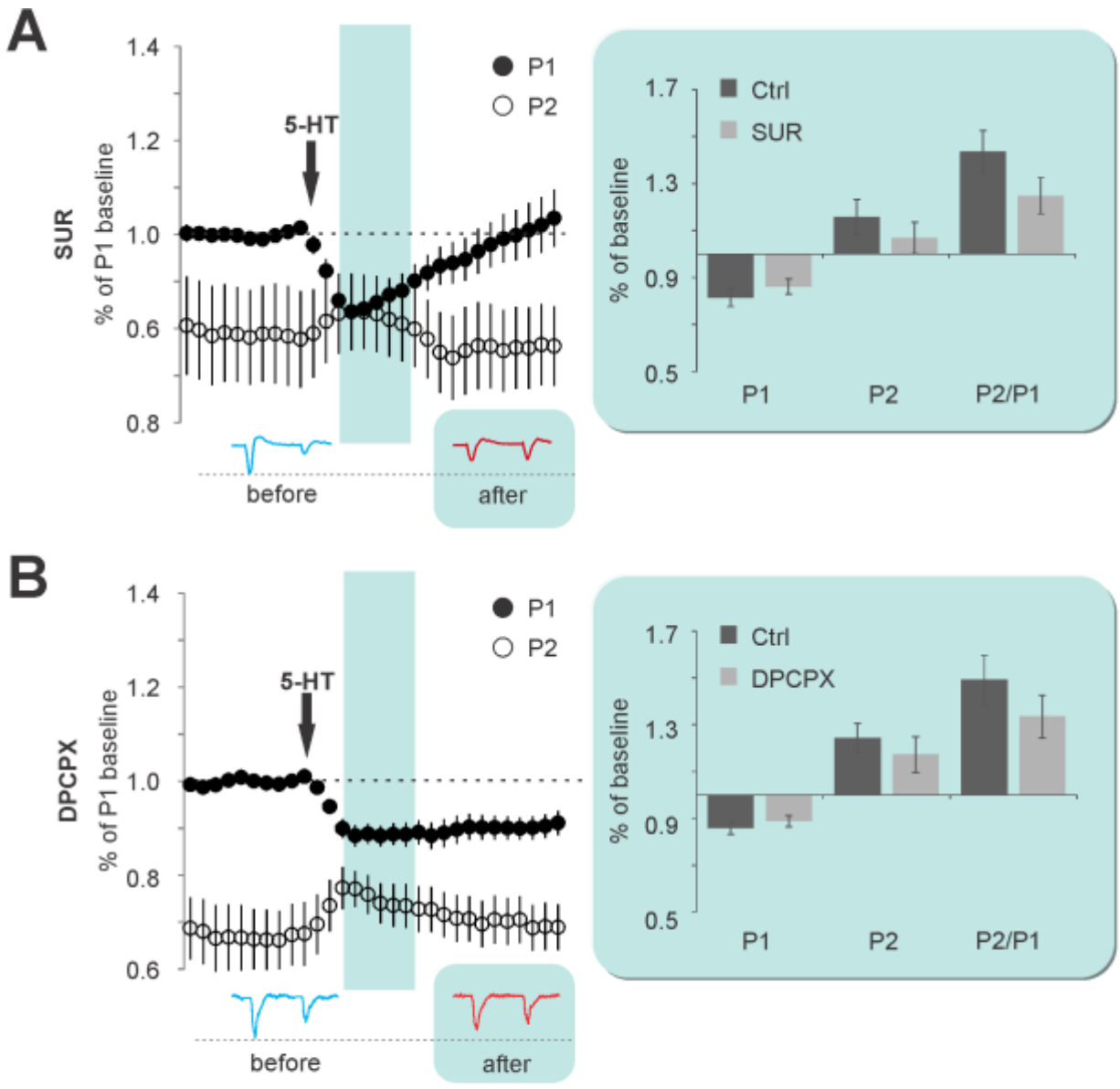


Figure 3.6: 5-HT effects on inhibition involve astrocytic ATP but not A1 receptors. *Left*, time series showing the amplitudes of P1 and P2 with the application of 5-HT in the presence of SUR and DPCPX over the course of an experiment. *Insets*, histograms showing the change in amplitude of P1, P2, and P2/P1 in response to 5-HT in the presence and absence of SUR and DPCPX. In the presence of SUR and DPCPX 5-HT caused no change in the amplitude of P1, P2, or P2/P1. * means $P < 0.05$, *paired t-test*, (SUR;n = 13 from 6 animals, DPCPX;n = 16 from 7 animals).

similar response to P1 as they both decreased. This resulted in an increase in the P2/P1, but it was reduced compared to controls. As the pharmacological agents used to interfere with astrocytic ATP reversed the effects of 5-HT, the mechanism by which 5-HT acts may involve the inhibition of ATP release from astrocytes. Thus, we next applied exogenous ATP and assessed its effects.

3.5 Exogenous ATP reduces P2 which is opposite to 5-HT

To further assess the potential role for ATP in cortical inhibition, we applied exogenous ATP to the slices using the same method used to apply 5-HT. When ATP was applied both P1 and P2 amplitudes decreased and the P2/P1 increased (Fig. 3.7A). Application of ATP resulted in a decrease in P2 amplitude compared to 5-HT that caused its increase. Thus, 5-HT seems to be inhibiting ATP release from astrocytes given that ATP effects show the inverse effect on P2.

In addition to applying exogenous ATP, we also used the protease-activated receptor 1 (PAR-1) agonist TFLLR. TFLLR has been used to activate astrocytes specifically as PAR-1 receptors are thought to be mainly found on astrocytes (Debeir et al., 1997, Han et al., 2011, Lalo et al., 2014). TFLLR was perfused through the slice for 3 minutes to allow it to reach a constant concentration of 10uM throughout the slice. After 3 minutes of perfusion, TFLLR was washed out with normal aCSF to simulate transient action. With the application of TFLLR, there was a decrease in P2 amplitude, similar to the effects seen with ATP and opposite to the effects of 5-HT (Fig. 3.7B). TFLLR acts on astrocytes to cause a rise in calcium (Debeir et al., 1997, Junge et al., 2004) which causes release of gliotransmitters, specifically ATP, resulting in an increase in evoked inhibition (Lalo et al., 2014).

