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Identification of the Neuronal and Astrocytic Mediators of the Hemodynamic Response to Dorsal Raphe Nucleus Stimulation

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February 2012

Integrated Program in Neuroscience

McGill University, Montreal

TABLE OF CONTENTS

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science.

TABLE OF FIGURES

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Edith Hamel, for taking me into her lab initially as a naïve undergraduate student and welcoming me further into the lab as a Master's student. She provided constant guidance and support over the last two years and for that I will always be grateful. I would also like to thank all members of my lab for their help along the way. To Dr. Baptiste Lacoste and Dr. Xavier Toussay, thank you for all of the laughs (and the coffee) since I've been here. You both not only taught me countless things, but truly made my time here enjoyable as well. I would also like to thank XingKang Tong for carefully instructing me on every technique under the sun, and for his seemingly endless knowledge of our lab. Without his help my time here would have been doubled. To Yiota Papadopolous and Aman Badhwar, I would also like to thank you both for the wonderful conversations and guidance along the way and for both of your caring help and support throughout our time together. Additionally, my committee members, Dr. Amir Shmuel and Dr. Bruce Pike provided many encouraging comments and suggestions and helped shape my project into something of which I am proud. Also, thank you to my mentor, Dr. Kathy Mullen for helping to guide me in the right direction along the way. My final thanks go out to my family, and particularly my dear friends Madeline Page, Shauna Mahajan and Nastasia Nianiaris and my loving boyfriend Phillip Neil for listening to me talk about this project for hours on end and believing in me throughout. Without your support, this would not have been possible.

This work was supported by grants from the Canadian Institutes of Health Research (CIHR) and a McGill Internal Medicine Returning Student Award.

ABBREVIATIONS

20-HETE	20-hydroxyeicosatetraenoic acid
5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin
AA	Arachidonic acid
AM	Amygdala
AP	Ascending pathways
BBB	Blood brain barrier
BF	Basal forebrain
BOLD fMRI	Blood oxygenation level-dependent functional magnetic resonance imaging
CBF	Cerebral blood flow
CBV	Cerebral blood volume
CCK	Cholecystokinin
ChAT	Choline acetyltransferase
COX	Cylooxygenase
COX-2	Cyclooxygenase-2
CP	Caudate-putamen
CRF	Corticotropin-releasing factor
CTX	Cortex
DβH	Dopamine-beta-hydroxylase
DH	Dorsal hypothalamus
DRN	Dorsal raphe nucleus
DSP-4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
EET	Epoxyeicosatrienoic acid
GABA	γ-aminobutyric acid
GH	Growth hormone
GHRH	Growth hormone releasing hormone

GP	Globus pallidus
HPC	hippocampus
IAP	[^{14}C] iodoantipyrine method
IEG	Immediate early gene
i.c.	Intracisternal injection
ILT	Intralaminar thalamic nuclei
i.p.	Intraperitoneal injection
IP	Interpeduncular nucleus
i.v.	Intravenous injection
K+	Potassium Ion
LC	Locus coeruleus
LDF	Laser Doppler Flowmetry
LH	Lateral hypothalamus
LS	Lateral septum
MAO	Monoamine oxidase
mGluR	Metabotropic Glutamate Receptor
MK-801	Dizocilpine
MT	Midline thalamic nuclei
NAc	Core of nucleus accumbens
NAs	Shell of nucleus accumbens
NE	Norepinephrine
NMDA	*N*-methyl-D-aspartate
NT	Neurotensin
NO	Nitric Oxide
NOS	Nitric oxide synthase
NVC	Neurovascular coupling
PAG	Periaqueductal grey
PCPA	p-chlorophenylalanine
PET	Positron emission tomography

PFC	Prefrontal cortex
PV	Parvalbumin
PFA	Paraformaldehyde
PGE_2	Prostaglandin E_2
PH	Posterior hypothalamus
s.c.	Subcutaneous injection
SERT	Serotonin transporter
SN	Substantia nigra
SOM	Somatostatin
STN	Subthalamic nucleus
TRH	Thyrotopin-releasing hormone
TPH	Tryptophan hydroxylase
VIP	Vasoactive intestinal peptide
VMH	Ventromedial hypothalamus

ABSTRACT

Neurovascular coupling (NVC), also known as functional hyperemia, is a term used to describe the increases in local cerebral blood flow (CBF) seen in areas of increased neuronal activity. Serotonin (5-HT) released from afferents of the dorsal raphe nucleus (DRN) is one neuromodulator thought to induce such a functional hyperemic response. However, the mechanisms underlying this response are largely unexplored. Here, we have attempted to identify *in vivo* the neuronal and glial mediators involved in the increases in cortical CBF observed during stimulations of the DRN. Electrical stimulation of the DRN was found to induce an approximately 30% bilateral increase in CBF in the somatosensory cortex. Selective depletion of 5-HT nerve terminals resulted in almost complete elimination of this evoked CBF increase (PCPA, -66%, $p<0.01$). Similarly, cortical noradrenergic denervation also largely eliminated this response (DSP-4, -80%, $p<0.01$). The bilateral increase in CBF was found to be unaltered by blockade of NMDA receptors (with MK-801) and 5-HT-2A receptors (with ketanserine). However, the evoked CBF response was decreased by blockade of GABA-A receptors (picrotoxin, -45%, $p<0.05$), nonselective α-adrenergic receptor antagonist (phentolamine, -45%, $p<0.05$) and nonselective β-adrenergic receptor blocker (propranolol, -55%, $p<0.05$) and had a trend towards a decrease, albeit not significant, upon blockade of group I metabotropic glutamate receptors (mGluR1 and 5; MPEP+ LY LY367385). The hyperemic response was found to also be reduced after selective inhibition of the epoxygenation reactions catalyzed by specific CYP450 isozymes (MS-PPOH, -42%, $p<0.05$). This was the first study of its kind to clarify not only the influence of the noradrenergic system on evoked CBF increases to DRN electrical stimulation, but also to

begin to characterize the cortical mediators involved in this response. These results demonstrate that the hyperemic response seen after electrical stimulation of the DRN depends heavily upon interactions between the serotonergic and the noradrenergic systems, and also largely on GABA interneurons and metabolically active astrocytes.

RÉSUMÉ

Neurovasculaire couplage est un terme utilisé pour décrire les augmentations du débit sanguin cérébral local (CBF) vus dans les domaines de l'activité neuronale accrue. La sérotonine (5-HT) a publié des afférences du noyau dorsal du raphé (DRN) est un neuromodulateur pensé pour induire une telle réponse fonctionnelle hyperémique. Cependant, les mécanismes sous-jacents de cette réponse sont largement inexploré. Ici, nous avons tenté d'identifier in vivo les médiateurs neuronales et gliales impliquées dans l'augmentation de la corticale CBF observées lors de stimulations de la DRN. La stimulation électrique du DRN a été trouvé pour induire une augmentation d'environ 30% bilatérale dans CBF dans le cortex somatosensoriel. Déplétion sélective des terminaisons nerveuses 5-HT a entraîné l'élimination presque complète de cette augmentation a évoqué CBF (PCPA, -66%, $p<0,01$). De même, la dénervation noradrénergique corticale aussi largement éliminé cette réponse (DSP-4, -80%, $p<0,01$). L'augmentation bilatérale dans CBF a été jugée non modifiée par le blocage des récepteurs NMDA (MK-801) et 5-HT-2A récepteurs (avec ketanserine). Cependant, la réponse évoquée CBF a été diminué par le blocage des récepteurs GABA-A (picrotoxin, -45%, $p<0,05$), antagoniste des récepteurs α non sélectif-adrénergique (phentolamine, -45%, $p<0,05$) et non sélectif des récepteurs β-adrénergiques bloqueur (propranolol, -55%, $p<0,05$) et a eu une tendance vers une diminution, bien que non significatif, sur le blocus du groupe I récepteurs métabotropiques du glutamate (mGluR1 et 5) (MPEP + LY LY367385). La réponse hyperémique a été trouvé également être réduit après l'inhibition sélective des réactions catalysées par epoxygenation spécifique du CYP450 isozymes (MS-PPOH, -42%, $p<0,05$). Cette étude était la première de son genre afin de clarifier non seulement

l'influence du système noradrénergique sur les augmentations de CBF évoqués à DRN stimulation électrique, mais aussi pour tenter de caractériser le réseau cortical impliqué dans cette réponse. Ces résultats démontrent que la réponse hyperémique observée après stimulation électrique de la DRN dépend fortement des interactions entre la sérotonine et les systèmes noradrénergiques, et aussi en grande partie sur les interneurones GABA et métaboliquement actives astrocytes.

GENERAL INTRODUCTION

RATIONALE

The first goals of this project were to carefully locate the placement of a tungsten electrode within the DRN to ensure proper site for electrical stimulation and then to characterize the baseline evoked CBF response seen as a result of this stimulation through the use of laser Doppler flowmetry (LDF). As the DRN is known for being a serotonergic nucleus and provide vast serotonergic afferents to the cerebral cortex, it was first important to confirm this involvement. Therefore, once a reproducible response was obtained, we aimed to determine the degree to which to the NVC response to electrical stimulation of the DRN depended upon the serotonergic system by pharmacologically blocking 5-HT nerve terminals with para-chlorophenylalanine (PCPA). If the serotonergic system was found to play an important role in the NVC response, as suspected, we then planned to pharmacologically block the 5-HT2A receptors, one of the most extensively expressed serotonin receptors, to determine if they were utilized in this pathway.

The DRN is known to have extensive connectivity with another brainstem nucleus, the locus coeruleus (LC), which is known for its noradrenergic afferent connectivity. Therefore, after illuminating the role of the serotonergic system, we thought it important to determine the respective contribution from the noradrenergic system by similarly performing noradrenergic cortical denervation with the use of the drug N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4). Once the influence of the noradrenergic system on the NVC response more clear, several adrenergic antagonistic drugs would be tested to further parse out which specific noradrenergic receptors are involved in the hyperemic

response. Once the contribution from both α- and β- adrenergic receptors was determined we would be able to begin to understand which serotonergic and noradrenergic afferent pathways are involved in the NVC response to electrical stimulation of the DRN.

It would then be important to identify the cortical excitatory and inhibitory neurons recruited *in vivo* by electrical stimulation of the DRN, determine their respective contribution in the coupled hemodynamic response, and assess whether astrocytes were also involved in transmuting neuronal signals into vascular responses. Our initial strategy was to use double immunostaining for c-Fos as a reporter of neuronal activity (Staiger, 2006) and markers of excitatory pyramidal cells (COX-2) and inhibitory GABA interneurons (parvalbumin, PV; somatostatin, SOM; vasoactive intestinal polypeptide, VIP; nitric oxide synthase, NOS and choline acetyltransferase, ChAT) as a way to identify the recruited cellular network. Ultimately, we only ended up assessing c-Fos in a few rats and were not able to proceed with this technique as c-Fos expression was not induced, despite the reproducible hemodynamic responses seen.

The project then concentrated on *in vivo* pharmacology (through use of MK-801, MPEP + LY367385, picrotoxin) in order to demonstrate that the evoked CBF response to electrical stimulation of the DRN resulted from a specific recruitment of selective populations of excitatory pyramidal cells and inhibitory GABA interneurons. Moreover, we wanted to see if metabolically activated astrocytes and p450 epoxygenase vasoactive messengers were also required for the full expression of this response with the use of N-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide (MS-PPOH). The ultimate goal of the project was to have a clearer understanding of how the serotonergic afferents from

the DRN, along with the interaction of the noradrenergic system from the LC influenced the NVC response to DRN electrical stimulation. Furthermore, we hoped to classify how these afferent systems resulted in a blood flow response by beginning to define the neuronal and astrocytic mediators involved.

LITERATURE REVIEW

NEUROVASCULAR COUPLING

It has been known for over a century that when there is increased neuronal activity in the brain, there is a corresponding increase in local CBF within the activated region (Roy, 1890). It is also clear that proper functioning of the brain requires a continuous blood supply (Girouard and Iadecola, 2006). NVC, or functional hyperemia, refers to this adjustment of local CBF, which ensures adequate supplies of glucose and oxygen reach neurons displaying increased activity. Therefore, it seems intuitive that the cerebral vasculature would contain control mechanisms which ensure that adequate blood would reach activated areas in correspondence with local energy needs. Somewhat surprisingly, although the precise mechanisms underlying NVC remain largely unknown, changes in the hemodynamic signals (Blood oxygenation level-dependent functional magnetic resonance imaging, BOLD fMRI response; CBF and cerebral blood volume, CBV) are relied upon by functional neuroimaging techniques to map changes in neuronal activity under physiological and pathological conditions and thus have an irrefutable clinical importance (Carmignoto and Gómez-Gonzalo, 2010; Cauli and Hamel, 2010; Iadecola, 2004; Koehler et al., 2009; Ogawa et al., 1990). Furthermore, NVC is known to be impaired in various pathological states such as hypertension, Alzheimer's disease and ischemic stroke, thus making it necessary to elucidate the mechanisms behind NVC in order to accurately interpret many basic and clinical functional neuroimaging studies. It is essential that we understand how increased neuronal activity correlates to local increases in CBF.

THE NEUROVASCULAR UNIT

The conjugation of neuronal activation into a hemodynamic response is thought to be mediated through a complex interplay between the activated neurons, the microvasculature and perivascular astrocytes (Hamel, 2006). This complex interplay comprises what is referred to as the neuronal-astrocytic-vascular tripartite functional unit (Cohen et al., 1996; Paspalas and Papadopoulos, 1996; Vaucher and Hamel, 1995) which acts to maintain a homeostatic microenvironment in the brain. The CBF changes seen in response to neuronal activation are thought to be mainly achieved at the arteriolar level within the brain (Hillman et al., 2007), whereas the intraparenchymal vessels are more involved in local CBF regulation and blood-brain-barrier (BBB) permeability (Cohen et al., 1996). The glial processes known as astrocytic end-feet enwrap synapses and blood vessels and allow for this close interaction of all three elements of the neuronal-astrocytic-vascular tripartite functional unit.

Thus far, several mechanisms have been postulated in the literature to account for the control of the microvasculature. There is a growing body of evidence suggesting that it is both excitatory and inhibitory neurons that govern this hemodynamic response to neuronal activity increases (Enager et al., 2009; Kocharyan et al., 2008; Niessing et al., 2005). Naturally, one potential site of regulation is via the activation of distinct functional networks of GABA interneurons (Kocharyan et al., 2008), which could mediate the NVC responses from the incoming afferent signals they receive and integrate, hence modulating pyramidal cell output and the activity of the overall cortical network. These interneurons may also act directly on the blood vessels themselves or through the activation of astrocytes and their subsequent release of vasoactive mediators, for example

the production of epoxyeicosatrienoic acids (EETs) by COX activation (Peng et al., 2002) by their perivascular end-feet. It has also been suggested the pyramidal cells are able to not only act indirectly through astroglial signaling, but also directly control NVC through the release of arachidonic acid (AA) metabolites, like the production of prostaglandin E2 (PGE_2) by COX activation (Lecrux et al., 2011; Niwa et al., 2001). Another suggestion has been that the neuronal or astrocytic products of synaptic signaling, like K+ or nitric oxide (NO) could also control the tone of the vasculature (Iadecola, 2004). One current theory postulates that NVC is controlled by the neurons and glia generating chemical signals which are released from perivascular nerves and astrocytes, thereby initiating the vasodilation of the microvessel, whereas it is the endothelial cells, pericytes and smooth muscle cells which act together to orchestrate the transduction of these signals into vascular responses which are temporally linked to the activated neurons (Girouard and Iadecola, 2006). However, the identities of the distinct neuronal populations (pyramidal neurons and/or GABAergic interneurons) which instigate these hemodynamic responses have largely been unexplored and remain unknown.

Functional and anatomical evidence indicates that the neuronal circuitry which is involved in NVC is afferent-specific and will largely depend on the cellular targets at the cortical level of the noradrenergic (NE), serotonergic (5-HT), cholinergic (acetylcholine, ACh), or GABAergic afferents from, for instance, the LC, the DRN and basal forebrain (BF) (Hamel, 2006). Evidence suggests that activity-evoked changes in CBF are driven by these incoming afferent pathways and their local processing within the activated area, resulting in an activated neuronal network which is afferent specific (Cauli and Hamel, 2010; Lauritzen and Gold, 2003). For example, cholinergic basalocortical afferents

arising from the BF are thought to target excitatory pyramidal cells (Henny and Jones, 2008; Houser et al., 1985), inhibitory GABAergic interneurons (Cauli et al., 2004), microvessels and astrocytes (Vaucher and Hamel, 1995). The cells which integrate the incoming afferent signals will then drive the response of the vasculature, which is thought to occur either by direct action on microvessels, or to be mediated through astrocytes (Hamel, 2006). It is therefore essential that we better understand which subsets of neurons are activated through the stimulation of various specific afferent pathways, for example the serotonergic projections coming from the DRN. Figure 1 illustrates the proposed "neurovascular unit" and the interaction of the afferent projections with the vasculature, the glial cells and the activated neuronal populations.

