Cholinergic modulation of neurovascular coupling in the rat somatosensory cortex

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ABBREVIATIONS

20-HETE	20-hydroxyeicosatetraenoic acid
2DG	2-deoxyglucose
ACh	acetylcholine
AChE	acetylcholinesterase
ANOVA	analysis of variance
BF	basal forebrain
BOLD fMRI	blood oxygenation level-dependent functional magnetic resonance imaging
CBF	cerebral blood flow
cGMP	cyclic guanosine monophosphate
ChAT	choline acetyltransferase
COX	cyclooxygenase
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
EDHF	endothelium-derived hyperpolarizing factor
EET	epoxyeicosatrienoic acid
fMRI	functional magnetic resonance imaging
G-protein	guanosine nucleotide binding protein
GABA	γ-aminobutyric acid
ICER	inducible cyclic adenosine monophosphate early repressor
IEG	immediate early gene
LDF	laser Doppler flowmetry
mAChR	muscarinic acetylcholine receptor
mGluR	metabotropic glutamate receptor
nAChR	nicotinic acetylcholine receptor
NMDA	*N*-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NPY	neuropeptide Y
PET	positron emission tomography
PFA	paraformaldehyde
PGE_2	prostaglandin E_2
PGI_2	prostacyclin
POm	posterior medial
PSP	postsynaptic potential
PV	parvalbumin
SOM	somatostatin
TPU	tissue perfusion units
TXA_2	thromboxane A_2
VPM	ventroposterior medial
VSMC	vascular smooth muscle cell

ABSTRACT

Neurovascular coupling, the increase in cerebral blood flow (CBF) seen in areas of increased neuronal activity, is thought to reflect levels of neuronal activation. Acetylcholine (ACh) is known to affect the processing of thalamocortical sensory information, but its effects on the CBF response to somatosensory stimulation are unknown. We sought to discover whether cholinergic modulation is reflected in the neurovascular coupling response, and to identify changes driving the altered response. In the barrel cortex, we measured blood flow by Laser Doppler flowmetry and identified activated cells using c-Fos. ACh enhancement increased the CBF response in a muscarinic-dependent manner, without altering activated cell populations or the extent of the c-Fos barrel. ACh decrease via selective cholinotoxic lesion diminished the CBF response as well as the area of c-Fos activation in the barrel cortex; activated cell types were again unchanged. These data show that cholinergic modulation of thalamocortical sensory information is reflected in the CBF response, and suggest that ACh effects are likely due to altered neuronal activity rather than to a shift in activated cell populations.

RÉSUMÉ

Le couplage neurovasculaire, l'augmentation de la perfusion cérébrale dans les régions ou l'activité neuronale est augmentée, est supposé refléter les niveaux d'activation neuronale. On sait que l'acétylcholine (ACh) a des effets sur la transmission des afférences sensorielles thalamocorticales, mais les effets de l'ACh sur la réponse neurovasulaire à la stimulation somatosensorielle sont encore mal connus. Notre but était de découvrir si la modulation cholinergique peut être détectée dans la réponse neurovasculaire, et d'identifier les changements cellulaires qui sous-tendent la réponse modifiée. Dans le cortex à tonneaux, nous avons measuré l'augmentation de débit sanguin induite par la stimulation des vibrisses par Laser Doppler, et utilisé l'immunocytochimie de c-Fos pour identifier les cellules activées. L'augmentation de l'ACh a potentialisé la réponse hyperémique d'une manière dépendante des récepteurs muscariniques, sans changer l'identité des cellules activées ni l'étendue de l'aire activée dans le cortex à tonneaux. La diminution de l'ACh suite à l'administration d'une cholinotoxine sélective a amoindri la réponse vasculaire tout comme l'aire corticale d'activation délimitée par le c-Fos. Les types de cellules activés n'étaient toujours pas changés. Nos résultats montrent que la modulation cholinergique de l'information sensorielle thalamocorticale est détectable dans la réponse cérébrovasculaire, et suggèrent que les effets de l'ACh sont liés à un changement d'activité neuronale et non à un changement de l'identité des populations de cellules activées.

1. GENERAL INTRODUCTION

Functional hyperemia, or neurovascular coupling, is the highly coordinated increase in cerebral blood flow (CBF) seen in areas of increased neuronal activity. CBF changes can be used in functional imaging methods such as positron emission tomography (PET) and functional magnetic resonance imaging (fMRI), where they serve as surrogates for local alterations in brain activity. Neurovascular coupling is altered in pathological conditions such as stroke, Alzheimer's disease (AD), and hypertension (*Girourd and Iadecola, 2006*). Current evidence suggests that the perfusion response is driven by the afferent neuronal signals to the target areas and, more importantly, by the local processing of this incoming input (*Attwell and Iadecola, 2002; Lauritzen and Gold, 2003*). The exact mechanisms by which this occurs, however, as well as the cell types and messengers involved, are still poorly understood.

The cholinergic system is involved in many brain functions, from memory to attention and arousal, and increasing acetylcholine (ACh) levels through basal forebrain (BF) stimulation leads to cortical activation. BF lesion decreases both sensory stimulation-dependent plasticity and whisker stimulation-evoked functional activity (*Juliano et al., 1991; Jacobs et al., 1991*); increased ACh has effects such as the enhancement of neuronal responsiveness (*Metherate et al., 1988*). ACh has thus been proposed as acting to increase the response possible to a given threshold of sensory stimulation: in this model, while increased ACh would facilitate a larger response to a given sensory input, ACh depletion would lessen the response achievable to the same threshold of sensory stimulation.

We hypothesized that BF-derived cholinergic tone modulates cortical sensory processing, potentially via altering mechanisms of neuronal processing through differential recruitment of

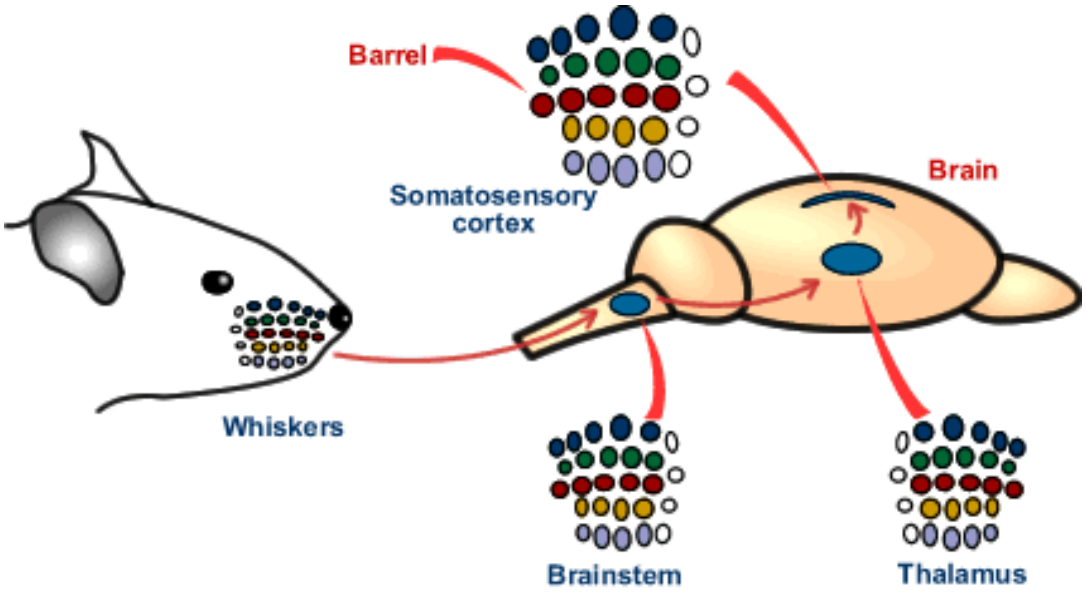
cholinoceptive interneurons. This role was investigated using CBF as an index of neuronal activity and c-Fos immunohistochemistry to identify the cellular basis of the modulation.

2. BACKGROUND

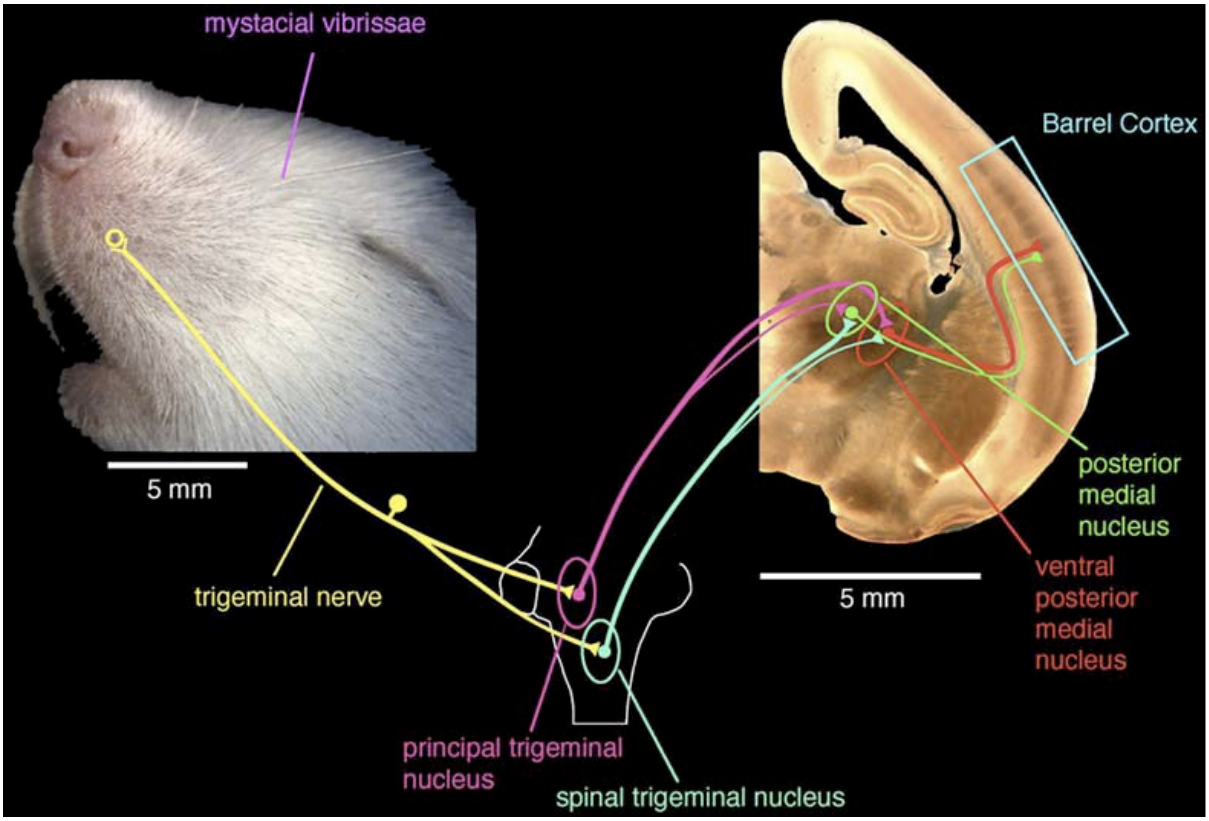
2.1 The whisker-to-barrel cortex pathway

The whisker-to-barrel cortex pathway is frequently used as a tool for studying neurovascular coupling, as information flows in a well-delineated and highly circumscribed manner (for a review, see *Lubke and Feldmeyer, 2007*) (**Figure 1)**. The so-called 'barrel cortex,' part of the somatosensory cortex, contains a topographical representation of each whisker, allowing activation of any given whisker to be accurately monitored in the brain. Five main rows of whiskers A-E have been defined, each containing 4-8 whiskers; an additional four whiskers are located caudally between the main rows and are designated α to δ (*Woolsey and Van der Loos 1970; Woolsey et al., 1975*). The main barrel input is via the lemniscal pathway: information travels from the whisker up via the trigeminal nerve to the trigeminal nucleus of the ipsilateral brainstem, across to the ventroposterior medial (VPM) nucleus of the contralateral thalamus, and from there to activate cells primarily in layer IV of the barrel cortex. Topography is preserved throughout, with anatomically distinct barrellettes at the level of the trigeminal nucleus and barreloids in the thalamus. Within the barrel cortex, most thalamocortical synapses are formed onto excitatory spiny stellate cells of layer IV (*Benshalom and White, 1986*); these then convey information primarily to pyramidal cells in layers II/III and IV within the same barrel (*Armstrong-James et al., 1992*). Pyramidal cells of laminae Vb and VI also receive some direct thalamocortical input (*Lu and Lin, 1993*).