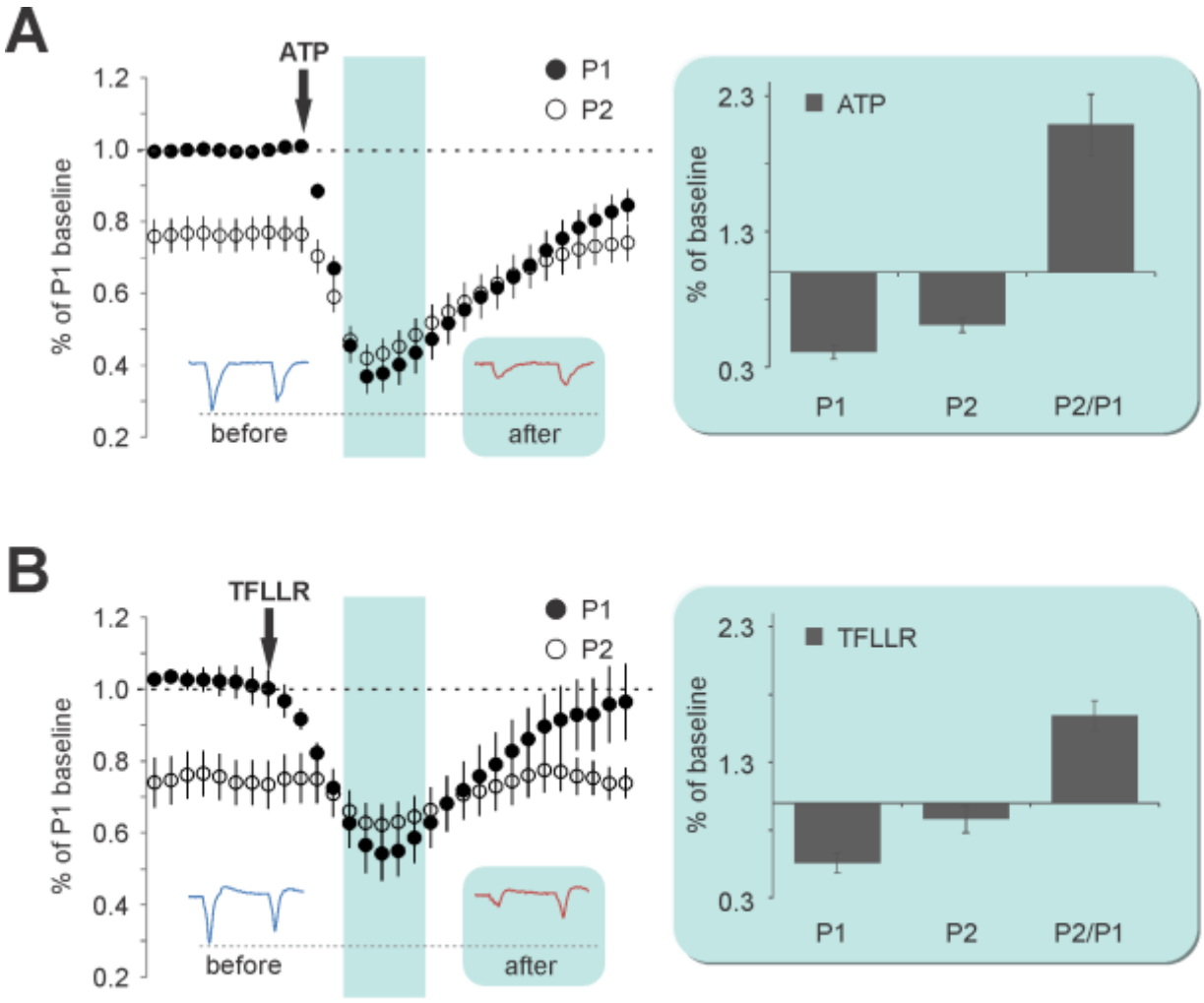


Figure 3.7: ATP and TFLLR showed the inverse of 5-HT. *Left*, time series showing the amplitudes of P1 and P2 with the application of ATP or TFLLR over the course of an experiment. *Insets*, histograms showing the changes in amplitude of P1, P2, and P2/P1 compared to baseline recordings in response to application of ATP or TFLLR. *A*, ATP caused a decrease in the amplitude of both P1 and P2. *B*, TFLLR showed similar results to ATP causing a decrease in the amplitude of both P1 and P2. $P < 0.05$, *paired t-test*, (ATP; n = 51 from 26 animals, TFLLR; n = 7 from 5 animals).

4.0 General Discussion

This is one of the first studies to assess the role of astrocytes in cortical inhibition and neuromodulation in a more integrated representation using extracellular field recordings. We assessed the astrocytic role and 5-HT effects on inhibition using a paired-pulse paradigm. The pharmacological agents, MPEP, IDA, 2-APB, CBX, FFA, and Ab129, were applied to interfere with astrocyte function and ATP release. In order to assess their effects, the amplitudes of P1, P2, and P2/P1 were compared before and after the application of each agent. MPEP was used to block mGluR5 glutamate receptors on astrocytes, IDA to inhibit glycolysis in astrocytes, 2-ABP to block IP_3-mediated calcium rise in astrocytes, CBX and FFA to block ATP release from hemichannels, and Ab129 to block P2Y receptors on interneurons. Application of each agent resulted in an increase in the P2/P1 which represents a decrease in inhibition and suggests that astrocytes and ATP are involved in cortical inhibition. To verify that all actions were mediated through effects on $GABA_A$ signaling, we applied the same agents in the presence of the $GABA_A$ antagonist BIC. In the presence of BIC, all significant changes in the P2/P1 were abolished, supporting the notion that astrocytes are involved in GABA-mediated inhibition. As adenosine has also been implicated in astrocyte-mediated inhibitory processes, we used the A1 receptor antagonist DPCPX to determine if the effects observed were partially mediated by adenosine. However, DPCPX showed no change in the P2/P1 ruling out involvement of adenosine A1 receptors. Accordingly, we proposed that in response to the first stimulation, glutamate is released from the presynaptic neuron and, in addition to acting on receptors on the postsynaptic neuron (P1), stimulates astrocytic calcium transients via mGluR5 glutamate receptors to release ATP to recruit surrounding inhibitory interneurons for suppression of the second stimulation (P2; Fig 4.1B). Thus, extracellular field recordings of integrated activity enables better understanding, as a single astrocyte is capable of regulating activity across multiple synapses. Taken together, we suggest that astrocytes modulate inhibition through the release of ATP to widely affect cortical neural function.