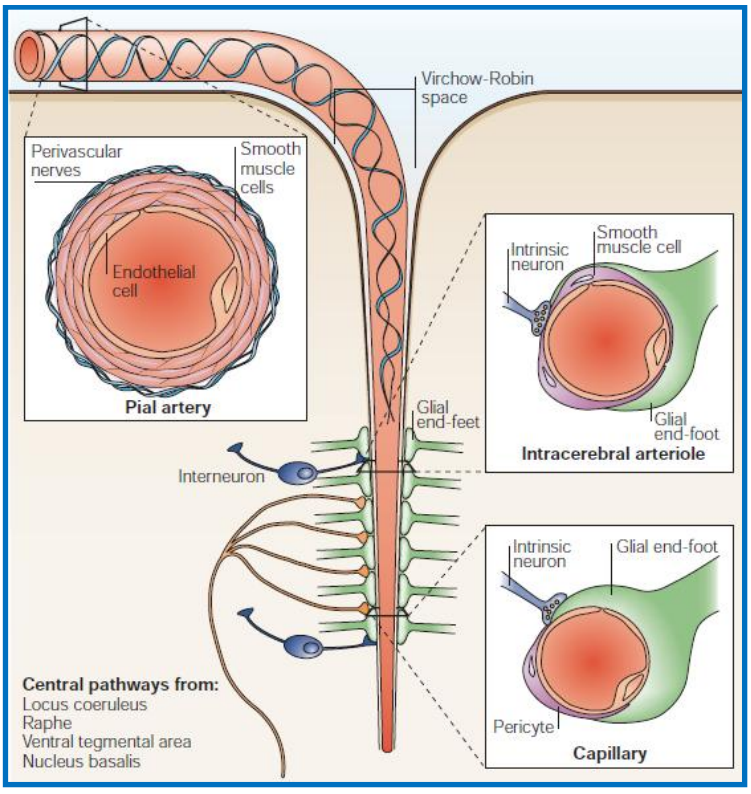

Figure 1: The Neurovascular Unit (Iadecola, 2004). It is now thought that the evoked hemodynamic response involves the coordinated interplay between activated neurons,

microvessels and astrocytes and this has been referred to as a neuronal-astrocytic-vascular tripartite functional unit. As you can see in the image, the intrinsic brain pathways (like the DRN) could relay neuronal signals which then interact with the vasculature, the glia, pyramidal cells and interneurons to somehow mediate a change in local CBF. However, the problem remains that we are still greatly unaware of the neuronal populations (pyramidal and GABAergic interneurons) that actually instigate CBF changes in response to these specific afferent pathways. Presently, the DRN is probably the least investigated of all of the aforementioned afferent pathways.

It has been shown that the serotonergic afferents of the DRN target the "vasomotor" interneurons in the cortex (Cauli et al., 2004); neurons which are capable of synthesizing and releasing vasoactive peptides which can act directly on the vasculature. Therefore the DRN is an attractive target for the study of NVC in the cerebral cortex. More interestingly, stimulation of 5-HT afferents from the DRN has been shown to increase cortical CBF, a response totally incompatible with the direct vasoconstrictor effect of 5-HT on cortical microvessels (Cohen et al., 1996). This observation further suggests that it is not the direct effect of a neurotransmitter or a neuromodulator on the microvessels that is driving the vascular response, but overall changes in the neuronal circuitry being selectively activated.

SEROTONERGIC AFFERENTS AND THE DORSAL RAPHE NUCLEUS

5-hydroxytryptamine or serotonin (5-HT) is an indoleamine synthesized from tryptophan hydroxylase (TPH). 5-HT is degraded by monoamine oxidase (MAO) and then dehydrogenated into 5-hydroxyindoleacetic acid (5-HIAA). 5-HT release in the brain occurs upon depolarization through a Ca^{2+}-dependent exocytosis and occurs as a function of the firing rate of serotonergic neurons. The effects of 5-HT release are mediated via

more than 15 presynaptic and postsynaptic serotonin receptors (Erritzoe et al., 2010) and the presence of most 5-HT receptor subtypes and their mRNAs have been well characterized in the cerebral cortex in varying distributions and gradients (Mengod et al., 2006). For instance, 5-HT1A and 5-HT1B receptor mRNA are greatly expressed in the cerebral cortex (Bonaventure et al., 1998; Bruinvels et al., 1994; Pompeiano et al., 1992), as well as 5-HT2A receptors whose expression follows a steep anteroposterior gradient (Mengod et al., 1990).

After 5-HT is released, it then either undergoes re-uptake by serotonin transporter (SERT) or is metabolized into 5-HIAA (Hamel, 2007). 5-HT has a profound role on a vast number of essential brain functions: it modulates mood, sleep, memory, sex, appetite, emotion and endocrine responses. Given its vast influence, it is not surprising that serotonin has been implicated in the etiologies of stroke, migraine and vasospasm (Edvinsson et al., 1983). However, the influence of the serotonergic system on regulation of CBF remains unclear.

The widespread neurochemical network of serotonergic neurons in the vertebrate central nervous system has remained stable across phylogeny in the rat, monkey and human brain (Jacobowitz and MacLean, 1978). For this reason it is possible to extrapolate the vast majority of cerebrovascular studies performed in the rat to applications in the human brain (Cohen et al., 1996), thus making this type of research applicable to humans. The majority of the 5-HT that innervates the forebrain comes from neurons whose perikarya reside within the midline raphe nuclei (Andén et al., 1966). Primarily in the rat, it has been shown that lesioning of these nuclei result in marked depletion of forebrain

serotonin (Kostowski et al., 1968; Kuhar et al., 1972; Lorens et al., 1971; Samanin et al., 1975), as well as cortical, hypothalamic and striatal 5-HT content (Jacobs et al., 1974). It is now known that more than 50% of the 5-HT neurons in the rat brain originate from the DRN, located in the periaqueductal gray (PAG) (Azmitia and Segal, 1978; Jacobs and Azmitia, 1992; Molliver, 1987). The DRN is thus the largest 5-HT-containing brainstem nucleus (Descarries et al., 1982; Steinbusch and Nieuwenhuys, 1983; Wiklund et al., 1981).

THE HETEROGENEITY OF THE DORSAL RAPHE NUCLEUS

The DRN is a bilateral and neurochemically heterogeneous nucleus which is bordered dorsally by the cerebral aqueduct and the rostral portion of the fourth ventricle and ventrally by the medial longitudinal fasciculus fiber bundles. Despite its well-characterized serotonergic afferents, only approximately 60% of the neurons within the DRN itself are serotonergic (Baumgarten and Grozdanovic, 1997; Jacobs and Azmitia, 1992; Molliver, 1987; Moore, 1981; Yasufuku-Takano et al., 2008). In addition to 5-HT, a plethora of other neurotransmitters and neuropeptides have also been identified within the DRN. Neurons expressing GABA (Belin et al., 1979; Gamrani et al., 1979; Mugnaini and Oertel, 1985; Nagai et al., 1983; Nanopoulos et al., 1982; Pfister et al., 1981), glutamate (Kaneko et al., 1990), dopamine (Nagatsu et al., 1979; Ochi and Shimizu, 1978), NE cell bodies and terminals (Grzanna and Molliver, 1980; Grzanna et al., 1978; Steinbusch et al., 1981), and NOS (Nakamura et al., 1991; Pasqualotto et al., 1991; Wang et al., 1995; Wotherspoon et al., 1994) have all been well characterized within the DRN. Interestingly, neuropeptidergic cell bodies have also been found inside the DRN, such as encephalin (Glazer et al., 1981; Hokfelt et al., 1977; Moss and Basbaum, 1983; Uhl et al.,

1979), VIP (Loren et al., 1979; Sims et al., 1980), Substance P (Chan-Palay et al., 1978; Hokfelt et al., 1978; Ljungdahl et al., 1978), cholecystokinin (CCK) (Bhatnagar et al., 2000; Otake, 2005; Vjanderhaeghen et al., 1980), neuropeptide Y (De Quidt and Emson, 1986), galanin (Cortes et al., 1990; Melander et al., 1986; Skofitsch and Jacobowitz, 1985), corticotropin-releasing factor (CRF) (Commons et al., 2003b), neurotensin (NT), SOM, thyrotopin-releasing hormone (TRH), growth hormone (GH) and growth hormone releasing hormone (GHRH), leu-enkephaline, metenkephalin, and gastrin (Descarries et al., 1986; Monti, 2010; Ochi and Shimizu, 1978). Some of these neuropeptides are co-expressed with other neurochemicals and some are synthesized by distinct cells of the DRN. It is therefore of importance to note that in addition to 5-HT, other neurotransmitter and neuropeptide systems contribute to the connectivity and modulation of the DRN 5-HT neurons (Descarries et al., 1982; Jacobs and Azmitia, 1992; Moore, 1981; Steinbusch et al., 1980; Tork, 1990; Wiklund et al., 1981).

The DRN has been divided based on cellular morphology into 5 major sub-regions: the most caudal aspect, the dorsolateral, the dorsomedial, the ventromedial and the interfascicular region (Steinbusch et al., 1981). The DRN has also been divided based upon expression of other neurotransmitters (Jacobs and Azmitia, 1992), its afferent and efferent connectivity (Imai et al., 1986; Peyron et al., 1997), and its functional properties (Adams and Moghaddam, 2001). The 5-HT cells within the DRN are found throughout its rostrocaudal axis, but are predominantly located along the midline of the rostral, dorsal and ventral subdivisions of the nucleus (Day et al., 2004; Molliver, 1987; Villar et al., 1988). The subdivisions of the DRN based on the characteristics of individual serotonergic neurons differ with respect to their morphologic features, membrane and

cellular properties, afferent regulation and efferent projection patterns. Anatomical studies have shown that the 5-HT neurons located more rostrally within the DRN project selectively to specific forebrain sties (e.g. the basal ganglia, substantia nigra (SN) and almost all neocortical regions), while 5-HT neurons located more caudally in the DRN project to other forebrain sites (e.g. hippocampus, entorhinal cortex, septum) (Köhler and Steinbusch, 1982; Steinbusch et al., 1981; Vertes, 1991). These topographically organized subdivisions of serotonergic neurons within the DRN suggest a potential link between structure and function; given that these subdivisions not only differ in regards to their serotonergic neurons but also co-express different neurotransmitters and neuropeptides.

DORSAL RAPHE NUCLEUS CONNECTIVITY

The serotonergic system within the DRN is a vast neurochemical network which provides dense innervation to the caudate-putamen (basal ganglia), SN, cerebral cortex (CTX), thalamus, hippocampus (HPC), amygdala (AM) and hypothalamus (Vertes, 1991) and is therefore the principle source of 5-HT innervation of the brain both in rats and humans (Monti et al., 2008; Vertes and Linley, 2008). An illustration of the DRN major projection sites is shown in Figure 2. Moreover, it has been shown that the DRN also receives projections from various other brain areas as well, which express monoamines, amino acids, ACh or neuropeptides and either act directly or indirectly via local circuits to modulate the 5-HT cellular activity (Abrams, 2004; Charara and Parent, 1998; Clements et al., 1991; Commons et al., 2003a; Jacobs and Azmitia, 1992; Johnson and Ma, 1993; Li et al., 2001; Lowry et al., 2008; Moss and Basbaum, 1983; Steinbusch, 1981). Additionally, the cerebral cortex, the limbic system, the BF bundle, the hypothalamus and the cholinergic, dopaminergic and noradrenergic nuclei of the brainstem have all been

shown to provide further inputs to the DRN 5-HT cell bodies. It is this multifaceted connectivity within the DRN, combined with the multitude of other neurotransmitter and neuropeptide systems and their concurrent connectivity's which amount to an extremely complicated modulatory role within in the vertebrate central nervous system and provides many routes through which the DRN could affect cortical activity and subsequently CBF.

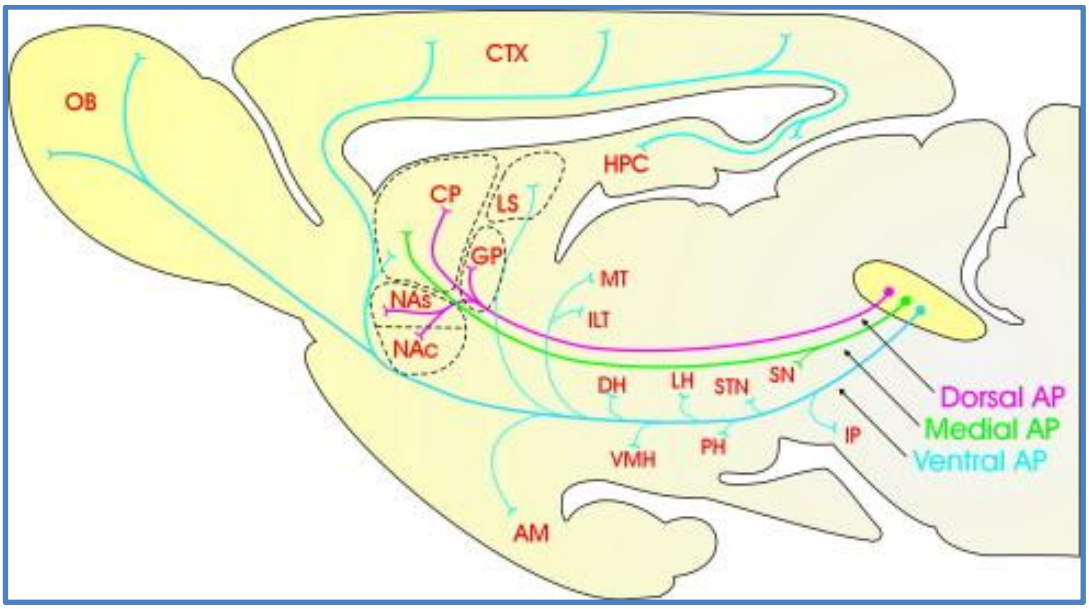

Figure 2: Major DRN Projection Sites. Figure taken from Michelsen et al., 2007. The diagram illustrates the three ascending pathways (AP's) of the rat DRN and their main targets. The dorsal and ventral ascending pathways are the two most important efferent projections of the DRN. They reach a multitude of targets throughout the forebrain, the most important one being the caudate-putamen. In addition, four descending projections leave the DRN: the bulbospinal pathway, cerebellar pathway, propriobulbar pathway and one that innervates the locus coeruleus, dorsal tegmental nucleus and pontine raphe nucleus. The dorsal ascending pathway rises from the medial and rostral DRN and innervates the striatum and globus pallidus. The main target of the medial ascending pathway is the substantia nigra, whereas the ventral ascending pathway targets many areas including the thalamic and hypothalamic nuclei, septum, amygdala, olfactory bulb, cerebral cortex and hippocampus. AM, amygdala; CP, caudate-putamen; CTX, cortex;

DH, dorsal hypothalamus; GP, globus pallidus; HPC, hippocampus; ILT, intralaminar thalamic nuclei; IP, interpeduncular nucleus; LH, lateral hypothalamus; LS, lateral septum; MT, midline thalamic nuclei; NAs, shell of nucleus accumbens; NAc, core of nucleus accumbens; SN, substantia nigra; STN, subthalamic nucleus; PH, posterior hypothalamus; VMH, ventromedial hypothalamus (Michelsen et al., 2007).

SEROTONIN AND NEUROVASCULAR COUPLING

Since the serotonergic system plays such an influential role in a variety of vital physiological activities, the question arose as to what, if any, influence it had on cerebral vascular changes. It has been known for over sixty years that 5-HT is a potent vasoconstrictor in blood serum (Rapport et al., 1948). Additionally, neuronal cells bodies and dendrites within the raphe nucleus have been shown to be in close apposition to local blood vessels (Itakura, 1985; Kapadia and de Lanerolle, 1984) and nerve fiber networks have been shown around major cerebral arteries and pial vessels that contain 5-HT (Chan-Palay, 1976; Cohen et al., 1995; Edvinsson et al., 1983; Scatton et al., 1985) and TPH (Chédotal and Hamel, 1990; Cohen et al., 1992; Mathiau et al., 1993). It is therefore not surprising that a direct role of the serotonergic system on NVC was suggested (Edvinsson et al., 1983; Reinhard et al., 1979). These observations provide the anatomical means for this system to functionally modulate the cerebral microcirculation through 5-HT. However, lesioning of the serotonergic neurons resulted in the observation of a resounding absence of vascular changes, irrespective of experimental paradigms and methods used to measure CBF (Cohen et al., 1996). One study showed that basal CBF was unaltered by an acute and specific lesion of the DRN serotonergic neurons (Underwood et al., 1992). The multitudes of studies showing similar results have led to the conclusion that the serotonergic system does not have significant tonic influence on

resting CBF and perhaps its main role is better demonstrated in periods of phasic activation.

In contrast to the results observed by removal of serotonergic neurotransmission, electrical stimulation of the DRN in both conscious and unconscious animals has been shown to significantly alter CBF (Cohen et al., 1996). However, the activation of 5-HT neurons either electrically or chemically has been shown to both increase (Cudennec, 1989; Goadsby et al., 1985a, b) and decrease CBF (Bonvento et al., 1989; Cao, 1992; Cudennec et al., 1993). It is important to consider that many studies have used different methodologies to measure blood flow, often which does not provide equivalent indices of CBF. The [^{14}C] iodoantipyrine (IAP) method is an autoradiographic technique which uses a radioactive tracer and results in a quantitative map of CBF values through the entire brain. This varies greatly from the LDF technique, which as used in this study and which provides qualitative flow values in a specific cortical area of about 1 mm^3 beneath the probe, thereby providing a measure of intracerebral blood flow. Studies in the cat and monkey have shown a vasodilatory effect of electrical stimulation with use of the LDF technique (Goadsby et al., 1985a, b). In contrast, the IAP technique has resulted in mainly global CBF decreases in the anesthetized rat (Bonvento et al., 1989), whereas in one study in the conscious rat, 9 out of 63 structures investigated showed an increased CBF (Cudennec, 1989).

Another important confounding factor in studies dealing with serotonin is the animals' state of vigilance (Bonvento et al., 1989) since the serotonergic system plays a profound role in the sleep-wake-arousal cycle (Jacobs and Azmitia, 1992). The DRN neurons have

also been shown to respond differently depending on their prior level of activity (Fornal et al., 1994). Therefore, it is likely that the effect of electrical stimulation of the DRN in awake vs. anaesthetized animals will result in different outcomes due to differing modulation of serotonergic neurotransmission.