Figure 1: Route of whisker-to-barrel cortex pathway information flow. Information travels from whisker to brainstem, contralateral thalamus, and finally to the barrel cortex; topographical organization is preserved throughout the pathway.



www.bris.ac.uk/synaptic/info/pathway/somatosensory.htm



Lubke and Feldmeyer, 2007. *Brain Struct Funct* 212:3-17

In addition to synapses with excitatory neurons, information is relayed to inhibitory interneurons both directly via thalamic input into layer IV (*Agmon and Connors, 1992*), and through synapses between stellate cells and interneurons in layers IV and II/III (*Beierlein et al., 2003; Helmstaedter et al., 2008*). These interneurons then project to excitatory and inhibitory neurons throughout the barrel and communicate with adjacent barrels. Interneurons can thus either inhibit or disinhibit pyramidal cell output, depending on whether their actions are directly on pyramidal cells or through intermediate interneurons, respectively. All neurons in a given barrel respond with shortest latency and longest amplitude to stimulation of their principal whisker.

Beyond the main lemniscal pathway, a subset of whisker information is conveyed through the separate paralemniscal pathway, which uses the posterior medial nucleus (POm) as its relay (*Koralek et al., 1998; Lu and Lin 1993*). Neurons in this pathway project primarily to pyramidal cells in cortical layer 5A (*Bureau et al., 2006*), as well as to the septae that separate individual barrels. While the lemniscal pathway responds optimally to stimulus frequencies below 8Hz, the paralemniscal pathway also responds to frequencies as high as 18Hz (*Melzer et al., 2006*), leading to the suggestion that the lemniscal and paralemniscal pathways are responsible for spatial and temporal coding, respectively (*Ahissar et al., 2000; Yu et al., 2006*).

At the cellular level, activation of individual neurons following whisker stimulation may be assessed using immunohistochemical detection of immediate-early genes (IEGs). IEGs, activated rapidly and transiently following external stimuli, regulate late-effector genes and thus couple stimulus to transcription, allowing cells to adapt to their external environments; they are vital for long-term neuronal plasticity (*Lanahan and Worley, 1998; Melzer and Steiner, 1997*). Of these, c-Fos is the most frequently used for immunohistochemical staining, although the

inducible cyclic adenosine monophosphate early repressor (ICER), the Jun-B proto-oncogene, and the early growth response 1 gene Krox-24 have also been used in some studies (*Staiger 2002; Staiger 2006*). C-Fos has low basal expression and is upregulated by (among others) novel sensory stimulation. In accordance with thalamocortical input, whisker stimulation induces layer-dependent c-Fos expression in the corresponding barrel, with the highest amount observed in stellate cells of layer IV and pyramidal cells of layers II/III and VI. Induction peaks at around 1 hour of stimulation and then steadily declines. Neuronal activity may also be monitored by 2-deoxyglucose (2DG) uptake and blood oxygenation level-dependent functional magnetic resonance imaging (BOLD fMRI), but c-Fos provides the best resolution at the combined cellular and morphological levels of individual neurons. C-Fos does have several limitations, including the fact that not all cell types express c-Fos following activation (*Curran and Morgan, 1985*); however, in the somatosensory cortex, it has been shown that c-Fos is a good indicator of changes in neuronal activity in awake rats under physiological conditions (*Staiger et al., 2002*).

While the majority of cortical neurons are excitatory pyramidal cells, ~20% are inhibitory interneurons, using γ-aminobutyric acid (GABA) as their neurotransmitter. Various different types of cortical interneurons exist, with different morphologies, firing patterns, connectivities, and immunohistochemical characteristics (for a review, see *Markram et al., 2004*). They form large numbers of inhibitory synapses with their targets and act to locally integrate cortical activity. Interneurons act through two types of receptors. GABA-A receptors are ionotropic chloride channels, therefore activation of these receptors under resting membrane conditions leads to hyperpolarization of the cell and thus decreases the probability of action potential firing. GABA-B receptors are metabotropic, their activation being coupled to guanine nucleotide-binding protein (G-protein) cascades which lead to effects such as the opening of K+ channels

(effectively hyperpolarising the neuron); they can also reduce cell Ca^{2+} conductance and decrease adenylyl cyclase activity. Interneuron activation has also been shown to be capable of directly stimulating changes in cortical microvessel tone (*Cauli et al., 2004*). In the cerebellum, stellate interneurons are responsible for the hyperemic response, as stellate simulation-induced nitric oxide (NO) release can drive dilations via vascular smooth muscle cell (VSMC) activation in cerebellar slices (*Rancillac et al., 2006*). Similarly, mice lacking cyclin D2, who therefore have a decreased number of cerebellar molecular layer stellate cells, display a reduced cerebellar CBF response to sensory stimulation (*Yang et al., 2000*).

GABA interneurons can be immunohistochemically subdivided based on their neuropeptide and calcium-binding protein content, and separated into four main families based on their content of parvalbumin (PV), somatostatin (SOM), vasoactive intestinal peptide (VIP), and cholecystokinin (*Kawaguchi and Kubota, 1997*); a subset of SOM interneurons also colocalize neuropeptide-Y (NPY) or nitric oxide synthase (NOS), while some VIP interneurons also contain choline acetyltransferase (ChAT) or calretinin (*Chédotal et al., 1994; Kubota et al., 1994; Kawaguchi and Kubota, 1997*). This fact allows identification of different interneuron populations activated by stimulation through double immunostaining for c-Fos and markers of interneuron subtypes. Following whisker stimulation, most activated interneurons are of the VIP and/or ChAT type, with very few colocalizing SOM, NPY or NOS (*Fernandes et al., 2007*). Interestingly, following overnight whisker exploration of a novel environment, VIP and also PV interneurons are activated (*Staiger et al., 2002*). Both VIP and PV interneurons receive significant thalamic afferent input (*Staiger et al., 1996a; Staiger et al., 1996b*). Their differential activation following whisker stimulation or overnight exploration of a new environment may thus be a reflection of the amount of afferent input they receive or, more likely, of the differences

between the two stimulation paradigms, as the temporal dynamics of stimulation, neuronal spiking and novelty have been shown to influence c-Fos upregulation (*Fields et al., 1997*). Interneurons of the same class are sometimes electrically connected via gap junctions; such connections exist rarely if at all between interneurons of different types (*Gibson et al., 1999; Hestrin and Galarreta, 2005*). It has been suggested that these gap junctions might play a role in cortical gamma oscillation (*Traub et al., 2001*), and hemodynamic signals have been shown to correlate with such synchronized oscillations (*Niessing et al., 2005*). Together, these results suggest that electrical coordination of interneuron networks may play a role in neurovascular coupling.

2.2 Why neurovascular coupling: metabolic and neurogenic hypotheses

Although neurovascular coupling has been recognized since the late 19th century, and changes in CBF are used in functional imaging methods such as PET and fMRI, the mechanism underlying the tight coupling between neuronal activity and CBF has not been fully explained. Two complementary hypotheses exist, the 'metabolic' and the 'neurogenic' (*Iadecola et al., 1987; Underwood et al., 1992*). The metabolic hypothesis originated in 1890 with the pioneering work of Roy and Sherrington (*Roy and Sherrington, 1890*). It posits a direct link between tissue energy demand and the amount of the hyperemic response, suggesting that vasoactive metabolites released by active neurons diffuse to vessels, where they mediate changes in vessel diameter. The more recent neurogenic hypothesis sees synaptic signaling itself as providing the impetus for CBF changes. Recent support for the neurogenic hypothesis was provided by work showing that while glucose consumption and blood flow both increased in the contralateral somatosensory cortex following unilateral forepaw stimulation, ipsilaterally, blood flow

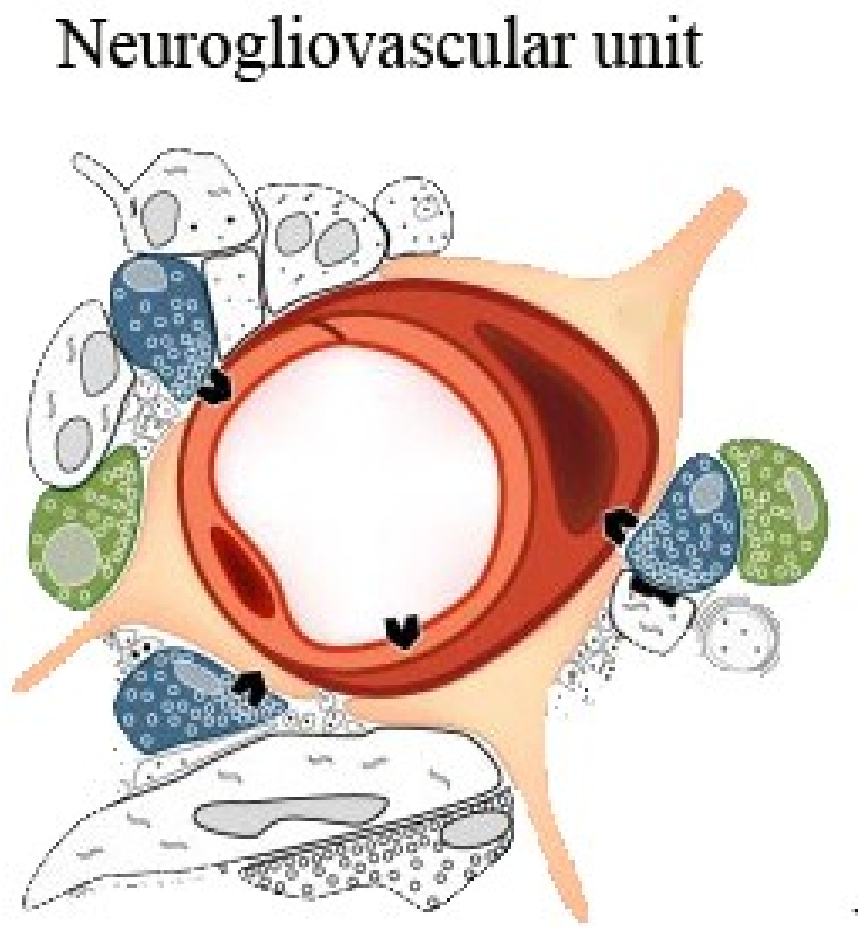
decreased but glucose uptake and neuronal activity increased (*Devor et al., 2008*). These findings are hard to explain using only the metabolic hypothesis, in which increased energy consumption (as through increased glucose uptake and neuronal activity) should lead to release of vasoactive metabolites and thus to increased CBF. A very recent study found anticipatory CBF responses linked to visual stimuli, that is, CBF responses that preceded the onset of stimulus-induced neuronal activity (*Sirotin et al., 2009*); these results too suggest that increased metabolic demand is not the sole force driving the CBF response, but rather that generation of the response may involve interplay between vasoactive metabolites and neuronal signals.

2.3 Anatomical aspects of neurovascular coupling

Blood reaches the brain via the anterior circulation, originating from the internal carotid arteries, and the posterior circulation, which arises from the two vertebral arteries meeting to form the basilar artery. The two circulations anastomose at the base of the brain in the Circle of Willis, which provides redundancy of flow in case one portion of the brain vasculature becomes blocked. From the Circle of Willis extend the large cerebral arteries, which give rise to smaller pial arteries and arterioles. These traverse the surface of the brain, giving off penetrating arteries and arterioles to the brain parenchyma. The penetrating vessels dive down through the Virchow-Robin space into the parenchyma itself, and become ensheathed with astrocytic endfeet. At this point the vessels lose their smooth muscle cell layer and are considered capillaries. The capillary endothelium forms the blood-brain barrier, whose tight junctions determine what kinds of molecules can pass from the blood into the brain. Astrocytic endfeet surround the capillaries, providing support, inducing blood-brain barrier properties in endothelial cells (*Janzer and Raff, 1987*), and participating in neurovascular coupling (see **Section 2.4**). Astrocytes are also in close

contact with neurons, each astrocyte maintaining its own spatially limited territory and overlapping little if at all with other astrocytes (*Halassa et al., 2007*); this close physical relation between blood vessels, astrocytes and neurons forms the basis of the neurovascular unit, which is at the heart of neurovascular coupling (**Figure 2)**. Subcortical neuronal projections are also implicated in the neurovascular coupling response, such as cholinergic projections from the basal forebrain, serotonergic from the raphe nucleus, and noradrenergic from the locus coeruleus (*Cohen et al., 1996; Iadecola, 2004; Hamel, 2006*).