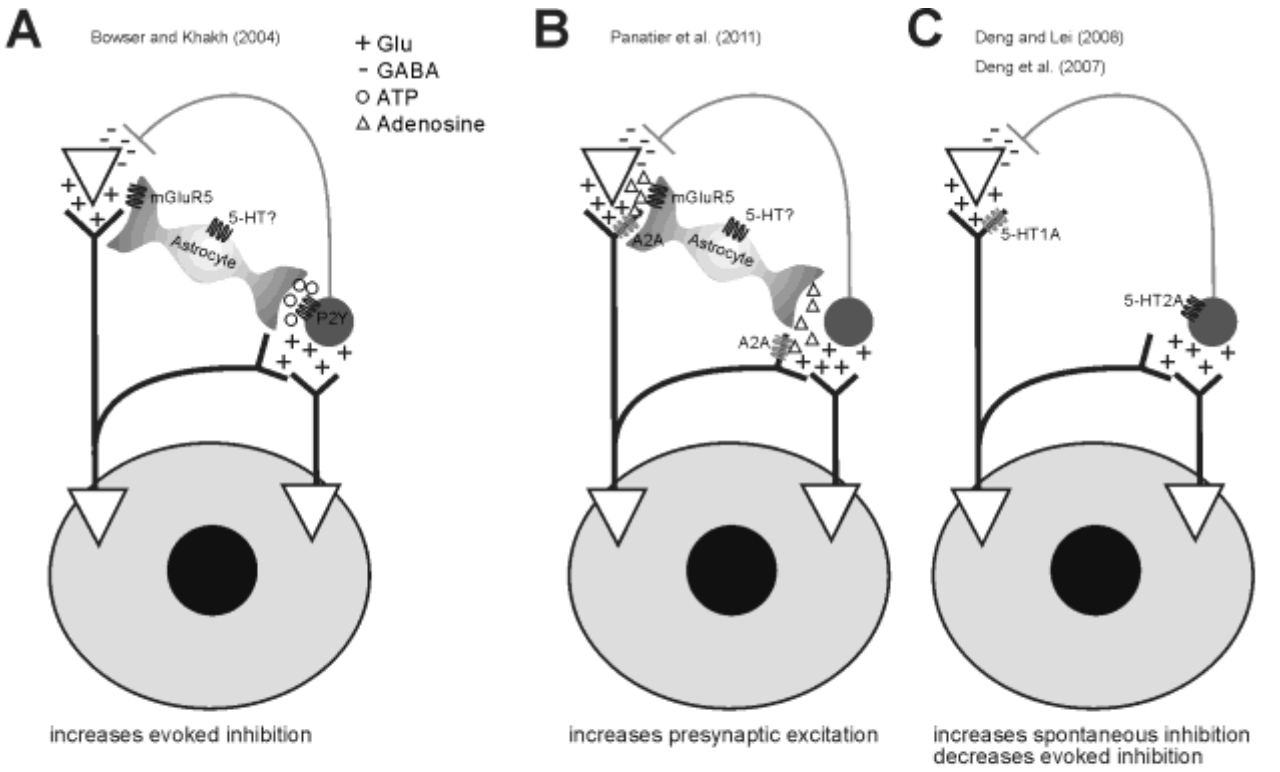


Figure 4.1 Schematic of possible mechanisms to modulate inhibition and 5-HT effects. A, Bowser and Khakh (2004) proposed that hippocampal astrocytes release ATP, in response to synaptic activity, which then acts on interneuron P2Y receptors to elicit GABA release. B, Panatier et al. (2011) suggested that astrocytes in the hippocampus sense synaptic activity via mGluR5 receptors and then release adenosine that acts on presynaptic A2A receptors to modulate glutamate release. C, Deng et al. (2007) found that 5-HT1A receptors on pyramidal neurons decrease excitation and in addition Deng and Lei (2008) demonstrated that 5-HT2A receptors on interneurons increase evoked inhibition through the release of GABA.

4.1 Astrocytes detect synaptic activity via mGluR5

We used the mGluR5 antagonist MPEP to block astrocyte activation in response to synaptic glutamate release. When we applied MPEP to our slices there was an increase in the paired-pulse ratio indicating a reduction in inhibition. Our results are consistent with the study done by Panatier et al. (2011). They demonstrated, with patch clamp, that astrocytes regulate basal transmission at hippocampal synapses by detecting glutamate release (Fig 4.1) (Panatier et al., 2011). When mGluR5 receptors on astrocytes were activated it caused an increase in astrocytic calcium, which in turn, enhanced basal synaptic activity (Panatier et al., 2011). Similar to our study, they used the mGluR5 antagonist, MPEP, at the concentration of 25 uM (Panatier et al., 2011). MPEP completely abolished calcium events in astrocytes thereby reducing synaptic efficacy (Panatier et al., 2011). This

