CALCIUM DEPENDENT
ASTROCYTE-NEURON COMMUNICATION

by

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Abstract

The discovery of direct communication between astrocytes and neurons has changed the perception of astrocytes from passive support cells to active partners in information processing. Astrocytes express myriad neurotransmitter receptors and have been shown to release neurotransmitters, allowing these cells to respond and signal to adjacent neurons, respectively. Astrocytes can rapidly respond to neurotransmitters with a rise in $[Ca^{2+}]_i$, and astrocyte neurotransmitter release has been shown to be calcium dependent. The purpose of this research was to investigate unknown aspects of astrocyte-neuron communication with in situ calcium imaging to improve our understanding of how these cells interact functionally.

The first objective of this research was to determine whether extracellular dopamine elicits astrocyte calcium transients in the prefrontal cortex (PFC). Astrocytes from this area express dopamine receptors and dopamine is an important neurotransmitter in the PFC. We found that astrocytes in PFC brain slices reliably respond to a high concentration of dopamine ([50μM]) with $[Ca^{2+}]_i$ transients, however these responses were due to the activation of adrenoreceptors, not dopamine receptors. The inability of a lower concentration of dopamine ([10μM]) to elicit astrocyte $[Ca^{2+}]_i$ transients questions the whether these cells can rapidly respond to PFC dopamine at physiological levels.

The second objective of this research was to investigate the extent that calcium dependent glutamate release from astrocytes is able to influence neural activity. The best studied mechanism of astrocyte gliotransmitter release is the calcium dependent release of glutamate which has been demonstrated in single astrocytes in situ. We used the
vasoactive peptide endothelin to preferentially elicit widespread astrocyte $[Ca^{2+}]_i$ transients astrocytes from hippocampal brain slices to determine whether the widespread calcium rise in astrocytes was associated with a change in glutamate sensitive synaptic transmission. Despite eliciting nearly ubiquitous astrocyte calcium responses, we observed no change in glutamate sensitive synaptic transmission as measured by extracellular field recordings. These results question the ability of astrocytes to acutely influence synaptic transmission of a brain region.

Our findings do not support an acute role of calcium dependent communication between astrocytes and neurons in rapid information processing in the systems we investigated.
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Dedication

This thesis is dedicated to Judy, David, Rosemary, and Henry.
Words cannot express how important you are to me.
Chapter 1: Background Information
Overview

This chapter is an introduction to the following two data chapters; Chapter 2 – ‘Dopamine elicits \([\text{Ca}^{2+}]_i\) transients from astrocytes in prefrontal brain slices by activating adrenergic receptors’, and Chapter 3 – ‘Endothelin elicits widespread astrocyte \([\text{Ca}^{2+}]_i\) transients without affecting neurotransmission in the rat hippocampus’. In this introduction, I focus on the intimate relationship between astrocytes and neurons. First I explain how astrocyte morphology is optimised to interact with its neural microenvironment. Next I cover how established astrocyte functions are necessary to support neural function, and do so in a passive way by not directly influencing neural activity. Then I introduce the direct communication between neurons and astrocytes and how this can actively influence information processing.

In Chapter 2, I investigated how dopamine in the prefrontal cortex (PFC) influences astrocyte signalling. Accordingly, in the second part of this background chapter I introduce the role of the prefrontal cortex in controlling higher cognitive function and the important influence of the neurotransmitter dopamine in the PFC. I then summarize what is known about the ability of dopamine to influence astrocytes by citing studies in culture and other tissue.

Chapter 3 of this thesis investigates whether the vasoactive peptide endothelin can elicit widespread astrocyte \([\text{Ca}^{2+}]_i\) transients in intact tissue and can influence synaptic transmission in an entire brain region. The third section of this chapter introduces what is known about the endothelin system, with emphasis given to its role in the central nervous system and the influence of endothelin peptides on astrocytes.
Astrocyte form and function.

From an evolutionary perspective, the nervous system collects and processes diverse sources of information to control complex bodily functions and behaviour. The roughly 100 billion neurons in the human brain that are specialised for this role use sensory adaptations to encode external information and elaborate morphology to rapidly transmit it along axons, across synapses, and through complex neural networks. Despite the importance of neurons, the glia and other non-neuronal cells of the brain are 10-50 times more numerous, and of these astrocytes are the most abundant (Bass et al., 1971; Nedergaard et al., 2003; Hatton and Parpura, 2004). Astrocyte morphology is intimately associated with neurons and from the time of their discovery the purpose of these cells has been considered supportive of neuronal function. Accordingly, the established functions of astrocytes support neural activity by allowing neurons to function optimally without directly affecting their signalling. This passive perception of astrocytes has changed dramatically in the last two decades. The direct communication between astrocytes and neurons is now being explored and astrocytes have been shown to actively influence neural activity. This dynamic two way communication between astrocytes and neurons suggests that astrocytes may have an active role in neural information processing; however the nature and extent of this communication is uncertain and is an area of substantial investigation (Ransom et al., 2003).
The morphology of astrocytes is well suited to its distinct microenvironment.

Distinct astrocyte subtypes are seen in different neuronal environments which suggests that the morphology of astrocytes is dependent upon its microenvironment (Reichenbach, 1989; Reichenbach and Wolburg, 2005). In the white matter of the CNS, 'fibrous' astrocytes have long smooth processes oriented parallel to axons that extend multiple finger-like outgrowths to axonal perinodal spaces (Reichenbach and Wolburg, 2005). In the grey matter of the brain where synapses are most concentrated, 'protoplasmic' astrocytes extend radial processes covered in submicroscopic lamellae and filopodia and predominantly contact synapses (Wolff, 1970; Chao et al., 2002). In regions with small, densely packed neurons, 'velate' astrocytes surround single, or groups of neuronal cell bodies with thin membrane sheets (Chan-Palay and Palay, 1972; Valverde and Lopez-Mascaraque, 1991). Glia simultaneously exposed to multiple environments can exhibit these distinct morphologies within the same cell. Specialised astrocytes spanning different layers of the retina, called Müller cells, have velate processes in the nuclear layer, protoplastic processes in the plexiform (synaptic) layer, and fibrous processes within the optic nerve head (Reichenbach and Wolburg, 2005). Astrocyte morphology is also influenced by non-neuronal elements within the brain. Astrocytes contact all basal laminae within the CNS (pia mater, blood vessels, and the vitreous body of the eye) with specialised endfeet (Reichenbach and Wolburg, 2005). Contact of closely related ependymoglial cells with cerebrospinal fluid (CSF) at the ventricular surfaces causes the expression of desmosomes and the outgrowth of microvilli and kinocillia (Reichenbach and Wolburg, 2005) and vascular astrocyte endfeet associate via gap
junctonal coupling to cover the entire cerebral vasculature (Reichenbach and Wolburg, 2005). The diversity of astrocyte processes in different brain regions correspond to and allow diverse functionally interactions of these cells with their particular microenvironment. **Protoplasmic astrocytes are intimately associated with their synaptic environment.**

The number of synapses in a human brain (~10^14) is greater than the number of stars in the galaxy (Kandel et al., 1991). Synapses are found predominantly in the grey matter of the CNS and are intimately associated with protoplasmic astrocytes. These cells are often characterized by their 5-10 major radial stem processes forming a star like shape that is the astrocyte namesake. Unique to protoplasmic astroglia are extensive lamellar extensions and finger-like protrusions from their stem processes and cell body which comprise ~50-60% of the cell’s volume and ~80% of its surface area (Chao et al., 2002). These peripheral astrocyte processes preferentially associate with synaptic membranes, often separated by only 20nm, and are the major site of glial-neuronal interactions ((Kuffer et al., 1984) cited in (Porter and McCarthy, 1997)). Synapses are usually ‘sealed’ at their margins by glial lamellae, although the coverage of synapses by astrocyte membranes can range from a complete sheath to no contact, even in adjacent synapses (Ventura and Harris, 1999). After the general shape of astrocyte stem processes is established during development, the peripheral processes develop in mutual dependence with synapses (Waxman et al., 1983). Signals released during neuronal activity, namely K+ ions (Reichelt et al., 1989) and neurotransmitters (Cornell-Bell et al., 1990a; Matsutani and Yamamoto, 1997), have been proposed to control the formation of astrocyte processes by attracting or repelling the growth of filopodia. Once the astrocyte
processes are established they continue to change their morphology in response to neural activity (Hirrlinger et al., 2004). Accordingly, changes in the morphology of astrocyte processes have been shown during long-term potentiation (Wenzel et al., 1991), kindling (Hawrylak et al., 1998), stimulation of afferents ((Guldner and Wolff, 1977) cited in (Reichenbach and Wolburg, 2005)), and by raising animals in ‘enriched’ environments (Stewart et al., 1986; Sirevaag and Greenough, 1991). Conversely, neurons are also dependent on astrocytes to contribute to the formation and maintenance of new synapses (Pfrieger and Barres, 1996). The intimate structural relationship of peripheral astrocyte processes with both pre- and post-synaptic neural elements forms a single functional element, a tripartite synapse, where all three elements are necessary for optimal synaptic function (Araque et al., 1999).

Astrocyte functions range from passive to active.

The originally function attributed to Glia was the structural support of the neural elements of the brain. Accordingly, Glia were named after the Latin word ‘gliok’ meaning ‘glue’ (Ransom et al., 2003). This limited view of astrocytes is no longer tenable and these cells are associated with such a wide range of functions in the CNS that it has been suggested they are “involved in virtually everything the brain does” (Ransom et al., 2003). Many astrocyte functions ensure optimal neuronal function by: increasing the efficiency of their signalling, ensuring that their metabolic needs are met, and protecting them from harm. Astrocytes are able to influence neural signalling by some of these interactions, but historically were not thought to directly influence neural activity. This view of has changed dramatically with the discovery that astrocytes can directly
communicate with neurons using neurotransmitters, the language of synaptic signalling, which raises the possibility that astrocytes directly participate in information processing.

It is interesting to consider that the ratio of astrocytes to neurons has steadily grown with the phylogeny of the nervous system (Nedergaard et al., 2003). The ganglia of a leech typically have 25-30 neurons per astrocyte, while the human cortex has an astrocyte-to-neuron ratio of 1.4:1 (Nedergaard et al., 2003). This increased astrocyte-to-neuron ratio suggests a corresponding increase in the importance of astrocytes for optimal CNS function. To fully appreciate neuronal and synaptic function in the human brain, it is necessary to understand the influence by astrocytes.

**By supporting optimal neural function, astrocytes may passively affect neural activity.**

Established astrocyte functions are necessary to support normal neuronal signalling, particularly at the synapse. Astrocytes maintain an optimal extracellular environment for synaptic transmission and provide important metabolic support for neurons (Simard and Nedergaard, 2004; Reichenbach and Wolburg, 2005). The rapid flux of ions during neural signalling causes the normally low ($\leq 2.5$ mM) extracellular potassium concentration ($[K^+]_e$) (Moghaddam and Adams, 1987) to undergo large fractional changes that can significantly alter neural excitability (Walz, 2000; Coles and Deitmer, 2005; Newman, 2005). Astrocytes can remove excess $[K^+]_e$ with a high potassium permeability and a considerable spatial buffering capacity (Walz, 2000). Astrocytes also maintain the extracellular environment by compensating for the pH changes during neuronal activity (Chesler and Kaila, 1992) and by rapidly controlling
extracellular osmolarity via water homeostasis (Simard and Nedergaard, 2004; Coles and Deitmer, 2005; Mulligan and MacVicar, 2006).

Astrocytes are necessary for sustaining the high energy demand of neural activity. In the rat brain under normal unanaesthetised conditions, approximately 80% of oxidative glucose consumption (the main energy source of the brain) is used during glutamatergic neurotransmission to actively take up released glutamate and restore ion gradients (Sibson et al., 1998; Pellerin and Magistretti, 2005). Astrocytes support and alleviate this energy demand by delivering energy and metabolites to where they are needed most. Astrocytes are thought to detect excitatory neural activity with glutamate transporters, which causes an increase in their aerobic glycolysis (glucose use) (Pellerin and Magistretti, 1994; Voutsinos-Porche et al., 2003; Pellerin and Magistretti, 2005). This increased glycolysis produces a metabolite in astrocytes that is released and preferentially taken up by adjacent, presumably active, neurons to fuel their oxidative metabolism, their main source of energy (Bouzier et al., 2000; Hassel and Brathe, 2000; Pellerin and Magistretti, 2005). The metabolite released by astrocytes in response to neural activity is thought to be lactate and the process whereby astrocytes to provide a useful energy supply to the neurons that need it most is known as the astrocyte-neuron lactate shuttle (Pellerin et al., 1998). Astrocytes can also limit the loss of neuronal energy and metabolites that is used to make released neurotransmitters by recycling them back to neurons before they are broken down in the extracellular space. The uptake of glutamate requires an energy cost of at least 1.5 adenine triphosphate (ATP) per glutamate transported and this cost is estimated to account for a large fraction of ATP used in the brain (Sibson et al., 1998). Astrocytes shift this energy cost away from neurons by taking
up the majority of glutamate released by neurons with selective transporters. Glutamate taken up by astrocytes is either metabolised into glutamine, a glutamate precursor that is recycled back to neurons (Broer and Brookes, 2001), or is oxidatively metabolised in the tricarboxylic acid cycle producing more than 30 ATP per molecule of glutamate (Swanson, 2005).

In addition to maintaining optimal synaptic functioning, altered neurotransmitter uptake and potassium buffering of astrocytes can alter neural signaling (Newman, 2005). By using transporters to take up neurotransmitters from perisynaptic sites, astrocytes influence the level of transmitters within the synapse, which can directly alter synaptic signalling and plasticity. Inhibition of glutamate transporters, which are mainly glial (Rothstein et al., 1996; Bergles and Jahr, 1998; Rauen et al., 1998), increases the size of recorded mini and evoked EPSCs in neurons (Barbour et al., 1994; Tong and Jahr, 1994; Takahashi et al., 1995). The passive influence of astrocytes on synaptic signalling can change dynamically over a time course of minutes or months with changes in the properties of perisynaptic astrocyte transporters, such as their desensitization or proximity to a synapse. During different stages of lactation, astrocytes in the supraoptic nucleus of the hypothalamus change their synaptic coverage (Theodosis and Poulain, 1993; Oliet et al., 2001; Theodosis and Poulain, 2001). During lactation, perisynaptic astrocyte processes retract slightly from synaptic margins, reducing their neurotransmitter uptake and allowing greater activation of extrasynaptic neuronal neurotransmitter receptors (presynaptic mGluRs), which reduces the level of subsequent transmitter release (Theodosis and Poulain, 1993; Oliet et al., 2001; Theodosis and Poulain, 2001).
Thus astrocytes can directly alter synaptic transmission by changing the extent of their influence on neural activity.

Astrocytes have many functions that are not directly involved in synaptic activity, but are still essential for normal brain function. Astrocytes affect synaptic transmission by directly controlling synaptogenesis (Pfrieger and Barres, 1996). In the adult brain, astrocytes maintain synaptic structure by supporting dendritic spines (Lippman and Dunaevsky, 2005) and have even been suggested to play a role in adult neurogenesis by providing a proper environmental niche (Alvarez-Buylla and Lim, 2004). Astrocytes also are important for protecting the brain in various ways. They selectively take up and detoxify ammonia (Coles and Deitmer, 2005) as well as bind free radicals with endogenous antioxidants (Makar et al., 1994). During injury to the brain, often seen during a stroke, astrocytes limit excitotoxic damage by removing excess extracellular glutamate and they are thought to limit the extent of nervous tissue damage by a process of reactive gliosis, an astrocytic scar around the area of injury (Eng and Lee, 2005). Astrocytes also protect the brain from potentially harmful agents in the rest of the body, including xenobiotics and pathogens, by contributing directly or indirectly to the maintainence of the blood brain barrier (Bauer et al., 2005). If this barrier is compromised, resulting in CNS infection, astrocytes attract both resident and systemic immune cells to clear the infection (Calvo et al., 1998; Ransom et al., 2003).

The direct signalling between astrocytes and neurons allows astrocytes to be active partners in information processing.