Attributing to the fact that we are now aware of the complexity and heterogeneity of the DRN, it is increasingly important to confirm the location of electrical stimulation within the nucleus itself. It was observed that in fact, depending on the anatomical sub-localization of the stimulation site within the DRN, different evoked CBF responses can be evoked, as illustrated in Figure 6 (Underwood et al., 1992). Selective stimulation of the rostroventral portion of the DRN results in decreases in CBF (as measured in the parietal cortex), while stimulation of the caudal portion elicits increases. Interestingly, very few studies investigating the hemodynamic response to DRN stimulation publish the location of their electrodes, making adequate interpretation of previous studies quite challenging.

Cao et al., showed that chemical stimulation of the DRN with L-glutamate could produce a decrease in CBF which was eliminated with application of either 5-HT1A or 5-HT2A receptor antagonists, methysergide or ketanserine (Cao, 1992), respectively. It has also been shown that pharmacological activation of 5-HT1A receptors through intravenous (i.v.) injection of a 5-HT1A receptor agonist, 8-OH-DPAT (McBean et al., 1991) or the subcutaneous (s.c.) injection of the 5-HT specific neurotoxin, methylenedioxyamphetamine (McBean et al., 1990), both result in an increase in CBF in several brain areas. These results in combination with electrical stimulation studies seem to suggest a role for 5-HT in the control of CBF, appearing mainly vasocontractile in

nature. Interestingly, it appears that the influence of 5-HT on CBF is independent of changes in cerebral metabolism, suggesting 5-HT has a direct effect on the cerebral circulation (Bonvento et al., 1991; Cohen et al., 1992; McBean et al., 1990, 1991).

SEROTONIN REGULATION OF CORTICAL CELLULAR ACTIVITY

Not many studies have looked into the mechanisms of action of 5-HT on the NVC response, but much is known about the effects of 5-HT on cortical neuronal activity. The neurophysiological effects of serotonin have been most extensively studied in the pyramidal cells of layer V of the prefrontal cortex (PFC). 5-HT application directly onto cortical brain slices has been shown to result in both depolarizing and hyperpolarizing responses in pyramidal and non-pyramidal cells (Aghajanian and Sanders-Bush, 2002), and has been shown capable of modulating active conductances (Andrade, 2011). This variation is now known to be due to the potential for 5-HT to activate multiple serotonin receptors which show differential expression throughout the cells of the cerebral cortex. For example, in young and adult rat brain slices, the hyperpolarizing effect that 5-HT has upon the pyramidal cells of layer V has been shown to primarily be mediated through activation of 5-HT1A receptors (Araneda and Andrade, 1991; Béïque et al., 2004). It is thought that these 5-HT1A receptors can be activated by endogenous 5-HT release due to the fact that 5-HT1A receptor-mediated inhibition is also seen in Layer V *in vivo* after DRN stimulation (Amargos-Bosch et al., 2004), consistent with the robust expression of these receptors in layers V and VI of the PFC (Pompeiano et al., 1992). In contrast, 5-HT2A receptors have been shown to have a depolarizing/excitatory effect on the pyramidal cells of Layer V (Araneda and Andrade, 1991; Villalobos et al., 2005) resulting in an increase in membrane excitability (Béïque et al., 2007). These results are supported

by *in situ* hybridization and receptor autoradiographic studies showing expression of 5-HT2A receptor mRNA in layer V of the PFC (Mengod et al., 1990; Wright et al., 1995). A recent study by Labonte et al. in the rat cortex further confirmed the *in vitro* results described above showing 5-HT2A and 5-HT1A receptor-mediated excitatory postsynaptic potential and inhibitory postsynaptic potentials, respectively (Labonte et al., 2009). Almost counter intuitively, these two receptor subtypes are found to be largely co-expressed in pyramidal cells (Amargos-Bosch et al., 2004; Araneda and Andrade, 1991; Béïque et al., 2004). Andrade et al. suggested that perhaps these two receptors regulate PFC pyramidal cell firing in a cooperative manner to modulate action potential firing (Andrade, 2011).

It has also been shown that DRN stimulation modulates the responses of barrel cortical neurons in layers IV and V of the rat (Aghajanian and Marek, 1997; D'Amato et al., 1987; Pazos et al., 1985). This occurs presumably either via the high density of serotonin receptors found in layers I and V of the barrel cortex in adult rats (Blue et al., 1988; D'Amato et al., 1987) or the high number of receptors localized to layers IV and VI in neonates (Arthurs et al., 2000). A recent study has also shown that while DRN stimulation has both excitatory and inhibitory effects on PFC neuronal activity (Puig et al., 2005), electrical stimulation of the DRN can inhibit the spontaneous activity of the PFC (Hajós et al., 2003; Mantz et al., 1990) and suppress the evoked responses of the PFC (Mantz et al., 1990) and cat somatosensory brain stem neurons (Chiang et al., 1989).

INTERACTION BETWEEN DRN AND LC

It is increasingly believed that there is a reciprocal relationship between the DRN and LC (Haddjeri et al., 1997; Singewald and Philippu, 1998) and that interactions between these two nuclei result in a significant influence of NE on the 5-HT system (Sakai et al., 1977). For instance, it is known that NE projections from the LC are able to influence DRN 5-HT neuronal activity in an excitatory manner (Luppi et al., 1995). It has been suggested based on pharmacological studies that the DRN 5-HT neurons are dependent on tonic activation by NE input which is mediated by α1-post-synaptic adrenoceptors.

However, it is not just the LC that influences the DRN; it is thought that the 5-HT from the DRN has an inhibitory role on the function of LC noradrenergic neurons (Cassano et al., 2009). For example, activation of 5-HT1 receptors has been shown to reduce glutamate-induced activation and the glutamatergic synaptic potentials of LC noradrenergic neurons (Aston-Jones et al., 1991; Bobker and Williams, 1989). It has also been seen that a 5-HT2 agonist induces increased activation of the GABAergic input from the DRN to the LC (Chiang and Aston-Jones, 1993), suggesting that 5-HT2 postsynaptic receptors which are located at presynaptic GABA neurons are a direct target of 5-HT released from DRN axons projecting to the LC (Celada et al., 2001). These findings are consistent with the suggested major inhibitory and minor excitatory effects displayed by serotonin at the LC (Lechin et al., 2006). A general overview of the interactions of the DRN and LC is illustrated in Figure 3, below.

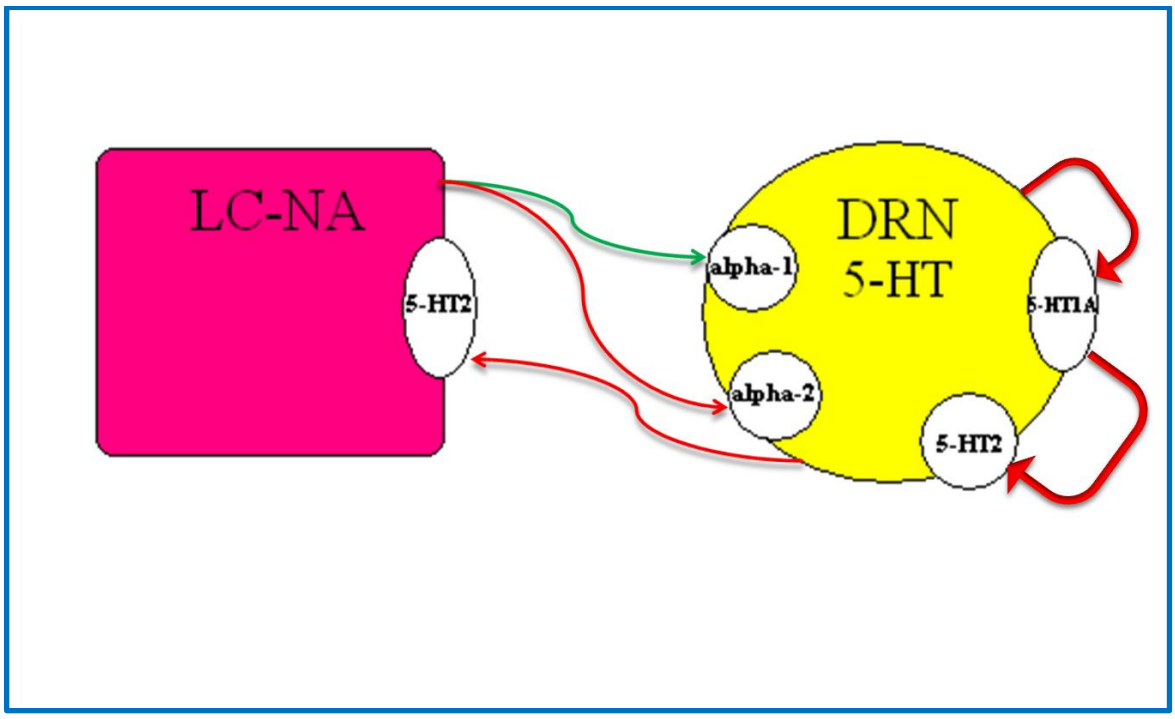

Figure 3: Interactions of the DRN and the LC. The above diagram illustrates some of the known interactions between the LC noradrenergic projections and the DRN 5-HT projections. Red arrows indicate inhibitory connections and green arrows indicate excitatory connections. Figure adapted based on findings by Lechin et al., 2006.

CURRENT STATE OF THE LITERATURE

At this point, investigation of the cellular mechanisms underlying the NVC response to DRN stimulation is minimal, at best. As described above, many studies have looked at determining the nature of the evoked CBF response but have mainly focused on whether it was a vasodilatation or vasoconstriction. Pharmacological studies in this field are presently quite scarce. Therefore, this project provides the necessary next step to elucidating firstly the contribution of the serotonergic and noradrenergic systems and secondly, beginning to determine the cortical network that conjugates these afferent signals into a blood flow response.

QUESTIONS AND OBJECTIVES

1. What is the reproducible hemodynamic response to electrical stimulation of the DRN?
    - Attempt to localize the electrode within the DRN and confirm its localization.
    - Classify what the evoked CBF response is in response to electrical stimulation, until it is reproducible.
2. Given that the DRN is a foremost a serotonergic nucleus, what is contribution of the serotonergic system to the evoked CBF response?
    - Pharmacologically destroy serotonergic nerve terminals with PCPA to determine the evoked CBF response after treatment, in order to determine the serotonergic systems contribution to the hemodynamic responses seen.
    - Confirm that PCPA treatment did selectively destroy 5-HT nerve terminals and had no effect on noradrenergic fibers with immunostaining.
    - Test 5-HT2A receptor antagonist to determine if these receptors specifically are involved in the hemodynamic response.
3. What is the contribution of the noradrenergic system on the evoked CBF response, given the extensive connectivity of the DRN with the LC?
    - Pharmacologically destroy noradrenergic afferent fibers with the use of DSP-4 in order to determine what effect this has on the evoked CBF response.

- Confirm that DSP-4 treatment successfully destroyed the noradrenergic fibers and had no effect of 5-HT nerve terminals, with the use of immunostaining.
- Test non-selective α- and β- adrenergic receptor antagonists in order to determine if specific sub-types of adrenergic receptors may be involved in this response.

4. What are the cortical excitatory and inhibitory neurons recruited *in vivo* by electrical stimulation of the DRN?
    - Attempt to identify the cellular ensemble that underlies the hemodynamic signals by discovering the subsets of neurons activated after *in vivo* stimulation of DRN in CBF-recorded rats (LDF) using double-immunostaining for c-Fos and markers of excitatory and inhibitory neurons.

5. What are the respective contributions of excitatory and inhibition neurons to the evoked hemodynamic response?
    - Identify *in vivo*, the mediators of the altered NVC response to DRN stimulation through pharmacological intervention with antagonists to glutamatergic receptors and GABA-A receptors.

6. Are astrocytes involved in transmuting these neuronal signals into vascular responses?
    - Perform additional *in vivo* pharmacology with a drug targeting the astroglial pathways.

HYPOTHESIS

Electrical stimulation of the DRN will result in the activation of a specific network of cortical pyramidal cells and GABA interneurons that drive the evoked CBF response either directly or through astroglial messengers, as measured with LDF.

METHODOLOGY

For all procedures adult male Sprague-Dawley rats (280-300 grams, Charles River) were used. Procedures were approved by the animal ethics committee of McGill University and followed the guidelines outlined by the Canadian Council on Animal Care. Rats were stereotaxically implanted under deep anesthesia (isoflurane, 5%, 2-3 minutes) with stimulating electrodes positioned within the DRN based on Bregma coordinates (AP:-1.33; V: -0.622; L:0) at a 40 degree posterior angle to avoid the superior sagittal sinus (Bonvento et al., 1989). Implantation was performed approximately 5 days before the experimental day in order to avoid cortical spreading depression and non-selective c-Fos activation. Electrical DRN stimulation and CBF measurements were performed in rats anesthetized with urethane (1.1 g/kg, i.p., in saline) as it does not affect autoregulation, CBF and cortical activity (Huttunen et al., 2008). A catheter filled with heparinized saline was inserted in the femoral artery for monitoring of blood pressure (PowerLab, ADInstruments). Blood gases and pH (Rapid Lab 348, Bayer) were also monitored throughout the experiment. Mean arterial pressure, heart rate, blood gases and pH remained normal throughout these experiments (see Table 1). Animals were then fixed in a stereotaxic frame (David Kopf Instruments) and body temperature maintained at 37˚C using a temperature control system (FHC). For electrical stimulation, the skull bone was thinned bilaterally (while intermittently being cooled with saline) in a 3 x 3 mm region

for cortical positioning of LDF needle-shaped probes (Transonic Systems) for CBF recording just below and lateral to the Bregma on each side over the left and right somatosensory cortex, positioned in areas free of large blood vessels.

Table 2: Physiological Parameters. Values shown are averages for each drug with standard error of the mean. For the PCPA experiment, at the time conducted the Blood Gas Analyzer was not properly reading pH, so only one value was accurately recorded and is shown in the table. The same thing is true for the saline and DSP-4 groups, of which only one blood sample was accurately read for saline and two for the DSP-4 group. However, because the values taken are within normal ranges and MABP was normal for all saline, DSP-4 and PCPA animals, it was deemed sufficient to display data.

	MABP (mmHg)					
	Baseline	**Vehicle**	**Drug**	**pH**	**pCO_2**	**pO_2**
i.p. PCPA	88±2			7.26	38±3	98±4
i.p. saline	79±3			7.43	29	152
i.p. DSP-4	105±9			7.43	36	120
i.c. ketanserine	84±7	67±3	65±3	7.39±0.04	45±4	89±7
i.c. phentolamine	84±6	76±8	77±7	7.39±0.02	39±3	101±3
i.c. propanolol	63±2	60±3	62±2	7.37±0.03	47±5	97±6
i.c. MK-801	71±5	66±5	63±6	7.29±0.08	48±3	97±6
i.c. MPEP + LY367385	76±6	75±5	74±3	7.36±0.03	44±3	100±4
i.c. picrotoxin	78±7	66±4	68±5	7.33±0.03	46±2	98±4
i.c. MS-PPOH	73±7	70±6	73±8	7.28±0.03	44±4	90±7

STIMULATION PARADIGM

Electrical stimulation parameters used were 100 Hz, 80 uA, 1 second on/1 second off for 20 seconds. Each stimulation was separated by a 10 minute interval to allow CBF to return to baseline. Approximately 2 baseline electrical stimulations were performed to verify a consistent change in CBF before injection of vehicle, subsequent stimulation to insure similar response, and then injection of drug after which stimulations were repeated at 10-, 20-, 30-, 40-, 50- and 60-minutes post-injection. An example of the stimulation protocol is illustrated below in Figure 4.

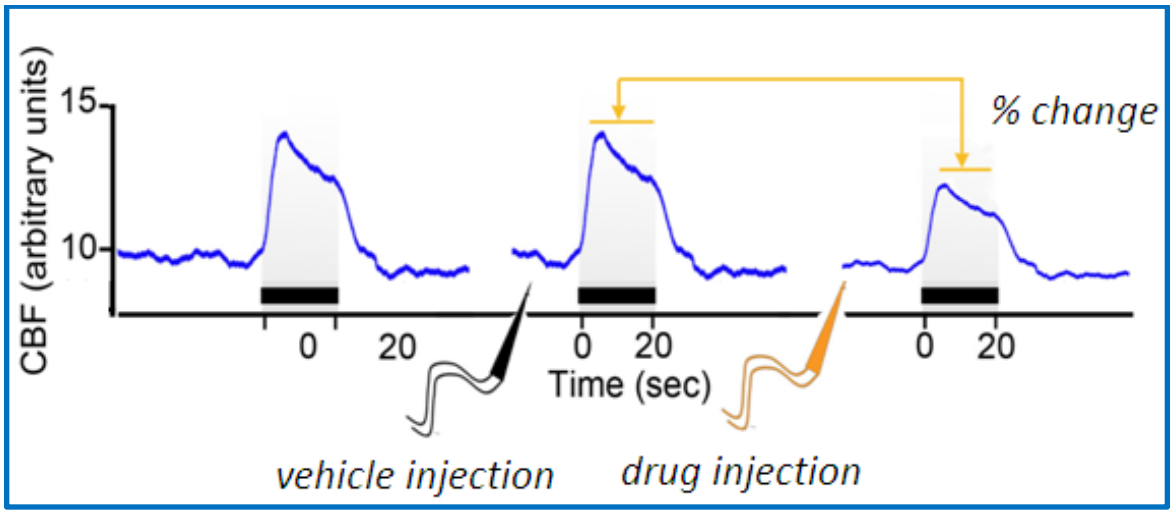

Figure 4: Illustration of Stimulation Protocol. As you can see from the diagram above, the first evoked CBF response is the baseline stimulus seen initially. Then after this is done twice to confirm its reproducibility, the vehicle is injected and 10 minutes later there is another stimulation to confirm that the vehicle i.c. injection had not altered the baseline response. Then the drug was injected and at 10 minute intervals following i.c. injection of the drug, the evoked CBF response was measured and compared with that seen with the vehicle. Figure adapted from Clotilde Lecrux with permission.