suggests that mGluR5 stimulated rises in astrocytic calcium is a critical step in modulating synaptic activity. However, although the major metabotropic glutamate receptor expressed on astrocytes is mGluR5 (Nakahara et al., 1997), these receptors have also been shown to be expressed on neurons and interneurons (Lopez - Bendito et al., 2002, Gereau and Swanson, 2008). This could account for the effects of MPEP observed in our study as well as Panantier et al. (2011). Blocking mGluR5 receptors would reduce the excitability of neurons and/or interneurons, which would, consequently, affect recruitment of inhibition. Panatier and colleagues (2011) recorded intracellular EPSCs evoked in pyramidal cells in the presence of MPEP (Panatier et al., 2011). MPEP resulted in the failure rate of EPSCs to increase, which could be indicative of effects on neurons (Panatier et al., 2011). However, they addressed this issue by applying MPEP in the presence of the calcium chelator BAPTA in the pipette of a patched astrocyte. When astrocytes were dialyzed with BAPTA, MPEP no longer showed an increase in the failure rate of EPSCs demonstrating that its actions are mediated through astrocytes and not neurons (Panatier et al., 2011). Thus, the results obtained by Panatier et al., using patch clamp, together with our results, using extracellular field recordings, demonstrates that astrocytes are recruited into synaptic circuits via mGluR5 (Fig 4.1).

4.2 Presynaptic A2A receptors to modulate glutamate release

In response to mGluR5 activation, astrocytes release gliotransmitters, including purines, to regulate synaptic transmission (Panatier et al., 2011). Given that astrocytes have been shown to be activated via mGluR5 (Panatier et al., 2011) and ATP is astrocyte currency (Coco et al., 2002, Anderson et al., 2004, Guthrie et al., 1999), we hypothesized that, in response to glutamate, astrocytes regulate evoked inhibition through ATP release. ATP released from astrocytes would then act on interneurons to modulate GABA release (Fig 4.1A). However, this is inconsistent with the Panatier et al. (2011) study as they demonstrated that A2A receptors increase pyramidal neuron excitability. They showed that in response to glutamate, mGluR5s are activated on astrocytes. Astrocytes then release purines which act on A2A adenosine receptors on the presynaptic terminal to increase glutamate release (Fig 4.1B) (Panatier et al., 2011). This could account for the effects observed in our study. Increasing glutamate release from the presynaptic neuron would

increase recruitment of inhibition by stimulating interneurons. By interfering with ATP levels and its release, there is less ATP available to be degraded into adenosine by ectonucleotidases. This, in turn, would decrease the efficacy of glutamate release, consequently decreasing interneuron stimulation. As Panatier et al. (2011) discovered using the A2A antagonist SCH 582621 (Panatier et al., 2011), blocking A2A receptors mimics the effects, on EPSC failure rates, seen with the mGluR5 antagonist MPEP (Panatier et al., 2011). We used the A1 antagonist, DPCPX, to block A1 receptors and assess the role of adenosine in cortical inhibition. However, DPCPX produced no change in the paired-pulse ratio, which lead us to believe that adenosine was not involved. However, we failed to consider adenosine A2A receptors. Thus, adenosine could nonetheless be playing a role. In addition, our data with SUR showed no effect on P1, P2, or P2/P1, which supports that inhibition is regulated through the mechanism described by Panatier et al. (2011). However, it could also be a consequence of the concentration we used as we applied 50 uM rather than 100 uM, which is the most commonly used concentration (Anderson et al., 2004, Deng and Lei, 2008, Tang et al., 2015). Ultimately, as Panatier et al. (2011) used patch clamp (Panatier et al., 2011) which is representative of a single cell, and we used extracellular field recordings which represents a more encompassing physiological environment, it is possible that astrocytic purines increase excitability but the overall effect is inhibitory.

4.3 Astrocytic purines are involved in mediating inhibition

As astrocytes release purines that affect synaptic transmission it is possible that ATP and adenosine are both involved in cortical inhibition. We addressed this possibility with the use of Ab129, a P2Y antagonist. These data showed a decrease in the paired-pulse ratio supporting that ATP mediates inhibition through P2Y receptors. This is in contrast to the Panatier et al. (2011) study, as their data support adenosine (Panatier et al., 2011) rather than ATP (Fig 4.1). However, it is known that adenosine acts on P1 as opposed to the P2 receptors on which ATP acts (Abbracchio et al., 2009). The best known P2 antagonists are Evans Blue, reactive blue 2, PPADS and suramin (Zimmermann, 1994). Ab129 is a derivative of reactive blue 2 and has been evaluated along with other reactive blue 2 derivatives for its efficacy and selectivity for P2 receptors (Brown and Brown, 2002).

Brown and Brown (2002) found that Ab129 was one of the most potent antagonists selective for P2Y receptors (Brown and Brown, 2002). Thus, we are confident that our data using Ab129 implicates P2Y receptors. Although the mechanisms described by Panatier et al. (2011) (Panatier et al., 2011) implicates adenosine, they failed to consider that ATP acts in addition to adenosine in the context of recruiting inhibition. Furthermore, our data are consistent with the studies done by Bowser and Khakh (2004) , and Lalo et al. (2014) on the involvement of ATP in synaptic transmission (Bowser and Khakh, 2004, Lalo et al., 2014). We demonstrated that inhibition can be altered by blocking P2Y receptors with Ab129. This is consistent with Bowser and Khakh who showed that ATP excites interneurons and astrocytes to increase synaptic inhibition (2004) (Bowser and Khakh, 2004) supporting our hypothesis that astrocytic ATP recruits interneurons (Fig 4.1A). They recorded from connected interneuron-pyramidal neuron pairs and used the ATP analog ATPyS to prevent its breakdown by ectonucleotidases (Bowser and Khakh, 2004). ATPyS caused patched interneurons to become excited and depolarize, subsequently increasing inhibition of pyramidal neurons (Bowser and Khakh, 2004). In addition, and consistent with our study, they applied P2 receptor agonists to determine which subtype was involved. P2Y agonists showed an increase in IPSCs in pyramidal neurons and P2X and adenosine agonists did not show any change (Bowser and Khakh, 2004), thereby implicating P2Y receptors. Our study, using extracellular field recordings, further supports Bowser and Khakh's (2004) as our results with Ab129 indicated a decrease in paired-pulse suppression. In addition, Bowser and Khakh used the P2 receptor antagonist PPADS and the P2Y selective antagonist MRS2179 which blocked the effects of P2Y agonists (Bowser and Khakh, 2004). This supports our hypothesis that inhibition is mediated through P2Y receptors and contests the involvement of P2X and adenosine receptors. Conversely, it has been shown that P2Y receptors are present on astrocytes and can induce ATP release (Anderson et al., 2004) which could account for our results and Bowser and Khakh's (2004) (Bowser and Khakh, 2004). By applying ATP or its analogs, astrocytic P2Y receptors are activated, triggering ATP-induced ATP release (Anderson et al., 2004). Consequently, ATP would be available for degradation by ectonucleotidases. Adenosine would then be available to act on presynaptic A2A receptors as in the mechanism described by Panatier et al. (2011) (Panatier et al., 2011). Due to the action of ATP analogs on astrocytes, they do