In addition to providing synaptic support, astrocytes may also be active partners in synaptic function. The direct participation of astrocytes in information processing has
become a possibility with the discovery of direct two-way communication between astrocytes and neurons. Astrocytes were widely considered passive after electrophysiology showed that their high resting $K^+$ conductance and extensive intracellular gap junctional coupling made these cells electrically non-excitable (Ransom and Sontheimer, 1992). Despite the expression of myriad neurotransmitter receptors in culture (Murphy and Pearce, 1987; Kimelberg, 1995) and in situ (Kettenmann and Steinhauser, 2005; Haydon and Carmignoto, 2006) the membrane potential of astrocytes varies only minimally in response to a wide variety of stimuli (Kang et al., 1998; Kang and Nedergaard, 1999). This non-excitable view of astrocytes changed dramatically following the visualisation of $[Ca^{2+}]_i$ in these cells. Bath exposure of glutamate to cultured astrocytes resulted in widespread $[Ca^{2+}]_i$ transients followed by the propagation of intercellular $[Ca^{2+}]_i$ waves (Cornell-Bell et al., 1990b). Since this landmark report, calcium imaging has revealed that astrocytes in culture and intact tissue respond in a dynamic fashion to diverse stimuli, including many neurotransmitters and neural activity. Stimulation of neurons in hippocampal brain slices has been shown to elicit $[Ca^{2+}]_i$ responses in neighbouring neurons by activating metabotropic glutamate receptors (Porter and McCarthy, 1996), GABA$_B$ receptors (Kang et al., 1998), and muscarinic acetylcholine receptors (Araque et al., 2002). Stimulation of parallel fibres in cerebellar slices can elicit calcium responses in Bergmann glia (a specialised astrocyte) via the release of nitric oxide (Grosche et al., 1999; Matyash et al., 2001). In astrocytes from hippocampal brain slices, stimulation of afferent Schaffer collaterals can lead to discrete $[Ca^{2+}]_i$ increases within astrocyte processes and cell bodies (Porter and McCarthy, 1996). Neuronal transmitter release in intact tissue therefore communicates with both neural and
glial cells resulting in astrocyte calcium responses that are both diverse and dynamic. Minimal stimulation (a single pulse) of parallel fibres is able to evoke discrete \([\text{Ca}^{2+}]_i\) increases in microdomains of Bergmann glia and if this stimulation in increased (10 pulses) it can elicit larger \([\text{Ca}^{2+}]_i\) responses able spread to the cell soma (Matyash et al., 2001; Newman, 2005). Astrocytes are seemingly able to integrate neural signalling received during stimulation of multiple neuronal afferents resulting in novel astrocyte \([\text{Ca}^{2+}]_i\) signalling not observed during stimulation of individual afferents (Perea and Araque, 2005). Astrocyte \([\text{Ca}^{2+}]_i\) responses to neural activity also show plasticity, as both the magnitude and frequency of astrocyte \([\text{Ca}^{2+}]_i\) oscillation elicited by afferent stimulation increases as the frequency of afferent stimulation is increased (Pasti et al., 1997). Intracellular oscillations have been observed in vivo (Hirase et al., 2004) and based on their sensitivity to minimal neural stimulation in brain slices, it is likely that astrocytes respond to neuronal activity in the intact brain. It is also interesting that astrocytes have been shown to spontaneously display calcium responses in hippocampal and thalamic slices (Parri et al., 2001; Nett et al., 2002), as this suggests that the excitability of astrocytes is not purely reactive. Perhaps the most interesting consequence of astrocyte calcium signalling is the ability of astrocytes to signal back to neurons by releasing chemical messengers, referred to as 'gliotransmitters' (Bezzi and Volterra, 2001; Volterra and Meldolesi, 2005). A calcium-dependent exocytotic mechanism of gliotransmitter release by astrocytes similar to that seen in neurons is supported by considerable evidence, but is controversial (Newman, 2005). Astrocyte glutamate release is elicited by stimuli that evoke \([\text{Ca}^{2+}]_i\) increases in astrocytes and is blocked by agents that prevent them (Araque et al., 1998a; Araque et al., 1998b; Bezzi et al., 1998; Kang et
Cultured astrocytes express the molecular machinery for exocytosis and glutamate release from astrocytes is sensitive to blockers and stimulants that target neuronal exocytotic machinery (Volterra and Meldolesi, 2005). Slight differences in the expression of exocytotic machinery in neurons and astrocytes may explain why the kinetics of exocytosis in astrocytes is not as fast as in neurons, but is more sensitive to $[\text{Ca}^{2+}]_i$ (Bezzi et al., 2004; Kreft et al., 2004). The recent observation of clear synaptic-like microvessels (SLMV) in small groups beneath astrocyte plasma membrane adjacent to neuronal terminals and dendrites is considered direct evidence of vesicular exocytosis in these cells (Bezzi et al., 2004). Furthermore, $[\text{Ca}^{2+}]_i$ dependent exocytotic fusion of vesicles in cultured astrocytes has been demonstrated in culture by total internal reflection fluorescence imaging (Bezzi et al., 2004) and by membrane conductance measurements (Kreft et al., 2004; Zhang et al., 2004). Other mechanisms of gliotransmitter release through transmembrane channels and transporters have been reported, however these are generally not calcium dependent and are unlikely to occur in the intact brain under normal conditions (Volterra and Meldolesi, 2005). The release of gliotransmitters from astrocytes has been shown to elicit currents and calcium rises in adjacent neurons, as well as influence their synaptic function. The most studied gliotransmitter is glutamate, the main excitatory neurotransmitter in the central nervous system (Kandel et al., 2000). In astrocyte-neuron co-cultures, electrical, mechanical, and chemical stimulation of astrocytes results in excitatory currents (slow inward currents; SICs) and calcium transients in neighbouring neurons, which are blocked by AMPA and NMDA glutamate receptor antagonists (Parpura et al., 1994; Hassinger et al., 1995; Araque et al., 1998a;
Sanzgiri et al., 1999). In the same cultures, astrocyte stimulation also affects synaptic function by decreasing evoked excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs, respectively) and increasing the spontaneous mini EPSCs and IPSCs by activating metabotropic glutamate (mGluRs) and presynaptic NMDA receptors, respectively (Araque et al., 1998a; Araque et al., 1998b; Mauch et al., 2001). Furthermore, activation of astrocytes has been directly shown to result in glutamate release in culture (Zhang et al., 2004). Stimulating astrocyte calcium responses also elicits neuronal responses in intact tissue preparations. In brain slices, spontaneous and stimulated astrocyte \([\text{Ca}^{2+}]_i\) increases evoke inward currents and \([\text{Ca}^{2+}]_i\) transients in adjacent neurons that are blocked by glutamate receptor antagonists (Pasti et al., 1997; Bezzi et al., 1998; Parri et al., 2001; Fellin et al., 2004; Newman, 2005). Glutamate released by astrocytes has also been shown to modulate synaptic transmission by increasing the frequency of spontaneous AMPA receptor currents in CA1 pyramidal neurons (Fiacco and McCarthy, 2004). In hippocampal brain slices, \(\text{GABA}_B\) receptor stimulation of astrocytes at synapses between inhibitory interneurons and pyramidal neurons, potentiate this inhibitory neurotransmission (increasing the mIPSC frequency and therefore the success rate of postsynaptic responses) by stimulating glutamate receptors on the \(\text{GABA}_B\)ergic interneuron (Kang et al., 1998). Astrocyte glutamate release may also encourage neuronal synchrony of unrelated neurons by increasing postsynaptic neuronal excitability (simultaneously eliciting SICs) in non-contiguous neurons up to 100\(\mu\text{M}\) apart (Angulo et al., 2004; Fellin et al., 2004). Astrocyte glutamate release is not the only gliotransmitter whereby astrocytes have been shown to modulate neuronal activity \textit{in situ}. The release of ATP by astrocytes and its conversion to adenosine by
membrane bound econucleotidases has an inhibitory influence on synaptic transmission (Volterra and Meldolesi, 2005). In the retina, stimulation of Muller cells (a specialised class of astrocyte) increases the K+ conductance in adjacent ganglion cells evoking a hyperpolarizing response by releasing ATP which activates A1 adenosine receptors following its conversion by ectoenzymes (Newman, 2003). In the CA1 region of hippocampal slices astrocytes have been shown to induce heterosynaptic depression of (Schaffer collateral-pyramidal neuron) synapses by sensing glutamate release at one synapse, which evokes an astrocyte [Ca²⁺]ᵢ rise that elicits ATP release, and depresses, an adjacent synapse (Zhang et al., 2003). While the majority of this research has focused on the ability of astrocyte gliotransmitters to signal to neurons, astrocytes probably communicate to most or all elements in the brain. Accordingly, a rise of [Ca²⁺]ᵢ in astrocyte vascular endfeet has been shown to signal to cerebral vessels and influence vessel diameter by increasing arachadonic acid formation that is converted to 20-HETE in underlying vascular smooth muscle cells (Mulligan and MacVicar, 2004). Astrocytes can dynamically respond to neural activity with forms of [Ca²⁺]ᵢ signalling that appear to be directly linked to the release of gliotransmitters able to influence adjacent neural and vascular elements in the central nervous system. The intimate association of protoplasmic astrocyte processes and synapses brings thousands of synapses are under the influence of a single astrocyte. The signalling between astrocytes and neurons is likely to have an important role in information processing, justifying further study.
Prefrontal cortex-dopamine function and a role for

Astrocytes

Prefrontal cortex function (PFC)

The cortex of the human brain, like the spinal cord, is anatomically separated based on its motor and sensory function (Fuster, 2001). The posterior neocortex, which includes the parietal, occipital, and temporal lobes, is considered sensory cortex, while the anterior neocortex of the frontal lobe is considered motor cortex. The motor cortex is further divided into motor, premotor, and prefrontal areas as it extends rostrally from the central sulcus. Generally speaking, the rostro-caudal order of these areas corresponds to their functional hierarchy for the execution of action. At the bottom of this hierarchy, the primary motor cortex executes elementary muscular movements, defined by single muscles or muscle groups. Anterior and adjacent to the primary motor cortex, the premotor cortex is concerned with more complex programs of movements defined by acts or goals. Generally speaking, the major anterior portion of the frontal lobe, known as the prefrontal cortex, is at the top of this hierarchy and is concerned with the representation and execution of schemas, plans, and concepts of action (Fuster, 2000, 2001). The prefrontal cortex in the human brain is further divided into orbital, medial, and lateral areas; although the function of the prefrontal cortex is distributed throughout these areas, there appears to be some functional differences between them (Fuster, 2001). The orbital and medial PFC are generally involved in emotional behaviour. Maximally developed in the human, the lateral PFC is responsible for the temporal organization of behaviour. Damage to the lateral PFC is characterised by an inability to formulate and carry out
sequences or plans of action, constituting what is now known as ‘dysexecutive syndrome’ ((Baddeley, 1986) cited in (Fuster, 2001)). Due to the profound disorder of attention that accompanies lateral PFC damage, it has been proposed that this area is responsible for ‘supervisory attentional control’ ((Shallice, 1988) cited in (Fuster, 2001)). The lateral PFC integrates information over time to guide future action (Fuster, 2001). A good test of this PFC function is with delayed response tasks that require the subject to remember task dependent contingencies over a delay phase before using them to solve a task. During the delay phase of these tasks, many neurons from the PFC show sustained activity (Niki1971 and (Fuster, 1973) from E.Miller chapter, Kandel Book). This delay activity was only seen when future action was necessary, it was not due to the expectation of reward, and the level of activity was positively correlated with the accuracy of task performance. These properties suggested that delay active cells, ‘memory cells’, can hold task contingencies online during in the absence of sensory information, during a task delay. Memory cells are considered the substrate for working memory, which is the short-term memory or scratch pad the brain uses to keep information in mind to guide future actions. Temporal integration is made possible by retrospective and prospective working memory in the PFC which keeps information ‘online’ in the absence of the associated stimuli (Niki1971 and (Fuster, 1973) from E.Miller chapter, Kandel Book; (Quintana and Fuster, 1999)). The ability to hold prior sensory information and future motor memory in mind allows the PFC to determine the best course of future action. All brain functions require the involvement of diverse and distributed brain areas including executive functions. While many brain areas are involved, the PFC appears to have special properties that allow it to contribute uniquely and importantly to executive
function. Namely the ability to plan future action by integrating events over time using
the delayed activity of memory cells

Dopamine in the PFC

The entire prefrontal cortex receives afferents of the mesocortical dopaminergic
pathway from the relatively small ventral tegmental area (VTA) (Seamans and Yang,
2004). These afferents allow dopamine to play an essential role in the regulation of PFC
function, without them behaviours associated with the PFC become severely disrupted.
Disruption of PFC DA levels in humans results in neurological and psychiatric disorders
making it a clinical priority to understand the role dopamine plays in PFC function (Yang
et al., 1999). Studies in primates and rats found that selective depletion of PFC DA by 6-
hydroxy-dopamine (6OHDA) disrupts working memory and higher cognition, confirming
the importance of these afferents in the behaviour of these animals and allowing
investigators to determine how DA affects PFC function (Le Moal and Simon, 1991).
Animal studies using micr...
receipt of reward, but is instead increased in the PFC to *solve* delayed response tasks. In this way DA appears to be helping the PFC to ‘work with memory’ rather than store it (Seamans and Yang, 2004). The level of dopamine in the PFC is also critical for its effect on working memory task performance, more is not always better.

Reducing PFC dopamine by lesion studies or local injection of dopamine receptor antagonists into the PFC of non-lesioned animals disrupts performance on delayed-response tasks (Sawaguchi and Goldman-Rakic, 1991; Seamans et al., 1998), however high rates of PFC DA turnover induced pharmacologically and injections of D1 agonists also produce deficits in delayed response tasks (Murphy et al., 1996; Zahrt et al., 1997; Floresco and Phillips, 2001). This same dose response relationship of dopamine is also observed for the length of delay-period activity of PFC neurons. Using extracellular single-unit recording and microiontophoresis of DA in freely behaving primates, DA increased the delay-period activity of PFC neurons up to 200% of pre-DA levels (Sawaguchi et al., 1988; Seamans and Yang, 2004). The increased delay-activity due to DA was contrasted by a similar effect of low current iontophoresis of DA antagonists, which decreased delay-activity at higher amounts (Williams and Goldman-Rakic, 1995). These results from primates suggest that an optimal range of PFC dopamine is necessary for maximal delay-activity and performance in delayed response tasks; these results have also been demonstrated in rats. Using a spatial delayed response task, Phillips and colleagues showed that maximal task performance observed after a 30-minute delay corresponded to ‘optimal’ D1 receptor stimulation as the performance of rats was disrupted by either D1 agonists or antagonists (Floresco and Phillips, 2001). Furthermore, as the task delay is increased from 30min to ≥4hr, task performance and PFC DA levels
were shown to drop sharply, below the ‘optimum’ level of 30 minutes. In support of an ‘optimum’ level of PFC dopamine, the same microinjection of D1 agonist that disrupted performance at 30 minutes if delivered at up to a 12 hour delay was found to actually improve performance (Floresco and Phillips, 2001). Hence if DA levels are low then performance is improved until DA levels are pushed beyond the optimal range where performance decreases. DA concentrations outside of this optimum range negatively affect PFC function and consequently the relationship of [DA] and PFC function is described by an inverted-U function (Goldman-Rakic et al., 2000). The general role of PFC DA seems to be tuning the selectivity of working memory representations (Williams and Goldman-Rakic, 1995; Sawaguchi, 2001). DA concentrations at either side of the optimal level of DA adversely affects delayed response task performance, but do so in qualitatively different ways. Over activation of DA receptors during a spatial delayed response task is characterised by preservation of earlier choices, possibly due to over representation of earlier memory fields. Induced under activation of DA receptors results in random choices, presumably without sufficient DA to maintain memory fields (Seamans and Yang, 2004).

Despite many detailed investigations at the in vitro or in situ levels, the effect of dopamine on prefrontal neural activity is still not fully understood. The action of dopamine on PFC neurons is complex with little direct effect on membrane potential and it is generally accepted that DA is a neuromodulator and not a classic excitatory or inhibitory neurotransmitter like Glutamate, Acetylcholine, or GABA. DA has been shown to act as a neuromodulator, in the striatum and nucleus accumbens, by potentiating glutamate excitation at lower concentrations and suppresses glutamate inputs at higher
concentrations (Seamans and Yang, 2004). DA's effects are complex and depend on many factors. In a recent review of dopamine modulation of the PFC, Seamans and colleagues described 18 key features of dopamine action (Seamans and Yang, 2004). While the importance of dopamine to PFC function is well established, understanding its mechanism of actions is far from complete and requires further study.

**Dopamine and astrocytes**

Evidence for functional dopamine receptor expression on astrocytes has been accumulating for decades and the majority of this has come from studies of cultured cells. *In vitro*, astrocytes demonstrate D1 and D2 receptor specific binding (Hosli and Hosli, 1986) as well as mRNA and protein expression for all five recognised dopamine receptor subtypes (Zanassi et al., 1999; Reuss et al., 2000; Reuss and Unsicker, 2001; Miyazaki et al., 2004). Activation of astrocyte dopamine receptors in culture results in a variety of functional responses from these cells, including adenylate cyclase/PKA activation (Zanassi et al., 1999), cAMP generation (Hansson et al., 1984; Hansson and Ronnback, 1988; Vermeulen et al., 1994), changes in astrocyte input resistance or resting membrane potential (Ogura and Amano, 1983; Hosli et al., 1987; Mudrick-Donnon et al., 1993; Biedermann et al., 1995), increased $[Ca^{2+}]_i$ (Reuss et al., 2000), increased CREB phosphorylation (Miyazaki et al., 2004), astrocyte growth factor release (Kinor et al., 2001; Ohta et al., 2003), and changes in astrocyte morphology (Alonso et al., 2003).

These *in vitro* results strongly suggest that astrocytes are able to express functional dopamine receptors and while astrocyte receptor expression is heavily influenced by extracellular conditions and can change dramatically in culture (Kastritsis and McCarthy, 1993; Shao and McCarthy, 1994), *in vitro* DAR expression in astrocytes
is unlikely to be a purely a culture artifact. In a study of primary astrocyte cultures with seemingly identical culture conditions evidence was found for dopamine receptors in striatal, but not cerebellar astrocytes (Bal et al., 1994).

These studies also suggest that astrocyte dopamine receptor expression is regionally variable. Dopamine receptor innervation throughout the brain is heterogenous and it has been suggested that astrocyte dopamine receptor expression might reflect afferent/neurotransmitter heterogeneity (Bal et al., 1994). It is even possible that DA afferents are responsible for inducing/regulating astrocyte DA receptor expression themselves. Astrocyte dopamine receptor expression can affected by the level of extracellular dopamine as well as other factors such as fibroblast growth factor (Reuss et al., 2000).

The most inconsistent factor across in vitro studies is the reported expression of dopamine receptor subtypes. This inconsistency is demonstrated by two studies from Reuss and colleagues who originally reported the expression of both D1 and D2 dopamine receptor subtypes contrasted one year later by a paper reporting only D2 subtype dopamine receptor expression the same cells without any noticeable differences in their experimental protocol (Reuss et al., 2000; Reuss and Unsicker, 2001).

Astrocyte properties are highly dependent on extracellular conditions and the variability of in vitro results is likely to be due in part to differences in experimental methods. Even if the reported results were consistent, it is not always possible to predict astrocyte properties in living animals with certainty based on results in culture. To gain reliable insight into the interaction between dopamine and astrocytes it is necessary to rely on studies using intact tissue preparations. Although the evidence for
Astrocyte dopamine receptor expression in vivo is limited, good immunological and possibly function evidence (from both rats and primates) has been published (Svingos et al., 1999; Khan et al., 2001; Alonso et al., 2003). A study by Kahn and colleagues showed D2 receptor immunoreactivity co-localised with the astrocyte marker GFAP in primate prefrontal cortex by using light and electron microscopy (Khan et al., 2001). The same study found that one third of D2 receptor binding in primate cortex is located on astrocytes/glia. Dopamine expression has also been found in intact tissue from cortex and pituitary (Svingos et al., 1999; Alonso et al., 2003). Furthermore, the Kahn study also presented evidence to suggest that astrocyte dopamine receptors are functional by showing that exposure to a selective D2 receptor agonist results in intracellular calcium signalling in acutely isolated astrocytes. As astrocyte calcium responses are strongly associated with astrocyte modulation of neural information processing, and in view of the limited understanding the mechanisms of dopamine signalling in the prefrontal cortex and its importance for normal and pathological neural function, the role of astrocyte dopamine receptors is worthy of further investigation.

**Endothelin system**

The endothelin peptides (ET-1, ET-2, and ET-3) are 21 amino acid endogenous peptides known for their potent vasoactive properties (Davenport, 2002). Non-vascular roles of endothelin are not clear despite the widespread distribution of endothelin binding sites throughout the central nervous system (MacCumber et al., 1990; Niwa et al., 1991). The expression of the two endothelin receptor subtypes in the brain is generally found on
different cell types: ET\textsubscript{A} receptor mRNA is mainly in cerebral vascular smooth muscle cells and ET\textsubscript{B} receptor mRNA is predominantly on glia (Hori et al., 1992). Nanomolar concentrations of endothelin peptides elicit $[\text{Ca}^{2+}]_i$ elevations in cultured hippocampal astrocytes (Blomstrand et al., 1999) and cerebellar Bergmann glia in brain slices (Tuschick et al., 1997). The calcium elevations elicited by endothelin are comparable in size those observed during glial glutamate release (Zhang et al., 2004). Furthermore, exposure of cultured hippocampal astrocytes to nanomolar ET\textsubscript{1} elicited glutamate efflux acting through the ET\textsubscript{B} receptor (Sasaki et al., 1997). The demonstration of glial $[\text{Ca}^{2+}]_i$ elevations and glutamate release induced by endothelin peptides make endothelin receptor activation a good potential target to elicit astrocyte glutamate release \textit{in situ} by either a calcium dependent, or independent mechanism.