Figure 2: The neurovascular unit. The neurovascular unit is composed of a capillary surrounded by astrocytic endfeet with closely apposed neuronal processes.



Hamel E 2006. *J Appl Physiol*, 100:1059-1064.

2.4 Neurovascular coupling mediators: general overview

Several vasoactive mediators have been identified, some produced by the endothelium itself (*Andresen et al., 2005*) and others by blood vessel smooth muscle cells, neurons, and astrocytes. Vasodilators released by the endothelium include NO, endothelium-derived hyperpolarizing factor (EDHF), prostacyclin (PGI_2), and prostaglandin E_2 (PGE_2); constrictors include endothelin, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and thromboxane A_2 (TXA_2). NO can be synthesized in endothelial cells by endothelial eNos, but also in neurons by neuronal nitric oxide synthase (nNOS) and, especially in some pathological conditions, in neurons by inducible iNOS (for a review of endothelial contributions, see *Andresen et al., 2005*).

Ions too can have effects on blood vessels. Potassium ions are vasodilatory, as their activation of VSMC inwardly rectifying Kir channels or Na^+ - K^+ -ATPase leads to the hyperpolarization of VSMCs and thus cell relaxation and vasodilation (*McCarron and Halpern, 1990*; for a review, see *Haddy et al., 2006*). In the cortex, it has been suggested that astrocytic endfoot BK channel release of K^+ mediates neurovascular coupling (*Filosa et al., 2006*); in the retina, no such role for K^+ has been observed. Indeed, mice lacking K^+ siphoning Kir4.1 channels showed normal vasomotor responses to light stimulation (*Metea et al., 2007*). The relative contribution of K^+ to neurovascular coupling may thus depend on the region under study. Another ion with potentially vasodilatory effects is H^+, which may also act through opening K^+ channels (*Faraci and Sobey, 1998*).

Various products of neuronal metabolism can have vasoactive effects. Among these is adenosine, produced by ATP catabolism, which has long been thought a player in mediating the hyperemic response. It has been shown to have vasodilatory effects both in the cortex and the cerebellum (*Ko et al., 1990; Li and Iadecola, 1994*), and has been implicated in the

neurovascular coupling response to whisker stimulation (*Dirnagl et al., 1994*). Similarly, lactate released as a product of neuronal oxidative metabolism could increase H^+ concentrations (*Attwell and Iadecola, 2002*).

Neurons release a number of vasoactive neurotransmitters. In particular, glutamate (especially acting through *N*-methyl-D-aspartate [NMDA] receptors) acts to increase NO levels, and can dilate pial arterioles *in situ* despite the lack of glutamate receptors on endothelial cells (*Bredt and Snyder, 1989; Busija and Leffler, 1989; Faraci and Breese, 1993; Morley et al., 1998*). Accordingly, inhibition of NO decreases the CBF response to NMDA (*Bhardwaj et al., 2000*). Indeed, NO has been implicated in controlling resting CBF and some neurovascular coupling responses (*Tanaka et al., 1991; Prado et al., 1992; Rancillac et al., 2006*). Notably, NO produced by nNOS acts as a permissive modulator of neurovascular coupling in response to whisker stimulation in the rat somatosensory cortex, likely through maintaining vascular smooth muscle cyclic guanosine monophosphate (cGMP) at a level high enough to allow vasodilation by other mediators (*Cholet et al., 1996; Lindauer et al., 1999*). Glutamate acting through metabotropic glutamate receptors (mGluRs) also prompts activation of phospholipase A_2, which in turn promotes the calcium-dependent release of arachidonic acid from plasma membrane phospholipids (*Aramori and Nakanishi, 1992*). Cyclooxygenase (COX) enzymes can then metabolize arachidonic acid to vasoactive prostaglandins (*Garavito and Mulichak, 2003*).

Astrocytes, as previously discussed, have extensive contact with both neuronal synapses and blood vessels, and are thus ideally positioned to mediate information transfer from one to the other (for a review, see *Haydon and Carmignoto, 2006*). They produce calcium waves in response to, among others, activation of their mGluRs. This can in turn lead to the release of vasoactive mediators such as the vasodilatory arachadonic acid metabolites epoxyeicosatrienoic

acids (EETs), and prostaglandins, which can effect either dilation or constriction (*Amruthesh et al., 1993; Alkayed et al., 1996, Mulligan and MacVicar, 2004*). Other vasoactive molecules released by astrocytes include ATP and d-serine (*Koehler et al., 2006*). Indeed, one study found that mGluR-induced Ca^{2+} increase in astrocytes occurred on the same time scale as the hyperemic response, and that blockade of mGluRs significantly decreased the expected neurovascular coupling response to somatosensory stimulation (*Zonta et al., 2003*). In the retina, astrocyte release of EETs has been demonstrated as the main effector of vasomotor responses, whose sign (dilation or constriction) is dependent on NO levels (*Metea and Newman, 2006*); astrocytic EETs are also implicated in the neurovascular coupling response to, among others, whisker and forepaw stimulation (*Peng et al., 2002; Peng et al., 2004*).

Several theories have been advanced to explain the potentially dichotomous effects of astrocyte activation on blood vessels. Some recent work suggests that oxygen availability is the determining factor for the sign of the hemodynamic response (*Gordon et al., 2008*). This study showed that under conditions of decreased O_2, external lactate accumulation due to decreased O_2 and increased astrocytic glycolysis led to increased transporter-mediated uptake of PGE_2 and thus to vasodilation, whereas when O_2 levels were high, the vasoconstrictor 20-hydroxyeicosatetraenoic acid (20-HETE), produced by smooth muscle cells (*Gebremedhin et al., 2000*), was the preferential end product of arachidonic acid metabolism. Another hypothesis, developed using arteries pressurized to various points and stimulated with a number of compounds including extracellular K^+, mGluR agonists, and EETs, is that existing vascular tone determines the sign of the astrocyte-mediated hemodynamic response to a given stimulus. In this view, arteries strive to attain an optimal set point, dilating when below and constricting when above this point in response to a given stimulus (*Blanco et al., 2008*). Support for the suggestion

that astrocytic Ca^{2+} waves primarily result in vasodilation under physiological conditions comes from two-photon imaging of anesthetized mice, in which photolysis of caged Ca^{2+} in astrocytic endfeet led to vasodilation via a COX product (*Takano et al., 2006*).

2.5 The cerebral blood flow response to whisker stimulation

Stimulation of a specific whisker follicle causes a stereotyped CBF increase in the corresponding contralateral barrel of the somatosensory cortex that may be detected by Laser Doppler flowmetry (LDF). Concordant with the high anatomical specificity of thalamic input, CBF is increased only in those barrels that correspond to stimulated whiskers (*Martin et al., 2002*). It has been suggested that surrounding areas experience negative hemodynamic activity, reflected as a decrease in the optical imaging signal (*Cox et al., 1993; Devor et al., 2005; Devor et al., 2007*). In general, neurovascular coupling involves highly elaborate communication between afferent inputs, various neuron types activated by the incoming stimuli, local vasculature, and perivascular astrocytes, although (as previously discussed) the exact cells and messengers involved may vary depending on the region and type of stimulus. In the whisker system, information is relayed via a glutamatergic thalamocortical pathway to the brain barrel cortex. Incoming glutamate activates excitatory and inhibitory neurons as well as astrocytes; these components of the pathway then communicate with each other and with blood vessels in elaborating the hemodynamic response (see **Section 2.4** for a more complete discussion). In the barrel cortex in particular, cyclooxygenase-2 (COX-2)-derived vasoactive prostanoids are involved, as inhibition of COX-2 attenuates the CBF response to whisker stimulation (*Niwa et al., 2000*). However, whether COX-2 derivatives originate from a subset of pyramidal cells expressing COX-2 (*Wang et al., 2005*) or from activated astrocytes is not known. Pyramidal cells

may thus have effects on neurovascular coupling that are both direct and indirect, through diffusible COX products and glutamate-mediated activation of astrocytes, respectively. Cyclooxygenase-1 (COX-1), on the other hand, has been shown important for the maintenance of resting CBF, but uninvolved in the neurovascular coupling response to whisker stimulation (*Niwa et al., 2001*). There is currently some debate as to the cellular localization of the two COX isoforms, but it seems that COX-2 is expressed by neurons and COX-1 by microglia (*Kaufmann et al., 1996; Hirst et al., 1998; Breder et al., 2004; Deininger and Schluesener, 1999*), providing anatomical support for their roles in functional hyperemia and resting vascular tone, respectively.

At the level of involved cell types, in addition to excitatory stellate and pyramidal cells activated by the incoming glutamatergic whisker input, astrocytes and interneurons play a role in the neurovascular coupling response to whisker stimulation. Indeed, astrocytes seem to be involved in neurovascular coupling regardless of the region of brain stimulated (for a review, see *Koehler et al., 2009*). As previously mentioned, interneurons of the same type are frequently coupled by gap junctions; similarly, gap junctions between astrocytes allow for fast electrical communication between networks of these cells. In mouse barrel cortex layer IV, gap junctions composed of connexins Cx43 and Cx30 have been shown to couple astrocytes primarily within individual barrels, with few to no gap junctions between adjacent barrels (*Houades et al., 2008*); this suggests that astrocytic networks too may contribute to the columnar organization of hemodynamic responses within the barrel cortex. Interneurons have been implicated in the neurovascular coupling response to whisker stimulation, as blockade of their ionotropic GABA-A receptors reduces the CBF response by 39% (*Fernandes et al., 2009*). Importantly, in the whisker-barrel system, blockade of muscarinic ACh receptors (mAChRs) via scopolamine has no

effect on the CBF response in either awake (*Nakao et al., 1999*) or anesthetized rats (*Fernandes et al., 2007*), implying no direct role for mAChRs in this process.

The presence of VIP in a subset of GABA interneurons activated by whisker stimulation is of interest as VIP has several effects that may be relevant to neurovascular coupling. It acts as a cortical neuromodulator, eliciting both excitatory and inhibitory post-synaptic potentials (PSPs). VIP interneurons are frequently in close apposition to blood vessels, although they rarely make direct contact with blood vessel laminae, but rather with perivascular astrocytes (*Chédotal et al., 2004*). Intracarotid infusion of VIP in rabbits induces an inrease in grey matter CBF (*Heistad et al., 1980*). Astrocytes possess the VIP receptors PAC and VPAC1, and superfusion of VIP onto preconstricted arteries in rat cortical slices (*Cauli et al., 2004*) or anesthetized cats (*Yaksh et al., 1987*) induces vessel dilation; this may also be mediated by VPAC1 receptors on cortical microvessel smooth muscle cells (*Yaksh et al., 1987; Fahrenkrug et al., 2000; Cauli et al., 2004*). VIP may also influence penetrating arteries, as many VIP interneurons project vertically towards the pial surface *(Chédotal et al., 1994; Bayraktar et. al., 2000)*. All these results suggest that VIP may play a role in whisker stimulation-induced neurovascular coupling. VIP release has been reported in the cortex following sciatic nerve stimulation (*Wang et al., 1985*); however, inhibition of VIP does not diminish the whisker CBF response (*Fernandes et al., in preparation*), thus it is possible that VIP may only play a role under certain conditions, as whisker stimulation may not provide a strong enough input to promote VIP release.

2.6 Acetylcholine and the basal forebrain system

The BF provides the majority of the cholinergic input to the rat cortex, with approximately 80% of cortical ChAT-positive fibres arising in the BF as compared to 20% from

intrinsic cholinergic neurons (*Johnston et al., 1981*). In humans, no such intrinsic source is found. Electrical stimulation of the BF increases CBF in the ipsilateral cortex; this response is significantly attenuated in both anesthetized and awake rats treated with muscarinic or nicotinic ACh receptor antagonists (*Biesold et al., 1989; Dauphin et al., 1991*). In addition, lesion of cholinergic BF fibers almost completely eliminates the CBF response to BF stimulation (*Kocharyan et al., 2008*). Although ACh can act directly on cerebral blood vessels via m5 mAChRs, (*Elhusseiny and Hamel, 2000*), recent morphological and pharmacological data from our laboratory has shown that pyramidal cells, GABA interneurons, and astrocytes are involved in the response to BF stimulation, and that pyramidal cells and interneurons can act at least in part independently of each other in mediating the CBF response (*Kocharyan et al., 2008; Lecrux et al., 2009*). Indeed, pharmacologically, the CBF response is significantly reduced by GABA-A and glutamate receptor antagonism, as well as through blockade of astrocyte-derived vasoactive EETs (*Kocharyan et al., 2008; Lecrux et al., 2009*). As assessed by c-Fos immunohistochemical staining, pyramidal cells and specific subtypes of GABA interneurons, particularly the sub-family of SOM, NPY and NOS interneurons, are activated by BF stimulation (*Kocharyan et al., 2008*). Indeed, SOM and NOS cells receive more BF cholinergic input than do other interneuron subtypes (*Cauli et al., 2004*), which might explain their preferential activation by BF stimulation. Since the cholinoceptive population of GABA interneurons differs from that activated by glutamatergic thalamic afferents (see **Section 2.1**), it is possible that additional cholinoceptive interneuron populations could be activated in the barrel cortex under conditions of cholinergic facilitation of sensory input.