LOCALIZATION OF ELECTRODE

Initial localization of electrode was achieved through the use of acute stimulations. The acute stimulations, which allowed simultaneous manipulation of the electrodes vertical depth, and monitoring of evoked CBF responses upon stimulation, allowed coordinates to be determined which gave the most consistent and reproducible CBF increase. To confirm that this location was in fact in the DRN, Toluidine blue dye injections were performed at these same coordinates to ensure the dye was present within the DRN in brain slices.

After it was determined that the DRN could be accurately and reproducibly targeted by repeated dye injections, the localization of the electrode was analyzed in each animal after the experiment to ensure accurate positioning. In each experiment, after final stimulations were performed, an electrolytic lesion was made using a higher current over the duration of 20 seconds. Brains were then cut on freezing microtome in 50 μm sections along the length of the DRN. If a small electrolytic lesion was seen to be positioned within the DRN, the localization of the electrode was deemed successful. An example of one section showing the electrolytic lesion is illustrated in Figure 5. Any experiments where the electrolytic lesion location was found outside of the borders of the DRN was discarded. Cresyl violet staining was performed on sections in which the lesion could not be seen with the naked eye in order to improve visualization.

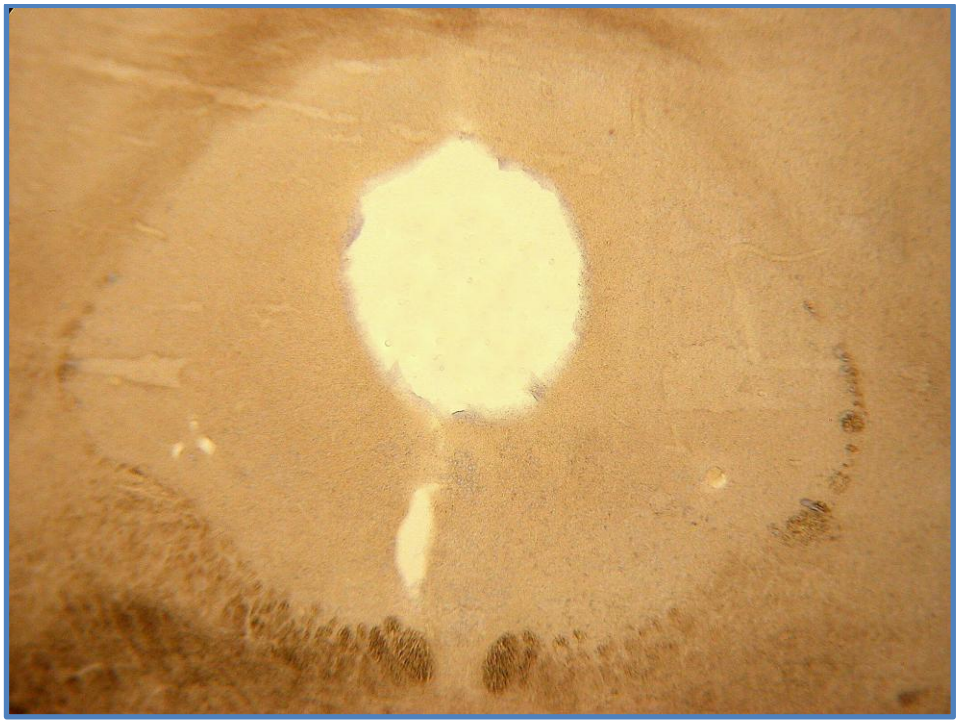

Figure 5: Localization of electrode within the DRN. This is an example of the size of the electrolytic lesion seen after experiments and used to determine accurate electrode location within the central DRN. When the electrolytic lesion was found outside of the DRN or deemed too lateral within the DRN, the experiment was discarded.

IMMUNOHISTOCHEMISTRY

C-FOS

At the end of the stimulation, rats were anesthetized with pentobarbital and their brains perfusion-fixed (500 mL of ice-cold 4.0% PFA in 0.1 M phosphate buffer, pH 7.4) and cut on a freezing microtome (25 μm slices). Sections were then immunostained for c-Fos (rabbit anti-c-Fos, 1:15,000; Oncogene), a marker of neuronal activation, detected with the ABC complex (45 min, Vectastain ABC kit, Elite PK-6100, Vector Laboratories) and visualized with the SG reagent (SK-4700, Vector Laboratories, blue-gray precipitate).

5-HT AND DBH

For all animals in the PCPA and DSP-4 experiments, rats were anesthetized with pentobarbital and their brains perfusion-fixed (500 mL of ice-cold 4.0% PFA in 0.1 M phosphate buffer, pH 7.4) and cut on a freezing microtome (25 μm slices). Sections were first immunostained for 5-HT (rabbit anti-5-HT, 1:8000; Immunostar, Inc), a marker of 5-HT nerve terminals, and detected with fluorescence (Anti-Rabbit CYTH-3, 1:3000 final). Separate sections were also stained for dopamine β-hydroxylase (DβH) (mouse anti-DβH, 1:2000, Millipore), a marker of noradrenergic nerve terminals and detected with the ABC complex (45 min, Vectastain ABC kit, Vector Laboratories) and visualized with a 0.05% solution of 3,3'-diaminobenzidine (DAB reagent, Vector Labs, brown precipitate). Species specific secondary antibodies (1:200, Vector, 45 minutes) were used; anti-rabbit for 5-HT and anti-mouse for DβH. Sections were observed under either epifluorescence (5-HT) or light (DβH) microscopy (Leitz Aristoplan, Leica), digital pictures taken (CoolPix 4500, Nikon), and then calibrated and edited with Adobe Photoshop 7 (Adobe Systems).

LESION PROCEDURE

We looked at depletion of 5-HT by inhibition of TPH through application of PCPA and cortical noradrenergic denervation by treatment with DSP-4.

For the PCPA treatment, implantation was performed on day 1, followed by drug injections on days 2, 3, 4 and 5 (200 mg/kg, i.p. 10 mL/kg saline). Electrical stimulations were performed on day 5 approximately 2 hours after the final drug injection. For this

experiment, treated animals were compared against saline-treated controls from the DSP-4 protocol.

For DSP-4, the first injection of the drug occurred on day 1 (60 mg/kg, i.p. 10 mL/kg saline), followed by the second injection on day 7 (50 mg/kg, i.p. 10 mL/kg saline). On the day following the second injection the implantation was performed and electrical stimulation occurred 4-5 days after this. Controls were treated with saline.

PHARMACOLOGY

In order to identify the neuronal, astroglial and/or vascular mediators of the perfusion response, we used *in vivo* pharmacology. The contribution of GABA interneurons, pyramidal cells and astrocytes were assessed through intra-cisternal (i.c.) injection (3 ul over 1 minute) of specific GABA and glutamate receptor antagonists, blockers of the serotonergic and noradrenergic systems, or inhibitors of synthesis of vasoactive messengers (EETs) under microscope monitoring (Kocharyan et al., 2008). Antagonists targeted group 1 and 5 metabotropic glutamate receptors (mGluR-1 and mGluR-5; MPEP+ LY367385), NMDA (MK-801), GABA-A (picrotoxin) and 5-HT2A receptors (ketanserine). Enzyme inhibitors targeted vasodilatory epoxyeicosatrienoic acids (EETs) and synthetic enzymes P450 epoxygenases (MS-PPOH). Furthermore to assess the interrelationship of the DRN with the noradrenergic systems of the brain, we tested the effects of a nonselective α-adrenergic antagonist (phentolamine) and non-selective β-adrenergic receptor blocker (propranolol).

In each rat, only one compound was tested in addition to the no-drug and vehicle conditions. CBF measurements were recorded and averaged from 4 to 6 stimulations

before and after either vehicle or drug administration (measured at 10, 20, 30, 40, 50 and 60 min after injection).

We used a 10^{-4} M concentration for most compounds except for MK-801 (4 x 10^{-3}M), MS-PPOH (10^{-3} M) and MPEP + LY367385 (10^{-3} M) and presented the most efficacious incubation time for each compound (usually at 20 or 30 minutes post-injection of drug). Concentrations and incubation times were determined from previous studies (Kocharyan et al., 2008; Lecrux et al., 2011) or from pilot studies based on demonstrated efficacy of the compounds after i.c. injection in other CNS-mediated functions. The GABA receptor antagonist was used at a dose that did not induce epileptic activity (Kocharyan et al., 2008; Lecrux et al., 2011). Unless otherwise stated, drugs were prepared in 0.5 M PBS. MK-801 and picrotoxin were purchased from Tocris Biosciences. MPEP (6-Methyl-2(phenylethynyl)pyridine), LY-367385 (vehicle 10^{-3} M NaOH in 0.5 M PBS equilibrated to pH 7.4 with 1N HCl), phentolamine and propranolol were from Sigma. MS-PPOH (vehicle 0.5% ethanol in 0.5M PBS) was obtained from Cayman Chemicals.

STATISTICAL ANALYSIS

All CBF results displayed are from the right somatosensory cortex. Bilateral responses were seen, and deemed equivalent in each hemisphere, therefore only one side of the cortex was used for all diagrams and analyses. It should be noted that the changes in the left cortex were not significantly different from those in the right. CBF changes induced by electrical stimulation were measured in arbitrary flux units, saved, and analyzed on a PC using Chart 7 software (ADInstruments). The percentage changes were averaged over the 20 second period of electrical stimulation obtained from all of the trials for that

particular compound. Drug effects were calculated by taking peak CBF response in drug condition vs. peak CBF response in vehicle condition. All Drug effects were tested by repeated-measures ANOVA and a post hoc Newman-Keuls test. All reported values are measure +/- SEM. All statistical analyses were performed with Prism4 (GraphPad Software), and $p<0.05$ was considered significant.

RESULTS

CHOICE OF DRN COORDINATES AND ESTABLISHMENT OF BASELINE RESPONSE

The first thing that needed to be confirmed was that the site of electrical stimulation was in fact the DRN itself and not an adjacent area. In order to verify this, Bregma coordinates were chosen according to previous literature so as to implant an electrode directly into the DRN. There is evidence that depending upon where you are stimulating within the DRN, you can evoke an increase or a decrease in CBF (Underwood et al., 1992) as seen in Figure 6, below. The coordinates that we chose are those suspected to give an increase in CBF, (AP:-1.33; V: -0.622; L:0) at a 40 degree posterior angle to avoid the superior sagittal sinus (Bonvento et al., 1989; Underwood et al., 1992).

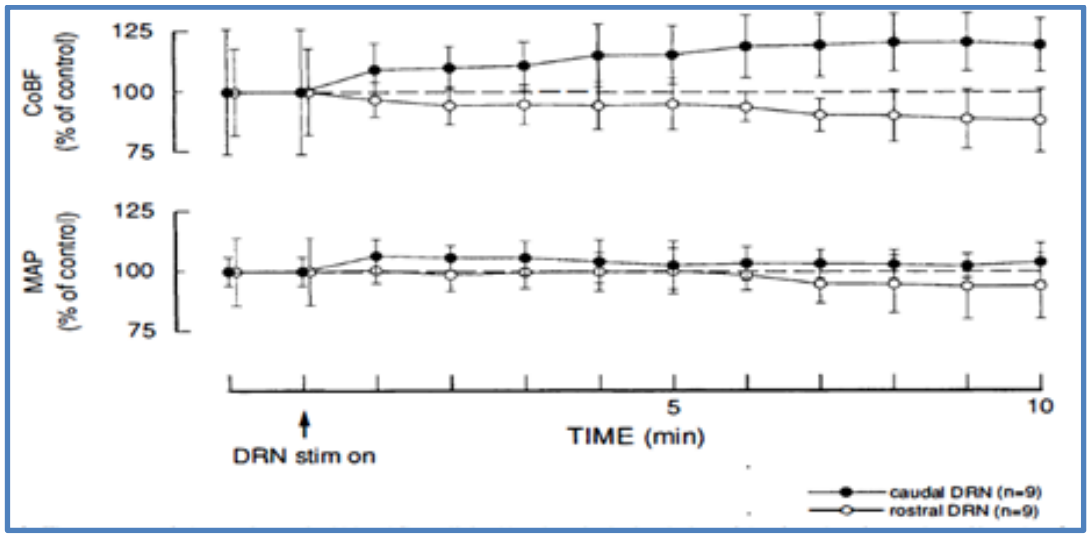

Figure 6: Time course of change in cortical blood flow elicited by electrical stimulation of the dorsal raphe nucleus (Underwood et al., 1992). Note that CBF either increased or decreased slowly as stimulus intensity was increased from 0 uA to approximately 75 uA. The changes in CBF were stable at approximately 2-3 minutes and were sustained with continued stimulation. This diagram illustrates the choice of stimulating the caudal DRN in order to obtain an increase in evoked CBF response.

Based upon these coordinates, in an attempt to evoke an increase in CBF by implanting in the caudal DRN, acute stimulations were then performed. The acute stimulations resulted in an approximate average of a 30% increase in CBF in response to the electrical stimulations at the aforementioned parameters. Our stimulation parameters were chosen such that they would give maximal CBF response without destroying the tissue. An example of a typical baseline stimulation which was seen in the acute stimulations and before i.c. administration of vehicle or drug in all of the pharmacological studies is illustrated in Figure 7, recorded from both the right and left somatosensory cortex.

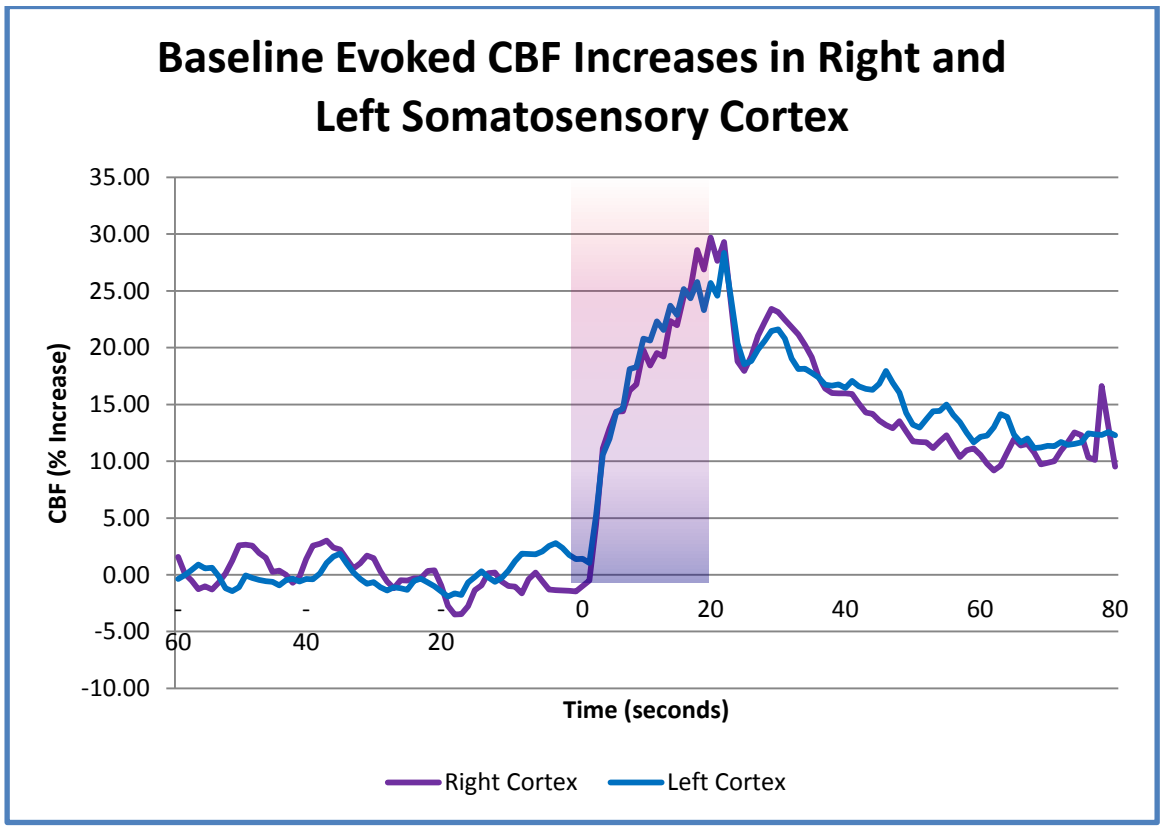

Figure 7: Baseline Evoked CBF Response in Right and Left Somatosensory Cortex. This is an illustration of typical evoked CBF increases in response to DRN electrical stimulation, averaged in eight animals over the right and left cortices, typically ranging between 25% and 35% change from baseline CBF measurement. The shaded bar

represents duration of electrical stimulus (20 seconds, 1 second on/1 second off). Due to the fact that the right and left cortex gave equivalent results in all experiments used, only the right cortex data was analyzed going forward as it was felt to represent the response seen bilaterally.