not necessarily isolate ATP effects as adenosine will still be present. Furthermore, P2Y antagonists block P2Y receptors on astrocytes, thereby reducing ATP release, in turn, decreasing adenosine. Thus, it is possible that astrocytes mediate inhibition through our proposed mechanism, which is consistent with the Bowser and Khakh study (2004), involving ATP and P2Y receptors on interneurons and astrocytes (Fig 4.1A) (Bowser and Khakh, 2004), through Panatier et al.'s (2011) described mechanism involving adenosine and presynaptic A2A receptors (Fig 4.1B) (Panatier et al., 2011), or a combination of both.

To further validate that purines released from astrocytes are involved in inhibition we used IDA to decrease the availability of ATP for release. IDA reduces ATP levels by inhibiting GAPDH, an enzyme essential for glycolysis (Schmidt and Dringen, 2009, Nodin et al., 2012). By reducing ATP concentration in astrocytes, less ATP is available for release to effect inhibition. In the presence of IDA P2/P1 increased, indicative of a decrease in inhibition. However, IDA is capable of affecting neurons as well as astrocytes. Lactate is the preferred energy substrate by neurons and astrocytes provide lactate to neurons through the astrocyte-neuron lactate shuttle (Pellerin et al., 2007). Lactate is essential in order to maintain neurotransmission (Pellerin et al., 2007), and interfering with glycolysis reduces the lactate available to neurons. Thus, this would appear as an effect on inhibition when in fact it is reducing neurotransmission. To counter this, we added 2mM lactate to aCSF in order to inhibit astrocyte ATP with IDA but also maintain neuronal function. In addition, IDA is a thiol reagent capable of disrupting glutathione metabolism resulting in oxidative stress (Liao et al., 2013). However, Schmidt and Dringen (2009) demonstrated the effects of IDA on glycolysis and glutathione in astrocytes and found that IDA is highly effective in inhibiting astrocyte glycolysis without substantially compromising glutathione metabolism (Schmidt and Dringen, 2009). Another study by Nodin et al. (2012) additionally demonstrated that IDA rapidly inhibits GAPDH leading to a resultant decrease in ATP levels without increasing oxidative stress (Nodin et al., 2012). Thus, we believe that our use of IDA in the presence of lactate allowed us to assess the role of astrocytic ATP without affecting neuronal function. The studies done by Panatier et al. (2011) and Bowser and Khakh (2004) study support our results with IDA that showed an increase in the P2/P1 indicative of a reduction in inhibition. However, as we used extracellular field recordings, it

remains unclear which mechanisms are responsible but the evidence strongly supports that purines are involved in cortical inhibition.

4.4 The mechanism of astrocytic purine release is uncertain

In addition to IDA, we used 2-APB which reduces intracellular calcium elevations and blocks hemichannels similar to the effects of CBX and FFA. 2-APB is an IP_3 receptor blocker, which inhibits intracellular calcium release from the endoplasmic reticulum (ER) (Peppiatt et al., 2003, Ozaki et al., 2013). When ligands bind to their G-protein receptors it induces PLC to form IP_3 and DAG from PIP2. IP_3 acts to open calcium channels on the ER resulting in a rise in intracellular calcium (Berridge, 1993). In addition, DAG and calcium activates PKC (Berridge, 1993). When we applied 2-APB, it resulted in a reduction in paired-pulse suppression represented by an increase in the P2/P1. Given that many neuromodulators use this signaling pathway 2-APB may be affecting activity via the inhibition of other neuromodulators. Furthermore, 2-APB is also capable of blocking connexin based hemichannels (Bai et al., 2006) and TRP channels (Bencze et al., 2015). This causes a further blockade of astrocyte ATP release as astrocytes express hemichannels containing connexin 43 (Brokamp et al., 2012) and there is evidence to support that ATP is released from astrocyte hemichannels (Stout et al., 2002). Thus, it is possible that our data with 2-APB is a result of either calcium blockade in astrocytes, release of ATP or its effects on neuromodulator signaling pathways. However, if 2-APB does indeed block hemichannels, this supports our CBX and FFA data. We proposed that because inhibition was reduced in the presence of CBX and FFA, ATP is released from astrocytes via gap junctions. This is consistent with Lalo and colleagues (2014) as they showed that ATP modulates inhibition in the cortex (Lalo et al., 2014), albeit through vesicular-mediated mechanisms. However, their study demonstrated, using patch clamp and sniffer cell approaches in dnSNARE mice, that ATP is released from astrocytes via exocytosis whereas we proposed that ATP is released via hemichannels as CBX and FFA increased the P2/P1. Thus, it is possible that CBX and FFA are eliciting their effects through a different mechanism than what we hypothesized. FFA is a large ion channel modulator (Guinamard et al., 2013) that blocks hemichannels and TRP channels (Stridh et al., 2008). In addition it blocks the enzyme cyclooxygenase (COX) responsible for the synthesis of inflammatory