2.7 Cholinergic modulation of thalamocortical input

ACh is implicated in brain 'on' states such as arousal, attention, and memory (*Miasnikov et al., 2008*), and decreasing ACh is required for the induction of sleep states. ACh has a facilitative or permissive role in the processing of thalamocortical glutamatergic afferents. In addition to its role in BF stimulation (see **Section 2.6**), increasing ACh levels through acetylcholinesterase (AChE) inhibition or cortical ACh iontophoresis heightens sensory evoked potentials, uncovers previously silent receptive fields, and lowers neuronal thresholds (*Ashe et al., 1989; Metherate et al., 1988; Metherate et al., 1990; Tremblay et al., 1990; Lamour et al., 1988*). In the auditory cortex, increasing ACh via BF stimulation lowers response threshold and facilitates field potentials and EPSPs following auditory stimulation, in a mAChR-dependent manner (*Metherate and Ashe, 1993; Chen and Yan, 2007*). In contrast, loss of cholinergic tone via BF lesion impairs the metabolic response to sensory stimulation, as assessed by 2DG (*Jacobs et al., 1991*); it also reduces sensory stimulation-dependent plasticity in the form of map expansion in the somatosensory cortex (*Juliano et al., 1991*). Similarly, in the visual cortex, cholinergic lesion or muscarinic inhibition decreases c-Fos activation following patterned visual stimulation (*Dotigny et al., 2008*). It has been suggested that ACh can act to enhance signal strength of incoming sensory information relative to cortico-cortical signals (*Oldford and Castro-Alamancos, 2003*).

In the whisker pathway, ACh could have effects at multiple levels depending on the differential distribution of its two receptor subtypes. Ionic nicotinic receptors (nAChRs), mediating fast flow of cations (including Na^+, K^+, and sometimes Ca^{2+}), are located presynaptically on the glutamatergic thalamocortical afferents (*Oldford and Castro-Alamancos, 2003*); their effects are generally excitatory. The receptors are formed of pentamers of 17

potential subunits, with great regional variation in receptor composition. In contrast, metabotropic mAChRs modulate neurons and interneurons within the cortex itself. Five isoforms (termed M1-M5) exist, and these can have a complex array of actions that result in either inhibition or excitation of synaptic transmission (*Hulme et al., 1990; Wei et al., 1994*).

Within the barrel cortex, similarly to that discussed above for the auditory and visual cortices, muscarinic receptors seem to be involved in cholinergic modulation, as agonism of muscarinic receptors has been shown to increase the strength of neuronal responses to minimally effective whisker stimuli (*Farkas et al., 1996*) and to enhance 'on' responses (*McKenna et al., 1988*). We therefore posited that muscarinic receptors might play an important role in any ACh-induced modulation of the whisker CBF response.

ACh is synthesized within cholinergic neurons from choline and acetyl-CoA by ChAT and degraded extracellularly within the synaptic cleft by acetylcholinesterase (AChE). Manipulating activity of AChE can thus increase or decrease the length of ACh action at the synapse, enhancing or inhibiting its effects.

2.8 Acetylcholine and cortical map representation

Alterations in barrel functional representation after ACh agonism or antagonism at both muscarinic and nicotinic receptors have been observed, as the muscarinic agonist carbachol or nicotinic agonist nicotine increased, and muscarinic antagonist scopolamine decreased, the area of intrinsic neuronal signaling following whisker stimulation (*Penschuck et al., 2002*). A similar effect has been observed in the auditory cortex, where the cortical area responding to a sound stimulus was increased by concurrent basal forebrain stimulation (*Kilgard and Merzenich, 1998; Mercado et al., 2001*). Likewise, increased ACh enhances odor discimination and sharpens

mitral cell receptive fields in the olfactory bulb, while cholinergic lesion decreases odor discrimination (*Doty et al., 1999; Chaudhury et al., 2009; Linster et al., 2001*).

3. RATIONALE AND HYPOTHESIS

ACh has long been known to modulate processing of glutamatergic thalamocortical sensory information. We hypothesized that this modulation would be evident in the CBF response to whisker stimulation, a well-characterized model of somatosensory input. We sought to determine whether a basal level of cholinergic tone was required for normal expression of the whisker-evoked CBF response, and whether we could increase this response by increasing ACh. As ACh does not affect this CBF response at basal conditions, we further hypothesized that neurons not normally involved in the response but possessing ACh receptors might become activated under conditions of inreased ACh. Although not tested in the present study, we hypothesized alternatively that changes in neuron electrical activity might be responsible for driving any observed alteration in the CBF response.

4. METHODS

4.1 Whisker stimulation

All experiments were approved by the animal ethics committee of McGill University, in keeping with the Canadian Council on Animal Care. Whisker stimulation manipulations were performed in both awake and anesthetized animals to permit evaluation of immunohistochemical and CBF changes, respectively. For the former, rats received five days of habituation, to reduce handling stress-related c-Fos induction (*Asunama et al., 1992*), for a 10-minute mechanical stimulation (~4Hz) on the sixth day. In order to prevent non-specific neuronal activation for animals to be immunohistochemically examined, rats were anesthetized with isoflurane (5%, 2-3 minutes) and all whiskers except for right C1-3 were trimmed as close to the face as possible the day prior to the experimental day. Following stimulation, rats were returned to their home cages for one hour to achieve maximal c-Fos accumulation (*Bisler et al., 2002*). To study CBF, animals were anesthetized with urethane (1.2g/kg, intraperitoneal [i.p.]), an anesthetic agent known to preserve neurovascular coupling responses to somatosensory stimulation (*Huttunena et al., 2007*), and continuously monitored by LDF at baseline and following mechanical stimulation (20s, ~10Hz) of all whiskers on the right side. At least 40s baseline was obtained between each stimulation.

4.2 CBF measurement

Animals were placed in a stereotaxic frame (Kopf Instruments) and the skull over the left barrel cortex (Bregma coordinates AP: -3mm, L: +7mm) was thinned to translucency to allow visualization of surface vessels. CBF was measured using a Transonic LDF microprobe

positioned away from large vessels and connected to a perfusion monitor. Body temperature was maintained at 37°C throughout procedures by means of a heating pad connected to a rectal temperature probe. In some rats, the femoral artery was catheterized for monitoring of blood pressure and heart rate (AD Instruments) and analysis of blood gases (pH, pO_2, pCO_2) (Rapid Lab 348, Bayer).

4.3 Immunohistochemistry

Rats were transcardially perfused through the ascending aorta under deep anesthesia (sodium pentobarbital, 100mg/kg, i.p.) with ice-cold 4% paraformaldehyde (PFA). Brains were removed, post-fixed in PFA for two hours at 4°C, then cryoprotected in 30% sucrose. Following snap freezing in isopentane (-40°C), 25µm-thick sections (or 35µm for ChAT fibres) were cut using a freezing microtome, then immunohistochemically stained for c-Fos (rabbit anti-c-Fos, 1:1500, Santa Cruz) either alone or together with markers of pyramidal cells and selected GABA interneuron subtypes using antibodies for COX-2 (rabbit anti-COX-2, 1:3000, Santa Cruz), ChAT (goat anti-Chat, 1:250, Chemicon), VIP (guinea pig anti-VIP, 1:15,000, Peninsula Laboratories), and SOM (rabbit anti-SOM, 1:20,000, Peninsula Laboratories) as previously described (*Kocharyan et al., 2008*). Following overnight incubation at room temperature, sections were rinsed in phosphate-buffered saline and incubated (45 min) with biotinylated species-specific secondary antibody (1:300, Vector, Burlingame CA), then rinsed and incubated with ABComplex (ABC kit, Vectastain Elite, Vector). Visualization of the reaction was achieved via the slate grey reagent (SG kit, Vector Labs) for c-Fos, or a 0.05% solution of 3,3'-diaminobenzidine (DAB, Vector Labs) for all other antibodies. For double immunolabeling, peptides or enzymes were detected in the first position, and c-Fos in second position.

4.4 Cholinergic lesion

Male Sprague-Dawley rats (250-270g) were anesthetized with isoflurane (5% for induction, 2% for maintenance) and injected with the selective cholinotoxin 192 IgG-saporin (4ug/2uL [Advanced Targeting Systems], or 2uL saline for controls) in the left lateral ventricle (Bregma coordinates AP: 0.8mm, L: 1.4mm, 4.3mm below skull surface) as previously described (*Kocharyan et al., 2008*). Intracerebroventricular injection of 192 IgG-saporin has been shown to selectively lesion cholinergic neurons located in the nucleus basalis magnocellularis of the BF while sparing those in the cortex (*Wenk et al., 1994*), leading to an almost total loss of cortical cholinergic fibres by 7-10 days after saporin injection (*Heckers et al., 1994; Kocharyan et al., 2008*). Following suturing, rats were returned to their home cages for 7-14 days to allow lesion development, then stimulated for c-Fos immunohistochemistry or CBF measurement before perfusion for ChAT immunostaining as described above.

4.5 Central nicotinic blockade

Male Sprague-Dawley rats (250-270g) were anesthetized with isoflurane (5% for induction, 2% for maintenance) and injected with the non-specific nicotinic antagonist chlorisondamine dichloride (12ug/5uL, [gift from Dr. Paul Clarke, Department of Pharmacology, McGill University], or 5uL saline for controls) in the left lateral ventricle as for cholinergic lesion (*Clarke, 1984; El-Bizri et al., 1995*). Rats were sutured and returned to their home cages for 1-4 weeks to ensure development of selective central nicotinic receptor blockade, which has been reported to last several months (*El-Bizri et al., 1995*), then whiskers were stimulated and blood flow measured by LDF as described above.

4.6 Pharmacology

ACh enhancement was accomplished either via linopirdine dihydrochloride (10 mg/kg, i.p., 20 min, Tocris) or physostigmine hemisulfate (0.1 mg/kg, s.c., 30 min, Tocris). MAChRs were blocked with scopolamine (0.1mg/kg, i.v., 20-40 min, Tocris).

4.7 Basal forebrain implantation and stimulation

Male Sprague-Dawley rats (280-300g) were chronically implanted with stimulating monopolar tungsten electrodes (0.35mm outer diameter, FHC, Bowdoinham, ME) in the substantia innominata of the BF as previously described (*Kocharyan et al., 2008; Lecrux et al., 2009*). Briefly, animals were anesthetized with isoflurane, placed in a stereotaxic frame, and electrodes positioned at bregma coordinates AP:-1.2mm, L:2.4mm, and V:-6.9mm. Animals were sutured, then returned to their home cages for 4-5 days. On the experimental day, animals were anesthetized by urethane (1.2mg/kg, i.p.), fixed in a stereotaxic frame, and an LDF probe placed over the barrel cortex as described above. After initial whisker stimulation, BF stimulation (100Hz, 0.5ms, 1s on/1s off, Isolated pulse stimulator, A-M systems) was performed alone using pulses at varying amplitudes (55-350μA, on average 150 ± 30μA) to find an amplitude at which baseline CBF increased only minimally in each rat. This BF stimulation was given for 30s to obtain a mean CBF value for BF stimulation alone. Stimulation was then performed at this amplitude for 10s alone, then for an additional 20s concurrently with whisker stimulation (~10Hz). Electrode placement in the BF was verified by the evoked CBF reponse to a 10s higher-amplitude (100-500μA, average 278 ± 49 μA) BF stimulation at the end of the experiment. Similarly, in scopolamine-treated animals, the same high amplitude stimulation was performed to confirm the expected reduction of the BF response by scopolamine.