PHARMACOLOGICAL BLOCKADE OF SEROTONERGIC SYSTEM

Because the DRN is known to primarily be a serotonergic nucleus and therefore the primary hub of 5-HT for the cerebral cortex, it was important to first establish whether or not serotonin was involved in the evoked CBF response. In order to do this we first eliminated the brain 5-HT content by pharmacological blockade with PCPA (n=6). As this nucleus is known for its serotonergic afferents first and foremost, it was suspected that this treatment would result in a diminution or removal of the evoked CBF response seen with electrical stimulation. This was precisely what was seen. The evoked CBF response was significantly decreased in PCPA-treated animals (-66%, $p<0.01$), which showed to us that, as suspected, the serotonergic system was a primary player in the NVC response to DRN electrical stimulation. The results are illustrated in Figure 8, below.

Additionally, in animals treated with PCPA, immunostaining was performed for 5-HT and DβH and compared with controls treated with saline. Immunostaining with DβH confirmed that there was no change in DβH content in PCPA treated animals. Immunostaining with PCPA confirmed that there was a clear loss of 5-HT nerve terminals in the cortical areas in which my CBF response was measured, therefore qualifying the treatment as successfully targeting the 5-HT content of the brain without affecting the noradrenergic content and thus providing validation that the decrease in

evoked CBF that was seen was directly a result of the decrease in serotonergic content in the brain. Immunostaining is also illustrated in Figure 8, below.

The next drug tested was the 5-HT2A antagonist (ketanserine, n=6) in order to determine if 5-HT2A receptors specifically played a role in the serotonergic component of the NVC response or if the influence of 5-HT was mediated through different receptors. The 5-HT2A receptors are one of the most abundant 5-HT receptors in the cortex (Hoyer et al., 1986; Pazos et al., 1985) and thought to mainly be expressed on glutamatergic pyramidal neurons, GABAergic interneurons (Morilak et al., 1994; Santana et al., 2004) and astrocytes. Interestingly, we saw no significant change in the evoked CBF response at any time point after administration of a ketanserine, suggesting that the evoked CBF increases to DRN stimulation, while involving the serotonergic system, were independent of 5-HT2A receptor activation. Results are illustrated in Figure 9, below.

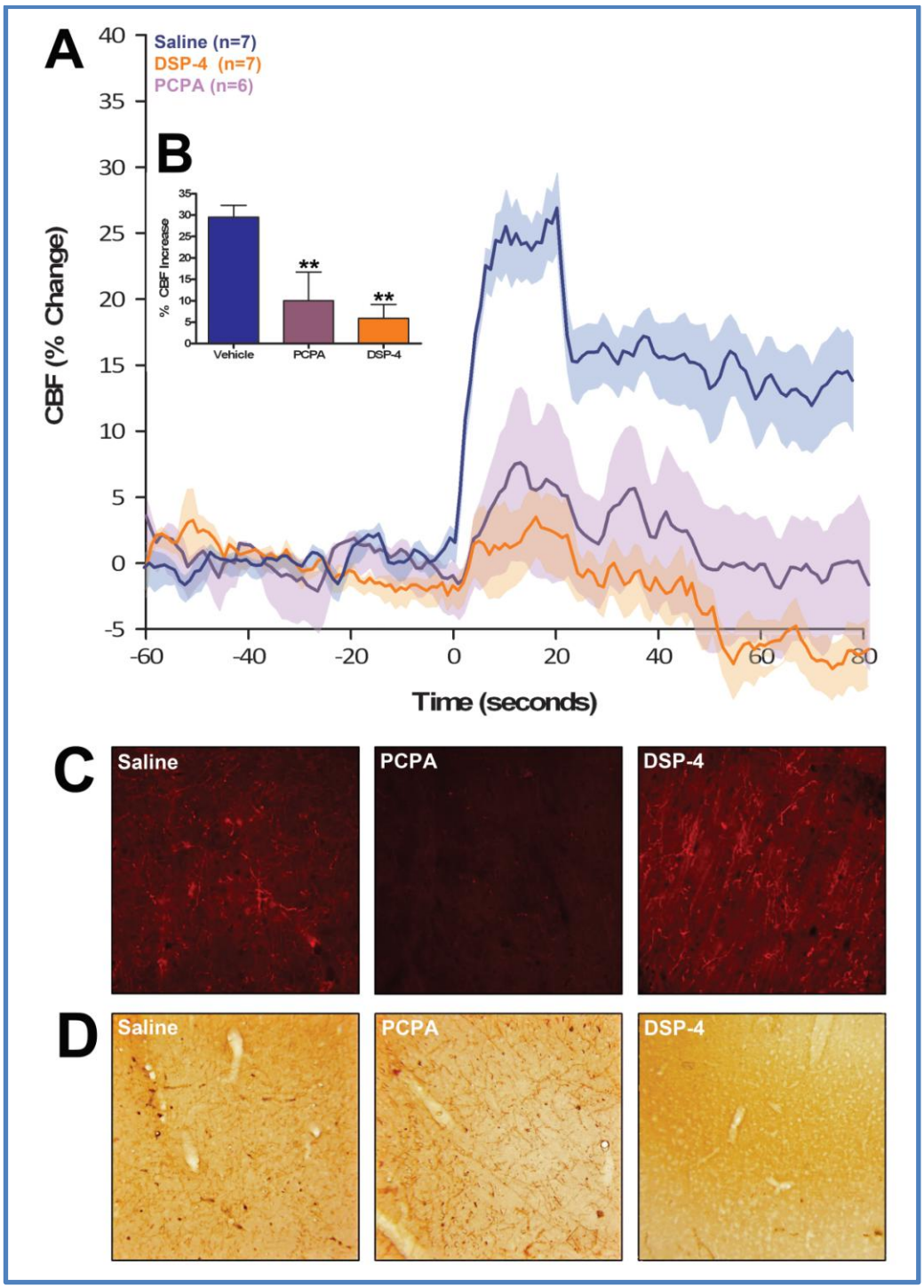

Figure 8: Effects of PCPA and DSP-4 treatment on the DRN electrical stimulation evoked CBF response. A-B PCPA (n=6) and DSP-4 (n=7) both decreased the CBF response to electrical stimulation of the DRN compared to saline treatment (n=7) **A** shows CBF responses at baseline and after vehicle and PCPA/DSP-4 injections, and **B** the

percent decrease in the evoked CBF response for each condition. **C** shows immunostaining for 5-HT nerve terminals. DSP-4 treatment did not affect 5-HT nerve terminal content, whereas PCPA significantly reduced the 5-HT, as desired. **D** shows immunostaining for DβH, the synthesizing enzyme of NE. PCPA treatment did not affect NE content, whereas DSP-4 treatment significantly reduced the NE content, as desired. Immunostaining was not quantified, as results were strikingly clear in all cases.

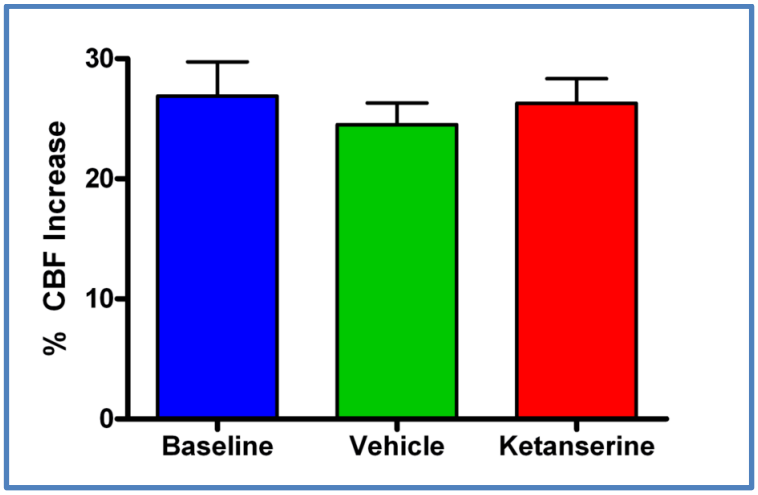

Figure 9: Effects of ketanserine on electrical stimulation of the DRN evoked CBF responses. Ketanserine is a 5-HT2A receptor blocker which was shown to not affect the CBF response to DRN electrical stimulation. The percent increase in the evoked CBF response at baseline and after vehicle and ketanserine injections are shown (n=7).

PHARMACOLOGICAL BLOCKADE OF NORADRENERGIC SYSTEM

It is now known that the DRN has vast interactions with another brainstem nucleus, the largely noradrenergic LC. Interactions between these two nuclei are of great functional importance as they are both known to be involved in the mediation of stress responses, sympathetic control, regulation of attentional processes and in memory consolidation (Calamandrei et al., 1992; Carli et al., 1983; Ferry et al., 1999). Interestingly, neurochemical experiments have shown the presence of NE and the enzymes which are

involved in its synthesis within the DRN itself (Saavedra et al., 1976). Similarly, autoradiographic studies have shown the presence of α1, α2 and β-adrenergic receptors within the DRN (Smith and Gallager, 1989). For these reasons, it was deemed important to also determine the potential contribution of the noradrenergic system on the NVC response to DRN stimulation. In order to assess this, in a similar manner to the PCPA experiments, cortical noradrenergic denervation was performed with the use of DSP-4 (n=7), a drug that significantly depletes the noradrenergic terminals in the brain. The evoked CBF response seen was dramatically decreased (-80%, $p<0.01$), in treated animals, which suggests that along with the serotonergic system, the noradrenergic system also plays a major role in facilitating the evoked CBF increases seen with DRN stimulation. In order to confirm that DSP-4 treatment was in fact selectively lesioning the noradrenergic system and not affecting the serotonergic system, immunohistochemistry was performed for all DSP-4 treated animals and controls. This confirmed that there was a dramatic loss of DβH fibers seen in the somatosensory cortex in the site of CBF recording. Additionally, there was no change in 5-HT nerve terminal content in treated animals. Evoked CBF results and immunostaining are illustrated in Figure 8, above. These results confirm that DSP-4 treatment selectively targeted the noradrenergic system and the decrease in the evoked CBF response seen in these animals was a result of this lesioning. As was done with the serotonergic system, we then attempted to gain an idea of the types of noradrenergic receptors that might play a role in this response. In order to do so, several pharmacological studies targeting the noradrenergic system were performed. The first compound tested was propranolol, a non-selective β-adrenergic receptor blocker. Administration of propranolol induced a significantly decrease in the evoked CBF

response seen (n=5, -55%, $p<0.05$). The second drug tested was phentolamine, which is a non-selective α-adrenergic receptor blocker. Phentolamine administration also induced a significant decrease in the evoked CBF response (n=6, -45%, $p<0.05$). Results are illustrated in Figure 10, below.

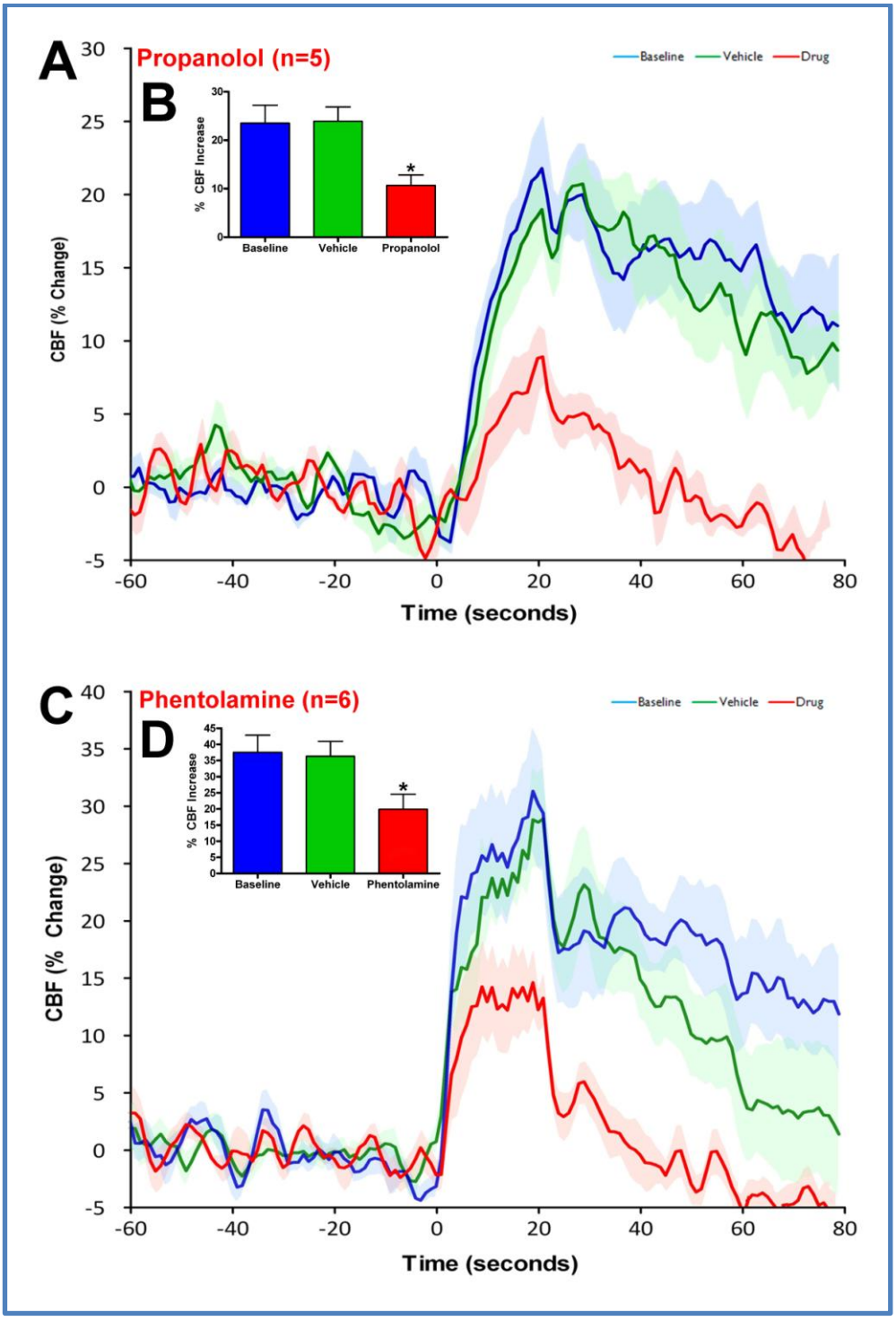

Figure 10: Effects of propanolol and phentolamine on the DRN electrical stimulation evoked CBF response. A-B The drug phentolamine is a non-selective α-adrenergic receptor blocker. Phentolamine decreased the CBF response to electrical stimulation of

the DRN. **A** shows CBF responses at baseline and after vehicle and phentolamine injections (n=6) and **B** the percent decrease in the evoked CBF response for each condition. **C-D** The drug propanolol is a non-selective β-adrenergic receptor blocker. Propanolol (n=5) decreased the CBF response to electrical stimulation of the DRN. **C** shows CBF responses at baseline and after vehicle and phentolamine injections (n=6), and **D** the percent decrease in the evoked CBF response for each condition. *p<0.05 drug versus vehicle (repeated measures ANOVA). Shaded areas represent SEM.

Together, these results suggest that the evoked CBF increases to DRN electrical stimulation involve both α- and β- adrenergic receptor activation. This result is not entirely surprising, given the vast contribution of the noradrenergic system which was shown with DSP-4 administration, and given the complicated interplay of α- and β-adrenergic receptors within the DRN and LC (see Discussion).

DETERMINATION OF THE CORTICAL NETWORK INVOLVED IN THE NVC RESPONSE

Once we had shown that the serotonergic and noradrenergic systems played major roles in the NVC response to DRN stimulation through their afferent projections, it became important to begin to determine what the cortical network was that conjugated the sub-cortical afferent connectivity's from the DRN and LC into hemodynamic changes within the cortex. We wanted to determine which sub-types of neurons in the cortex were being contacted by these afferent projections and if there was an astrocytic involvement. Our initial plan was to first perform c-Fos double immunocytochemistry on animals electrically stimulated in order to parse out the specific neuronal populations and then perform pharmacological experiments by blocking cortical receptors to see their respective contributions to the hemodynamic responses.

C-FOS IMMUNOHISTOCHEMISTRY

It is possible to assess activation of individual neurons at the cellular level using immunohistochemical detection of immediate-early genes (IEGs) (Staiger, 2006; Staiger et al., 2002), as has been shown following whisker stimulation, for example (Lecrux et al., 2011). IEGs are known to be activated both rapidly and transiently in response to external stimuli and to regulate late-effector genes, therefore coupling stimulus to transcription allowing cells to adapt to their changing external environments (Lanahan and Worley, 1998; Melzer and Steiner, 1997). Thus, IEGs are essential for long-term neuronal plasticity. Of the known IEGs, c-Fos is the most commonly used for immunohistochemical staining and has been proven as a successful marker of cellular activation in previous studies (Kocharyan et al., 2008; Lecrux C, 2012; Lecrux et al., 2011). c-Fos was also an attractive IEG choice because it has been shown to be a good indicator of changes in neuronal activity in the somatosensory cortex of awake rats under physiological conditions (Staiger et al., 2002). However, one limitation of c-Fos is the fact that not all cell types express c-Fos following activation (Curran and Morgan, 1985).

When electrical stimulations were performed in the DRN, as per the aforementioned protocol, it was extremely surprising to find that no c-Fos activation was seen in the somatosensory cortex of these animals (n=4), despite the presence of the normal bilateral hemodynamic response measured in this area. The exact protocol used has previously shown to be effective in detecting c-Fos expression in the somatosensory cortex after electrical stimulation of the LC (Toussay et al., Abstract 2011), making this result rather unexpected.