**General information on the endothelin system**

The mammalian endothelin system consists of three isopeptides, ET-1, ET-2, and ET-3, (Yanagisawa et al., 1988; Inoue et al., 1989) and two specific receptors, ET\textsubscript{A} and ET\textsubscript{B} ((Arai et al., 1990; Sakurai et al., 1990), respectively). ET-1 was the first element of the system to be characterised and was isolated from the vascular endothelium (Yanagisawa et al., 1988). Since this initial discovery, the entire endothelin system has been found throughout the mammalian vasculature where it has been shown to play a key role in vascular physiology. The endothelin system is also found in many non-vascular tissues including: kidney, lung, brain, endocrine organs, reproductive organs, and gastrointestinal tract (Highsmith, 1998). The physiological significance of endothelin in nonvascular systems is limited, although unique interactions have been revealed, notably in the lung, kidney and CNS. Endothelin plays an important role during mammalian
development (Baynash et al., 1994; Kurihara et al., 1994) and considerable evidence suggests that endothelin is important in the pathophysiology of many diseases, particularly in cardiovascular, pulmonary, and renal disease (Highsmith, 1998). The extensive expression and many proposed functions of the endothelin system underscore its importance in the body and justify further study, which has thus far been focused primarily on the molecular biology and vascular physiology of endothelin.

The endothelin system is more extensively understood at the molecular level. The three endothelin isopeptides arise from distinct genes encoding precursor proteins (~200 amino acids) that are cleaved by proteases to inactive 'big endothelins' (Bloch et al., 1989a; Bloch et al., 1989b). A final unique cleavage that releases the active 21 amino acid isopeptides is attributed to specific endothelin-converting enzymes (ECEs) (Yanagisawa et al., 1988). It is generally accepted that ET-1 and ET-2 are produced by intracellular ECE activity (Russell and Davenport, 1999; Lambert et al., 2000), however this has not been shown for ET-3 (Warner, 2004). Once processed, ET1 is primarily released through constitutive secretory pathways (Haynes and Webb, 1994) but can also be released via a regulatory pathway, which is unusual for mammalian bioactive peptides (Russell et al., 1998). In the regulated pathway, ET-1 is released from endothelial cell-specific storage granules (Weibel-Palade bodies) in response to external physiological, or possibly pathological stimuli (Russell et al., 1998). The nature of the stimuli necessary to induce regulated ET-1 release is not clear; however the predominant constitutive release of ET-1 means that peptide synthesis and therefore release is largely dependent on the level gene transcription. Once released into the extracellular space, endothelin is thought to act as an autocrine or paracrine factor because serum levels are too low to activate ET
receptors (<5pM) at distant sites and plasma ET is rapidly degraded and cleared, with a half life on the order of roughly 1 minute (Anggard et al., 1989; Highsmith, 1998). Endothelin peptides elicit their effects through seven transmembrane g-protein coupled receptors (GPCRs) and two endothelin receptor subtypes have been fully characterised in mammals (Arai et al., 1990; Sakurai et al., 1990). The ET$_A$ receptor binds ET peptides in order ET-1 ~ ET-2 > ET-3 ($K_i$ ~ 20-60pM > 6500pM), while the ETB receptor binds ET peptides with equal affinity ($K_i$ ~ 15pM) (Takayanagi et al., 1991). Under physiological conditions, these binding affinities mean the ET-3 peptide is selective for the ET$_B$ receptor while ET-1 and ET-2 bind both receptors equally (Davenport, 2002). Endothelin receptors can couple to multiple g-proteins ($G_{q1}$, $G_{111}$, $G_6$ and $G_{12}$) (Aramori and Nakanishi, 1992; Takigawa et al., 1995) and activate multiple signal transduction pathways and including phospholipase (PL)A$_2$, PLC and PLD, as well as cytosolic protein kinases (PKC) (Pollock & Highsmith 1998). Specific signalling pathways of ET receptor subtypes may be shared, or distinct depending upon the cell-type, species, and post-translational endothelin receptor modifications such as palmitoylation (Highsmith, 1998). The most common consequence of activation of either endothelin receptor subtype is an increase in intracellular calcium (Pollock et al., 1995). Calcium responses are usually bi-phasic, starting with an initial fast phase due to PLC induced inositol hydrolysis that results in the release of IP$_3$ gated intracellular calcium stores. This primary cytosolic calcium rise results in a secondary [$Ca^{2+}]_i$ response dependent upon the influx of extracellular [$Ca^{2+}]$ through voltage gated calcium channels and capacitative calcium entry (Pollock et al., 1995; Tuschick et al., 1997). The functional outcome of ET signalling depends on the cell type, the tissue, and the stage of development.
Given the widespread expression of endothelin receptors, it is not surprising that research has discovered many important developmental, physiological, and pathological roles of endothelin (Warner, 2004). During development, selective disruption of either the ET-1 or the ET\textsubscript{A}-receptor results in failed craniofacial tissue formation and elevated blood pressure (Kurihara et al., 1994; Clouthier et al., 1998), while similar disruption of either ET-3 or the ET\textsubscript{B}-receptor results in failed gastrointestinal development with aganglionic megacolon (Baynash et al., 1994; Hosoda et al., 1994). In adult mammals, the endothelin system has different functions in different organ systems and the majority of research efforts have focused on its role in the vasculature. ET-1 is the most ubiquitous, potent, and long lasting endogenous vasoconstrictor known in mammals (Yanagisawa et al., 1988). This potent vasoconstrictor was first reported in the culture medium of bovine endothelial cells (ECs) (Hickey et al., 1985). Endothelin receptors in vascular smooth muscle cells (vSMCs) are predominantly the ET\textsubscript{A} subtype (85%) (Davenport et al., 1995), which is largely responsible for ET-1 induced vasoconstriction (Maguire and Davenport, 1995). The only expression of ET\textsubscript{A} receptors on endothelial cells is in the cerebral vasculature (Stanimirovic et al., 1994) where they are suggested to increase capillary permeability following ET-1 exposure (Purkiss et al., 1994).

Endothelin-B receptors are widely expressed on the endothelium and their stimulation releases endothelium derived relaxing factors such as nitric oxide and prostanoids that cause the relaxation of underlying vSMCs (Warner et al., 1989). Constitutive release of ET-1 from endothelial cells is thought to maintain vascular tone by acting in a paracrine fashion on ET\textsubscript{A} receptors in the underlying smooth muscle (Albassam et al., 1999). ET release by ECs can also act in an autocrine fashion through ET\textsubscript{B} receptors to produce
endothelium derived relaxing factors that counteract excessive vasoconstriction (de Nucci et al., 1988). By acting through both endothelin receptor subtypes, bolus intravenous injections of ET-1 typically result in a brief, small vasodilation followed by a strong, prolonged vasoconstriction. The endothelin system therefore is an important bi-directional modulator of vascular tone and its disregulation has been implicated in the pathophysiology of vascular diseases including hypertension (Highsmith, 1998). The vasoconstrictive properties of endothelin also have protective functions by preventing excessive bleeding or the spread of infection (Warner, 2004). In the kidney the vasoconstrictive action of endothelin is an order of magnitude greater than angiotensin II (Cairns et al., 1989). ET-1 causes an initial transient increase in glomerular filtration rate (GFR) in some species and dose dependent decreases in GFR at higher concentrations (Warner, 2004). At low concentrations ET-1 can also induce natriuresis and (Clavell et al., 1995), allowing ET-1 to control sodium and water handling in the kidney. The lung has some of the highest concentrations of ET-1 (Matsumoto et al., 1989) and a major source of ET-1 is from airway epithelial cells (MacCumber et al., 1989; Rozengurt et al., 1990). Endothelin can mimic asthma by causing potent and sustained contractions of airway smooth muscle (Uchida et al., 1988) and by potentiating neurogenic contraction of airway smooth muscle (Fernandes et al., 1996). In the endocrine organs, ET-1 is thought to stimulate aldosterone release from the adrenal gland (Naruse et al., 1994) and to have an autocrine role in the regulation of antidiuretic hormone release in the neurohypophysis (Yoshizawa et al., 1990). The endothelin system is expressed in the supporting cells of the male reproductive system (Ergul et al., 1993) and endothelin can induce uterine contractions in females (Tsunoda et al., 1993). In the gastrointestinal (GI) system, the
ET-2 is known as vasoactive intestinal contractor (VIC) and is found in high concentrations in GI smooth muscle (Highsmith, 1998) where ET_A and ET_B are found in enteric and myenteric neurons (Eaker et al., 1995).

ET-1 and ET-3 peptides are detected throughout the mammalian brain. Immunoreactivity for ET-1 is found in the 17 non-human and 24 human brain regions examined, namely: the cerebral cortex, diencephalon, brainstem, basal nuclei, cerebellum, limbic system, pineal and pituitary glands (Giaid et al., 1989; Matsumoto et al., 1989; Yoshizawa et al., 1989; Lee et al., 1990; MacCumber et al., 1990; Yoshizawa et al., 1990; Giaid et al., 1991; Takahashi et al., 1991a, b; Naidoo et al., 2004a). ET-1 mRNA has been detected by in situ hybridization and RT-PCR in 22 of the 24 human brain regions where ET-1 immunoreactivity is detected (Giaid et al., 1989; Lee et al., 1990; Giaid et al., 1991; Naidoo et al., 2001) as well as several brain regions in porcine and rodent brain (Giaid et al., 1989; MacCumber et al., 1989; MacCumber et al., 1990; Yoshizawa et al., 1990). ET-3 expression is not as widely reported as ET-1 expression possibly because sources of ET-3 in the brain have not been studied as extensively.

Immunoreactivity for ET-3 is abundant in the rat brain, particularly in the neostriatum, hypothalamus, and pituitary (Matsumoto et al., 1989; Shinmi et al., 1989; Lee et al., 1990; Fuxe et al., 1991; Takahashi et al., 1991a) and in the human brain is reported in the hypothalamus and the pituitary (Takahashi et al., 1991a). ET-3 mRNA is found in the rodent brain (Shiba et al., 1992) as well as in human pituitary and hypothalamus (Bloch et al., 1989a; Takahashi et al., 1991a). Although ET-3 is abundant in the brain, with the exception of the posterior pituitary, it is reported in lesser amounts compared to the ET-1 (Matsumoto et al., 1989; Takahashi et al., 1991a). Despite an apparently smaller profile,
ET-3 is considered the major extra-vascular ET within the brain (Barnes and Turner, 1997; Highsmith, 1998) because a considerable fraction of cerebral ET-1 is from a vascular source while ET-3 does not appear to be released by the cerebral vasculature (Stanimirovic et al., 1994). Accordingly, ET-3 is 150% higher than ET-1 in human CSF (Ando et al., 1991). More ET-3 in the brain might also be due to the extrapolation of misrepresentative culture results. Regardless of which isopeptide is predominant, their presence throughout the brain and inability to cross the blood-brain barrier (Koseki et al., 1989), suggest that these peptides have non-vascular sources and functions in the brain. Cultured neurons can synthesise and release ET-1 (MacCumber et al., 1990; Franco-Cereceda et al., 1991; Maggi et al., 2000), but whether they synthesise ET-3 has not been determined. In intact tissue, ET-1 mRNA and immunoreactivity have been observed in many neuronal cell types in rat, porcine, and human brain (Giaid et al., 1989; Shinmi et al., 1989; Lee et al., 1990; Yoshizawa et al., 1990; Giaid et al., 1991; Naidoo et al., 2001; Naidoo et al., 2004a). Conversely reports of neuronal ET-3 expression are limited to neostriatal neurons (Fuxe et al., 1991). Cultured astrocytes synthesise and release both ET-1 and ET-3 in primary culture (MacCumber et al., 1990; Ehrenreich et al., 1991; Ehrenreich et al., 1993), but do not appear to be a major source of endothelin peptides in the normal brain (Jiang et al., 1993; Nie and Olsson, 1996; Schmidt-Ott et al., 1998). ET-1 and ET-3 immunoreactivity is markedly increased in reactive astrocytes following injury or disease (Jiang et al., 1993; Yamashita et al., 1993; Nie and Olsson, 1996). It is possible that observed in vitro endothelin peptide expression represents the reactive astrocyte phenotype (Nie and Olsson, 1996). The cellular sources of endothelin peptide expression in the brain are still not clear and conflicts in expression are seen with
different tissue sources. In Purkinje cells from human post-mortem brain tissue (obtained between 6 and 22 hours after death) ET-1 immunoreactivity was widely observed in the cell soma (Naidoo et al., 2001), but single cell RT-PCR of the same cells in fresh rodent brain slices found no evidence of any ET mRNA (Schmidt-Ott et al., 1998). Assuming this discrepancy is not due to a species difference, it shows the influence different tissue preparations have on the expression of endothelin peptides, making it difficult to come to conclusions about their cellular sources. The localization of ET synthesizing enzymes is also indicative of the cellular sites of ET synthesis in the brain. Accordingly, ECE-1 immunoreactivity has been widely observed in neural cell types from rat and human brain (Giaid et al., 1991; Barnes et al., 1997; Nakagomi et al., 2000; Naidoo et al., 2004b; Warner, 2004). Glial ECE expression is intense in the fibres of the glial limitants and is detected to a lesser degree in astrocytes from grey and white matter, but is not seen in oligodendrocytes (Schmidt-Ott et al., 1998; Warner, 2004). Receptors for endothelin peptides are widely expressed in the mammalian brain. In healthy human and rodent brain the ET\textsubscript{B} receptor subtype accounts for about 90\% of endothelin receptors in the cerebral cortex (Hori et al., 1992; Harland et al., 1995; Naidoo et al., 2004b). ET\textsubscript{B} receptors are prominently expressed in glia as well as epithelial cells of the choroid plexus and ependymal cells lining the ventricles. A smaller number of ET\textsubscript{A} receptors are found on the pial surface and microvessels of the brain with much lower, but detectable, levels in the grey and white matter (Hori et al., 1992; Harland et al., 1995). Generally speaking, neuronal endothelin receptors in the brain parenchyma are the ET\textsubscript{A} subtype and glial receptors are the ET\textsubscript{B} subtype (Morton and Davenport, 1992; Pomonis et al., 2001). The functional significance of the endothelin system in the brain is poorly understood.
although a diversity of functions reported is not surprising considering its extensive
distribution in the brain. In the cerebral vasculature, endothelin peptides help to maintain
vascular tone through either constriction or dilation of the vasculature. In brain areas
involved in the regulation of circulatory and renal function, the localization of endothelin
components is considered evidence that ET has a role in the CNS control of fluid-volume
status and blood pressure (Ouchi et al., 1989; Mosqueda-Garcia et al., 1993; Highsmith,
1998). Since the localisation of endothelin peptides is within neurons it has been
suggested that endothelin may have a role in neurotransmission by acting as a
neuropeptide (Giaid et al., 1989) or by affecting neuronal excitability (Yoshizawa et al.,
1989). Endothelins can stimulate the release of glutamate from striatal brain slices
(Koizumi et al., 1994) and dopamine from the rat striatum in vivo (van den Buuse and
Webber, 2000); endothelin also inhibits the spontaneous release of norepinephrine from
neurons in the hypothalamic slices (Di Nunzio et al., 2002). Endothelin has recently been
shown to bi-directional modulate pain perception in the spinal cord by directly
stimulating sensory neurons through ETA receptors while indirectly inhibiting
(hyperpolarizing) the same neurons by eliciting opioid release from adjacent glial cells
through ETB receptors (Khodorova et al., 2003). Endothelins cause the mobilization of
several signal transduction pathways in glial cells, invariable leading to an increase in
intracellular calcium (Blomstrand et al., 1999) resulting in many biological effects
including: increased glucose uptake (Tabenero et al., 1996), glutamate efflux (Sasaki et
al., 1997), stimulation of proliferation and mitogenesis (MacCumber et al., 1990;
Stanimirovic et al., 1995), immediate early gene expression (Schinelli et al., 2001), actin
reorganization (Koyama and Baba, 1994), reactive gliosis (Baba, 1998), and potent gap
junctional uncoupling (Blomstrand et al., 1999). The physiological or pathological significance of the extravascular endothelin system in the brain is still not clear; however its extensive expression and numerous biological effects are a promising avenue for future research.

Hypotheses and Objectives

Calcium imaging has radically changed our perception of the roles of astrocytes by revealing the two-way communication between astrocytes and neurons. However the function of bidirectional interactions between astrocytes and neurons is still far from understood.

Despite the ubiquitous presence of glial cells in the CNS, studies examining the communication between neurons and astrocytes in many brain areas, including the cortex, have been relatively sparse. The influence of the neurotransmitter dopamine on the function of the prefrontal cortex (PFC) is both elusive and complex (Testa et al., 2005), however the actions of dopamine in this area are almost exclusively focused on neural, not astrocytic, responses. Dopamine receptors are expressed on astrocytes from the prefrontal cortex, but the effect of dopamine on astrocyte calcium in intact tissue, which is important for astrocyte-neuron communication, has not been demonstrated. To understand how dopamine affects PFC function, we investigated how dopamine affects the \([\text{Ca}^{2+}]_i\) of astrocytes in PFC brain slices.

Additionally, studies to date investigating the influence of glial glutamate release on synaptic transmission have focused on changes at the level of single cells (Araque et al., 1998a; Araque et al., 1998b; Kang et al., 1998; Newman and Zahs, 1998; Fiacco and
McCarthy, 2004); as a result, the influence of glial glutamate on the activity of large neural assemblies is not known. To investigate this, we tested whether widespread \([\text{Ca}^{2+}]_i\) elevations elicited by the vasoactive peptide endothelin in hippocampal astrocytes \textit{in situ} could acutely influence synaptic transmission of neural assemblies measured by glutamate-sensitive extracellular field potentials.

\textbf{Hypothesis 1:} Dopamine receptor activation evokes calcium transients from prefrontal cortex astrocytes in intact tissue.

\textbf{Objective:} Image astrocyte \([\text{Ca}^{2+}]_i\) in prefrontal cortex brain slices to determine whether bath exposure to dopamine elicits intracellular calcium responses.

\textbf{Hypothesis 2:} Widespread astrocyte calcium transients can be elicited by endothelin peptides in intact tissue and can influence the synaptic transmission of an entire brain region.