mediators which affects neurotransmission (Yang, 2009). CBX blocks astrocytic hemichannels but has also been shown to interfere with voltage-gated calcium channels (Vessey et al., 2004). This reduces synaptic activity, which would explain why we see a decrease in inhibition. However, it has also been shown that ATP can be released from astrocytic hemichannels and that both mechanisms in combination, (release via hemichannels and exocytosis) can facilitate ATP release (Garré et al., 2010, Lalo et al., 2014, Wei et al., 2014). In addition, astrocytes use hemichannels to propagate calcium signals (Dermietrel et al., 1991). Blocking calcium would further disrupt astrocyte signaling resulting in a reduction in ATP release, thus supporting our hypothesis of astrocytic involvement in inhibition. However, we cannot rule out that ATP is being released from astrocytes via hemichannels, exocytosis, or a combination of both. The fact that Lalo et al. (2014) found that astrocytic ATP modulates inhibition (Lalo et al., 2014) in the cortex supports our hypothesis that it plays a role in mediating cortical inhibition.

4.5 ATP effects are GABA dependent

Since we found that ATP is involved in modulating inhibition, we investigated whether it required GABA to elicit its effects. Consistent with the studies done by Bowser and Khakh (2004), and Lalo et al. (2014) we found that astrocytic mediation of inhibition involved GABA (Bowser and Khakh, 2004, Lalo et al., 2014). When we applied MPEP, IDA, CBX, and Ab129 in the presence of the $GABA_A$ antagonist BIC we no longer observed an effect on inhibition, suggesting that inhibition mediated through astrocytes depends on the release of GABA from interneurons. These results are in line with Lalo et al. (2014) as well as Bowser and Khakh (2004) as they found that IPSCs in pyramidal neurons mediated by ATP were significantly reduced in the presence of BIC (Bowser and Khakh, 2004, Lalo et al., 2014). This supports our hypothesis that interneurons are excited by ATP to release GABA onto their postsynaptic targets (Fig 4.1A). In contrast to our study, Lalo and colleagues (2014) found that ATP down-regulated $GABA_A$ receptors resulting in a decrease in inhibition (Lalo et al., 2014), whereas our data suggest that ATP increases inhibition in the cortex. They used P2X knock out mice to show that these effects were mediated through ATP and P2X receptors (Lalo et al., 2014). In comparison, our study suggested that ATP is acting through P2Y receptors to increase GABA release from interneurons. However, the

results obtained in the study done by Lalo et al. (2014) were acquired using patched pyramidal neurons, and ATP actions on interneurons were not accounted for. Thus, ATP release from astrocytes could be acting on pyramidal neurons through P2X receptors and on interneurons via P2Y receptors. This may explain the large effect we observe on the first stimulation with exogenous ATP administration. In addition, as we used extracellular recordings instead of patch clamp recordings, it is possible that ATP excites pyramidal neurons but the overall effect on the neural circuitry remains inhibitory through facilitation of GABA release from interneurons.

4.5.1 $GABA_A$ and not $GABA_B$ receptors are responsible for the observed results

It is possible that GABA acts on $GABA_A$ and $GABA_B$ receptors to mediate effects on inhibition (Rozas et al., 2001). Carlos Rozas and colleagues (2001) have demonstrated that GABA, does indeed, act through $GABA_B$ receptors but its response peaks considerably later than $GABA_A$ (Rozas et al., 2001). They assessed the effects of $GABA_A$ and $GABA_B$ on paired-pulse suppression by using a range of interstimulus intervals, the $GABA_A$ antagonist picrotoxin, and the $GABA_B$ antagonist saclofen (Rozas et al., 2001). They found that picrotoxin reduced paired-pulse suppression at shorter intervals including 20, 40 and 80 ms intervals between P1 and P2, and that saclofen reduced paired-pulse suppression at longer intervals including 80, 160, and 320 ms intervals between pulses (Rozas et al., 2001). We used an interstimulus interval of 50 ms which is too short for $GABA_B$ to have an effect, and therefore did not evaluate a $GABA_B$ antagonist. In contrast to Rozas et al. (2001), we did not observe an effect on the P2/P1 in the presence of BIC. However, it was able to effectively block the effects of MPEP, IDA, CBX, and Ab129. In line with Rozas et al. (2001) we applied a sub-saturating concentration of $GABA_A$ antagonist to prevent excessive excitation. The fact that BIC had no effect on the P2/P1 may have been a result of adding lactate to our aCSF. We added lactate in order to maintain neurotransmission in the presence of the agents used in our study. Studies have shown that lactate seems to be involved in maintaining GABAergic receptor activity (Laschet et al., 2004), synaptic vesicle cycling (Morgenthaler et al., 2006), and enhancing the accumulation of GABA vesicles (Tarasenko et al., 2006). Rozas et al. (2001) did not use lactate in their solutions which would account for why they observed a decrease in paired-pulse suppression in the

presence of a $GABA_A$ antagonist and we did not. In addition, in prior studies without lactate we also observed an effect on paired-pulse suppression (Ming et al., 2015) similar to that shown by Rozas et al. (2001) (Rozas et al., 2001). Interestingly, Rozas et al. (2001) also showed that GABAergic inhibition increases in the presence of the $GABA_A$ modulator diazepam (Rozas et al., 2001). The study done by Rozas et al. (2001) in addition to the studies done by Bowser and Khakh (2004), and Lalo et al. (2014) support our results that astrocytic mediation of inhibition involved $GABA_A$ signaling pathways (Rozas et al., 2001, Bowser and Khakh, 2004, Lalo et al., 2014).