A second stimulation paradigm was attempted (100 Hz, 50 uA, 0.5 ms, 1 second on/1 second off for 5 minutes) which had a lower amplitude and longer duration and was shown in BF electrical stimulation to induce c-Fos expression (Kocharyan et al., 2008). The use of this different stimulation paradigm was deemed equivalent to pharmacological studies as the evoked CBF response was of the same magnitude (~25-35%) as with the initial stimulation paradigm. In all animals (n=8), only a weak c-Fos signal was seen throughout the cortex. Results are illustrated in Figure 11. Results obtained from both stimulation protocols were not sufficient in order to proceed with c-Fos immunostaining and therefore double-staining for other specific neuronal markers would have been futile. These findings were overall unexpected since we effectively detected c-Fos upregulation in the somatosensory cortex following various electrical stimulation protocols involving the basal forebrain (Kocharyan et al., 2008), the infraorbital nerve or transcallosal afferents (Enager et al., 2009) and the LC (Toussay et al., Abstract 2011). Hence, we then chose to move on from c-Fos experiments and instead focus on pharmacological studies in order to parse out the cortical network involved in the NVC response to DRN stimulation.

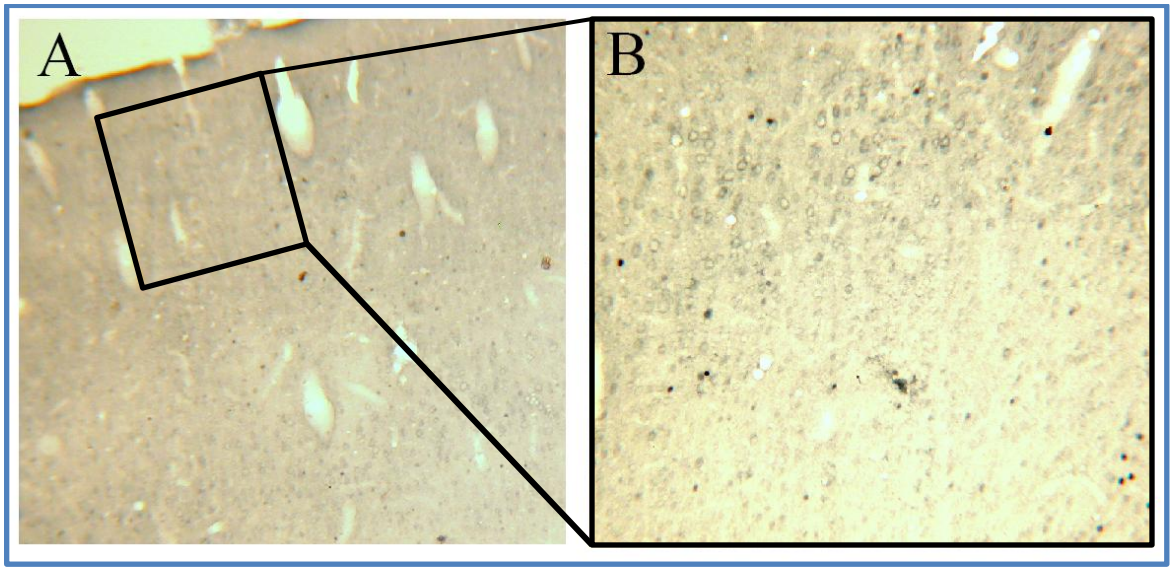

Figure 11: c-Fos Immunocytochemistry in response to electrical stimulation of the DRN. A-B Sample pictures taken from somatosensory cortex shown, under region of LDF probe during electrical stimulations. When the DRN was stimulated, there was very little c-Fos expression throughout the somatosensory cortex, the site of LDF probes. **A** shows the somatosensory cortex and **B** shows a close-up of what was seen throughout the cortical layers I-VI.

PHARMACOLOGICAL BLOCKADE OF GLUTAMATERGIC SYSTEM

Excitatory pyramidal cells are the most abundant cortical neurons and are known to play a vital role in the NVC response to sensory stimulation that is driven by glutamatergic thalamocortical afferents (Enager et al., 2009; Lecrux et al., 2011). They are also known to be activated following stimulation of cholinergic basalocortical afferents (Kocharyan et al., 2008). In both of these pathways, pyramidal cells are thought to act, in part, through the release of glutamate.

We wanted to assess the possible implication of excitatory glutamate-releasing pyramidal cells in the NVC response to electrical stimulation of the DRN. The first pharmacological

compound tested related to the glutamatergic system was MK-801, a non-competitive NMDA receptor antagonist. Indeed, a role for NMDA receptors has been previously reported in the evoked CBF response or generation of the BOLD signal after sensory stimulation (Gsell, 2006; Nielsen and Lauritzen, 2001). Similarly, MK-801 has been shown to decrease the evoked CBF response to whisker stimulation of the barrel cortex (Lecrux et al., 2011). Blocking NMDA receptors is thought to block NMDA receptor activation which is induced by synaptic glutamate and results in a decrease in pyramidal neurons spontaneous firing rate. The various serotonin receptors have a diverse expression throughout the cortex. Some of the most abundant serotonin receptors in the cerebral cortex include 5-HT1A, 5-HT2A and 5-HT3A receptors, all of which are selectively expressed in distinct populations of pyramidal neurons and inhibitory interneurons and play important roles in the modulation of cortical activity (Puig and Gulledge, 2011). Therefore, it was postulated that the serotonergic system could directly influence pyramidal neurons, which could then release glutamate and act through NMDA receptors to potentially control the hemodynamic response.

However, when MK-801 was administered, no significant changes in the evoked CBF response were seen at any time point (n=8), suggesting that the evoked CBF increases to DRN stimulation do not involve NMDA-receptor mediated glutamatergic pathways. Results are illustrated in Figure 12 at time point 20 minutes post-injection of MK-801. This result was surprising; as it was anticipated the 5-HT afferents from the DRN might project largely onto pyramidal neurons, and therefore could involve an NMDA-mediated NVC response.

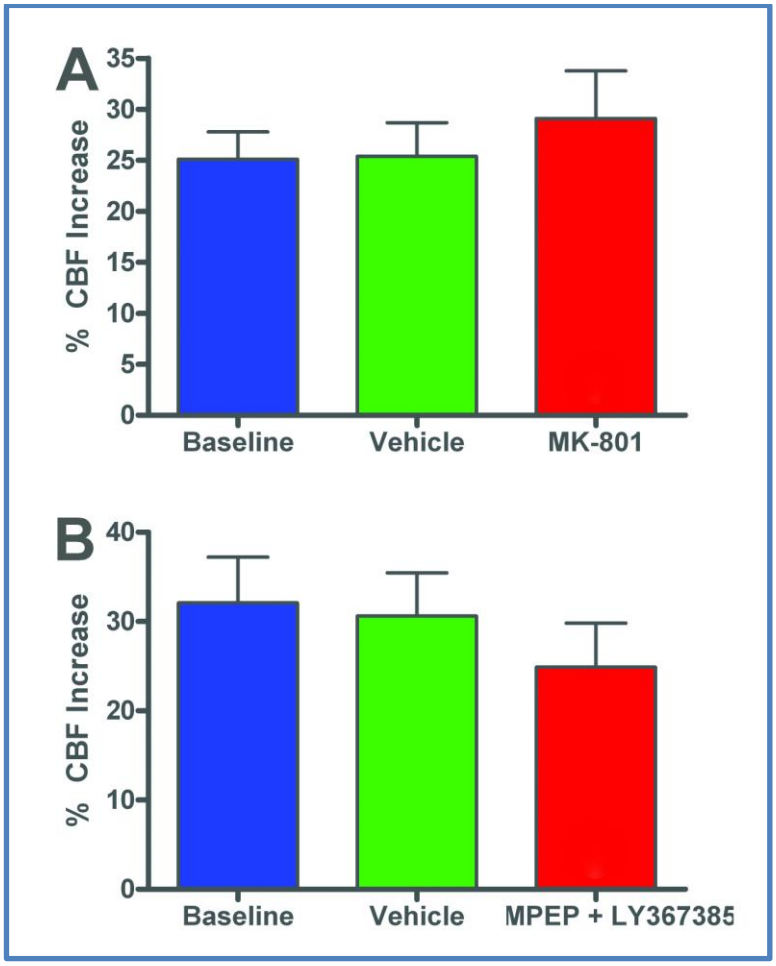

Figure 12: Effects of MK-801 and MPEP + LY367385 on electrical stimulation of the DRN evoked CBF responses. A MK-801 is an NMDA receptor antagonist which was shown to not affect the CBF response to DRN electrical stimulation. The percent increase in the evoked CBF response at baseline and after vehicle and MK-801 injections is shown (n=8). **B** Combined application of MPEP + LY367385 was also shown to not affect the CBF response to DRN electrical stimulation. The percent increase in the evoked CBF response at baseline and after vehicle and MPEP + LY367385 injections are shown (n=8).

As it is highly unlikely that pyramidal neurons are not involved in the NVC response at all, we sought to pharmacologically block additional glutamate-mediated receptors. We then decided to assess the implication of group I mGluRs, through the use of MPEP + LY367385, which have been identified to be involved in the required signaling cascade

for the NVC response to sensory stimulation (Shi et al., 2007; Zonta et al., 2003). It was suspected that the serotonergic afferents from the DRN could recruit pyramidal neurons, which once activated could then affect the CBF either directly or through the release of COX-2 derived dilatory prostaglandins or indirectly via astrocytic synthesis and release of vasodilatory EETs, which has been suggested to occur via mGluRs (Lecrux et al., 2011; Shi et al., 2007).

When MPEP + LY367385 was tested, similar to MK-801 experiments, there was no significant change in evoked CBF seen at any time point post-injection of MPEP + LY367385 (n=8), but there was a trend towards a decrease. Together, these results suggest that the glutamatergic contribution to the evoked CBF response may involve a small contribution from group I metabotropic glutamatergic receptors (mGluR1+5), but does not appear to be NMDA-receptor mediated.

PHARMACOLOGICAL BLOCKADE OF GABA-ERGIC SYSTEM

After the surprising results of the MK-801 and MPEP + LY367385 administration, it was decided to next test the response to blockade of GABA-A receptors with picrotoxin. GABAergic inhibitory interneurons represent approximately twenty percent of all neurons in the cerebral cortex. It is thought that GABAergic interneurons are key players in the timing of pyramidal cell firing, synchronizing network activity and in the generation of cortical rhythms (Markram et al., 2004). GABAergic interneurons are known to play a vital role in the influence and control of cortical activity and they appear to act through feed-forward and feed-back inhibition. They are known to form vast inhibitory synaptic connections with their targets and act in such a way as to locally

integrate cortical activity. It is also known that cortical GABA interneurons provide rich innervation to the local microvasculature and perivascular astrocytes (Cauli et al., 2004; Vaucher, 2000) and in fact, GABAergic interneurons are able to alter the tone of local microvessels following their activation (Cauli et al., 2004; Kocharyan et al., 2008). These interneurons are therefore in an ideal position to integrate the CBF in response to local changes in neuronal activity (Fergus and Lee, 1997), as was suggested by their stimulation in isolated cortical slices which resulted in vascular responses (Cauli et al., 2004), in addition to their actions in modulating pyramidal neuron firing.

GABAergic inhibitory interneurons are known to act through two separate receptors. The GABA-A receptors are ionotropic chloride channels. Activation of these receptors leads to hyperpolarization of the cell and decreases the potential for action potential firing. The other GABAergic receptor is the GABA-B receptor. These metabotropic receptors are guanine nucleotide binding protein (G-protein) coupled receptors which lead to the opening of K^+ channels which also result in hyperpolarization of the cell. Additionally, they can reduce Ca^{2+} conductance and reduce adenylyl cyclase activity. It has been shown that the full expression of the CBF response to BF stimulation requires GABA-A receptor activation by specific subsets of GABA interneurons (Kocharyan et al., 2008; Lecrux C, 2012). Because serotonergic afferents also have the potential to contact inhibitory interneurons via 5-HT receptors, it was suspected that the full NVC response to electrical stimulation of the DRN may also involve these neurons.

As anticipated, a significant decrease (~45%, $p<0.05$) in the evoked CBF response after administration of a non-competitive antagonist to GABA-A receptors (picrotoxin, n=8)

was seen. This result suggests that GABA, presumably released from recruited interneurons and acting via GABA-A receptors, is involved in cortical network mediating the hyperemic response. Results are illustrated in Figure 13, below.

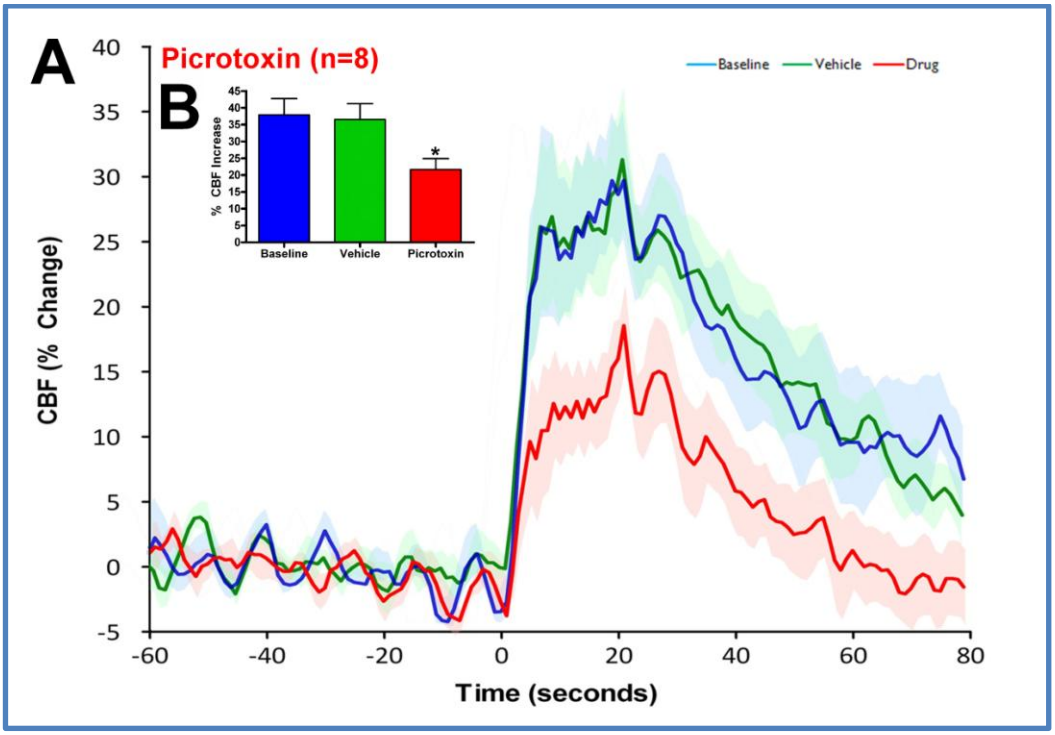

Figure 13: Effects of picrotoxin on the DRN electrical stimulation evoked CBF response. A-B The drug picrotoxin is a non-competitive antagonist to GABA-A receptors. Picrotoxin decreased the CBF response to electrical stimulation of the DRN. **A** shows CBF responses at baseline and after vehicle and picrotoxin injections (n=8), and **B** the percent decrease in the evoked CBF response for each condition. .*$p<0.05$ drug versus vehicle (repeated measures ANOVA). Shaded areas represent SEM.

PHARMACOLOGICAL BLOCKADE OF ASTROCYTIC MEDIATORS

Having determined that the GABA-A receptors, but not the NMDA receptors play a significant role in the evoked CBF response to DRN stimulation, it was of interest to determine if astrocytes could be involved in the cortical network activated with DRN electrical stimulation. Additionally, this was of interest considering the fact that astrocyte activation cannot be probed by c-Fos induction (Kocharyan et al., 2008; Staiger et al., 2002).

Pyramidal cells are known to play a role in the NVC response to other incoming afferent systems, for example thalamocortical afferents activated via sensory stimulation (Lecrux et al., 2011). Furthermore, glutamate released from pyramidal cells in this pathway is thought to induce calcium transients in astrocytes (Zonta et al., 2003), resulting in the synthesis and release of vasoactive EETs (Koehler et al., 2009). Similarly, pyramidal cells are also known to be activated after BF stimulation via cholinergic afferent pathways (Kocharyan et al., 2008). This NVC response has been recently shown to be largely mediated by glutamate-releasing pyramidal cells and arachidonic acid (AA)-derived cytochrome P450 epoxygenase EETs (Lecrux C, 2012). Additionally, given the fact that astrocytes express both GABA-A and GABA-B receptors, there has been the suggestion that GABA may also be able to induce calcium transients within astrocytes leading to the production of EETs (Lecrux C, 2012). We therefore wanted to investigate whether EETs, products of the AA pathway synthesized via cytochrome P450 epoxygenase could play an important role in mediating the NVC response to electrical DRN stimulation, as they have been seen to be involved in the functional hyperemic response to BF and sensory stimulation (Lecrux C, 2012; Lecrux et al., 2011; Leithner et al., 2009; Peng et al., 2004).

MS-PPOH is a drug which is thought to selectively inhibit the epoxygenation reactions which are catalyzed by specific CYP450 isozymes. The proposed pathway on which it acts is thought to be selective to astrocytes and responsible for the release of vasodilatory EETs (Alkayed et al., 1996; Koehler et al., 2009). Therefore, astrocytes are ideally positioned in order to regulate synaptic transmission and the functional hyperemic response. Astrocytic processes contact thousands of synapses and their end-feet are complexly associated with the cortical microvasculature. Involvement of astrocytic CYP450 epoxygenase in NVC has been shown and EETs are now known to play a key role in mediating this (Koehler et al., 2009; Lecrux C, 2012; Leithner et al., 2009; Peng et al., 2002; Peng et al., 2004; Shi et al., 2007).