\textbf{Objective:} Image astrocyte \([\text{Ca}^{2+}]_i\) in hippocampal slices during bath perfusion of endothelin peptides to confirm widespread \([\text{Ca}^{2+}]_i\) transients in intact tissue. Determine whether widespread astrocyte \([\text{Ca}^{2+}]_i\) responses are associated with a change in synaptic transmission by measuring extracellular field recordings from hippocampal brain slices.
Summary

Astrocytes are well suited to support neural function and while extensive, ever-increasing literature supports this role, the extent that astrocytes influence of neural function is still an open question. An important aspect of astrocyte-neural interactions is the direct control of synaptic transmission by perisynaptic astrocytes. The synapse is a focal point for understanding the mechanisms of neural information processing. Studying the influence of protoplasmic astrocytes processes, which surround the synapse, holds promise for a more complete understanding of information processing.

The two-way communication between synaptic and glial elements is the objective of this thesis and consequently the protoplasmic astrocytes, which are intimately associated with synapses by their perisynaptic processes, are the object.
Chapter 2: Dopamine elicits \([\text{Ca}^{2+}]_i\) transients from astrocytes in prefrontal brain slices by activating adrenergic receptors.
Astrocytes have been shown to express myriad neurotransmitter receptors *in vitro* and in intact tissue. By expressing neurotransmitter receptors, astrocytes can directly respond to neural activity, which raised the possibility that these cells are involved in information processing. Astrocytes in the prefrontal cortex have been shown to express receptors for dopamine, an important neurotransmitter in this region. To understand how astrocytes might be involved in dopamine signalling in the prefrontal cortex, we investigated the ability of dopamine to elicit $[\text{Ca}^{2+}]_i$ transients from astrocytes in prefrontal cortex brain slices.

Bath perfusion of dopamine [50μM] elicited $[\text{Ca}^{2+}]_i$ transients from 41% of cells visualized and 71% of slices exposed compared to a lower concentration of dopamine [10μM] which only elicited responses from 4% of cells and 11% of slices exposed. $[\text{Ca}^{2+}]_i$ transients to [50μM] dopamine were not prevented by pre-incubating slices with the Na$^+$ channel blocker TTX, which suggests that astrocyte responses were not subsequent to activation of neuronal receptors. Astrocyte $[\text{Ca}^{2+}]_i$ transients were due to the activation of adrenoreceptors, not dopamine receptors. Dopamine receptor agonists were unable to elicit astrocyte responses in slices that were responsive to [50μM] dopamine, despite comparable activation of dopamine receptors. Astrocyte $[\text{Ca}^{2+}]_i$ transients were not prevented by antagonists for dopamine receptors, but were blocked by preincubation of responsive slices with $\alpha_1$- and $\beta$-adrenoreceptor antagonists.

In addition to its effects on astrocyte $[\text{Ca}^{2+}]_i$, dopamine [50μM] caused the constriction of pial and penetrating arterioles in the superficial layers of rodent prefrontal cortex slices. This constriction was fully reversed by $\alpha_2$-adrenoreceptor antagonists, but unaffected by antagonists for dopamine receptors.
In conclusion, astrocyte $[\text{Ca}^{2+}]_i$ transients and vessel constrictions elicited by dopamine in the prefrontal cortex are due to activation of adrenoreceptors not dopamine receptors, in disagreement with previous reports. The inability of more physiological concentrations of dopamine to elicit astrocyte $[\text{Ca}^{2+}]_i$ transients questions their ability to rapidly respond to changes in dopamine in the prefrontal cortex.
Introduction

Astrocytes have historically been considered structural and metabolic support cells for neurons, however reports of these cells directly communicating with neurons and influencing information processing has changed this passive view. Astrocytes are able to communicate with neighbouring neurons by expressing myriad neurotransmitter receptors and by releasing neurotransmitters themselves (Porter and McCarthy, 1997; Volterra and Meldolesi, 2005). Accordingly, astrocytes have been shown to enhance, depress, and synchronize synaptic transmission (Volterra and Meldolesi, 2005). When exposed to neurotransmitters directly, or indirectly during neural activity, astrocytes respond with elevations in their intracellular calcium that vary dramatically in their pattern and extent (Volterra and Meldolesi, 2005). While many studies of astrocyte 

$[\text{Ca}^{2+}]_i$, responses to neurotransmitters have been made in vitro, culture conditions alter the properties of these cells. The responses of astrocytes to neurotransmitters in an intact tissue are more likely to represent in vivo responses, but not all neurotransmitter responses of astrocytes have been investigated in intact tissue preparations. Accordingly, further studies of astrocyte responses to neurotransmitters in intact environments are necessary to determine the extent and types of functional relationships between neuron and astrocytes in vivo.

Working memory processes in prefrontal cortex (PFC) are central to the executive functions of the frontal lobes (Fuster, 2000). Accuracy in working memory tasks is dependent on the release of dopamine into the PFC by mesocortical afferents (Robbins, 2000) and is optimal when dopamine receptor occupancy is within a critical range of an
inverted U-shaped function (Zahrt et al., 1997; Floresco and Phillips, 2001; Seamans and Yang, 2004). Despite considerable research, the cellular mechanisms by which dopamine affects PFC function are not clear. While most of this work has been concerned with the subtle modulatory effects of dopamine on PFC neurons (Seamans and Yang, 2004), the effects on non-neuronal elements have received considerably less attention. Astrocyte fractions from the cortex of rodent, monkey, and human brains comprise roughly one third of the cortical D2-dopamine receptor binding sites and *in vivo* immunoreactivity for these dopamine receptors has been demonstrated on perineuronal astrocyte processes in the monkey PFC (Khan et al., 2001). Activation of astrocyte dopamine receptors *in vitro* results in multiple responses including cAMP generation (Hansson et al., 1984; Zanassi et al., 1999; Facchinetti et al., 2004), membrane potential changes (Hosli et al., 1987), growth factor release (Kinor et al., 2001; Ohta et al., 2003), and intracellular calcium responses (Reuss et al., 2000; Khan et al., 2001). Despite the variety of responses elicited from astrocytes by dopamine, its effects on astrocytes in intact tissue have not been investigated. Understanding how astrocytes respond to dopamine in the intact PFC is necessary for a complete understanding of its influence on prefrontal cortex function.

To study the effect of dopamine in prefrontal astrocytes *in situ* we used two-photon laser scanning microscopy to record astrocyte $[Ca^{2+}]_i$ levels in PFC brain slices during dopamine perfusion (6 min). Exposure of prefrontal slices to [50μM] dopamine frequently elicited $[Ca^{2+}]_i$ transients from astrocytes in the superficial cortical layers that were unaffected by pretreatment with the Na$^+$ channel blocker TTX (1.2μM). In contrast, [10μM] dopamine perfusion rarely had an effect on astrocyte $[Ca^{2+}]_i$; despite being a saturating concentration for D1 and D2 receptors *in vitro* (Tiberi and Caron, 1994;
Moreland et al., 2004). Incubating brain slices with specific receptor antagonists revealed that adrenoreceptors, not dopamine receptors, were responsible for the astrocyte $[\text{Ca}^{2+}]_i$ transients elicited by dopamine [50μM]. Dopamine exposure also elicited vessel constrictions of pial and penetrating vessels in the same area by activation of $\alpha_2$-adrenoreceptors. The activation of vascular and astrocyte adrenoreceptors by high dopamine concentrations in the prefrontal cortex may partially underlie the disruption of working memory processes at higher PFC dopamine receptor occupancy, or even neuropathology of the frontal cortex associated with excessive prefrontal dopamine release.
Methods & Materials

Brain slice preparation and AM-ester dye loading

In accordance with institutional guidelines, brain slices (Fig1) were prepared from male Sprague Dawley rats (postnatal days 13-21). Rats were deeply anesthetized with halothane and rapidly decapitated. Brains were quickly removed from the skull and cooled for 2 minutes in 0-4°C sucrose cutting solution (in mM): sucrose (230), KCl (2.5), NaHCO₃ (26), glucose (10), MgSO₄ (10), NaH₂PO₄ (1.25), and CaCl₂ (0.5), bubbled with O₂/CO₂ (95/5%). Cooled brains were ‘blocked off’ with an oblique cut to minimize the extent of transected dendrites from deep layer prefrontal pyramidal neurons and to reduce cutting time as described previously (Yang and Seamans, 1996). Prefrontal cortex (PFC) brain slices (350-400μm thick) were cut with a vibratome (Leica VT1000S) and transferred to a room-temperature incubation chamber containing oxygenated artificial cerebral spinal fluid, aCSF (in mM): NaCl (126), KCl (2.5), NaHCO₃ (26), glucose (10), MgSO₄ (2), NaH₂PO₄ (1.25), and CaCl₂ (2), bubbled with O₂/CO₂ (95/5%). Slices were allowed to recover for at least 30 minutes before incubation with calcium sensitive dye.

After recovery, slices used for imaging were incubated for 1½-2 hours in aCSF containing the membrane permeable, AM-ester form, of the Ca²⁺ indicator Rhod-2 AM (10μM), Molecular Probes (Eugene, OR) (final DMSO concentration: 0.3%). Rhod-2 incubation has been shown to preferentially load astrocytes in rodent brain slices (Fig1), demonstrated previously by co-localization of Rhod-2 with green fluorescence protein driven by the astrocyte promoter for glial fibrillary acidic protein (Mulligan and MacVicar, 2004). Loaded slices were transferred to a recording chamber which was
Figure 2.1 Loading of the fluorescent calcium indicator Rhod-2 AM in astrocytes from the superficial layers (I-II) of the prefrontal cortex.

(A) A rectangle surrounding an isolated rat brain indicates the brain region and plane from which cortical brain slices were prepared. The arrow points to a schematic of a coronal cross section representative of slices prepared to isolate the prefrontal cortex. Black rectangles on the left hemisphere of the schematic near the midline of the slice indicate the range of cortical fields imaged. No preference was made for hemisphere in experiments. (B) A transmitted light image taken with a 2.5x objective showing the midline of a prefrontal slice from a 14 day old rat. (C) A transmitted image taken with a 40x objective focused 60μm below the slice surface showing a penetrating arteriole and the surrounding parenchyma. C and D were taken from the visual field demarcated in B with an orange outline. (D) Rhod-2 fluorescence in a two dimensional projection of an image stack (20 images separated by 1μm Z-plane step) from the same field as C. Arborized cell bodies with projections extending to the walls of nearby vessels is typical of protoplasmic astrocyte morphology, (mean cell body diameter: 6.5μm±0.23). Images in B-D were taken after 1.5 hours of dye loading. (Scale bar = 20μm)
gravity perfused with oxygenated aCSF at a rate of 1-2mL per minute, maintained at
25±0.5°C with an inline heater (Warner instruments). Slices were allowed to settle for
≥20 minutes in the recording chamber before use.

**Two photon calcium imaging**

Imaging was performed using a two-photon laser-scanning microscope (Zeiss-
LSM 510 scan head coupled to a Zeiss-Axoscope-2 fitted with a 40X-W/0.80NA
objective lens) directly coupled to a Ti:sapphire laser (Mira;Coherent) providing
~100fsec pulses, at ~80MHz, pumped by a 5W laser source (Verdi;Coherent). Rhod-2
fluorophores were excited at a wavelength of 835-840nm and the epifluorescence was
detected by an external (non descanned) detector with a 605nm (55nm bandpass) filter.
Photodynamic damage was reduced by using an acoustic optical modulator, AOM (Zeiss)
to attenuate the laser intensity to the minimum necessary to achieve adequate signal-to-
noise. Images were acquired from the superficial layers of the medial prefrontal cortex,
typically 60-120μm from the cut surface to avoid slice damage.

**Analysis of calcium signals and vessel diameter changes**

Images were analysed offline using Zeiss LSM (version 3.2) software.
Fluorescence signals were converted to relative fluorescence changes over time and
expressed in percentages; defined as \( \Delta F/F_0 = ((F_1-B_1)-(F_0-B_0))/(F_0-B_0) \), where \( F_1 \) and
\( F_0 \) are fluorescence in the loaded astrocyte soma at a given time point or at the beginning
of an experiment, respectively, and \( B_1 \) and \( B_0 \) are the background fluorescence at a given
time point or at the beginning of an experiment, respectively. Background values were
taken concurrently from an adjacent area free of loaded cells, or processes. Only dye
loaded cell bodies in the plane of two photon excitation were used to quantitate calcium changes to avoid any differences in calcium changes that might arise from tissue movement. Individual cells were considered responsive if they had a fluorescence change ($\%\Delta F/F_0 \geq 50$). To account for spontaneous astrocyte calcium transients, brain slices were only considered responsive if $\geq 20\%$ of dye loaded cells in the field of view were responsive.

Pial surface vessels at the cortical midline and arterioles penetrating into superficial cortical layers were visualized using infrared differential contrast (IR-DIC) optics. Vessels were selected by the appearance of smooth muscle cells and a healthy appearance over an extended region; collapsed vessels were excluded. Vessel diameter changes were obtained by IR-transmitted image detection with an external PMT. Image series were analyzed off-line with Zeiss (version 3.2) software by measuring peak diameter changes compared to a stable control period.

**Statistics and drugs used**

Sample means were reported +/- the standard error of the mean (SEM). Statistically significant differences between population means were assessed using the Students t-test with a confidence level of $p<0.05$ unless indicated otherwise.

Dopamine, SKF38393, Quinpirole, SCH23390, Sulpiride, Norepinephrine, Propranolol, Prazosin, and Yohimbine were obtained from Sigma-Aldrich (St. Louis, MO). All drugs were prepared fresh, within 2-5 minutes of use with the exception of SCH23390 which was stored as frozen aliquots until being thawed and vortexed prior to use. Dopamine, norepinephrine, quinpirole, SKF38393, SCH23390, and Yohimbine were dissolved in dH$_2$O, Sulpiride and Propranolol were dissolved in ethanol and
Prazosin was dissolved in methanol; all drugs were diluted 1000 fold prior to their use.

Brain slices were continually exposed to antagonists for at least 10 minutes prior to agonist exposure; exposure to agonists was approximately 6 minutes.
Results

Dopamine elicits calcium responses from astrocytes in the superficial layers of mPFC brain slices.

Bath perfusion of dopamine (10 to 50μM) elicited calcium transients in dye loaded astrocytes from the superficial cortical layers of mPFC brain slices (Fig2). Elicited astrocyte calcium transients were dose dependent and were rarely seen during [10μM] dopamine perfusion. During exposure to DA[10μM], 11% of slices responded, with 4±3% of cells responding per slice when both responsive and non-responsive slices were combined (115cells, 9slices, Fig2D,E). During [50μM] dopamine exposure, 71% of slices responded, with 41±5% of cells responding per slice when both responsive and non-responsive slices were combined (444cells, 31slices, Fig2D,E). In cells that responded to dopamine there was no significant difference between the size of the calcium transients elicited by either concentration of dopamine used, DA[10μM]: %ΔF/F0 154±29 (4cells, 1slice), DA[50μM]: %ΔF/F0 176±6 (173cells, 22slices), (Fig2F).

To determine whether the observed astrocyte calcium responses were dependent on activation of neural receptors, we blocked action potential dependent neural signaling by pretreating slices with the Na⁺ channel blocker tetrodotoxin (TTX, 1.2μM) prior to dopamine exposure. Compared to dopamine exposure without TTX, blocking Na⁺ channels did not significantly change the percentage of cells per slice that responded to [10μM] or [50μM] dopamine, 1.9±1.3% (2/94cells, 7slices) and 25.3±7.3% (20/88cells, 7slices), respectively (Fig2H). In the presence of TTX, the percentage of responding cells per slice during [50μM] dopamine exposure was significantly larger than during exposure
Figure 2.2 Dopamine elicits \( [\text{Ca}^{2+}]_i \) transients in astrocytes from superficial layers of the prefrontal cortex that are independent of \( \text{Na}^+ \) channel activation.

(A) Example of fluorescence changes (\( \Delta F \), pseudocolor) in Rhod-2 loaded astrocytes at five time points before and during bath perfusion of 10 and 50\( \mu \)M dopamine. (B,C) Representative \( [\text{Ca}^{2+}]_i \) elevations from Rhod-2 loaded astrocytes responding to bath perfusion of dopamine (10 and 50\( \mu \)M). Images in A correspond to fluorescence changes during dopamine perfusion shown in B. For traces, normalized fluorescence changes are expressed as \( \Delta F/F_0 \); scale bar = 2min. (D) The likelihood of a slice responding to dopamine was dose dependent and was not always observed; DA[10\( \mu \)M]: 11% of slices responded (1/9slices), DA[50\( \mu \)M]: 71% of slices responded (22/31slices). (E) The percentage of cells responding per slice to 10\( \mu \)M dopamine (4%±3 of cells responding per slice, 9slices) was significantly lower than the percentage of responding cells during exposure to 50\( \mu \)M dopamine (41%±5 of cells responding per slice, 31slices) (p>0.05). (F) In the cells that did respond to dopamine, there was no significant difference in the normalized peak change of astrocyte \( [\text{Ca}^{2+}]_i \), DA[10\( \mu \)M]: \%\( \Delta F/F_0 \) 154±29 (4cells, 1slice), DA[50\( \mu \)M]: \%\( \Delta F/F_0 \) 176±6 (173cells, 22slices) (p<0.05). (G-I) To prevent action potential dependent neural signaling, slices were continuously exposed to the \( \text{Na}^+ \) channel blocker tetrodotoxin (TTX, 1.2\( \mu \)M) ≥10 min prior to 6min bath perfusion with dopamine. (G) Astrocyte \( [\text{Ca}^{2+}]_i \) transients observed in response to dopamine resembled those seen without TTX (B). Normalized fluorescence changes are expressed as \( \Delta F/F_0 \), scale bar = 2min. (H) The percentage of cells per slice that responded to 50\( \mu \)M dopamine was lower, but not significantly different in the presence of TTX; DA[50\( \mu \)M] alone:
41±5% of cells responding per slice, (444cells, 31slices); DA[50M] with TTX: 25.3±7.3% (88cells, 7slices) (p<0.05). (I) The normalized peak change of astrocyte [Ca$^{2+}$]i during 50M dopamine exposure was not significantly different when alone or in the presence of TTX; DA[50μM]: %ΔF/F0 176±6 (173cells, 22slices); DA[50μM] with TTX: %ΔF/F0 150±19 (13cells, 3slices, Fig2I) (p<0.05).
to [10μM] dopamine (Fig2H); this difference was similar to the responses to dopamine without TTX. The peak fluorescence change of astrocytes responding to dopamine in TTX was not significantly different from astrocyte responses to dopamine alone, DA [50μM] with TTX: %ΔF/F0 150±19 (13 cells, 3 slices, Fig2I), DA [10μM] with TTX: %ΔF/F0 171±26 (18 cells, 1 slice).