4.8 Data Analysis

4.8.1. Laser Doppler

CBF was measured in arbitrary tissue perfusion units (TPU). Only animals with baselines stable throughout the duration of the experiment were included in the analysis. Stimulus-induced CBF changes were taken as the mean across the peak response, and expressed as percent change from baseline. Repeated measures analysis of variance (ANOVA) followed by post-hoc Newman-Keuls comparison test was used to analyze all data except that from control and saporin lesioned rats, which were compared by Student's t-test. No significant difference was observed between baseline and vehicle stimulations for any condition. For average CBF responses, 3 representative stimulations were chosen for baseline, vehicle, and drug conditions (where applicable) and were averaged for each second, beginning 20 seconds before stimulation onset and ending 20 seconds after. Data were normalized to set each resting baseline (defined as the average TPU value over the 20 seconds prior to stimulation) equal to 0% CBF increase, and values for each second expressed as percent change relative to the standardized baseline. Since CBF values may depend on absolute CBF, which is not measurable by LDF, we also compared resting baseline values between treatment groups. No significant difference in resting baseline was found between conditions.

4.8.2. Choline acetyltransferase fibre immunohistochemistry

For ChAT fibre semi-quantitative analysis, digital pictures from the barrel cortex region (3 sections/rat) were acquired (Nikon Eclipse E800, Nikon), randomized, and ranked by two observers on a scale from 0 to 5 (0 being no visible fibres and 5 being the highest observed fibre density). Density rankings were then averaged by animal and by group, and compared by Student's t-test.

4.8.3. C-Fos barrel size and extent

Quantitative analysis of c-Fos barrel area sizes was accomplished using digital pictures taken (Nikon Eclipse E800) using NeuroLucida software (MBF Bioscience) to trace the area occupied by c-Fos positive nuclei in layer IV and NeuroExplorer (Nex Technologies) to quantify the corresponding cortical area. For quantification of rostral-to-caudal extent of lesioned barrels, every third section was acquired throughout the entire barrel cortex and stained for c-Fos as described above. Sections were then observed by light microscopy (Leitz Aristoplan, Leica) and compared to bregma levels (Paxinos and Watson, 1970) to determine earliest barrel appearance, level of maximal extent, and level of disappearance.

4.8.4. Identification of activated neurons by double immunohistochemistry

For quantification of cell subtype activation, 2-3 sections/rat were observed by light microscopy and cells counted in layers II/III, IV, V, and VI (or in layers II/III and IV for COX-2). Almost no c-Fos-positive cells were ever observed in layer I, so they were not included in this analysis. The number of each cell type colocalizing c-Fos was compared to the total number of cells of that type, both by layer and in overall barrel. Data were expressed as percent of the total population of each cell type. Data from the ipsilateral barrel cortex were subtracted from contralateral barrel data to obtain the specific percent activation for each cell type, and zero percent activation represented no specific activation or higher activation on the ipsilateral side. Comparisons were made using Student's t-test. For each rat, an average of 634 ± 44 cells were counted for COX-2, 243 ± 14 for VIP, and 246 ± 21 for SOM.

Editing of digital photos was done using Adobe Photoshop 7 (Adobe Systems, San Jose, CA). Data are presented as mean ± SEM. All statistical analyses were conducted using GraphPad Prism4 software (San Diego, CA), with $p<0.05$ considered significant.

5. RESULTS

5.1 Pharmacological ACh enhancement augmented the whisker CBF response

Following ACh increase via the ACh release-enhancing drug linopirdine, the CBF response to whisker stimulation was augmented by 31 ± 3.6 % relative to saline vehicle injection ($p<0.001$) (**Figures 4A,B)**. Since linopirdine may also increase release of other neurotransmitters, the AChE inhibitor physostigmine was used in another group of rats in order to control for the specificity of the ACh effect. Physostigmine increased the whisker-evoked CBF response by 40 ± 8.1% relative to vehicle ($p<0.05$) **(Figures 4C,D)**. Mean arterial pressure, heart rate, blood gases and pH remained normal throughout these experiments (see **Table 1**).

5.2 ACh augmentation did not increase extent of c-Fos barrel activation

The immediate-early gene c-Fos can be used to mark neurons activated by whisker stimulation. In the somatosensory cortex, c-Fos expression is low at basal conditions, but its transcription has been shown to increase in both pyramidal cells and interneurons functionally activated following stimuli such as direct whisker manipulation (*Fernandes et al., 2007*) or exploration of a novel environment (*Staiger et al., 2002*). In an attempt to identify the neuronal changes driving the enhanced perfusion response under conditions of increased ACh, we traced the area of the c-Fos barrel in contralateral layer IV at its fullest extent (from bregma ~-2.3 to ~-3.3). Immunostaining for c-Fos in animals given either linopirdine or physostigmine revealed no change in the area of the c-Fos barrel relative to saline-treated controls (**Figures 5A-D**).

5.3 Decreasing ACh through cholinergic lesion decreased the whisker CBF response

In order to ascertain whether the potentiating effects of linopirdine and physostigmine could indeed be ascribed to ACh, we next investigated the consequences of ACh deprivation on

the hemodynamic response to sensory stimulation. Efficacy of the selective cholinotoxin 192 IgG-saporin was confirmed by immunostaining of cortical ACh innervation using the ACh-synthesizing enzyme ChAT. Semi-quantitative analysis revealed a decrease in fibre density of ~70% in the area of the barrel cortex (**Figures 6A,B**). In contrast to results obtained under conditions of increased ACh, specific lesion of ACh afferents to the somatosensory cortex decreased the whisker-evoked CBF response by 28% ($p<0.001$) (**Figures 6C,D**). Physiological parameters remained normal for the duration of these experiments (**Table 1**).

5.4 Cholinergic lesion decreased area and extent of c-Fos barrel activation

In contrast to the unaltered area of c-Fos activation observed following ACh increase, decreasing ACh afferent input to the cortex reduced the area of the c-Fos barrel measured in layer IV at its largest extent by 39% ($p<0.05$) (**Figures 7A,B**). We next attempted to determine whether the rostral-to-caudal extent of the barrel was altered under conditions of decreased cholinergic tone, staining every third section throughout the entire barrel cortex. Cholinergic lesion decreased the rostral-to-caudal area of cortex occupied by the c-Fos barrel by ~25% ($p<0.01$) (**Figure 7C**), with the saline barrel starting on average at bregma level -1.8 and the lesioned barrel at ~-2.2; the control barrel reached its maximal extent at bregma ~-2.1, while the lesioned barrel did not until ~-2.5. In both conditions, the barrel could no longer be observed at bregma ~-3.7 (**Figure 7D**).

5.5 Changes in cholinergic tone did not alter neurons activated by whisker stimulation

To identify the specific cell types activated under our two conditions of opposite cholinergic tone, we performed double immunohistochemical staining for c-Fos and markers of different neuron subtypes. We chose to focus on two of the main intereuron subtypes, VIP and SOM, as VIP but not SOM interneurons are known to be activated following whisker stimulation

in awake animals, while SOM but not VIP interneurons are primarily responsive to BF stimulation (*Staiger et al, 1996a; Fernandes et al., 2007; Kocharyan et al., 2008*). Pyramidal cells expressing COX-2 were also examined, as these cells are not only activated by both whisker and BF stimulations (*Fernandes et al., 2007; Lecrux et al., 2009*), but COX-2 has been shown to be an important mediator of the whisker-evoked CBF response (*Niwa et al., 2000*). No significant differences in activated cell types were observed between controls and animals treated with either linopirdine or saporin. In all conditions, ~21% of COX-2 pyramidal cells and ~13% of VIP interneurons, as compared to less than 8% of SOM interneurons, were specifically activated in the contralateral barrel cortex. (**Figures 8A-C**).

5.6 BF stimulation augmented the whisker CBF response through mAChRs

A more physiological increase of ACh, as compared to pharmacological enhancement via linopirdine or physostigmine, was tested to better mimic conditions of increased cholinergic tone *in vivo*. This was achieved using simultaneous stimulation of whiskers with electrical stimulation of the BF at an amplitude determined for each rat to have a minimal effect on baseline CBF in the barrel cortex (+1.7 ± 0.5%) (**Figure 9A**). The mean CBF response to whisker stimulation was increased by 52 ± 18% when paired with this BF stimulation ($p<0.05$) (**Figures 9B,C**). Following blockade of mAChRs with scopolamine, the CBF response to paired whisker + BF stiulation was decreased by 75 and 90% relative to whisker + BF stimulation after 20 and 40 minutes, respectively ($p<0.01$) (**Figure 9C**). No effect of scopolamine was seen on whisker stimulation alone (**Figure 9D**), in accordance with previous results (*Nakao et al., 1999; Fernandes et al., personal communication*), clearly indicating that mAChRs are implicated in the heightened CBF response observed following paired whisker + BF stimulation.

5.7 No nicotinic component to the cerebral blood flow response under conditions of increased acetylcholine

Having found a muscarinic component to the facilitation of the CBF response following BF stimulation *in vivo*, we sought to determine whether nicotinic receptors are also involved under conditions of enhanced ACh neurotransmission. Nicotinic receptors were blocked through intracerebroventricular injection of the non-subtype specific nicotinic antagonist chlorisondamine, which is known to create a powerful central nicotinic blockade lasting several months without affecting peripheral nicotinic receptors (*Clarke, 1984; El-Bizri et al., 1993*). We observed no effect of chlorisondamine treatment on the CBF increase induced by linopirdine injection (**Figures 10C,D**) relative to saline-treated animals (**Figures 10A,B**). Indeed, a trend (albeit non-significant) was observed towards a higher percent facilitation of the response in chlorisondamine-treated animals, but the sample size was small.

6. FIGURES

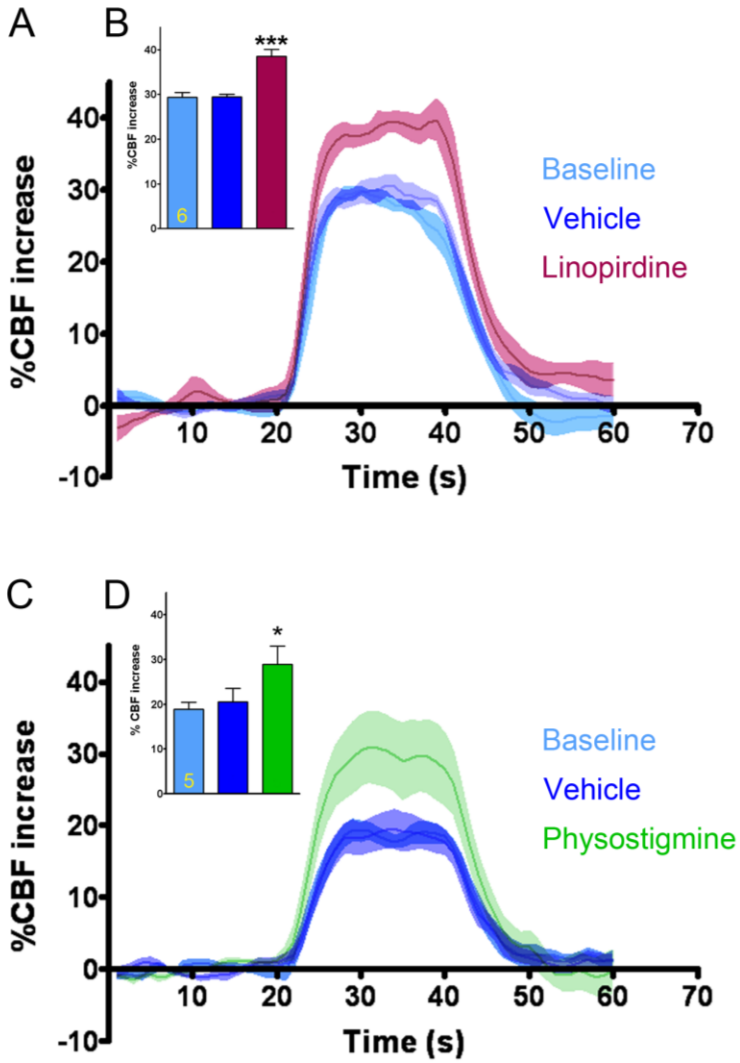


Figure 3: Effects of linopirdine and physostigmine on whisker-evoked CBF responses
A-B The ACh release-enhancing drug linopirdine increased the CBF response to whisker stimulation. **A** shows CBF responses at baseline and after vehicle and linopirdine injections, and **B** the percent increase in the evoked CBF response for each condition. **C-D** Similarly, physostigmine, an acetylcholinesterase inhibitor, enhanced the CBF response to whisker stimulation. Averaged CBF responses are shown in **C** and the effects of physostigmine compared to baseline and vehicle on the whisker-induced CBF response in **D**. ***p<0.001, *p<0.05 drug versus vehicle (repeated measures ANOVA). The number of animals is indicated at the base of each histogram. Shaded areas represent SEM.