When MS-PPOH (n=6) was tested, significant decreases (~42%, $p<0.05$) in the evoked CBF response were seen, suggesting a contribution of vasoactive EETs, presumably released from astrocytes, in the evoked CBF response to electrical stimulation of the DRN. Results are illustrated in Figure 14, below.

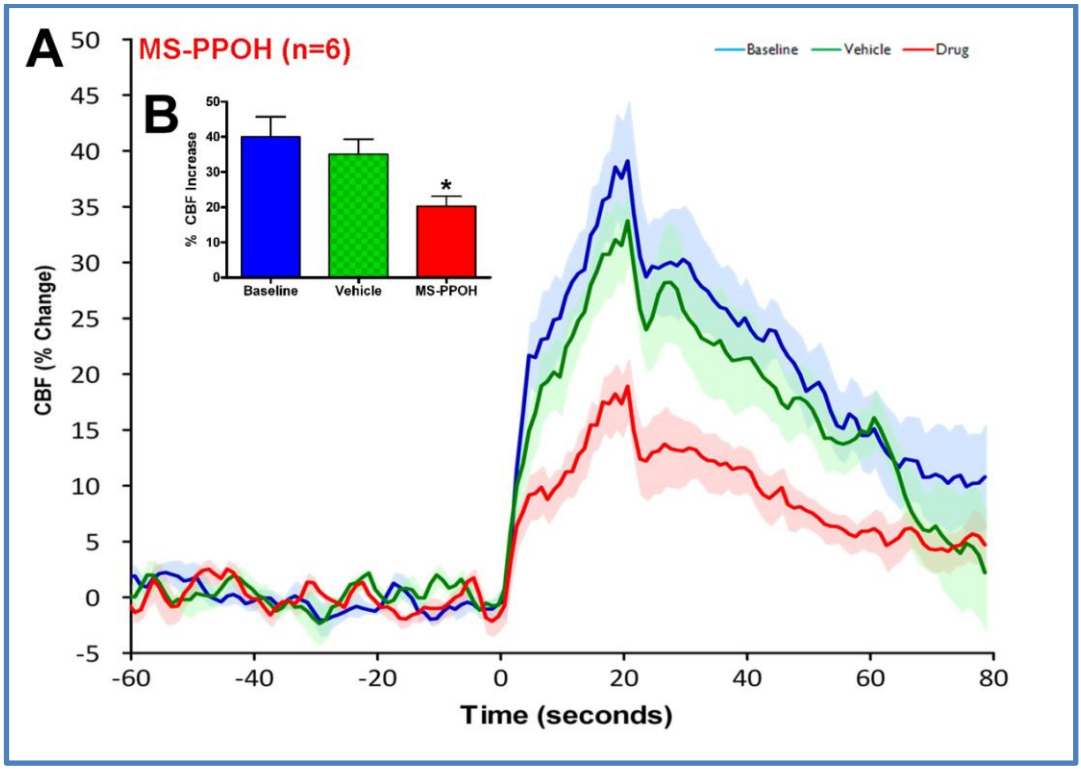

Figure 14: Effects of MS-PPOH on the DRN electrical stimulation evoked CBF response. A-B The drug MS-PPOH is thought to selectively inhibit the epoxygenation reactions which are catalyzed by specific CYP450 isozymes. MS-PPOH (n =6) decreased the CBF response to electrical stimulation of the DRN. **A** shows CBF responses at baseline and after vehicle and MS-PPOH injections, and **B** the percent decrease in the evoked CBF response for each condition. *p<0.05 drug versus vehicle (repeated measures ANOVA).

DISCUSSION

Pharmacological depletion of the 5-HT and NE content of the brain, with PCPA and DSP-4, respectively, showed marked reduction of the evoked CBF responses in both conditions, suggesting heavy involvement of both neuromodulators. Additionally, it was determined that both α- and β- adrenergic receptors were needed in the contribution from the noradrenergic system, while 5-HT2A receptors were not significantly involved in the serotonergic modulation. c-Fos immunohistochemistry was not able to be completed due to a lack of c-Fos induction, despite bilateral hemodynamic responses in the somatosensory cortex. At this time glutamate does not appear to be significantly involved in the CBF response; as blocking NMDA receptors and mGluRs did not result in a significant change in blood flow. However, GABA-A receptors do seem to contribute to this response, as do vasoactive EETs; presumably released from astrocytes. A summary of the results from all pharmacological compounds tested is illustrated in Figure 15, below.

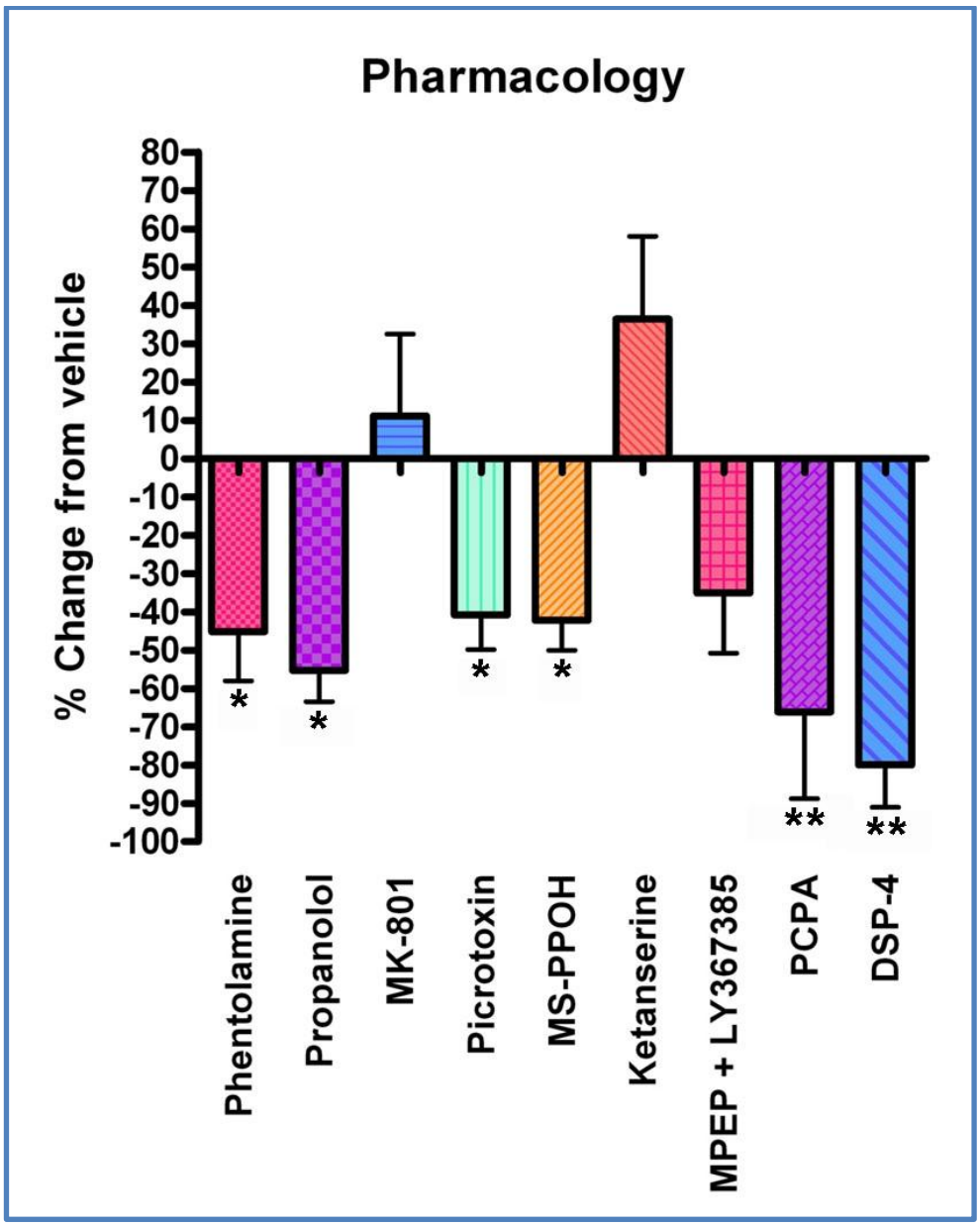

Figure 15: Summary of Pharmacological Experiments. *p<0.05, **p<0.01 drug versus vehicle (repeated measures ANOVA). Compounds tested included phentolamine (n=6), propranolol (n=5), MK-801 (n=8), picrotoxin (n=8), MS-PPOH (n=6), ketanserine (n=7), MPEP + LY367385 (n=8), PCPA (n=6) and DSP-4 (n=7).

PHARMACOLOGICAL BLOCKADE OF SEROTONERGIC SYSTEM

The first goal of this project was to determine if a reproducible hemodynamic response to electrical stimulation of the DRN could be obtained, and if so, what its magnitude was. The DRN was successfully targeted by stimulating electrodes and the evoked CBF response to electrical stimulation was determined to be in the range of 25-35% increase in CBF in both the right and left somatosensory cortex.

The second objective was to determine the contribution of the serotonergic system in the evoked CBF response. Given that the DRN is traditionally identified by its robust 5-HT projections, the question remained as to what extent the 5-HT afferents projecting from the DRN played in the NVC response. It was decided to determine the contribution of the serotonergic system in the evoked CBF response by selectively depleting 5-HT throughout the brain through inhibition of tryptophan hydroxylase with PCPA. This procedure resulted in a decrease in evoked CBF of ~66%, which strongly suggests a role for serotonergic afferents in this pathway.

The next serotonin-related drug which was tested was ketanserine, a 5-HT2A receptor antagonist. It has been shown that close associations exist between 5-HT nerve terminals and intracortical penetrating arteries, microarterioles and capillaries in the frontoparietal cortex (Cohen et al., 1995). This suggests that the 5-HT afferents from the DRN have the potential functional associations to affect the microvasculature directly.

Given the abundance of 5-HT2A receptors in the cortex and their complicated interplay with 5-HT1 receptors, blocking 5-HT2A receptors was desirable. Another reason for the choice of ketanserine was that this was one of the only drugs tested in the literature for its

effect on DRN stimulation. In a study by Cao et al. where the DRN was chemically stimulated with L-glutamate resulting in a decrease in CBF, the response was eliminated with intravenous injection of ketanserine, suggesting a role for 5-HT2A receptors in this response (Cao, 1992). However, when ketanserine was tested in the present study it was found to have no significant effect on the evoked CBF response. There are several potential explanations for this result. First of all, it is possible that 5-HT2A receptors do not play a significant role in the NVC response to electrical stimulation of the DRN. Secondly, it is possible that the complex interplay between 5-HT2A and 5-HT1A receptors which modulate cortical neuronal activity somehow resulted in a compensatory mechanism which gave a null effect. In the rat brain prefrontal cortex, it has been shown that 60% of pyramidal neurons express both 5-HT1A and 5-HT2A receptors (De Almeida and Mengod, 2007; López-Giménez et al., 1997; Martin-Ruiz et al., 2001; Pazos et al., 1985; Pompeiano et al., 1992; Pompeiano et al., 1994; Santana et al., 2004; Willins et al., 1997) and around 80% of these neurons co-express both (Amargos-Bosch et al., 2004; Puig et al., 2010; Santana et al., 2004). This co-expression is quite interesting considering the seemingly opposing influences that these two receptors exert on neuronal excitability (Puig and Gulledge, 2011). Although the functional interaction of these two receptors within single neurons is not currently understood, it was suggested by Andrade et al., in 2011, that 5-HT1A and 5-HT2A receptors regulate in a cooperative manner how pyramidal neurons encode excitatory inputs into action potential firing (Andrade, 2011). It was proposed that 5-HT2A receptors modulate neuronal gain through their control of the slow after-hyperpolarization while 5-HT1A receptors control the input intensity range over which the cell will encode excitatory inputs into firing activity via their regulation of

the cell's membrane potential (Andrade, 2011; Araneda and Andrade, 1991; Higgs et al., 2006; Zhang and Arsenault, 2005). One important anatomical difference between these two receptors is their differential localization within cellular compartments. In pyramidal neurons, 5-HT1A receptors have been shown to be densely expressed in the initial segments of axons where they act to hyperpolarize the cell (Azmitia et al., 1996; Cruz et al., 2004; Czyrak et al., 2003; Gerecke et al., 2001; Martin-Ruiz et al., 2001). In contrast, 5-HT2A receptors are mainly expressed on apical dendrites (Jakab and Goldman-Rakic, 1998; Martin-Ruiz et al., 2001) where they can act to further depolarize the cell. Similarly, GABAergic interneurons in the cortex also express 5-HT1A, 5-HT2A, or 5-HT3A receptors (Andrade and Weber, 2010; De Almeida and Mengod, 2007; Jakab, 2000; Morales and Bloom, 1997; Puig et al., 2004; Puig et al., 2010; Santana et al., 2004; Vucurovic et al., 2010; Willins et al., 1997; Zhou and Hablitz, 1999). However, unlike in pyramidal neurons where there is a great deal of co-expression, two separate populations of PV-expressing fast-spiking interneurons express either 5-HT1A or 5-HT2A receptors, separately (Morales and Bloom, 1997). Therefore, there remains the possibility that by blocking 5-HT2A receptors, we could be blocking not only pyramidal neuron synaptic activity, but also interneuron excitability, resulting in an overall null effect. Without testing additional 5-HT receptor antagonists, this remains to be proven and will need to be further investigated in future studies.

Ideally, if it were possible to target 5-HT2A receptors within specific cell types, we would be able to see the pyramidal neuron vs. interneuron contribution to the CBF response. At the present time this is not yet a feasible solution and so alternative methods will have to be used. Figure 16, taken from Puig et al., 2011 illustrates the differential

expression of 5-HT1A, 5-HT2A and 5-HT3 receptors within the neocortex. Given the vast co-expression of 5-HT1A receptors on pyramidal neurons and astrocytes, it could be of great interest going forward to test a 5-HT1A receptor antagonist as well.

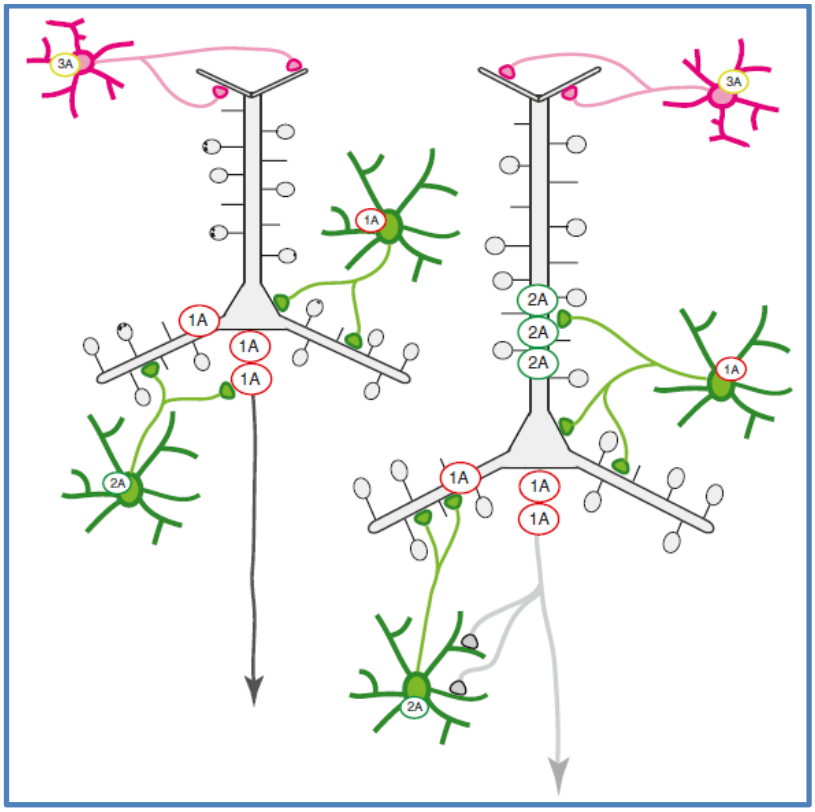

Figure 16: Localization of 5-HT receptors within the prefrontal cortex microcircuit. Figure taken from Puig et al., 2011. This diagram illustrates that many pyramidal neurons in deeper cortical layers (i.e. layer V) co-express 5-HT1A and 5-HT2A receptors. Specific populations of GABAergic interneurons within these layers also express 5-HT receptors which have differing innervation of pyramidal neurons: 5-HT1A- and 5-HT2A-expressing fast-spiking interneurons are preferentially located in deep layers where they contact pyramidal neurons at the soma and proximal dendrites; slow-spiking interneurons that express 5-HT3A receptors are located in superficial layers where they innervate pyramidal neurons at the distal dendrites (Puig and Gulledge, 2011).

Another 5-HT receptor that may be worth testing is the 5-HT3A receptor, which is the only known ionotropic 5-HT receptor (Barnes and Sharp, 1999; Chameau and van Hooft, 2006). This gives it the potential to act quickly on the hemodynamic response and may be of interest going forward. In the mature neocortex, it has been found that the 5-HT3A receptor is only present on GABAergic interneurons (Chameau and van Hooft, 2006; Morales and Bloom, 1997) including VIP- and/or CCK-expressing bipolar cells and multipolar neuropeptide Y (NPY)-expressing cells (Férézou et al., 2002; Inta et al., 2008; Varga et al., 2009; Vucurovic et al., 2010). Since VIP- and NPY- expressing neurons are GABAergic interneurons capable of modulating the dilatation and contraction of nearby microvessels in cortical brain slices (Cauli et al., 2004), the 5-HT3A receptors therefore have some potential to regulate local blood through their effect on the activity of these neurons.