**Dopamine induced astrocyte calcium transients result from adrenoreceptor activation.**

Dopamine receptor agonists and antagonists were unable to elicit and prevent astrocyte calcium transients, respectively. Exposing PFC brain slices to D1 and D2 dopamine receptor agonists, SKF38393 and Quinpirole [10μM], had little effect on astrocyte calcium, 6.3±6.3% cells responded per slice (3/38 cells 3 slices), (Fig3B). The same slices were responsive to dopamine [50μM] following agonist exposure, 60.4±10.7% (24/38 cells 3 slices), (Fig3B). These results suggested that calcium responses elicited by DA [50μM] were not due to dopamine receptor activation.

To determine which receptor was mediating the observed calcium transients we measured the responses of slices to multiple dopamine exposures. This approach allowed us to confirm whether a slice was responsive to dopamine before attempting to block the response to a second exposure with receptor antagonists. Multiple exposures to dopamine [50μM] without antagonists elicit multiple responses (Fig3C-E). In initially responding slices, a second exposure to dopamine resulted in a reduced, but not significantly different, percentage of responding cells per slice; 1st exposure: 57.9±11.6% (57/91 cells, 5 slices); 2nd exposure: 46.9±12.2% of cells per slice (47/89 cells, 5 slices; Fig3D). When responsive slices were exposed to selective dopamine receptor antagonists (SCH23390 &
Figure 2.3 Dopamine induced astrocyte calcium transients result from adrenoreceptor activation.

(A) Normalized fluorescent changes of astrocytes from a brain slice had did not change during exposure to the selective D1 and D2 type dopamine receptor agonists, SKF38393 & Quinpirole [15μM], respectively (i) in slices that were confirmed to be responsive to subsequent exposure to 50μM dopamine (ii). (B) The percentage of responding cells per slice for the brain slices exposed to dopamine agonists and [DA50μM] was 6.3% ±6.3 (3/38cells 3slices) and 60.4% ±10.7 (24/38cells 3slices), respectively. (C-E) Astrocyte [Ca^{2+}]_i transients elicited during 50μM dopamine perfusion were also observed during subsequent exposures. (C) The normalized fluorescence changes from seven astrocytes in the same brain slice during two exposures to dopamine 50μM. All seven cells responded during the first exposure to dopamine, but during a second exposure only six of these seven cells responded. Slices were allowed to recover for twenty minutes between exposures. (D) The percentage of cells that responded per slice to a second dopamine [50μM] exposure was reduced, but not significantly different from initial responses when the second exposure was dopamine [50μM] alone or in the presence of the dopamine receptor antagonists SCH23390 and Quinpirole [15μM] (SCH & Quin); DA alone: 1^st response 57.9% ±11.6, 2^nd response 46.9% ±12.2 (5slices); DA with SCH & Quin: 1^st response 56.8% ±11.9, 2^nd response 35.4% ±10.8 (4slices). The presence of the adrenoreceptor antagonists Propranolol and Prazosin [15μM] (Prop & Praz) during a second exposure to dopamine completely prevented responses from previously responding slices; DA with Prop & Praz: 1^st response 36.0% ±9.3, 2^nd response 2.2% ±2.2 (3slices). (E) The percentage of responding cells per slice for subsequent dopamine
responses was normalized to percentage of responding cells from a preceding exposure to dopamine alone. Compared to subsequent responses without antagonists, the normalized percentage of responders during a second dopamine response was not significantly different in the presence of D1 and D2 dopamine receptor antagonists (SCH23390 & Quinpirole [15μM]), but almost completely prevented by the presence of α1- and β-adrenoreceptor antagonists (Prazosin and Propranolol [15μM]); DA alone: 83.9% ±15.9 (5slices), SCH&Quin: 59.1 ±7.3, (4slices), Praz&Prop: 4.1% ±4.1 (3slices). Normalized fluorescence changes are expressed as ΔF/F₀, scale bar = 2min. Statistical differences were assessed with a students t-test (confidence interval p<0.05).
Dopamine receptor antagonists:
SCH 23390 [1μM] (D1-type)
Sulpiride [1μM] (D2-type)

Adrenoreceptor antagonists:
Prazosin [15μM] (α1-type)
Propranolol [15μM] (β-type)
Sulpiride, [1μM]) for ten minutes or more, a second exposure to dopamine still elicited calcium responses, 35.4±10.8% of cells responded per slice (34/89 cells, 4 slices; Fig 3D). In contrast, when responsive slices were exposed to antagonists selective for the α1 and β-adrenergic receptors (Prazosin & Propranolol, [15μM]), no calcium responses were observed during a second exposure to dopamine [50μM], 2.2±2.2% of cells responded per slice (1/45 cells, 3 slices; Fig 3D). When responses to a second dopamine exposure were normalized to responses from a first exposure, no significant differences were seen between control responses and those in the presence of dopamine receptor agonists, in marked contrast to adrenergic receptor antagonists which almost completely blocked responses to dopamine (Fig 3E). These findings strongly suggest that calcium responses elicited by dopamine [50μM] were due to activation adrenergic receptors on astrocytes from the medial prefrontal cortex.

**Dopamine constricts pial and penetrating cortical vessels by activating adrenergic receptors.**

In addition to its effect on astrocyte [Ca^{2+}]_{i}, [50μM] dopamine perfusion caused pial and penetrating cortical blood vessels to constrict to 68.7%±5.1 of control diameter (7 slices, Fig 4). Constrictions were persistent in the presence of dopamine (up to 6 minutes) and vessel diameters were reversed to control levels following dopamine washout. Immediately following 3 minutes of dopamine perfusion, co-perfusion of dopamine with selective D1 and D2 dopamine receptor antagonists (SCH23390 and Sulpiride [10μM]) did not significantly change the diameter of constricted vessels (3 slices; Fig 4B). A similar exposure with the α2-adrenoreceptor antagonist Yohimbine [10μM] fully reversed vessel dopamine induced constrictions to control diameters (4 slices; Fig 4A,B).
Figure 2.4 Dopamine elicits vessel constrictions in pial and penetrating cortical arterioles by activating α2-adrenoreceptors.

Vessel constrictions induced by dopamine perfusion [50μM] were reversed by antagonists for α2-adrenoreceptors, while dopamine receptor antagonists had not effect. (A) A transmitted-IR image of a pial vessel from the midline of a prefrontal slice (vertically orientated) with an arteriole penetrating into superficial cortical layers (horizontally orientated); brain slices were prepared from a 15 day old rat. Bath perfusion of dopamine [50μM] for 3 minutes caused a maximal vasoconstriction to 65.5% of the control diameter (46.4μm) which was fully reversed by co-perfusion of dopamine with the α2-adrenoreceptor agonist Yohimbine [10μM]. Scale bar = 50μm. (B) The percentage of the vessel diameter normalized to a stable baseline level was significantly decreased from following 3 minute perfusion of dopamine [50μM] (7slices). Immediately following this response, co-perfusion of dopamine with antagonists for dopamine receptors (Sulpiride and SCH23390 [10μM], 3slices) had no significant effect on vessel diameter, while antagonists for the α2-adrenoreceptors (Yohimbine [10μM], 4 slices) fully reversed vessel diameter to control values. This effect of receptor antagonists suggests that vessel constrictions evoked by dopamine are due to activation of α2-adrenoreceptors. Statistical differences between vessel diameters were assessed with a students t-test (* confidence interval p<0.05).
A

Control (0min)  DA [50µM] (3min)  DA [50µM] & Yohimbine [10µM] (5min)

B

% vessel diameter normalized to baseline

- Control
- DA50µM
- DA50µM with Antagonist 10µM

SCH23390 & Sulpiride 10µM (3slices)  Yohimbine 10µM (4slices)

* Significance indicated
This experiment strongly implicates α2-adrenoceptors in mediating the observed vessel constrictions of pial and penetrating vessels by [50μM] dopamine in the superficial layers of the prefrontal cortex.
Discussion

Dopamine is an important neurotransmitter for the optimal functioning of the prefrontal cortex (PFC). Accordingly, when levels of extracellular dopamine fall outside of an optimal range, performance on PFC dependent tasks is adversely affected (Zahrt et al., 1997; Yang et al., 1999; Seamans and Yang, 2004). Most research of PFC function has been concerned with how dopamine affects neural activity and behavior in the PFC (Seamans and Yang, 2004). The effects of dopamine on astrocytes in the PFC have not been studied as intensely despite a growing body of knowledge that these cells can influence information processing (Volterra and Meldolesi, 2005). Cultured astrocytes have been shown to express dopamine receptors directly by immunohistochemistry, receptor binding, mRNA and protein expression, as well as indirectly with functional assays (Henn et al., 1977; Hosli and Hosli, 1986; Hosli et al., 1987; Bal et al., 1994; Luo et al., 1999; Zanassi et al., 1999; Reuss et al., 2000; Kinor et al., 2001; Ohta et al., 2003; Miyazaki et al., 2004). In intact tissue, astrocyte dopamine receptors have been reported in the striatum, prefrontal cortex, and pituitary (Svingos et al., 1999; Khan et al., 2001; Alonso et al., 2003). Light and electron immunocytochemistry in monkey PFC revealed D2-dopamine receptors on astrocyte processes surrounding interneurons (Khan et al., 2001). The same study reported that approximately one third of the total D2-dopamine receptor binding in human, monkey, and rodent cortex was found on astrocytes (Khan et al., 2001). Despite the strong evidence for astrocyte dopamine receptor expression in the prefrontal cortex, the responses of astrocytes to dopamine have not been investigated in intact tissue. We investigated astrocyte calcium responses to dopamine in freshly
prepared brain slices, as [Ca$^{2+}$]$_i$, calcium transients are associated with many astrocyte functions including their direct communication with neurons (Fiacco and McCarthy, 2006). Activation of astrocyte dopamine receptors in vitro with selective receptor agonists results in various functional responses including astrocyte [Ca$^{2+}$]$_i$ transients (Reuss et al., 2000; Kinor et al., 2001). Also, in acutely isolated cortical astrocytes from mice, the D2 dopamine receptor agonist Quinpirole elicits [Ca$^{2+}$]$_i$ transients (Khan et al., 2001). In our experiments, acute dopamine exposure resulted in astrocyte [Ca$^{2+}$]$_i$ transients and these responses were not significantly affected by the Na$^+$ channel blocker TTX (Fig 2). The lack of an effect by TTX is evidence against an indirect effect on astrocyte following the activation of neuronal dopamine receptors. Dopamine receptor agonists and antagonists failed to elicit or prevent these calcium transients, respectively, (Fig 3). Elicited [Ca$^{2+}$]$_i$ responses were prevented by adrenergic antagonists (Fig 3) indicating that they were due to activation of adrenoreceptors, not dopamine receptors. The failure of dopamine to elicit [Ca$^{2+}$]$_i$ transients through astrocyte dopamine receptors deviates from reports of similar responses to selective dopamine receptor agonists in vitro (Reuss et al., 2000; Khan et al., 2001; Kinor et al., 2001). A possible reason for this discrepancy is that the astrocytes we studied in situ do not express dopamine receptors. Culture conditions have been shown to dramatically influence astrocyte properties including the extent of dopamine receptor expression in astrocytes (Bal et al., 1994); therefore astrocyte calcium responses elicited by dopamine agonists in vitro may have been due to the activation of dopamine receptors expressed in response to culture conditions. It is also possible that reported astrocyte [Ca$^{2+}$]$_i$ transients elicited by dopamine in vitro were due to dopamine receptor expressing cells obtained outside of the
PFC as these cultures were prepared using the entire rodent cortex from which only a subset of cells responded (Reuss et al., 2000; Khan et al., 2001). Regional variability of astrocyte receptor expression within the PFC is also a possible reason why the astrocytes imaged did not have dopamine receptors as our study focused on superficial cortical layers while the majority of dopamine receptor binding in this area is reported in deeper layers (Vincent et al., 1993). On the other hand, if the astrocytes we imaged did express dopamine receptors, it is possible their activation did not result in \([\text{Ca}^{2+}]_i\) signaling. Reports of \([\text{Ca}^{2+}]_i\) signaling coupled to D1- and D2-dopamine receptors are conflicting and the variability of signal transduction is attributed to the heterologous expression systems used to study dopamine receptor responses (Missale et al., 1998). By showing that dopamine receptor activation does not result in a change in astrocyte \([\text{Ca}^{2+}]_i\), our results cannot rule out the expression of these receptors on prefrontal astrocytes. Reports of predominantly neuronal dopamine receptor expression in the rodent mPFC may have attributed perisynaptic glial receptor expression to neurons due to the resolution used (Vincent et al., 1993). High resolution immunocytochemistry using electron microscopy, similar to that used in primate PFC (Khan et al., 2001), is necessary to determine whether astrocytes from the superficial layers of the rodent PFC express dopamine receptors and their absence would explain why dopamine receptor agonists failed to elicit astrocyte \([\text{Ca}^{2+}]_i\) responses in brain slices.

It is not surprising that dopamine elicited calcium responses from adrenoreceptors. It is well known that noradrenergic receptor activation can elicit calcium signalling in astrocytes (Duffy and MacVicar, 1995) and dopamine has been shown bind adrenoreceptors due to its structural similarity to other catecholamines (Swaminath et al., 62).
In the periphery, dopamine successively activates D1, β1, and α1/α2 receptors at relative plasma concentrations of 1, 3, and 10, respectively (Ooi and Colucci, 2001). Dopamine can also elicit functional responses from adrenoreceptors. Dopamine stimulates cAMP generation through β-adrenoreceptors expressed artificially in HEK293 cells (EC50 ~ [10 μM]) (Swaminath et al., 2004) and naturally in cultured astrocytes ([10-100 μM] dopamine) (Hansson et al., 1984; Zanassi et al., 1999; Facchinetti et al., 2004). In intact tissue, similar concentrations of dopamine have been shown to modulate the electrophysiological properties of neurons by stimulating α-adrenoreceptors in brain slices from the rat hippocampus (10 μM) and quail preoptic area (10-100 μM) (Malenka and Nicoll, 1986; Cornil et al., 2002). We observed calcium transients in response to [10 μM] and [50 μM] dopamine, but these responses were infrequent at [10 μM] dopamine (1/9 and 21/31 slices, respectively; Fig2). The efficacy of these dopamine concentrations to stimulate adrenoreceptor responses was similar, or slightly lower than the effective concentrations of previous reports (Malenka and Nicoll, 1986; Zanassi et al., 1999; Cornil et al., 2002; Facchinetti et al., 2004). The higher dopamine concentration necessary to elicit adrenoreceptor responses in mPFC brain slices may be due to greater quantities of dopamine metabolizing enzymes in this brain region, or a higher concentration of dopamine necessary for eliciting [Ca2+]i transients compared to other responses. The possibility that observed [Ca2+]i transients were due to the indirect activation of adrenoreceptors following the conversion of exogenous dopamine to norepinephrine by dopamine-β-hydroxylase (DbH) is unlikely as previous reports of adrenoreceptor responses elicited by dopamine in culture and in brain slices were unaffected by DbH inhibition (Cornil et al., 2002; Facchinetti et al., 2004).
The significance of our findings hinges on whether dopamine can activate adrenoreceptors in vivo. During a PFC dependent working memory task, basal levels and task dependent increases in the concentration of extracellular dopamine ([dopamine]_e) were reported within the picomolar range in the rat mPFC (Phillips et al., 2004); however the microdialysis method used to determine [dopamine]_e may have underestimated the size of changes in confined extracellular spaces (i.e. the synaptic cleft) by sampling a relatively dilute volume (spanning a 2mm length of extracellular space) (Phillips et al., 2004). Tissue damage surrounding microdialysis probes may also reduce the [dopamine]_e recorded (Bungay et al., 2003). Despite the potential underestimation of neurochemicals by in vivo microdialysis, the activation of adrenoreceptors by dopamine in the PFC is unlikely during normal behaviour. Optimal performance in working memory tasks is seen within a narrow range of PFC [dopamine]_e, described by an ‘inverse-U’ function, meaning that either low or high dopamine receptor occupancy disrupts performance on working memory tasks (Zahrt et al., 1997; Floresco and Phillips, 2001; Seamans and Yang, 2004). The concentration of dopamine necessary to activate adrenoreceptors would saturate dopamine receptor binding and if this occurred during normal behaviour then exposure to high levels of dopamine receptor agonists should not have an effect on task performance. The activation of astrocyte adrenoreceptors is more likely to occur when PFC dopamine is abnormally elevated during pathological states, such as stress. Stress has been shown to preferentially increase [dopamine]_e and dopamine turnover in the PFC by more than 300% over basal levels (Thierry et al., 1976; Dunn and File, 1983; Roth et al., 1988; Abercrombie et al., 1989; Imperato et al., 1991; Kaneyuki et al., 1991). Sensitive voltammetry studies have reported that restraint induced stress in rats elevates...
mPFC dopamine levels in the submicromolar range (Doherty and Gratton, 1996). The increased levels of dopamine in the prefrontal cortex during acute stress decreases performance on working memory tasks in rodents and primates, and this decrease is ameliorated by dopamine receptor antagonists (Arnsten and Goldman-Rakic, 1998). The activation of astrocyte adrenoreceptors in the PFC may contribute to this disruption of working memory performance, or may have more long term effects on PFC function. Dopamine induced adrenoreceptor signaling differs from epinephrine and norepinephrine induced signaling by preventing the adrenoreceptor internalization (Swaminath et al., 2004). The inability of adrenoreceptors to internalize can have significant consequences on downstream signaling as it does for mu-opioid receptor signaling where the agonist morphine, which fails to cause receptor internalization, is significantly more addicting than methodone and other agonists which do result in the receptor internalization and rapid signal termination (Whistler et al., 1999). Disruptive astrocyte signaling due to aberrant astrocyte adrenoreceptor activation by dopamine might change the properties of these cells following periods of repeated stress and may have serious consequences for brains vulnerable to psychiatric dysfunction. Stress is thought to precipitate, or exacerbate neuropsychiatric disorders including schizophrenia and depression (Mazure, 1995). Post-mortem evidence also suggests that glia are selectively disrupted in neuropsychiatric disorders such as depression and schizophrenia (Rajkowska, 2000; Cotter et al., 2001). Further study of the effects of high concentrations of dopamine on astrocytes similar to those seen during stress may advance our understanding of the pathogenesis behind these neuropsychiatric disorders.
Dopamine in the medial prefrontal cortex has also been reported to act directly on the microvasculature in superficial cortical layers. Krimer and colleagues have reported that penetrating arterioles and capillaries in the mPFC of the rhesus monkey are apposed by varicosities of dopaminergic fibers (Krimer et al., 1998). The same study found that perivascular iontophoresis of dopamine in brain slices from the ferret mPFC caused cortical vessels to constrict which the investigators interpreted as functional evidence for the control of cortical blood flow by dopamine (Krimer et al., 1998). We also observed constrictions of penetrating arterioles from rodent mPFC brain slices during bath perfusion of dopamine, however these responses were the result of α2-adrenoreceptor activation and not dopamine receptors (Fig4). This suggests that the receptor responsible for the cortical vessel constrictions observed by Krimer and colleagues might have been the result of adrenoreceptor activation due to the very high concentration of dopamine [200mM] used (iontophoretically) to elicit them (Krimer et al., 1998). It is unlikely that dopamine controls cortical microcirculation under normal conditions when the same control could be achieved by adrenergic fibers found on the cortical circulation (Raichle et al., 1975). The disruption of normal vascular function by dopaminergic activation of adrenoreceptors may be significant for the pathology of hemodynamic and neuropsychiatric disorders associated with changes in regional cortical blood flow (Goldberg, 1984; Weinberger et al., 1988; Krimer et al., 1998). Further work is necessary to understand the significance of the close anatomical relationship between dopaminergic fibers and the cortical microvasculature.