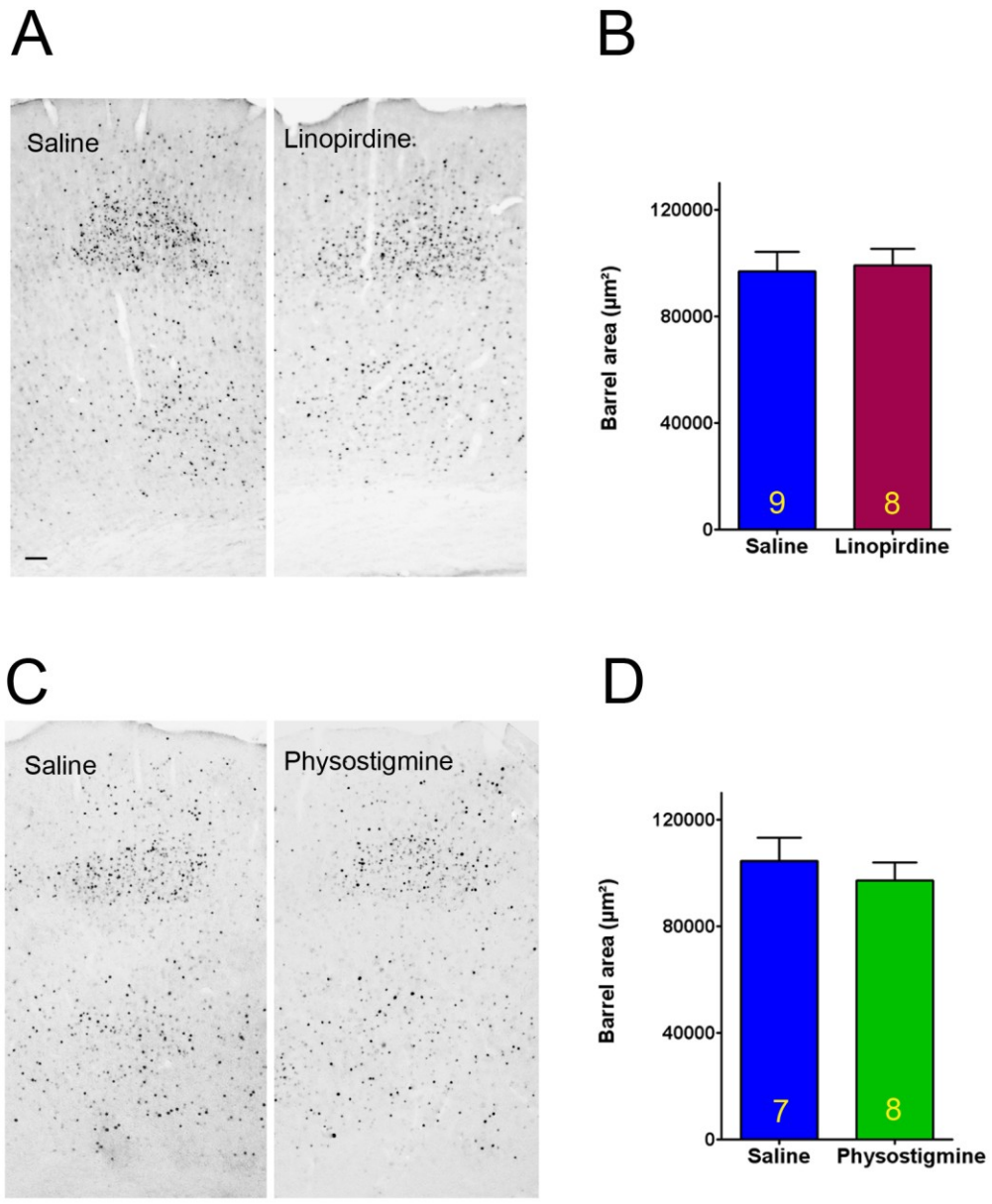


Figure 4: Analysis of c-Fos barrel size following linopirdine or physostigmine treatment
Neither linopirdine (**A-B**) nor physostigmine (**C-D**) altered the cortical area occupied by c-Fos immunoreactivity in layer IV at the full extent of the activated barrel (bregma levels ~-2.3 - -3.3). **A** Immunostaining for c-Fos in the barrels of representative saline- and linopirdine-treated animals. **B** Analysis of barrel size showed no change between the two conditions. **C** C-Fos immunostaining within barrels in representative saline- and physostigmine treated animals. **D** As with linopirdine, physostigmine did not alter the c-Fos barrel area. The number of animals is indicated at the base of each histogram. Scale bar: 50µm

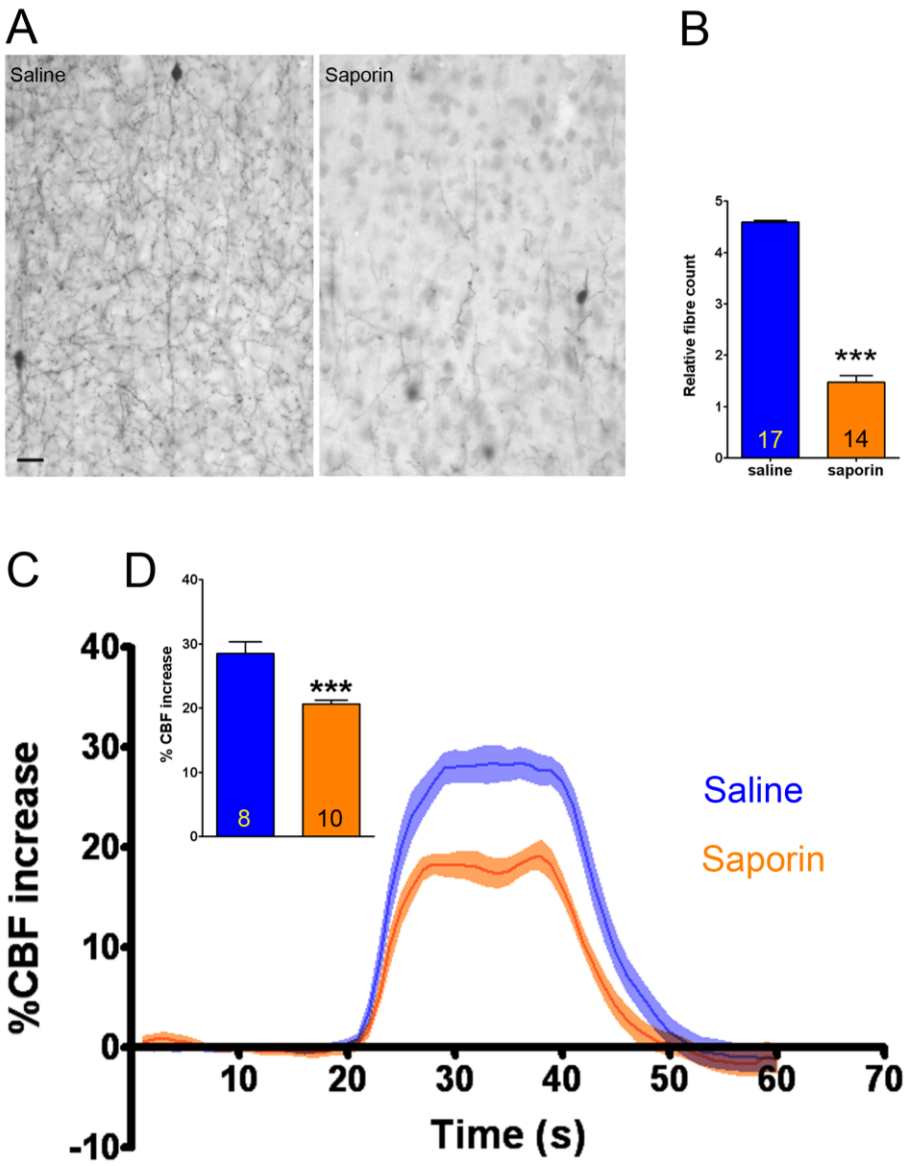


Figure 5: Effect of 192 IgG-saporin on the evoked CBF response

A ChAT fibre immunostaining decreased considerably in the barrel cortex region following 192 IgG-saporin injection. **B** Semi-quantitative analysis of fibre density (see **Methods** ***4.8.2***) showed a decrease of ~70% following lesion. **C** Averaged CBF responses in saline- and saporin-treated animals. **D** Lesioned animals showed a significant decrease in the whisker-evoked CBF response. ***p<0.001 by Student's t-test. The number of animals is indicated at the base of each histogram. Shaded areas represent SEM. Scale bar: 20µm

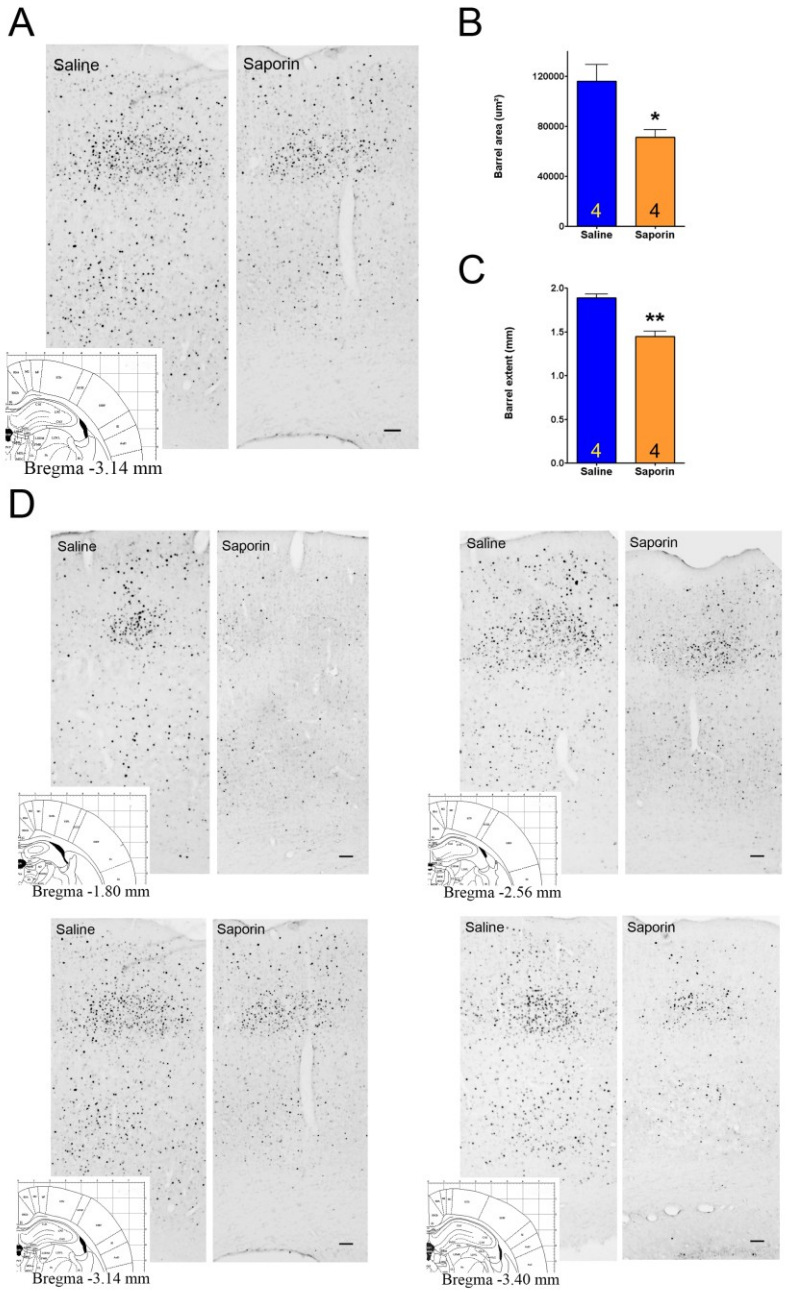


Figure 6: Analysis of barrel size and extent following saporin injection

A Representative c-Fos immunostained barrels at their full extent in saline- and saporin-treated animals. **B** Analysis of c-Fos barrel size revealed a decrease of 39% in lesioned relative to control animals. **C** The rostral-to-caudal extent of the c-Fos barrel was decreased by ~0.4mm (~25%) in lesioned animals. **D** Representative c-Fos immunostaining showing rostral-to-caudal extent of barrels in saline- and saporin-treated animals. Control barrels appeared at bregma ~-1.8 and reached their maximal extent at bregma ~-2.1; lesion barrels were not observed until bregma ~-2.2, and did not reach their full extent until bregma ~-2.5. In both conditions the c-Fos barrel disappeared around bregma -3.7. *$p<0.05$, **$p<0.01$ by Student's t-test. The number of animals is indicated at the base of each histogram. Scale bars: 50µm