PHARMACOLOGICAL BLOCKADE OF NORADRENERGIC SYSTEM

Once the contribution of the serotonergic system was confirmed, the next objective was to determine if the noradrenergic system also played a role in the NVC response. This was an important point of study given the vast interconnectivity of the DRN and the LC. Pharmacological destruction of the noradrenergic afferent fibers with DSP-4 confirmed that the noradrenergic system also had a heavy influence on the NVC response, which greatly reduced the evoked CBF response (-80%) to DRN stimulation. It is surprising how substantial the role of the noradrenergic system was shown to be, as it was initially anticipated that the serotonergic system would largely mediate this hemodynamic response. It thus appears that functional interactions between the 5-HT and NE neurons

mediate the evoked CBF responses to DRN stimulation, resulting in interdependence on both serotonergic and noradrenergic mechanisms.

There was also a significant reduction in the evoked CBF response seen after administration of non-selective α- and β- adrenergic receptor antagonists. Given the complex expression of specific α-adrenergic receptors within the DRN and LC itself, it would be interesting to target these specifically in order to better understand their role in mediating this response.

DETERMINATION OF THE CORTICAL NETWORK INOLVED IN THE NVC RESPONSE

The noradrenergic and serotonergic systems were both found to play large roles in the afferent connectivity from the DRN so it became important to then determine which cortical excitatory and inhibitory neurons were recruited *in vivo* by electrical stimulation of the DRN. In an attempt to elucidate the cellular ensemble underlying the hemodynamic response, c-Fos double-immunohistochemistry was attempted. However, despite observation of bilateral evoked CBF increases, no c-Fos signals were seen in the animals tested and these experiments were not pursued further. This was a surprising result given previous findings shown for BOLD responses (Lu et al., 2004) and in other LDF studies (Enager et al., 2009; Lecrux et al., 2011).

It is known that stimulus-induced c-Fos expression depends on various factors, but largely on firing patterns (Fields et al., 1997; Yi et al., 2007) and it is likely that its induction reflects a strong Ca^{2+} influx (De Kock and Sakmann, 2008; Karagiannis et al., 2009; Porter et al., 1998). There exists the possibility that these stimulation paradigms

were not strong enough to induce c-Fos, despite being strong enough to illicit a hemodynamic response. It is possible that depolarization's in the areas surrounding the recruited region were not able to induce sufficient synchronized action potentials (Ferezou et al., 2006; Petersen et al., 2003) to illicit the induction of c-Fos.

In going forward, it could be of interest to try to repeat these experiments with varying stimulation parameters and potentially look at IEGs other than c-Fos to see if their up-regulation could be seen instead. For example, the inducible cyclic adenosine monophosphate early repressor (ICER), the Jun-B proto-oncogene, and the early growth response 1 gene Krox-24 (Erg-1 or Zif268) could all be noteworthy candidates, as they have been used successfully in other studies (Staiger, 2006; Staiger et al., 2002). It may also be important in future investigations to look at electrophysiological recordings in response to electrical stimulation of the DRN. This could be an important tool for gathering the information regarding specific types of neuronal cell populations activated in response to stimulation, since this was not able to be determined with c-Fos double-immunocytochemistry despite this procedures success with other incoming afferent pathways.

PHARMACOLOGICAL BLOCKADE OF GLUTAMATERGIC SYSTEM

The project then relied on pharmacological studies in order to determine the respective contributions of excitatory and inhibitory neurons on the evoked hemodynamic response. As it is suspected that the 5-HT afferents project heavily onto pyramidal neurons within the cortex, it was anticipated that the NVC response occurred via a glutamate-mediated mechanism. Several AA metabolites have been implicated as possible mediators of the

glutamate-induced CBF changes because of their vasodilatory properties, so it was of interest to determine if this was a pathway through which the DRN stimulation resulted in local blood flow changes.

It has previously been shown that systemic administration of MK-801 dramatically increases 5-HT DRN neuron firing activity (Lejeune et al., 1994), which has been suggested to be mediated by the blockade of presynaptic NMDA receptors projecting to the DRN. It has been shown (Labonte et al., 2009) that the blockade of glutamatergic neurotransmission via MK-801 is able to modify the 5-HT excitatory extra-synaptic activity in the medial prefrontal cortex (mPFC) of anesthetized rats. The acute administration of MK-801 decreased NMDA-evoked neuronal discharge but increased the percentage of spikes in bursts of presumed pyramidal neurons (Labonte et al., 2009). Evidence was also shown that MK-801 selectively potentiates 5-HT-induced excitations among pyramidal neurons that show 5-HT2A/2C receptor-mediated excitatory responses but that it does not produce any modification in those pyramidal neurons expressing 5-HT1A-mediated inhibition (Labonte et al., 2009).

Despite the expected role of NMDA receptors in the evoked CBF response to DRN stimulation, our results did not show this. The selective NMDA receptor antagonist MK-801 appeared to have no effect on the evoked CBF increase at any time point post-injection (up to 60 minutes). Considering the large number of animals confirming this result (n=8) and the fact that the solutions used in this study were shared with concurrent studies that found highly significant decreasing effects of MK-801 on the hyperemic

responses to whisker or LC stimulation (Lecrux et al., 2012; Lecrux et al., 2011; Toussay et al., Abstract 2011); we can discard any obvious experimental artifact.

One possible explanation for the lack of an effect in this study could come from the fact that NMDA receptors are also localized in sub-cortical regions such as the DRN (Hajós et al., 2003; Hajós et al., 1998; Peyron et al., 1997; Puig et al., 2005; Roche et al., 2003) where MK-801 administration, as suggested earlier, could act to increase 5-HT DRN neuronal firing. Moreover, glutamatergic inputs to the DRN originating in the medial prefrontal cortex (mPFC), amygdala, several hypothalamic areas and the parabrachial nucleus have been described (Lee et al., 2003). Therefore, the glutamatergic regulation of the evoked CBF response to DRN stimulation could come from the excitatory glutamatergic inputs from both cortical and subcortical areas and the glutamatergic neurons located in the DRN itself. It is thus possible that either NMDA receptors do not play a role in this response at all, or that the action of MK-801 on NMDA receptors within the DRN itself acts to increase 5-HT neuron firing leading towards an increased CBF response, while blockade of NMDA receptors in the cortex leads towards decreasing the CBF response due to altered reception of glutamate released from pyramidal neurons, overall giving a null effect on blood flow.

The next drug tested was MPEP + LY367385. It was found that the use of MPEP + LY367385 to block mGluR5, which is expressed by most cortical neurons and astrocytes (Cahoy et al., 2008; Cauli et al., 2000) along with mGluR1, which is restricted to a subset of cortical GABAergic interneurons (Baude et al., 1993) including those which express

VIP (Cauli et al., 2000), did not significantly block the evoked CBF response to DRN stimulation, although there was a slight trend towards a decrease.

Based on this study it appears that following its release from pyramidal neurons, glutamate does not act on NMDA receptors, but may to a small extent act through group I mGluRs which are expressed by several cortical neurons (Baude et al., 1993) and by astrocytes (Lalo et al., 2006a). Since under normal conditions, mGluRs are recruited for selective circuit activation during times of glutamate spillover (Iserhot et al., 2004), this could offer a possible explanation for their rather small contribution to the DRN-stimulation-driven evoked CBF response, similar to what has been reported for the cholinergic BF system (Lecrux C, 2012), as opposed to their important contribution in glutamatergic-mediated pathways activated through whisker stimulation (Lecrux et al., 2011; Zonta et al., 2003).

It is still probable that pyramidal cells are involved in the hemodynamic response, but perhaps the complex co-expression 5-HT1A and 5-HT2A receptors on pyramidal neurons may play a part in masking the role of glutamate in these studies. Perhaps the depolarizing effects of 5-HT2A receptors combined with the hyperpolarizing effect of 5-HT1A receptors, both expressed alone and together on pyramidal neurons, control the NVC response through different mechanisms and are somehow able to compensate for each other when NMDA receptors or mGluRs are blocked. The complex relationship between these two serotonin receptors and how they may mediate pyramidal and non-pyramidal cell firing and ultimately result in blood flow changes is yet to be determined and speculative at best. Alternatively, the possibility also exists that glutamate release

from pyramidal neurons is mediated through AMPA and/or kainate receptors. It will be important in future studies to pharmacologically block both of these and determine their respective contributions.

PHARMACOLOGICAL BLOCKADE OF GABA-ERGIC SYSTEM

Cortical interneurons are also suspected to be heavily targeted by 5-HT afferents and therefore thought to play a role in the hemodynamic response. As noted by Ascoli et al., 2008, GABAergic interneurons differ with respect to their expression of neurochemical markers, electrophysiological properties, morphology and cellular and subcellular location of output synaptic contacts (Ascoli, 2008; Klausberger and Somogyi, 2008). Administration of 5-HT to cortical slices has been shown to result in large enhancements in ongoing spontaneous GABAergic synaptic transmission (Zhou and Hablitz, 1999). Furthermore, it is known that cortical "vasomotor" VIP- and ACh-containing GABAergic interneurons are richly innervated by brainstem 5-HT afferents (Férézou et al., 2002). This would suggest that serotonin is capable of exciting at least a certain subpopulation of GABAergic interneurons (Andrade, 2011). It has been suggested that this increase in spontaneous synaptic activity in GABAergic interneurons seen after serotonin application is mediated by activation of 5-HT2A and 5-HT3A receptors, which have been shown to be expressed mainly on fast spiking PV expressing (Andrade and Weber, 2010) and VIP/CCK expressing interneurons (Férézou et al., 2002) respectively. Serotonin has also been shown to inhibit fast spiking interneurons specifically through activation of 5-HT1A receptors (Puig et al., 2010), but at the current time it is unknown if 5-HT1A receptors are co-expressed with 5-HT2A receptors in this subgroup of fast spiking interneurons, or if these receptors are expressed on different subgroups of fast spiking interneurons

(Andrade, 2011). Even though it was not possible to determine the specific sub-populations of GABAergic interneurons involved in the evoked CBF response to DRN stimulation via c-Fos immunocytochemistry, it was still important to discover if GABA plays a role in mediating this response to begin with.

Pharmacological blockade of GABA-A receptors showed a significant decrease (~45%) in the evoked CBF response, confirming a role for GABAergic interneurons in NVC. Since GABAergic interneurons are inhibitory in nature, this result appears counterintuitive at first. However, it is likely a result of altered inhibition of the activated cortical network involved in the NVC response, as has been suggested in the hemodynamic responses to BF and sensory stimulation (Kocharyan et al., 2008; Lecrux et al., 2011). Overall, this could result in the inhibition of pyramidal cells and therefore could explain the decrease in evoked CBF response. In this system, it is hard to confirm that this is the mechanism of action since we do not have the data from c-Fos double immunostaining which could confirm which populations of excitatory and inhibitory neurons are recruited, however it is not unlikely that a similar pathway could explain this data. At this time point, it is suspected that the inhibition of GABA-A receptors results in a change in the way in which cortical interneurons inhibit pyramidal cells, resulting in a decrease in cortical output and hence, cortical CBF.

Interestingly, despite the fact that within the rat frontal cortex serotonergic fibers are present throughout all cortical layers, synaptic specializations are only associated with about 25% of 5-HT release sites, which suggests that the greater majority of 5-HT release sites are non-synaptic (Seguela et al., 1989). These findings have been similarly found in

both the cat and primate cortex as well (DeFelipe and Jones, 1988; Mulligan and Tork, 1988). The 5-HT release sites which contain these synaptic specializations have been shown to predominantly contact GABAergic interneurons within more superficial cortical layers (DeFelipe et al., 1991; Hornung and Celio, 1992; Smiley and Goldman-Rakic, 1996). Therefore, this suggests that signaling to pyramidal neurons within deeper cortical layers acts mostly via volume transmission from non-synaptic 5-HT release sites, while signaling is synapse-specific mainly in higher cortical layers onto inhibitory interneuron populations (Hornung and Celio, 1992). This pattern would allow 5-HT3 receptors expressed on GABAergic interneurons in higher cortical layers to respond rapidly to 5-HT release and act quickly to induce a change in CBF via their action on pyramidal neurons and/or astrocytes. It is possible that the 5-HT regulation of pyramidal neuron excitability directly is more of a tonic action, while the phasic control of 5-HT regulation lies mainly in the hands of the GABAergic interneurons in more superficial cortical layers. 5-HT afferents from the DRN projecting to the cortical "vasomotor" GABAergic interneurons, like those containing VIP, could lead to the dilatation of cortical microvessels causing a CBF increase. Therefore, when GABA-A receptors are blocked, this increase does not occur and the CBF response is diminished. This could explain in part why picrotoxin blockade would decrease CBF, while blocking NMDA receptors and mGluRs did not appear to have significance. However, it should be noted that this pathway of GABA-A receptor action directly upon blood vessels has been shown in BF stimulation to only account for about 10% of the evoked CBF response (Lecrux et al., 2011).

Going forward, it could also be of interest to also test a GABA-B receptor antagonist, to better clarify the role of these interneurons. However, it should be noted that the contribution of GABA-B receptors was tested in BF stimulation and in the whisker-to-barrel cortex pathway and neither system found a significant reduction in evoked CBF (Kocharyan et al., 2008; Lecrux et al., 2011).

PHARMACOLOGICAL BLOCKADE OF ASTROCYTIC MEDIATORS

Finally, it was of interest to see if there was an astrocytic component to the CBF responses. GABA-A receptors (Fraser et al., 1995; Meier et al., 2008; Nilsson et al., 1993), NMDA receptors and mGluRs (Lalo et al., 2006b; Schipke et al., 2008) are present on astrocytes and known to increase intracellular Ca^{2+}, providing a key pathway towards the release of vasoactive messengers (Carmignoto and Gómez-Gonzalo, 2010). MS-PPOH i.c. administration significantly reduced (~42%) the evoked CBF response, suggesting that vasoactive EETs, which have been reported to be solely located within astrocytes (Alkayed et al., 1996) play a major role in the NVC response to DRN stimulation. It is possible that these astrocytes are activated through glutamate from pyramidal neurons, directly from 5-HT or by GABAergic interneurons. In future studies, it would be of interest to test various other blockers of astrocytic metabolism in order to confirm the role of astrocytes in this response, and perhaps test these in combination with glutamate or GABA receptor blockers in order to determine their pathway of activation.

FUTURE DIRECTIONS

Although this study provided a necessary starting point for investigation into the cortical network underlying the NVC response to DRN electrical stimulation, there is a great deal more that could be done in future studies to help illuminate this picture. Firstly, it will be important to determine the means by which the serotonergic system controls pyramidal neuron and/or GABAergic interneuron activation. This could be done by continuing the pharmacological studies by blocking 5-HT3 receptors or 5-HT1A receptors. Since 5-HT3 receptors are the sole ionotropic serotonin receptors in known in the cortex, this could be an interesting target, as their location on GABAergic interneurons merits them potential functional significance in conjugating a rapid blood flow response. 5-HT1A receptors are heavily co-expressed with 5-HT2A receptors, and their inter-relationship on pyramidal neurons may prove important. Depending upon the results of these pharmacological studies, it could also be interesting to block both 5-HT1A and 5-HT2A receptors at the same time.

Concurrently, it would be beneficial to better classify the role of the noradrenergic system by selective blockade of specific adrenergic receptors. As there appears to be a complex combinatorial relationship of adrenergic receptors between the DRN and LC, it would be of great use to discover precisely how these systems interact in their control of CBF.

It is suspected that apparent insignificance of glutamatergic-based receptor blockade seen in this study could have been due to confounding serotonergic regulation. Once the serotonergic afferent control is better determined, it may clarify some of the results seen. However, it could be interesting to also block AMPA and kainate glutamate receptors as

well, to see if either plays a major role. It would also be interesting to fully investigate the role that astroglial messengers play as intermediaries for both inhibitory and excitatory neurons. This could be done by selectively impairing astrocyte oxidative metabolism by blocking mitochondrial aconitase with fluorocitrate or fluoroacetate.

Finally, it would be extremely helpful to have a means to discover the excitatory and inhibitory cortical neurons involved in this response. Future studies would be wise to look into other IEGs as a means for doing so, and/or different stimulation paradigms in order to find a way to induce activation of an IEG and determine neuronal populations. This would help elucidate the exact types of pyramidal and GABAergic interneurons involved in the response and would add a great deal to the present pharmacological data. Additionally, the investigation of the hemodynamic role of serotonin released from the serotonergic terminals during whisker stimulation will be an interesting and informative follow-up study that will be conducted by our laboratory going forward.

CONCLUSIONS

This study is able to demonstrate that the NVC response to DRN electrical stimulation is largely controlled by the neuromodulation of the serotonergic and noradrenergic systems, through the involvement of both α- and β-adrenergic receptors. Furthermore, it was shown that DRN afferents likely act through their effects on inhibitory interneurons, and potentially through excitatory pyramidal neurons, although the exact mechanism of action remains to be determined. This control of the vascular response occurs through the release of EETs, likely from astrocytes, most probably following their activation by GABA released from GABAergic interneurons, although a small contribution from glutamatergic neurons cannot be totally disregarded in view of the small inhibitory effect of Group 1 mGluR blockade, which are known to act at least partly through astrocytes (Koehler et al., 2009). The proposed mechanisms are yet to be confirmed. Therefore, further pharmacological and immunohistochemical studies will be imperative in determining the precise cortical network activated in the NVC response to DRN stimulation. By beginning to understand the cellular ensembles mediating blood flow responses from afferent pathways, we will not only begin to have a deeper understanding of the brain's physiology, but could ultimately improve the interpretation of basic and clinical functional neuroimaging studies.

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