4.6 Astrocytes release purines to modulate GABAergic inhibition

In line with previous studies (Rozas et al., 2001, Bowser and Khakh, 2004, Panatier et al., 2011, Lalo et al., 2014), our results suggest astrocytic purines are involved in inhibition and that their effects are dependent on GABA. We proposed that astrocytes sense synaptic activity through mGluR5 receptors, resulting in an intracellular rise in calcium and ATP release. This was consistent with Panatier et al. (2011). Further, we suggested that ATP would then act on interneurons to enhance GABAergic output onto pyramidal neurons to increase inhibition. This is in line with the study done by Bowser and Khakh (2004) (Bowser and Khakh, 2004), however, there are multiple ways in which inhibition is mediated (Fig 4.1). These mechanisms include adenosine A2A receptors on presynaptic terminals (Fig 4.1B) as described by Panatier et al.(2011) (Panatier et al., 2011), P2Y receptors on interneurons (Fig 4.1A) as described by Bowser and Khakh (2004) , and P2X receptors as described by Lalo et al. (2014) (Bowser and Khakh, 2004, Panatier et al., 2011, Lalo et al., 2014). Thus, it is clear that astrocytic ATP and its metabolites play a role in mediating inhibition but multiple mechanisms are involved.

4.7 5-HT effects on inhibition

5-HT effects were compared in the presence and absence of the same pharmacological agents. In the absence of pharmacological agents, application of 5-HT caused a decrease in P1, an increase in P2, and an increase in P2/P1 that is completely abolished in the presence of BIC demonstrating that 5-HT mediates its actions exclusively via inhibition. Thus, we investigated whether 5-HT reduced inhibition by acting on

astrocytes. We then applied ATP and TFLLR in the absence of pharmacological agents to compare their effects to 5-HT effects. ATP application produced a decrease in both P1 and P2, and TFLLR generated similar results. This supports that ATP is being released from astrocytes to affect inhibition as TFLLR selectively activates astrocytic PAR-1 receptors to facilitate gliotransmitter release (Panatier et al., 2011). ATP and TFLLR showed a decrease in P2 as opposed to an increase, opposite to the effects observed with 5-HT. This suggests that 5-HT is acting on astrocytes to inhibit ATP release, in turn causing a decrease in evoked inhibition.

4.7.1 5-HT1A and 5-HT2A can alter inhibition

Our results are consistent with a study done by Deng and Lei (2008) (Deng and Lei, 2008). They found that 5-HT increased spontaneous IPSCs and reduces evoked IPSCs (Deng and Lei, 2008) which accounts for our observations in which 5-HT initially decreased the amplitude of P1 but increased the amplitude of P2. In addition to affecting inhibition, Deng et al. (2007) demonstrate that 5-HT directly inhibits pyramidal neuron excitation through 5-HT1A receptors (Deng et al., 2007). Deng and Lei (2008) also found that 5-HT2A receptors are responsible for increasing the excitability of interneurons (Deng and Lei, 2008) which increased spontaneous inhibition (Fig 4.1C). However, they found that 5-HT reduced evoked IPSCs which was explained in part by the fact that 5-HT decreased action potentials through the inactivation of interneuron sodium channels (Deng and Lei, 2008). This is consistent with our hypothesis as we proposed that interneurons regulate 5-HT effects on inhibition through $GABA_A$ receptors which we confirmed using BIC. In line with Deng and Lei (2008), in which they determined the effects of 5-HT on IPSCs to be sensitive to BIC (Deng and Lei, 2008), our study showed that 5-HT no longer produced an increase in the P2/P1 and P2 amplitude in the presence of BIC. However, we believe that in addition, 5-HT alters astrocytic ATP release, in turn, effecting evoked inhibition.

4.7.2 Astrocytic purines are involved in 5-HT effects

We found that by interfering with astrocytes we were able to reduce the effects of 5-HT on inhibition. As previously discussed, our data suggests that astrocytic release of ATP increases inhibition in the cortex by exciting interneurons and increasing GABA release.

Therefore, we alluded that 5-HT is inhibiting ATP release to cause a decrease in inhibition. Our results are consistent with the study done by Panatier et al. (2011) as they showed that inhibition of astrocytic activity can reduce presynaptic excitability leading to reduced excitability (Panatier et al., 2011) which includes interneurons (Fig 4.1B). In addition, Bowser and Khakh (2004) showed that astrocytic ATP mediates inhibition through interneurons (Fig 4.1A) (Bowser and Khakh, 2004). In the Deng and Lei (2008) study they did not consider a role for astrocytes (Fig 4.1C) (Deng and Lei, 2008). They used SUR as a G-protein inhibitor, but it is also known to be a non-selective P2 antagonist (Deng and Lei, 2008), which implicates ATP in 5-HT effects. Thus, as we observed a decrease in inhibition when 5-HT was applied which was reversed in the presence of ATP limiting agents, lead us to the conclusion that 5-HT is inhibiting ATP resulting in decreased inhibition.

4.8 ATP effects on cortical inhibition

We applied ATP in order to compare its effects to 5-HT and assess whether 5-HT inhibits its release from astrocytes to affect inhibition. When ATP was applied we observed a decrease in P2 amplitude in comparison to 5-HT application where we observed an increase. Thus, we suggested that 5-HT is blocking the release of ATP would result in the inverse effect. To test whether ATP was being released from astrocytes we applied TFLLR to selectively activate astrocytes through the PAR-1 receptor. In the presence of TFLLR we observed similar effects as we had with the application of ATP, supporting that ATP is released from astrocytes. This is in line with the study done by Lalo et al. (2014) as they found that application of TFLLR elevated calcium in astrocytes and elicited currents in ATP sensitive sniffer cells (Lalo et al., 2014). Thus, it is evident that astrocytes release ATP in response to TFLLR (Lalo et al., 2014). Therefore, it is possible that 5-HT decreases inhibition through inhibition of astrocytic ATP. However, we were not able to block the effects of ATP, when applied exogenously, with Ab129, PPADS, or SUR (data not included), thus it is unclear how exogenous ATP is affecting our slices. This could be a result of the technique we used as we used extracellular field recordings compared to patch clamp studies (Bowser and Khakh, 2004, Lalo et al., 2014). In these studies, they were able to observe currents in single cells induced by the application of ATP or its analogs and were able to block these currents with ATP antagonists. Through the use of specific agonists and

antagonists Bowser and Khakh (2004) deduced that ATP excites interneurons through P2Y receptors (Bowser and Khakh, 2004). In contrast, Lalo et el. (2014) deduced from their data that ATP effects were mediated through P2X receptors (Lalo et al., 2014). However, we were unable to inhibit ATP effects which may be a consequence of the fact that our recordings included more complex circuitry rather than the single cell recordings used in the studies done by Bowser and Khakh (2004), and Lalo et al. (2014) (Bowser and Khakh, 2004, Lalo et al., 2014).