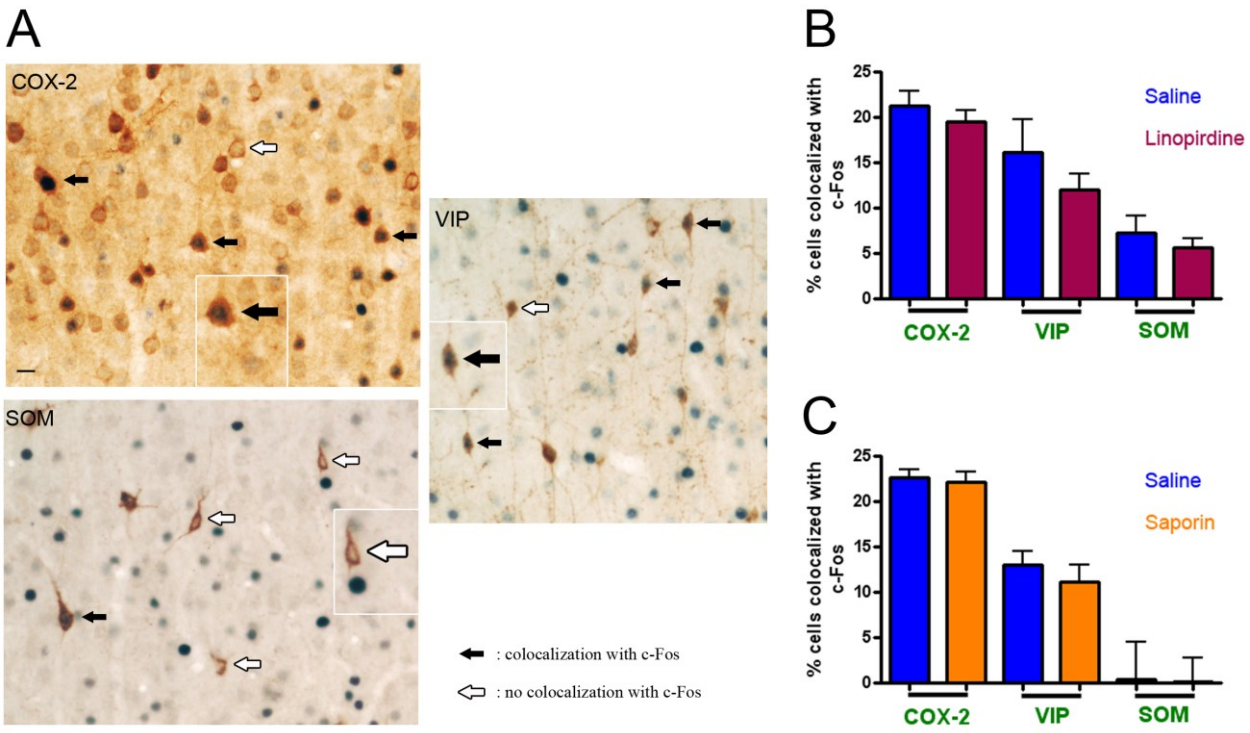


Figure 7: Immunohistochemical identification of activated neurons within the barrel
A Double immunohistochemistry revealed specific activation (c-Fos, blue nuclei; see **Methods**) of COX-2 pyramidal cells and VIP interneurons, with few to no SOM interneurons activated following whisker stimulation. **B** Quantitative analysis following saline or linopirdine treatment, with no significant differences between control and linopirdine-treated animals. **C** Quantitative analysis following saline or saporin injection, again showing no significant differences between the two conditions. $p>0.05$ by Student's t-test. Scale bar: 5μm
black arrowheads = colocalization with c-Fos white arrows = no colocalization with c-Fos

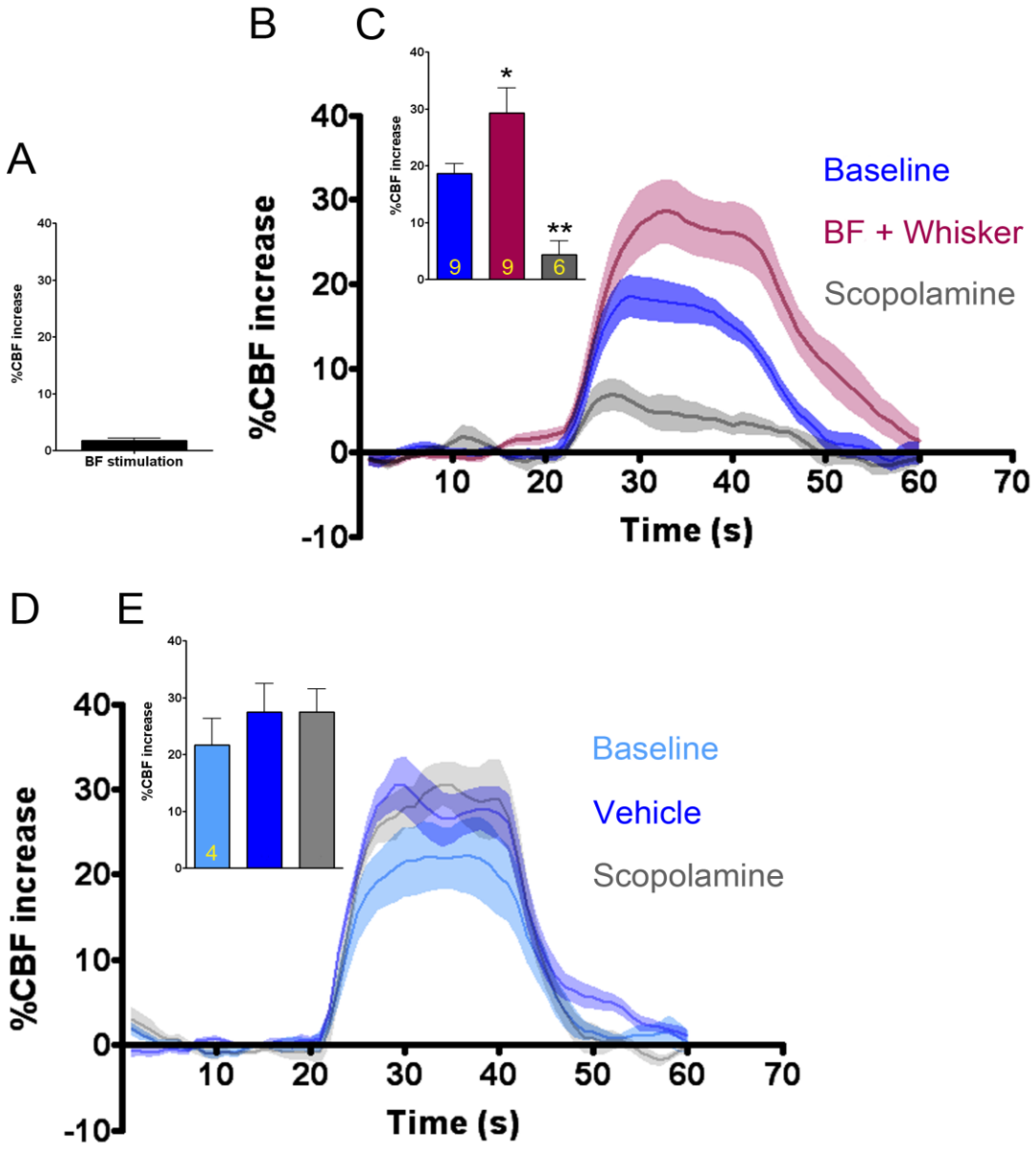


Figure 8: CBF response following concurrent whisker and BF stimulation, before and after mAChR blockade

A The amplitude of BF stimulation (150 ± 30μA) was determined for each animal to not significantly increase baseline CBF in the barrel cortex (+1.7 ± 0.4%). **B** Average CBF responses following whisker stimulation alone, whisker stimulation coupled with BF stimulation, or paired whisker and BF stimulation after scopolamine. **C** The CBF response to paired whisker and BF stimulation was 52 ± 18% greater than that to whisker stimulation alone. Scopolamine reduced the CBF response to paired whisker and BF stimulation at 20 and 40 minutes. **D** Scopolamine had no effect on whisker stimulation alone. The number of animals is indicated at the base of each histogram. Shaded areas represent SEM. *p<0.05 versus whisker stimulation alone and **p<0.01 versus whisker + BF stimulation by repeated measures ANOVA

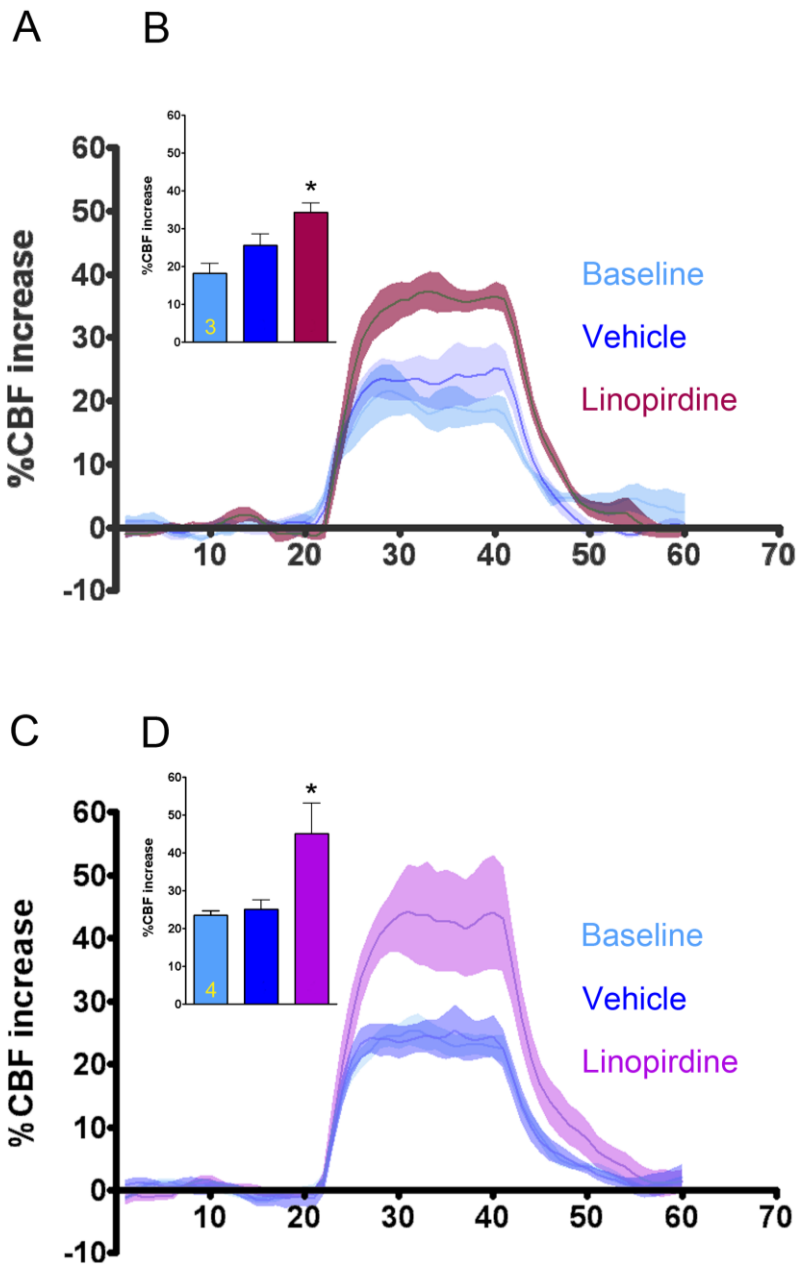


Figure 9: CBF response to whisker stimulation following linopirdine treatment and nicotinic receptor blockade