In summary, our results suggest that high [dopamine]c can activate adrenoreceptors in the superficial layers of the mPFC resulting in astrocytic calcium
signaling and vascular constriction, which may contribute in part to disruption and ultimately to dysfunction of PFC function. Confirmation of aberrant adrenoreceptor activation in vivo is necessary to determine whether this signaling might underlie neuropsychiatric disorders and serve as a potential therapeutic target for preventing their progression.
Chapter 3: Endothelin elicits widespread astrocyte \([Ca^{2+}]_i\) transients without affecting neurotransmission in the rat hippocampus
Astrocytes can influence synaptic activity in both culture and in intact tissue through the \([Ca^{2+}]_i\) dependent release of glutamate. The physiological impact of this phenomenon is not known and studies of its effects on synaptic activity are limited to small numbers of cells or synapses. To understand the extent that astrocyte glutamate release affects neural activity, we investigated the influence of widespread astrocytic \([Ca^{2+}]_i\) transients, induced by endothelin peptides, on synaptic transmission in hippocampal brain slices.

Endothelin peptides cause widespread astrocyte \([Ca^{2+}]_i\) transients and glutamate efflux in culture. Using two-photon laser scanning microscopy to visualize \([Ca^{2+}]_i\) changes in Rhod-2 dye loaded astrocytes, we confirmed that these endothelin peptides could elicit widespread \([Ca^{2+}]_i\) transients in hippocampal brain slices (P16-19). Five minute bath exposure to the endogenous endothelin peptides ET-1 and ET-3 \([100nM]\) elicited calcium transients in 97% and 94% of visualized astrocytes, (66cells 7slices and 88cells 9slices, respectively). The specific ET\(_B\) receptor agonist, \([Ala^{1,3,11,15}]ET-1\) \([100nM]\), caused \([Ca^{2+}]_i\) transients in 100% of astrocytes visualized (97cells 8slices). The \([Ca^{2+}]_i\) transients elicited by ET-3 and \([Ala^{1,3,11,15}]ET-1\) were prevented by preincubation with the ET\(_B\) receptor antagonist BQ-788 \([1\mu M]\) alone (0% responders: 45cells 4slices, 36cells 3slices, respectively) while the response to ET-1 was only prevented by co-application of ET\(_A\) and ET\(_B\) receptor antagonists \([1\mu M]\), BQ-123 and BQ-788 respectively (0% responders: 26cells 3slices). The ET\(_B\) receptor is highly expressed in astrocytes and its activation may be a relatively selective inducer of astrocyte \([Ca^{2+}]_i\) transients and possibly glial glutamate efflux.
We used the selective ET$_B$ receptor agonist [Ala$^{1,3,11,15}$]ET-1 to test the effect of widespread astrocyte [Ca$^{2+}$]$_i$ transients on hippocampal synaptic transmission measured by extracellular field recordings. Surprisingly, 5min [Ala$^{1,3,11,15}$]ET-1 perfusion did not noticeably influence the glutamate sensitive mossy fiber-CA3 field potential (peak 102.2%, slope 102.8% of control; 5slices), or glutamate dependent LTP induction at the CA3-CA1 synapse (peak 106%, slope 104% of control; 3slices). In conclusion, endothelin peptides cause widespread activation of astrocytic [Ca$^{2+}$]$_i$ transients in situ with no obvious influence on hippocampal synaptic transmission measured by extracellular fields.
Introduction

Evidence has been accumulating for over a decade in support of the bi-directional communication between neurons and astrocytes however the significance of this signaling is not established. The close anatomical association of astrocytes and nerve terminals (Ventura and Harris, 1999) allows for a potentially major influence of glial-neuronal signaling on the physiology, or pathophysiology of synaptic function, therefore it is important to understand the consequences of this signaling on neurotransmission.

Astrocytes respond directly to glutamate released from stimulated neural afferents with [Ca^{2+}]i transients and oscillations in brain slice cultures (Dani et al., 1992) and in fresh brain slices (Porter and McCarthy, 1996; Pasti et al., 1997). Conversely, spontaneous and stimulated [Ca^{2+}]i elevations in astrocytes can induce calcium-dependent glial glutamate release which in turn elicits [Ca^{2+}]i elevations and slow inward currents in surrounding neurons in culture (Parpura et al., 1994; Araque et al., 1998a; Araque et al., 1998b) and in situ (Bezzi et al., 1998; Parri et al., 2001; Fellin et al., 2004). Additionally, astrocyte glutamate release can modulate synaptic transmission in culture by enhancing the frequency of spontaneous mini excitatory postsynaptic potentials, or by reducing the amplitude of evoked postsynaptic currents (Araque et al., 1998a; Araque et al., 1998b).

Recently, glial glutamate release in situ has been shown to increase the frequency of spontaneous AMPA receptor currents in CA1 pyramidal neurons (Fiacco and McCarthy, 2004), and synchronize their activity (Fellin et al., 2004). Studies to date investigating the influence of astrocyte glutamate release on synaptic transmission have focused on changes at the level of a single cell (Araque et al., 1998a; Araque et al., 1998b; Kang et
al., 1998; Newman and Zahs, 1998; Fellin et al., 2004; Fiacco and McCarthy, 2004); as a result, the influence of glial glutamate on the activity of large neural assemblies is not known. To investigate this, we tested whether widespread $[\text{Ca}^{2+}]_i$ elevations in hippocampal astrocytes in situ could acutely influence synaptic transmission of neural assemblies in hippocampal brain slices measured by glutamate-sensitive extracellular field potentials.

To elicit widespread calcium responses in astrocytes we briefly exposed brain slices to endothelin peptides. The endothelin peptides (ET-1, ET-2, and ET-3) are 21 amino acid endogenous peptides known for their potent vasoactive properties (Davenport, 2002). Non-vascular roles of endothelin are not clear despite the widespread distribution of endothelin binding sites throughout the central nervous system (MacCumber et al., 1990; Niwa et al., 1991). While $\text{ET}_A$ receptor mRNA expression in the brain is mainly in cerebral vascular smooth muscle cells, the expression of $\text{ET}_B$ receptor mRNA is predominantly on glia (Hori et al., 1992). Exposure to nanomolar concentrations of endothelin peptides elicits $[\text{Ca}^{2+}]_i$ elevations in cultured hippocampal astrocytes (Blomstrand et al., 1999) and cerebellar Bergmann glia in situ (Tuschick et al., 1997). These calcium elevations were comparable in size to $[\text{Ca}^{2+}]_i$ increases observed during glial glutamate release (Zhang et al., 2004). Furthermore, acute exposure of cultured hippocampal astrocytes, preloaded with L-$[\text{H}]$ glutamate, to nanomolar ET-1 elicited glutamate efflux by acting preferentially through the $\text{ET}_B$ receptor (Sasaki et al., 1997). The demonstration of glial $[\text{Ca}^{2+}]_i$ elevations and glutamate release induced by endothelin peptides make endothelin receptor activation a good potential tool for eliciting
astrocyte glutamate release in situ by either a calcium dependent, or independent mechanism.

Two-photon fluorescence imaging confirmed rapid and widespread astrocyte 
$[\text{Ca}^{2+}]_i$ elevations in rat hippocampal brain slices when exposed to the endogenous endothelin peptides, ET-1 & ET-3, or the selective ETB receptor agonist $[\text{Ala}^{1,3,11,15}]\text{ET-1}$ [100nM]. We measured extracellular field potentials to test whether these astrocytic calcium transients were associated with widespread astrocyte glutamate release capable of affecting the synaptic transmission of large neural assemblies. Despite the widespread astrocytic calcium responses observed in imaging experiments, endothelin exposure had no measurable effect on the field potentials of glutamate sensitive processes: mossy fiber-CA3 presynaptic inhibition and CA3-CA1 LTP induction. The inability of endothelin exposure to measurably influence field potentials questions either the straightforward dependence of astrocyte glutamate release on elevated $[\text{Ca}^{2+}]_i$, or the ability of this glutamate release to directly influence synaptic transmission of large neural assemblies.
Methods & Materials

Brain slice preparation and AM-ester dye loading

In accordance with institutional guidelines, brain slices were prepared from male Sprague Dawley rats (postnatal days 13-21). Rats were deeply anesthetized with halothane and rapidly decapitated. Brains were quickly removed and cooled for 2 minutes in 0-4°C sucrose cutting solution (in mM): sucrose (230), KCl (2.5), NaHCO₃ (26), glucose (10), MgSO₄ (10), NaH₂PO₄ (1.25), and CaCl₂ (0.5), bubbled with O₂/CO₂ (95/5%). Horizontal hippocampal slices (350-400μm thick) were cut with a vibratome (Leica) and transferred to a room-temperature incubation chamber containing oxygenated artificial cerebral spinal fluid, aCSF, (in mM): NaCl (126), KCl (2.5), NaHCO₃ (26), glucose (10), MgSO₄ (2), NaH₂PO₄ (1.25), and CaCl₂ (2), bubbled with O₂/CO₂ (95/5%). Slices were allowed to recover for at least 30 min before dye incubation or electrophysiological recordings.

After recovery, slices used for imaging were incubated for 1½ to 2 hours in aCSF containing the membrane permeable, AM-ester form, of the Ca²⁺ indicator Rhod-2 AM (10μM), Molecular Probes (Eugene, OR) (final DMSO concentration: 0.3%). This technique results in preferential loading of astrocytes in rodent brain slices demonstrated previously by co-localization of Rhod-2 with green fluorescence protein driven by the astrocyte promoter for glial fibrillary acidic protein (Mulligan and MacVicar, 2004). Dye loaded slices (Fig1) were transferred to a recording chamber and gravity perfused with oxygenated aCSF at a rate of 1-2mL per minute, maintained at 25±0.5°C with an inline
Figure 3.1 Astrocytes in the stratum radiatum of the hippocampus loaded with the Ca\textsuperscript{2+} indicator Rhod-2 AM.

(A) A transmitted light image of the stratum radiatum in a hippocampal brain slice from a 15 day old rat. In this field a bifurcating vessel is shown at the top and left of the image. Insert, a schematic of the hippocampus shows the imaged area outlined in red. (B) Rhod-2 fluorescence (pseudocolor) of loaded astrocytes overlaid on the transmitted image shown in A. Cell bodies and processes contact the vessel wall, typical of normal astrocyte morphology; the cell body diameter of the loaded cells was 7.02\mu m \pm 0.22. (C) A two-dimensional projection of an image stack (20 images separated by 1\mu m Z-plane step) of rhod-2 fluorescence ranging \pm 10\mu m from the image plane in A and B. Loaded cells display stellate morphology of protoplasmic astrocytes with projections and endfeet on the basal vessel wall. Scale bar = 20\mu m.
heater (Warner instruments). Slices were allowed to settle for ≥20 minutes in the recording chamber before use.

Two photon calcium imaging

Imaging was performed using a two-photon laser-scanning microscope (Zeiss-LSM 510 scan head coupled to a Zeiss-Axoscope-2 fitted with a 40X-W/0.80NA objective lens) directly coupled to a Ti:sapphire laser (Mira;Coherent) providing ~100fsec pulses at ~80MHz and pumped by a 5W laser source (Verdi;Coherent). Rhod-2 fluorophores were excited at a wavelength of 835-840nm and the epifluorescence detected by an external (non descanned) detector after passing through a 605nm (55nm bandpass) emission filter. Photodynamic damage was reduced by using an acoustic optical modulator, AOM (Zeiss) to attenuate the laser intensity to the minimum necessary to achieve adequate signal-to-noise. Images were acquired from the stratum radiatum of the hippocampal CA1 region of slices, typically 60-120μm from the cut surface to avoid slice damage.

Electrophysiological Field Recordings

Prior to recordings, slices were perfused with aCSF containing picrotoxin (50 μM) to block GABA\textsubscript{A} receptor-mediated inhibitory synaptic currents. Extracellular recordings were obtained with glass micropipettes filled with aCSF (resistance, 1-3 MΩ). Extracellular recordings were filtered at 5 kHz, digitized at 10 kHz using a Digidata1200 interface (Axon Instruments, Foster City, CA), and stored on a Pentium III computer for offline analysis using Clampfit (Axon Instruments).
Excitatory postsynaptic responses were evoked by stimulating either the Mossy fiber pathway for Mossy fiber-CA3 potentials, or the Schaffer collateral pathway for the CA3-CA1 synaptic potentials. Extracellular stimulation was achieved with a constant current pulse (0.1 ms; ~5mV the voltage necessary to achieve a 0.5mV field potential) delivered by a tungsten bipolar electrode positioned at the surface of the slice. Synaptic responses were evoked at 0.05 Hz stimulation except during the induction of LTP. Mossy fiber-CA3 and CA3-CA1 field excitatory postsynaptic potentials (fEPSPs) were recorded from glass pipettes in the stratum lucidum or radiatum, respectively, at least 50 μm away from the cell body layer. After obtaining a stable baseline for ≥ 10 min, agonists were added directly to the aCSF reservoir and bath applied. In a subset of experiments, LTP was induced by high frequency stimulation (HFS, 100 pulses at 100 Hz) of the Schaffer collateral pathway with the same stimulating strength used during baseline recording. For LTP experiments, [Ala$^{1,3,11,15}$]ET-1 was continuously present in aCSF 6 min before, during, and 6 min after the induction of LTP by HFS.

**Analysis of calcium signals and field recordings**

Images were analysed offline using Zeiss LSM (version 3.2) software. Fluorescence signals were converted to relative fluorescence changes over time and expressed in percentages; defined as ΔF/F0 = ((F1-B1)-(F0-B0))/(F0-B0), where F1 and F0 are fluorescence in a loaded astrocyte soma at the peak of an induced fluorescence change or at the beginning of an experiment, respectively, and B1 and B0 are the background fluorescence at the peak fluorescence change of a cell or at the beginning of an experiment, respectively. Background values were taken concurrently from an adjacent area free of loaded cells, or processes.
Field recordings were analysed with Clampfit (version 9.0) software (Axon instruments). The peak and initial slope of fEPSPs were normalized to a stable baseline value and this was used for comparison of different treatments. For LTP experiments, stable fEPSP peaks and slopes normalized to baseline were used to obtain a ratio that normalized the extent of LTP induction across slices, referred to as the LTP ratio. The LTP ratio was derived by the quotient of the peak or slope values after (a) and before (b) HFS; e.g. for slope values: \( \frac{\text{slope}_a}{\text{slope}_b} \) where ‘slope\(_a\)’ and ‘slope\(_b\)’ are the slopes after and before HFS, respectively.

**Statistics and Drugs**

Sample means are reported +/- SEM. Statistically significant differences between population means were assessed using the Students t-test with a confidence level of \( p<0.05 \) denoted by a single asterisk (*). Unless otherwise indicated, statistical comparisons from imaging experiments were made between trials with the same endothelin peptide, either with or without an antagonist.

Endothelin-1, Endothelin-3, [Ala\(^{1,11,15}\)]Endothelin-1, BQ-123, BQ-788, and picrotoxin were obtained from Sigma-Aldrich (St. Louis, MO); L-CCG-1 was obtained from Tocris (Ellisville, MO). Drugs were prepared in dH\(_2\)O and stored as frozen aliquots at 1000 times the final concentration used. Aliquotes were thawed and vortexed just prior to dilution in aCSF. The final concentrations of all endothelin peptides was [100nM] and for all endothelin receptor antagonists was [1\(\mu\)M].
Results

Six minute bath perfusion of endothelin peptides ([100nM]) elicited widespread 
$[\text{Ca}^{2+}]_i$ transients from hippocampal astrocytes in all slices imaged (Fig 2,3; n=24 slices),
confirming responses reported from primary cultured astocytes (Blomstrand et al., 1999).
Despite eliciting nearly ubiquitous calcium responses, the endothelin B receptor agonist
$[\text{Ala}^{1,3,11,15}]\text{ET}-1$ (AlaET1) had no noticeable effect on extracellular field potentials of
two major hippocampal synapses: the fEPSP of the mossy fiber-CA3 synapse and LTP
induction at the CA3-CA1 synapse (Fig 4). In summary, the widespread activation of
astrocyte $[\text{Ca}^{2+}]_i$ transients observed in situ was not associated with a significant change
in hippocampal synaptic transmission measured by extracellular potentials.

Endothelin peptides elicit widespread $[\text{Ca}^{2+}]_i$ responses in hippocampal
astrocytes in situ.

Bath perfusion of endothelin peptides ([100nM]) evoked virtually simultaneous
calcium responses in visualized cells. The observed calcium changes were characterized
by a rapid rise in $[\text{Ca}^{2+}]_i$ which remained elevated for minutes before slowly returning to
roughly baseline levels (Fig 2). The normalized peak fluorescence change elicited was
similar for endogenous agonists (ET:1 $\%\Delta F/F_0$ 194±15, 64cells 7slices; ET3: $\%\Delta F/F_0$
175±11, 81cells 9slices; Fig 2A,D), but was significantly larger in response to the
selective endothelin B receptor agonist $[\text{Ala}^{1,3,11,15}]\text{ET}-1$ [100nM] (AlaET1: $\%\Delta F/F_0$
254±17, 97cells 8slices; Fig 2C,D,3). The similar size of calcium responses during
exposure to the endogenous agonists is likely explained by their nearly identical binding
Figure 3.2 Endothelin peptides elicit \([\text{Ca}^{2+}]_{i}\) transients in hippocampal astrocytes by dissimilar endothelin receptor activation.