5.0 Conclusion

- Astrocytes are involved in cortical inhibition
- Astrocytic purine release plays a role in mediating cortical inhibition
- 5-HT affects astrocytes to alter inhibition
- ATP is altered by 5-HT to affect inhibition

We hypothesized that inhibition in the cortex is mediated through astrocytic release of ATP and that it is altered by neuromodulators such as serotonin. Upon critical examination of our data and the evidence presented by others (Bowser and Khakh, 2004, Deng and Lei, 2008, Panatier et al., 2011, Lalo et al., 2014), it is evident that astrocytic ATP plays a role in mediating inhibition in cortical networks. In addition, it seems that 5-HT acts on astrocytes to alter ATP release, thereby affecting inhibition. However, the mechanism remains unclear. The study done by Bowser and Khakh (2004) supports our idea that astrocytes release ATP to excite interneurons for an overall inhibitory effect (Bowser and Khakh, 2004), but Panatier et al. (2011) suggests that adenosine acts to increase the efficacy of presynaptic release of glutamate (Panatier et al., 2011). Moreover, Lalo, et al. (2014) suggest that ATP release from astrocytes enhances excitability of pyramidal neurons (Lalo et al., 2014). Thus, it is evident that astrocytes are involved but the mechanism of how ATP or adenosine is acting to induce these effects remains unclear. However, our results are consistent with these studies as the effects on inhibition as well as 5-HT effects described are mediated through interneuron release of GABA. Furthermore, we found that 5-HT may be acting to alter these mechanisms and change inhibition in the cortex. Based on our data and the evidence presented by Deng and Lei (Deng and Lei, 2008), we suggest that 5-HT is acting to inhibit ATP release from astrocytes. They suggested that there is an initial increase in inhibition mediated via 5-HT2A receptors on interneurons (Deng and Lei, 2008), and a decrease in excitation mediated through 5-HT1A receptors on the presynaptic terminal (Deng et al., 2007). They found that spontaneous inhibition was increased and evoked inhibition was decreased with the application of 5-HT.

We showed that by interfering with astrocytes with the application of MPEP, IDA, 2-APB, CBX, FFA, and Ab129, 5-HT effects on evoked inhibition were reduced. Thus, we maintain that astrocytes are involved in mediating inhibition in the cortex and 5-HT can alter it. However, the mechanisms remain unclear. The use of extracellular field recordings represents a more physiological environment which gives us a better understanding of overall activity. However, it makes it difficult to isolate specific mechanisms at play as it includes all aspects that may be interacting to modulate inhibition. The studies discussed use whole cell patch clamp techniques that provide a more reductionist approach allowing them to isolate such mechanisms, but this is not necessarily representative of what is occurring overall. Therefore, it is possible that a combination of the proposed mechanisms described is eliciting effects on inhibition in cortical networks. More research is needed to determine the specifics of astrocyte influence on synaptic inhibition and how 5-HT affects astrocytes to modify this.

6.0 Future Directions

As there are many possible mechanisms by which astrocytes may mediate inhibition in cortical networks, future research is needed in order to determine the combinations of mechanisms that are involved and how they interact to elicit the effects observed on inhibition. Panatier et al. (2011) suggested that inhibition is mediated through the release of adenosine from astrocytes to act on presynaptic terminals affecting the efficacy of glutamate release (Panatier et al., 2011). In order to determine if this is consistent in our study we have plans to repeat experiments in the presence of the A2A blocker SCH 582621 and examine its effects on paired-pulse suppression and the 5-HT response. However, in line with our hypothesis, Bowser and Khakh (2004) suggested that it is through astrocytic release of ATP that inhibition is mediated (Bowser and Khakh, 2004). In order to test this in the more complex extracellular field recordings that are more representative of physiological conditions, it would be interesting to apply an ectonucleotidase inhibitor such as apyrase. Apyrase prevents the degradation of both exogenous and endogenous ATP and would allow us to observe the effects of elevated ATP and rule out the action of its metabolites.

Extracellular field recordings that include all activity in the area surrounding the recording pipette (~100-200 um diameter) enable an integrated understanding of the overall effects but made it difficult to conclusively determine the mechanism by which ATP is involved in cortical inhibition and the 5-HT effects. Thus, it would be useful to perform further studies using patch clamp techniques to measure inhibitory synaptic events directly. Patch clamp provides many options to study inhibition in the cortex, including but not limited to, the direct measure of inhibitory and excitatory currents in pyramidal neurons and interneurons in the presence and absence of pharmacological agents and neuromodulators. We could then examine the changes in currents in specific cells in response to pharmacological agents and 5-HT using a more reductionist model such as in the studies done by Deng and Lei (2008), Panatier et al. (2011), Bowser and Khakh (2004), and Lalo et al. (2014) (Bowser and Khakh, 2004, Deng and Lei, 2008, Panatier et al., 2011, Lalo et al., 2014). Patch clamp studies would be useful in determining the specific receptors

and mechanisms involved in cortical inhibition and 5-HT effects on neurons, interneurons, and astrocytes. This would allow us to compare results using patch clamp to results using extracellular field recordings in order to determine specific mechanisms and how they interact to result in the overall effects.

7.0 References

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