(A-C) (i) Representative traces of normalized fluorescence changes (%ΔF/F0) of astrocytes in hippocampal brain slices exposed (6min) to endothelin peptides (100nM) ET-1 (Ai), ET-3 (Bi), and Ala\(^{[1.3,11,15]}\) ET-1 (AlaET1; Ci). (ii) Traces of normalized fluorescent changes preincubated (≥10min) with endothelin receptor antagonists able to efficiently prevent the astrocyte \([\text{Ca}^{2+}]_{i}\) responses elicited by the endothelin peptides in (i). Antagonists for both endothelin receptors (BQ123 and BQ788 [1μM]) were necessary to prevent the fluorescent changes elicited by ET1 exposure (Aii), while ET3 and the selective ETB receptor agonist AlaET1 induced responses were prevented by BQ788 [1μM] alone, (Bii) and (Cii), respectively. Time scale = 2min. (D) Mean normalized fluorescence change (%ΔF/F0) of rhod-2 loaded cells during exposure to endothelin peptides alone or in the presence of endothelin receptor antagonists. ET-1, ET-3, and AlaET-1 (100nM) caused a marked rise in astrocyte \([\text{Ca}^{2+}]_{i}\) (%ΔF/F0: 194±15, 64cells 7slices; 175±11, 81cells 9slices; 254±17, 97cells 8slices; respectively). The response ET-1 alone was significantly increased in the presence of BQ-123 [1μM] and was not changed by BQ-788 [1μM] (%ΔF/F0: 255±24 and 209±24, respectively). The response to ET-1 was only blocked by co-perfusion of both ETA and ETB endothelin receptor antagonists (BQ123 and BQ788 [1μM]). The calcium response elicited by ET-3 alone was also significantly larger in the presence of BQ-123 [1μM] (%ΔF/F0: 244±27, 34cell 3slices) and blocked in the presence of BQ-788, or by co-perfusion of both antagonists (%ΔF/F0: -1.3±3 and -0.8±2, respectively). The response elicited by AlaET-1 alone was
significantly larger than the endogenous peptides (%ΔF/F0: 254±17, 97 cells/8 slices), but was not different than their responses in the presence of BQ-123. The AlaET-1 response was also blocked in the presence of BQ-788, or by co-perfusion of both antagonists (%ΔF/F0: 1.8±4.9 and -0.17±1.8, respectively). Statistical differences of responses in the presence of antagonists are compared to responses of the same agonist alone (* represents a confidence value p<0.05).
ET-1 [100nM]

BQ123 [1μM] & BQ788 [1μM]
(ETA & ETB receptor antagonists, respectively)

BQ788 [1μM]
(ETBr antagonist)

BQ123 & 788
(ETA & ETB receptor antagonists, respectively)

Alone

BQ123
(ETAr antagonist)

BQ788
(ETBr antagonist)

BQ123 & 788
(ETA & ETB receptor antagonists, respectively)

Number of slices
affinity for the ETB receptor, despite a hundred fold difference in their affinity for the ETA receptor (Saeki et al., 1991).

The calcium responses elicited by endothelin peptides were observed in almost all visualized astrocytes (ET-1: 97%±3 of visualized cells per slice responded, 66cells 7slices; ET-3: 94%±7 of cells/slice responded, 88cells 9slices; AlaET-1: 100% of cells/slice responded, 97cells 8slices; Fig3)

The astrocyte calcium responses elicited by the endogenous endothelin peptides were similar in size and extent, despite acting through dissimilar endothelin receptors. Pre-incubating slices with a selective antagonist for the \textit{ET\textsubscript{A}} receptor, BQ123 [1\mu M], did not prevent calcium responses during ET-1 or ET-3 exposure. (ET-1 & BQ123: \%ΔF/F0 255±24, 100% cells/slice responded, 59cells 3slices; ET-3 & BQ123: \%ΔF/F0 244±27, 90%±9.5 of cells/slice responded, 38cells 3slices; Fig2D,3). In fact compared to responses without an antagonist, the presence of BQ123 significantly increased the size of the calcium responses to endogenous agonists, to the level elicited by AlaET-1 alone (Fig2). Pre-incubating slices with a selective antagonist the \textit{ET\textsubscript{B}} receptor, BQ788 [1\mu M], did not block the response to ET-1 but did block the response to ET3 (ET-1 & BQ788: \%ΔF/F0 209±24, 100% of cells/slice responded, 34cells, 3slices; ET-3 & BQ788: \%ΔF/F0 -1.4±3.0, 0% of cells/slice responded, 45cells 4slices; Fig2B,3). AlaET-1 responses were also prevented by pre-incubation with BQ788 [1\mu M] (0% of cells/slice responded, \%ΔF/F0 1.8±4.9, 36cells 3slices; Fig2C,3). The response to ET-1 was only blocked by co-incubation with both BQ123 and BQ788 [1\mu M], (0% of visualized cells responded, \%ΔF/F0 3.0±2.2, 26cells, 3slices; Fig2A, 3). ET3 and AlaET1 elicited responses were also blocked by co-exposure of slices to BQ123 and BQ788 (ET3: 0% of
Figure 3.3 Endothelin receptor activation causes widespread astrocyte calcium $[Ca^{2+}]_i$ transients.

Exposure of hippocampal brain slices to endothelin agonists alone elicited calcium responses ($\%\Delta F/F0>0.05$) in essentially every cell visualized (responding cells per slice: ET-1, 97%±3, 66 cells 7 slices; ET-3, 94%±7, 88 cells 9 slices; AlaET-1, 100%, 97 cells 8 slices). The presence of endothelin antagonists either had not effect on, or completely prevented the percentage of responding cells to ET-1, ET-3, and AlaET1. Statistical differences of responses to peptides in the presence of antagonists were compared to responses to peptides alone (* represents a confidence interval of p<0.05).
ET1  | ET3  | AlaET1

% of cells responding per slice

(7)  (3)  (3)  (9)  (3)  (8)

- Alone
- BQ123 (ETAr antagonist)
- BQ788 (ETBr antagonist)
- BQ123&788

(#) Number of slices

(3)  (4)  (3)  (3)  (3)
cells/slice responded, %ΔF/F0 -0.7±2.0, 26cells 3slices; AlaET1: 0% of cells/slice responded, %ΔF/F0 -0.2±1.8, 26cells 3slices), but this was due to the inhibitory effect of the latter (Fig2Bi,Ci,Di,3). Antagonist experiments showed that ET-1 induced astrocyte calcium transients were elicited through both endothelin receptor subtypes, while ET-3 and AlaET1 elicited calcium responses were the result of ETB receptor activation.

**Endothelin B receptor activation did not influence the hippocampal field recordings.**

To determine whether the calcium rise elicited by endothelin peptides could alter synaptic transmission, we tested the influence of ET$_B$ receptor activation on hippocampal field potential recordings sensitive to extracellular glutamate. The mossy fiber-CA3 hippocampal synapse is sensitive to presynaptic inhibition by activation of group II/III metabotropic glutamate receptors (mGluRs) (Kamiya et al., 1996). The selective ET$_B$ receptor agonist AlaET-1 [100nM] had no appreciable influence on the mossy fiber-CA3 fEPSP slope and peak values compared to baseline recordings (% of normalized to baseline: 101.8%±2.6 and 102.3%±2.6, respectively; 5 slices; Fig4A-C). In contrast to the effect of AlaET-1, the group II/III mGluR agonist L-CCG-1 [20µM] caused repeatable inhibition of the fEPSP slope and peak (% of normalized to baseline: 62.5%±3.6 and 42.6%±3.8, respectively; 5 slices; Fig4A-C). Astrocyte calcium transients induced by AlaET-1 in the stratum lucidum of the hippocampal CA3 region (the site the recording electrode) were confirmed separately. Synaptic transmission at the CA3-CA1 synapse in the hippocampal stratum radiatum was also unaffected by AlaET-1 exposure. Long-term potentiation (LTP) of the CA3-CA1 synapse was induced in hippocampal brain slices by high frequency stimulation (HFS) of the afferent Schaffer collateral.
Figure 3.4 Endothelin B receptor activation did not influence the hippocampal field recordings.

(A-C) The influence of the endothelin B receptor agonist Ala[^1,3,11,15]ET-1 [100nM] (AlaET1) and the mGluR1/5 agonist L-CCG-1 [20μM] on mossy fiber-CA3 fEPSPs. (A) Raw traces of mossy fiber-CA3 fEPSP recordings; (A_i) black and orange traces represent the field potential before and directly after L-CCG-1 exposure, respectively, show marked presynaptic inhibition of the fEPSP, (A_ii) two overlaid traces before and during AlaET-1 perfusion overlap nearly identically showing no noticeable influence of the agonist on presynaptic inhibition. (B) L-CCG-1 caused reversible and repeatable inhibition of the mossy fiber-CA3 fEPSP slope (normalized to a stable baseline level) that was not mimicked by ≥10 minute exposure of AlaET-1. The arrows indicate the time points of raw traces represented in A_i and A_ii (1,2 and 3,4, respectively). (C) Slope fEPSP values normalized to baseline levels during stable control levels, exposure to AlaET-1, and subsequent exposure to L-CCG-1. Compared to a stable control period AlaET-1 had not effect on fEPSP values which were significantly reduced by L-CCG-1. (D-F) The influence of AlaET1 exposure on the induction of LTP in the CA1-CA3 region of hippocampal brain slices. (D) Representative traces of CA3-CA1 stratum radiatum fEPSPs before and after LTP induced by HFS (D_i and D_ii, respectively). (E) The fEPSP slope, normalized to baseline, after LTP was induced at the CA3-CA1 synapse (HFS indicated by the solid arrow) in the presence of AlaET1. The black arrows labeled ‘1’ and ‘2’ indicate the time points of raw traces in D_i and D_ii, respectively. (F) The quotient of the fEPSP slope values (normalized to baseline) before and after HFS represents the degree of synaptic potentiation, denoted as the ‘LTP ratio’. There was no significant
difference in the slope LTP ratios induced in the presence of AlaET-1 compared to controls. Statistical differences were determined by a confidence interval of p<0.05 (*).
Post-HFS
10msec

A) L-CCG-1 [20µM]  ii) AlaET1 [100nM]

B) HFS (High Frequency Stimulation; 100Hz, 1sec)

C) Control
● AlaET1 100nM
● LCCG-1 Stable Peak

D) Pro-HFS  ii) Post-HFS

E) HFS (High Frequency Stimulation; 100Hz, 1sec)

F) Control
● AlaET1

Normalized EPSP peak and slope

Normalized EPSP slope

Slope (5 slices)
pathway after 6 minutes of AlaET-1 exposure. HFS corresponded temporally to the elicited astrocyte [Ca\textsuperscript{2+}]\textsubscript{i} elevation observed in imaging experiments in this area. AlaET1 exposure had no effect on the LTP ratio of slope or peak values compared to control experiments (normalized to stable baseline: 104.0%±5.0, 106.5%±9.8, respectively; 3 slices; Fig4D-F). Additionally, AlaET-1 did not noticeably affect the CA3-CA1 field potential prior to HFS (data not shown). Agonist induced [Ca\textsuperscript{2+}]\textsubscript{i} responses in astrocytes surrounding the recording electrode (in the CA1 stratum radiatum) were confirmed separately during field recordings. No effect of AlaET-1 perfusion on glutamate dependent hippocampal field recordings, despite widespread activation of astrocyte calcium transients, questions the direct influence of [Ca\textsuperscript{2+}]\textsubscript{i} dependent astrocyte glutamate release on synaptic transmission in situ.
Considerable evidence supports a mechanism of vesicular astrocyte glutamate release similar to in neurons whereby elevated $[\text{Ca}^{2+}]_i$ is the primary requirement for vesicular release (Innocenti et al., 2000). Stimuli that have been shown to elicit glutamate release from astrocytes are ineffective when $[\text{Ca}^{2+}]_i$ elevations are prevented by depleting internal stores by thapsigargin, or by buffering $[\text{Ca}^{2+}]_i$ with BAPTA-AM, showing that this process is dependent on intracellular calcium elevations (Bezzi et al., 1998; Innocenti et al., 2000). Furthermore, astrocyte glutamate release has been elicited in culture and in fresh brain slices, by directly increasing astrocyte $[\text{Ca}^{2+}]_i$ with various methods, i.e. calcium ionophores, mechanical stimulation, direct photostimulation (Parpura et al., 1994), electrical stimulation (Araque et al., 1998a; Innocenti et al., 2000), and uncaging of calcium and IP$_3$ by photolysis (Fellin et al., 2004; Fiacco and McCarthy, 2004). The numerous careful studies that have examined the effects of astrocyte glutamate release on surrounding neurons have been focused on the stimulation of and recording from a small number of cells, respectively. Due to the focused nature of these studies, it is not known to what extent calcium-dependent glutamate signaling by astrocytes can influence neural activity. To study this, we tested whether the widespread astrocyte $[\text{Ca}^{2+}]_i$ rise elicited by endothelin peptides could affect synaptic transmission measured by glutamate sensitive extracellular hippocampal field potentials (fEPSP), presumably by calcium-dependent astrocyte glutamate release. Surprisingly, acute exposure to the endothelin B receptor agonist AlaET1 [100nM], did not affect the mossy fiber-CA3 fEPSP despite its sensitivity to presynaptic inhibition by glutamate acting through group II metabotropic
glutamate receptors (mGluRs) on the mossy fiber terminals (Kamiya et al., 1996) (Fig 4). AlaET-1 was also unable to affect the level of CA3-CA1 long-term potentiation (LTP), which is dependent on the activation of postsynaptic glutamate receptors (Fig 4). The interpretation of these results depends upon whether endothelin induced calcium transients were able to elicit calcium-dependent vesicular glutamate release from astrocytes. The observed calcium rise elicited by endothelin peptides (Fig 2) was comparable to the change seen during calcium-dependent glial glutamate release in vitro (Zhang et al., 2004). The elicited calcium responses observed in hippocampal astrocytes was also similar to the responses elicited by endothelin peptides in Bergmann glia from cerebellar brain slices which involved the release of IP3 gated intracellular calcium stores (Tuschick et al., 1997); uncaging of IP3 is also able to elicit glial glutamate release (Fiacco and McCarthy, 2004). The likeness of elicited $[Ca^{2+}]_i$ responses to those that elicit astrocyte glutamate release, together with the abundance of evidence for a $[Ca^{2+}]_i$ dependent release mechanism in these cells, suggests that endothelin exposure in our experiments was able to elicit widespread glutamate release from astrocytes corresponding in extent to the observed $[Ca^{2+}]_i$ responses. If endothelin did result in astrocyte glutamate release then the lack of an effect of AlaET1 on field recordings questions the significance of the influence of astrocyte glutamate release on information processing. Calcium-dependent glutamate release from astrocytes in situ has been shown to activate extrasynaptic NMDARs on hippocampal pyramidal cells (Fellin et al., 2004), increase the frequency of sIPSCs on hippocampal interneurons (Liu et al., 2004), and cause slow inward NMDAR mediated currents in thalamic neurons (Parri et al., 2001). These effects may have a subtle influence on synaptic transmission not measured by.
extracellular fields. The physiological role of astrocyte glutamate release might also be important during discrete periods of an animal’s behavior or life span, such as development. Synaptic organization is greatest during early development, coinciding with the greatest number of spontaneous calcium oscillations in astrocytes (Parri et al., 2001). Focal astrocyte glutamate (vesicular release) may aid in synaptogenesis by attracting or directing axonal growth cones (Zheng et al., 1996). Astrocyte glutamate release may also aid synaptic stability of established synapses by using glutamate to communicate to extra or perisynaptic glutamate receptors to influence glio-synaptic coupling, which may stabilize dendritic spines allowing astrocytes to provide improved metabolic support to synapses (Lippman and Dunaevsky, 2005). In order to be sure that widespread astrocyte glutamate release does not acutely affect synaptic transmission in our study, endothelin induced astrocyte glutamate release must be directly confirmed in situ. Despite eliciting widespread \([Ca^{2+}]_i\) transients, it is possible that endothelin did not cause astrocyte glutamate release if the requirement for glutamate release is more involved an elevation in \([Ca^{2+}]_i\) alone. Indeed, Muyderman and colleagues showed that group I metabotropic glutamate receptor (mGluR I) and \(\alpha_1\)-adrenergic receptor activation resulted in a similar \([Ca^{2+}]_i\) rise in astrocytes, but only the former was able to elicit glutamate release (Muyderman et al., 2001). Furthermore, pre-incubation of astrocytes with norepinephrine (NE; 1 min) prevented mGluR I induced \([Ca^{2+}]_i\) oscillations and glutamate release in astrocytes (Muyderman et al., 2001). Metabotropic glutamate receptor induced calcium oscillations are due to oscillating protein kinase C activity and IP_3 production downstream of phospholipase-C (PLC) signaling (Hofer et al., 2002; Zur Nieden and Deitmer, 2005). NE also signals via PLC (Delumeau et al., 1991; el-Etr et al., 1992) and
it was suggested by the authors of this study that NE is disrupting mGluR I induced
[Ca^{2+}]_i oscillations and glutamate release by affecting PLC signaling. Endothelin
signaling also occurs through the PLC pathway (Aramori and Nakanishi, 1992) and the
[Ca^{2+}]_i responses elicited by endothelin peptides (Fig2,3) resemble responses to NE in
brain slices (Duffy and MacVicar, 1995). It is possible that differences between
metabotropic glutamate receptor signaling and endothelin/adrenergic receptor signaling
have an important influence on astrocyte glutamate release irrespective of [Ca^{2+}]_i
elevations.

This study is also the first report of astrocyte [Ca^{2+}]_i transients elicited by
endothelin peptides in an intact tissue preparation. Astrocyte receptor expression in vitro
can change dramatically depending on culture conditions (Juurlink and Hertz, 1985;
Barres et al., 1989); therefore in situ studies are more likely to reflect the normal
astrocyte expression in vivo. The receptors responsible for to observed responses to
endothelin peptides were due to the activation of both ET_A and ET_B endothelin receptor
subtypes (Fig2), in agreement with previous reports in cultured hippocampal astrocytes
(Blomstrand et al., 1999). The endothelin receptors responsible for observed [Ca^{2+}]_i
responses differed from endothelin peptide evoked responses in Bergmann glia which
only exhibited [Ca^{2+}]_i responses through the ET_B receptor in rat cerebellar brain slices
(Tuschick et al., 1997). This difference in glial receptor expression presumably underlies
differences in the physiological or pathological roles of the endothelin system on these
cells. Despite the widespread expression of endothelin receptors throughout the CNS,
particularly on glia and cerebral vasculature (Ehrenreich et al., 1991; Hori et al., 1992),
the role of astrocytic endothelin receptors is not well understood. The high affinity of
endothelin peptides for their receptors (Ki: ~20-60pM and ~15pM for ETA and ETB receptors, respectively (Takayanagi et al., 1991)), necessary for their paracrine function on the vascular endothelium for the maintenance of vascular tone, together with the responsiveness of astrocytes to endothelin peptides, implicates the endothelin system as a potential candidate for the communication between the vasculature and the intimately associated adjacent perivascular astrocyte endfeet (Simard et al., 2003).

The lack of an effect of endothelin on glutamate sensitive hippocampal field recordings, despite widespread astrocyte calcium responses, questions the simplicity of the mechanism or the extent of the influence of calcium-dependent astrocyte glutamate release. Further studies of calcium-dependent astrocyte glutamate are necessary to understand the complex signaling and subtle effects of this phenomenon.
Chapter 4: General Discussion and Future Directions
Introduction

The focus of this thesis is to investigate unknown aspects of astrocyte-neuronal signalling in order to improve the understanding of functional interactions between these cells. We investigated the two-way communication between neurons and astrocytes primarily by imaging astrocyte calcium in brain slices. The aspects of this signalling that we investigated were, firstly, the ability of dopamine in the prefrontal cortex (PFC) to signal to astrocytes by eliciting intracellular calcium transients and secondly, the ability of astrocytes in the hippocampus to influence neural synaptic transmission after widespread activation of astrocyte calcium transients.

For both of these studies, we used two-photon imaging to non invasively monitor astrocyte calcium changes (Potter, 1996; Nakamura, 1999; So et al., 2000). The superior penetrating depth by the infrared beam allows cells at greater depths to be investigated avoid damaged superficial cells, whilst maintaining excellent optical resolution provided by focal excitation of fluophores.

To consider the status each hypothesis in turn:

**Hypothesis one, “Dopamine receptor activation evokes calcium transients from prefrontal cortex astrocytes in intact tissue.”**

A previous study had shown that astrocytes expressed dopamine receptors in primate PFC and exposure to dopamine receptor agonists elicits calcium responses in acutely isolated astrocytes (Khan et al., 2001). Our objective was to image astrocyte calcium in prefrontal cortex brain slices to determine whether there are intracellular
calcium responses during bath exposure of dopamine. We observed astrocyte [Ca\textsuperscript{2+}]\textsubscript{i} changes in prefrontal cortex brain slices during bath exposure to dopamine, but these responses were not due to dopamine receptor activation. We had expected that the astrocyte [Ca\textsuperscript{2+}]\textsubscript{i} responses would be due to the activation of dopamine receptors; however, the [Ca\textsuperscript{2+}]\textsubscript{i} responses we observed the result of norepinephrine receptor activation. Therefore in the intact PFC, dopamine elicits [Ca\textsuperscript{2+}]\textsubscript{i} responses from astrocytes as hypothesised, but not by activating dopamine receptors.

**Hypothesis two  'Widespread astrocyte calcium transients can be elicited by endothelin in intact tissue, influencing the synaptic transmission of an entire brain region.'**

Reports of glutamate release in astrocytes strongly support a calcium-dependent exocytotic release mechanism, similar to that in neurons (Volterra and Meldolesi, 2005). While astrocyte glutamate release has been shown to affect synaptic transmission, the extent of this influence has not been established. Our objective was to image the astrocyte [Ca\textsuperscript{2+}]\textsubscript{i} response to bath perfusion of endothelin peptides in hippocampal slices to demonstrate widespread calcium release and then measure extracellular field recordings from hippocampal brain slices to determine whether widespread astrocyte calcium responses are associated with a change in synaptic transmission.

We found that widespread astrocyte calcium transients elicited by activation of endothelin receptors were not associated with a change in extracellular hippocampal field potentials. This result suggests that calcium-dependent glutamate release either cannot
acutely influence synaptic transmission of an entire brain region or is dependent on more complex conditions than a rise in $[\text{Ca}^{2+}]_i$ alone.

**Synthesis of results:**

Our work showed that dopamine failed to elicit rapid calcium responses and that ubiquitous activation of astrocyte calcium transients did not obviously effect of the neural activity of brain slices.

To evaluate our findings further, we shall first discuss the strengths and weaknesses of our studies and then the overall significance of our findings in the field of astrocyte-neuron signalling before considering directions for future research.

**Comments on strengths and weaknesses of the thesis research**

**Strengths**

A major problem with studying astrocytes in brain slices is how to obtain meaningful data within a complex tissue preparation from electrically non-responsive cells (Cornell-Bell et al., 1990).

**The importance of calcium**

Astrocytes have evaded the majority of attention in neuroscience research on account of their relatively non-responsive electrophysiological properties. This perception of astrocytes started to change with the report of dynamic calcium signalling in astrocyte cultures exposed to glutamate (Cornell-Bell et al., 1990). Astrocytes have
now been shown display dynamic and complex calcium signalling either spontaneously or in response to a wide range extracellular signals, including neurotransmitters (Volterra and Meldolesi, 2005). Astrocyte calcium changes also play a central role in inter- and intracellular astrocyte signalling as well as signalling by astrocytes to neural and vascular elements in the brain (Volterra and Meldolesi, 2005). Monitoring astrocyte calcium changes by calcium imaging techniques has become a powerful tool for studying astrocytes-neuron signalling and is a strength of this research.

**What astrocyte preparation to study?**

In deciding how to study the astrocyte calcium responses, we knew that primary astrocyte cultures have been the main model used to study mammalian astrocytes ever since the recognition that ~95% of the cells from primary cultures of 1-2 day old rats express the astrocyte marker GFAP (Kimelberg, 1983; Federoff and Vernadakis, 1986). Despite the widespread use of primary cultures, the expression of transporters, receptors, ion channels, structural markers, and enzymes is markedly different in cultured astrocytes compared to those left *in situ* (Kimelberg et al., 2000). Freshly isolated astrocytes (FIAs) have more similarities with *in situ* astrocytes, but considerable differences are still found (Kimelberg et al., 2000). These differences have made it difficult to reach conclusions about the functional properties of astrocytes in the intact brain from studies using primary cultures or FIAs. The other major disadvantage to studying isolated or cultured astrocytes is the loss of all cell-cell interactions because the intimate hetero- and homocellular interactions of astrocytes are important for their form and function. To properly study
astrocyte functions, especially their interactions with other cells, intact tissue preparations must be used.

A major strength of this thesis research is the study of astrocyte properties in freshly prepared brain slices, which preserve the intimate interactions of astrocytes with neighbouring cells. The expression of neurotransmitter receptors in astrocytes has been shown to correspond to the neurotransmitter expression of the intact brain region they are from making it important to characterise receptor expression (i.e. dopamine and endothelin receptor expression) in intact tissue. Furthermore, investigating the relationship between widespread astrocyte calcium transients and extracellular neural field activity requires the intact association between astrocytes and neurons preserved in fresh brain slices.

**How to measure the intact astrocyte calcium release?**

In order to study astrocytes in intact brain slices, we used of two-photon laser scanning microscopy (TP-LSM). This approach enabled me to measure astrocyte calcium changes deep in fresh brain slices using the acetoxymethyl (AM) ester derivative of the fluorescent calcium indicator Rhod-2. Two-photon laser scanning fluorescence microscopy allows the restricted excitation of fluorescent probes deep within tissue slices, requiring less power and a better signal to noise ratio than other imaging approaches, namely confocal laser scanning microscopy. Consequently, the use of TP-LSM allows imaging deeper into tissue with less photo damage and photo bleaching to tissue and fluorescent probes, respectively. The AM form of the fluorescent calcium indicator Rhod-2 increases the lipophilicity of the probe, allowing it to enter cells after
which the AM group is cleaved by intracellular esterases trapping the probe within a cell. Incubation of acute brain slices with this calcium probe for >1hr results in the selective loading of cells positive for the astrocyte marker GFAP (Mulligan and MacVicar, 2004). While the reason for the selective loading of astrocytes by Rhod-2 is not clear, this approach allows the selective imaging of astrocyte calcium changes deep within intact tissue avoiding the damaged cut surface of brain slices.

Hence, the major strength of this thesis research was the ability to study aspects of astrocyte-neuron signalling by monitoring dynamic astrocyte calcium responses from healthy semi-intact tissue.

**Weaknesses**

**Age of the rat**

One weakness caused by our approach was the exclusive use of young (≤ 2-3 weeks) animals that was necessary to achieve proper loading of the fluorescent probe Rhod-2. Evidence suggests mesocortical dopamine afferents to the rodent PFC undergo considerable and long lasting postnatal maturation (Sullivan and Brake, 2003). As astrocyte receptor expression responds to its neuronal environment, it is possible then that dopamine receptor mediated responses were not observed because mesocortical DA afferents in the superficial PFC layers had not developed sufficiently to induce DA receptor expression. However, this should not have affected the ability of astrocytes to release glutamate as this has been demonstrated in rodent brain slices at a similar
developmental age (Pasti et al., 1997; Bezzi et al., 1998; Parri et al., 2001; Fellin et al., 2004).

**Implying astrocyte receptor expression**

While our choice of techniques, specifically TP-LSM, provided powerful insight into aspects of astrocyte signalling *in situ*, our approach also had some weaknesses. Namely, by measuring intracellular calcium responses, we can only imply the expression of astrocyte receptors. If no calcium response is observed, the agonist used may still be activating astrocyte receptors and eliciting responses that are not dependent on intracellular calcium. Furthermore, observed calcium responses in astrocytes may be secondary to activation of a non-astrocyte cell type. The best method for determining cell specific receptor expression in intact tissue is high-resolution immunolabelling or receptors and cellular markers, but this was outside of the scope of the present research.

**Agonist concentrations**

In both studies we used high agonist concentrations in line with previous research in culture to determine whether these same effects occurred in an intact tissue model.

In our initial dopamine study we used lower concentrations that elicited very infrequent $[\text{Ca}^{2+}]_i$ responses. We therefore increased the dose to a concentration that reliably elicited a response. Although these are high doses compared to reports of prefrontal dopamine$_e$ (Phillips et al., 2004), the same concentration of dopamine has been used in culture to elicit astrocyte calcium responses (Reuss et al., 2000) and in intact tissue to study neural dopamine responses (Seamans and Yang, 2004). While the high
concentrations of dopamine used may not be reached in the PFC during delayed response
tasks, but it is still possible that responses at these concentrations may be reached during
pathological situations such as during stress (Doherty and Gratton, 1996).

In the endothelin study we used a high endothelin concentration to elicit a
widespread response throughout the tissue; the concentration chosen was in line with
previous research from culture (Blomstrand et al., 1999). The endothelin concentrations
used to elicit widespread calcium responses in hippocampal slices were likely saturating
endothelin receptors (Highsmith, 1998) and might only be seen by astrocytes during
pathological conditions such as stroke where endothelin levels is known to be increased
(Yamashita et al., 1993).

The high concentrations of agonists used in these studies should be considered
before applying our findings to normal astrocyte physiology.

**Specific strength and weakness in the Dopamine study**

The major strength of our dopamine response study in chapter 2 was the
evaluation of astrocytes responses in intact healthy tissue. By imaging the superficial
synaptic cortical layers we were able to obtain less harmful imaging in deeper, healthier
tissue because the lack of pyramidal cell bodies causes less scattering of light.
Consequently, we decided to study the superficial layers because of this superior imaging
achieved and because preliminary observations found calcium transients in some
astrocytes exposed to dopamine.

However restricting our investigation to the superficial cortical layers could be
considered a weakness because the expression of dopamine receptors in these layers is
sparse compared deeper (V-VI) layers (Vincent et al., 1993). It is possible that deeper layer PFC astrocytes might express functional dopamine receptors linked to calcium signalling, which was not observed in our study of superficial layers.

Specific strength and weakness in the Endothelin study

The purpose of the endothelin response study in chapter 3 was to investigate the extent that astrocytes can influence neural activity in an entire brain region; for this purpose, extracellular hippocampal field recordings are well suited because they measure the synaptic transmission in an entire brain region. From previous studies we know that endothelin [100nM] produces a ubiquitous calcium response from culture in astrocytes and endothelin receptors are predominately found on glia in the intact brain (Hori et al., 1992). A rise in intracellular calcium has been shown to release glutamate from astrocytes, making endothelin a strong potential tool for eliciting [Ca$^{2+}$]$_i$ dependent glutamine release from astrocytes without directly affecting neurons. Furthermore, endothelin has been shown to elicit glutamate release from astrocytes in culture preloaded with glutamate (Sasaki et al., 1997). The lack of an effect of endothelin exposure on synaptic transmission questions whether endothelin was actually able to elicit glial glutamate release from astrocytes in situ. Not confirming in situ glutamate release although technically difficult, could be considered a weakness of this study.
Discussion of the overall significance of the thesis research to the field of study

Rapid communication between astrocytes and neurons is a relatively new concept in the field of neuroscience that has challenged the traditional perception of glia as passive, support cells. Astrocytes have now been shown to respond to and release neuroactive substances including ATP and Glutamate as well as influence synaptic signalling and plasticity in situ (Volterra and Meldolesi, 2005). The result of these discoveries has been a dramatic shift in the perception of astrocytes. Astrocytes are now being seen from a perspective with similarities to neurons; as discrete signalling partners with the ability to detect and affect both neural signalling and information processing.

Our understanding of the communication between these cells types is building considerable momentum, but is still far complete. In order to fully understand CNS function, we must understand how its elements interact. The majority of research in the field of astrocyte-neuron signalling is from culture studies, or is confined to a small number of cells from well-studied regions of the brain such as the hippocampus or cerebellum. My research contributes to the field of neuroscience by expanding the existing knowledge about astrocyte-neuron communication, by looking at interactions outside of these traditional areas and by investigating the influence of many astrocytes on large neural assemblies.
**First study - Dopamine elicits [Ca\(^{2+}\)]\(_i\) responses from PFC astrocytes.**

Our first study investigated the ability of dopamine to elicit calcium responses in astrocytes from intact prefrontal brain slices and expands knowledge of astrocyte calcium excitability in intact prefrontal cortex, an area that has received less attention in the context of astrocyte-neuronal signalling.

Specifically, our study has shown that in intact tissue dopamine elicits calcium signalling in astrocytes and this is unaffected by blocking action potential propagation in neurons. Dopamine is an important signal in the PFC and by showing that it can elicit responses from astrocytes in intact PFC tissue our results suggest PFC dopamine release as another example of signalling between astrocytes and neurons. The finding that norepinephrine receptors are activated by dopamine was unexpected, but raises that interesting possibility that astrocytes may be a target of pathological signalling by excessive dopamine release in the PFC, as might occur during times of stress.

It is interesting that in schizophrenia, post mortem studies show dysfunction in PFC glia (Cotter et al., 2001) and it is known that high dopamine release induced in the PFC can induce psychotic symptoms. These reports raise the possibility that astrocytes may be a salient target in PFC dysfunction. Further study will be necessary to determine the role of astrocytes in PFC pathophysiology.
Second study - Endothelin elicits widespread $[\text{Ca}^{2+}]_i$ responses from hippocampal astrocytes, which are not associated with a change in hippocampal synaptic transmission.

Our study showed that astrocytes respond to endothelin in intact hippocampal tissue with an elevation in their $[\text{Ca}^{2+}]_i$; however because the widespread calcium transients observed were not associated with a change in hippocampal synaptic transmission (measured by extracellular field potentials), this raises some questions.

Previous reports of a direct relationship between astrocyte calcium and glutamate release in both culture and brain slices (Pasti et al., 1997; Parri et al., 2001; Fellin et al., 2004), caused us to hypothesize that a widespread calcium rise in hippocampal astrocytes could elicit sufficient glutamate release to influence hippocampal synaptic transmission. According to the extracellular field potentials we measured, this was not the case and our result questions the extent that astrocyte glutamate release can influence neural activity. Due to the difficulty of eliciting astrocyte calcium responses in intact tissue without affecting their neuronal neighbours, the extent that astrocyte gliotransmitter release can affect neural activity had previously not been investigated. Using patch clamp analysis to stimulate or record from single cells, astrocyte calcium transients result in gliotransmission that has been shown to elicit neural responses and alter synaptic activity (Kang et al., 1998; Fellin et al., 2004). The extent of astrocyte calcium signalling can range from intracellular microdomains to intercellular calcium waves, able to spread for hundreds of micrometeres throughout astrocyte syncitium (Cornell-Bell et al., 1990; Grosche et al., 1999). It was therefore reasonable to investigate the effect of astrocyte calcium signalling on a corresponding range of neural activity, namely extracellular field...
recordings that measure the postsynaptic potentials from a population of neurons that may also extend hundreds of micrometers (Moser, 1995). No effect of elicited calcium responses on glutamate sensitive fields suggests that astrocytes are unable to acutely influence the synaptic transmission of a large neural population, which should be useful for studies concerned with the influence calcium dependent astrocyte gliotransmission has on neural function and information processing. It is also possible that hippocampal synaptic transmission was unchanged during endothelin exposure if the elicited rise in astrocyte $[\text{Ca}^{2+}]_i$ did not result in the release of glutamate. If this is the case, the widely reported ‘direct’ relationship between astrocyte calcium and gliotransmitter release may be more complex than that seen in the synaptic terminals of neurons. A more complex relationship between astrocyte $[\text{Ca}^{2+}]_i$ and glutamate release is found in cultured astrocytes which release glutamate when exposed to glutamate agonists, but not adrenergic agonists (Muyderman et al., 2001) despite the ability of both agonists to elicit calcium rises from these cells (Duffy and MacVicar, 1995; Zur Nieden and Deitmer, 2006).

**Conclusion:**

Firstly, astrocytes in the prefrontal cortex react to dopamine with intracellular calcium transients in intact tissue. However this appears to be due to the activation of norepinephrine receptors on astrocytes, rather than dopamine receptors. As calcium responses were elicited by a relatively high concentration of dopamine, this result suggests that astrocyte adrenoreceptors might be a target of dysfunctional dopamine signaling in the PFC.
Secondly, widespread astrocyte \([\text{Ca}^{2+}]_i\) transients can be elicited by endothelin in intact hippocampal tissue, however these were not associated with a change in hippocampal synaptic transmission measured by extracellular field recordings. This result suggests new avenues for research by questioning either the extent that astroyte gliotransmission influence neural activity, or the simplicity of its calcium dependence.

There is a need to further evaluate our findings and future research possibilities are outlined below.

**Comments on future research**

Although this research has given some advance on present knowledge, it has also raised many questions that need to be assessed in future research.

Firstly, in the PFC, the ability of dopamine to elicit dopamine responses from deeper cortical layers needs to be assessed because there are greater densities of dopamine receptors in these layers (Vincent et al., 1993).

Furthermore, it will be useful to directly confirm dopamine receptors in PFC using high-resolution fluorescent immunocytochemistry made possible by TP-LSM, to correlate with our findings.

Higher levels of dopamine released into the PFC, possibly during periods of stress, may adversely affect PFC function due, in part, to the non-specific activation of astrocyte norepinephrine receptors. Hence it would be interesting to determine whether norepinephrine receptor antagonist infusion by microdialysis can lessen the decrease in PFC function seen in animal models of chronic stress.
With respect to endothelin, it is important to confirm that the astrocyte calcium responses observed during endothelin exposure are able to elicit glutamate release from astrocytes as our conclusions about astrocytes-neuron signalling were based on this assumption.

To determine whether astrocyte calcium transients during endothelin exposure elicit subtle responses from hippocampal neurons, in line with previous reports, we can use sensitive patch clamp electrophysiology to measure pyramidal neuron responses in intact tissue during endothelin exposure.

Finally, as endothelin elicits widespread responses from hippocampal astrocytes, it would be interesting to directly determine cell specific endothelin receptor expression in this area using fluorescent immunocytochemistry to correlate with our findings and more fully understand the interaction between the endothelin system and astrocytes.
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