Rats treated with chlorisondamine displayed linopirdine-enhanced CBF responses to whisker stimulation similar to saline controls. **A, C** Averaged CBF responses at baseline and following vehicle or linopirdine injection in **A** saline- and **C** chlorisondamine-treated animals. **B, D** Mean of the peak CBF response for **B** saline- and **D** chlorisondamine-treated rats. The number of animals is indicated at the base of each histogram. Shaded areas represent SEM. *p<0.05 by repeated measures ANOVA

Table 1: Physiological Parameters

	MABP (mmHg)			Heart rate		
	Baseline	*Vehicle*	*Drug*	*Baseline*	*Vehicle*	*Drug*
i.p. linopirdine	62 ± 4	56 ± 2	81 ± 5	252 ± 30	292 ± 19	326 ± 12
i.c.v. saline	75 ± 7			343 ± 41		
i.c.v. saporin	65 ± 5			253 ± 18		
i.c.v. saline + linopirdine	76 ± 1	69 ± 1	86 ± 3	386 ± 1	398 ± 7	526 ± 44
i.c.v. chlorisondamine + linopirdine	66 ± 2	65 ± 1	62 ± 5	300 ± 20	314 ± 2	333 ± 19

	pH			pCO_2		
	Baseline	*Vehicle*	*Drug*	*Baseline*	*Vehicle*	*Drug*
i.p. linopirdine		7.42 ± 0.01	7.40 ± 0.01		43 ± 2	44 ± 2
i.c.v. saline	7.42 ± 0.01			38 ± 3		
i.c.v. saporin	7.42 ± 0.01			42 ± 6		
i.c.v. saline + linopirdine	7.37 ± 0.02	7.35 ± 0.02	7.36 ± 0.02	50 ± 2	52 ± 4	49 ± 2
i.c.v. chlorisondamine + linopirdine	7.34 ± 0.01	7.32 ± 0.01	7.29 ± 0.01	52 ± 2	55 ± 3	58 ± 4

7. DISCUSSION

The present results demonstrate a role for ACh in the expression of the neurovascular coupling response to whisker stimulation. While increasing cholinergic tone enhanced the CBF response to whisker stimulation, decreasing cortical ACh innervation diminished this hemodynamic response, indicating that a basic level of cholinergic tone is required. Furthermore, the data show that these ACh effects are primarily mediated by mAChRs, as muscarinic but not nicotinic blockade significantly reduced the response facilitation induced by *in vivo* BF stimulation or an ACh release-enhancing agent. At the neuronal level, ACh enhancement did not alter overall cortical area of activation or activated cell types, suggesting that an increase in the neuronal activity of target neurons might be responsible for the increased whisker-evoked CBF response. Decreasing ACh, however, decreased the cortical area activated by whisker stimulation, further indicating that a basal level of cholinergic tone is required to enable some cells to respond to whisker stimulation.

7.1 Methodological Considerations

Pharmacological ACh increase was achieved using the drugs linopirdine or physostigmine. Linopirdine has been shown to enhance evoked ACh release without altering basal efflux, thus its effects are truly to augment normal release rather than cause non-specific activation (*Zaczek et al., 1997*). Given that ACh application may have differing effects when applied via endogenous and exogenous routes, depending on whether synaptic or extrasynaptic receptors are being activated (*Oldford and Castro-Alamancos, 2003*), linopirdine's effects of augmenting endogenous release should faithfully recreate normal pathways. However, since its

method of action is through blocking voltage-gated K+ channels, linopirdine *in vitro* increases release not only of ACh but also of dopamine, serotonin, glutamate, and d-aspartate; effects *in vivo* have not been fully studied (*Zaczek et al., 1998*). To ensure that the CBF effects we observed following linopirdine treatment were indeed due to ACh, the potent acetylcholinesterase inhibitor physostigmine was used in different rats. Importantly for this study, neither linopirdine nor physostigmine affect c-Fos expression in the barrel cortex in young rats (*Dent et al., 2001; Messamore et al., 1993; Milivojevic et al., 2001*), thus c-Fos immunostaining should detect only the specific effects of whisker stimulation on neuronal activation. One potential consideration is that ACh increase through linopirdine, physostigmine, and BF stimulation enhanced the whisker-evoked CBF response to different extents; this may be due to the different mechanisms of ACh augmentation employed by each method. Linopirdine augments normal ACh release while physostigmine slows the degradation of previously released ACh; the greater percent CBF response enhancement seen following BF stimulation may simply reflect a higher amount of ACh reaching the cortex under these conditions.

7.2 Effects of cholinergic modulation on CBF responses and neuronal activation

It is possible that the ACh facilitation of the whisker CBF response might have been due in part to a direct effect on blood vessels, rather than to an effect on neuronal processing. Blood vessels do contain dilatory ACh receptors, most notably m5 muscarinic receptors (*Elhusseiny and Hamel, 2000; Yamada et al., 2001*). However, we did not observe changes in baseline CBF following injection of the ACh-enhancing compounds linopirdine and physostigmine. Furthermore, in our combined BF and whisker stimulation paradigm, the chosen amplitude of BF

stimulation increased resting CBF by only ~2%, while the whisker response was increased by 55%. We suggest, therefore, that the majority of the response increase cannot be attributed to a direct effect on blood vessels, but rather to interactions between ACh and activity of the neuronal network targeted by thalamocortical afferent input.

Neurovascular coupling is thought to reflect levels of neuronal activity. It has previously been shown that cholinergic lesion decreases whisker evoked functional activity (*Jacobs et al., 1991*), thus it is not surprising that a decreased CBF response should accompany decreased brain activation. Similarly, the effects of ACh on inreasing neuronal activity and lowering firing thresholds have been well characterized (*Lamour et al., 1988; McKenna et al., 1988; Metherate et al., 1990; Tremblay et al., 1990*). The increased CBF response we observed under conditions of increased ACh corresponds well with the expected ACh-mediated increase in neuronal responsiveness.

In an attempt to identify the neuronal changes driving the increased perfusion response when ACh was increased, we examined neuronal activation with c-Fos. A previous study using intrinsic optical imaging found that agonism of either muscarinic or nicotinic receptors increased the area activated by whisker stimulation (*Penschuck et al., 2002*). However, we did not see an increase in the area of barrel c-Fos activation when ACh was increased, despite the fact that the concomitant CBF response was augmented. This may perhaps be explained by the differences between intrinsic signals and c-Fos activation. Both subthreshold synaptic potential and spiking activity are thought to contribute to intrinsic imaging signals (*Grinvald et al., 2001*). C-Fos activation, on the other hand, is thought to require synaptic activation and not merely increased spiking activity (*Luckman et al., 1994*). The area of barrel cortex experiencing small electrical

fluctuations in response to whisker stimulation may thus be greater than that undergoing outright activation and synaptic transmission.

Consistent with the significantly decreased CBF response, the extent of the c-Fos barrel was smaller following cholinergic lesion. This reduction was evident both in coronal sections and in rostral-to-caudal extent. Mean response probability of layer II/III interneurons has been shown to decrease away from the barrel centre (*Kerr et al., 2007*). As ACh may tonically lower the threshold of input required to promote action potential firing in neurons in the barrel periphery, neurons lacking cholinergic tone may have not been able to fire in response to whisker input, which would explain their decreased c-Fos activation.

As we could not find any difference in overall neuronal activation that might explain the increased CBF response after linopirdine and physostigmine, which are known to facilitate ACh neurotransmission, we turned to identification of activated cell types, hypothesizing that activated populations might have changed. Under conditions of novel environment exploration or awake mechanical whisker stimulation, interneurons colocalizing VIP and/or ChAT are activated, with little to no activation of SOM, NPY or NOS interneurons (*Staiger et al., 2002; Fernandes et al., 2007*). This is consistent with the fact that VIP interneurons are a main target of thalamocortical afferents (*Staiger et al., 1996a*). In contrast, BF stimulation activates different interneuron populations, namely SOM, NPY and NOS cells (*Kocharyan et al., 2008*), NOS and SOM interneurons being the most frequent targets of cholinergic afferents from the BF (*Cauli et al., 2004*). We therefore posited originally that these cell populations might become activated by whisker stimulation under conditions of increased cholinergic tone. However, no such shift in activated cell types was found in this study. We observed similar proportions of activated VIP interneurons, and little to no activation of those containing SOM, both under normal conditions

and following linopirdine treatment. COX-2 pyramidal cell activation was also similar between control and linopirdine-treated animals. It has previously been shown that for some neuron types, such as cultured dorsal root ganglion cells, the temporal pattern of stimulation can affect c-Fos upregulation (*Fields et al., 1997*). Our BF electrical stimulation paradigm (100Hz pulses, 1s on/1s off for 20s/stimulation) did differ temporally from that used in the whisker pathway (continuous ~4Hz stimulation for 10 minutes). However, the differential activation of interneuron subtypes in these two systems is well explained by their pathway-specific network innervation, suggesting that the temporal stimulation patterns we used were not the main determinants of c-Fos upregulation in these two cortical areas. It is possible that cholinoceptive interneurons may have been only weakly activated, at a level too low to upregulate c-Fos. Taken together, our c-Fos and double immunohistochemistry results suggest that increased ACh facilitates the whisker CBF response through effects on the activity of thalamocortical target cells. This corresponds well with previous work showing that ACh iontophoresis can increase the magnitude of neuronal discharge to a given dose of glutamate or strength of sensory stimulus (*Metherate et al., 1988*), and that pharmacological ACh agonism or BF stimulation enhances visual evoked potentials (*Kang and Vaucher, 2009*).

7.3 Contribution of ACh receptor subtypes to the CBF response facilitation

We investigated the respective contributions of muscarinic and nicotinic receptors to the ACh facilitation, to determine whether the site of ACh action was on cortical pyramidal cells and interneurons (*McCormick and Prince, 1985, Woolf, 1993*) or on the thalamocortical afferents (*Oldford and Castro-Alamancos, 2003*), respectively, notwithstanding the expression of nicotinic

receptors by VIP interneurons (*Porter et al., 1999*). In the hippocampus, nicotinic receptors have been shown to enhance glutamatergic synaptic transmission (*Radcliffe and Dani, 1998*), suggesting they might play a similar role in the cortex. However, studies in the auditory cortex have found mAChRs to mediate many or even all ACh effects on decreasing response thresholds (*Metherate et al., 1990; Chen and Yan, 2007*). Similarly, in the somatosensory cortex, mAChRs have been shown to increase neuronal response strength to minimal stimuli (*Farkas et al., 1996*). Indeed, neonatal ocular dominance plasticity in the visual cortex has been shown dependent on muscarinic but not nicotinic receptor activation (*Gu and Singer, 1993*). In agreement with these observations, we were able to block ~70% of the BF-induced facilitation of the CBF response to whisker stimulation using the mAChR antagonist scopolamine.

Nicotinic receptors are notoriously difficult to study, as many different subtypes exist and nAChRs also mediate peripheral autonomic nervous system transmission; nicotinic blockade can thus have effects on blood pressure which might confound analysis of CBF responses. We therefore chose to use chlorisondamine, as it can selectively block central nicotinic receptors (*Clarke, 1984; El-Bizri et al., 1995*), and no significant effect on mean blood pressure was observed in the present study under any condition. ACh enhancement via linopirdine was able to augment the whisker-evoked CBF response in chlorisondamine-treated animals to levels slightly, but not significantly, higher than in control animals. Thus, nicotinic receptors appear not to be important in facilitating the CBF response, and may even provide a tonic inhibition of activation under control conditions. ACh overall seems to act on neuronal processing within the somatosensory cortex, rather than on the strength of glutamatergic thalamocortical inputs.

7.4 Summary

The predominantly mAChR agonist carbachol increases functional barrel representation, as measured by intrinsic optical imaging (*Penschuck et al., 2002*). Laser Doppler flowmetry measures local tissue perfuson to a depth of only ~1mm from the bone surface, corresponding to layers I, II/III, and upper layer IV at best. The increased CBF response we observed following ACh enhancement must therefore correlate with neuronal activity in these layers. This is consistent with the thalamocortical input in the barrel cortex spreading rapidly from layer IV stellate cells to layers II/III (*Benshalom and White, 1986; Armstrong-James, 1992*). As our overall area of c-Fos barrel activation was not increased by ACh augmentation, it seems likely that ACh acts through mAChRs to directly modulate the responsiveness of cells previously shown to be important in the whisker response, namely pyramidal cells, including those that express COX-2, and thalamocortical target VIP interneurons. Lack of recruitment of cholinoceptive SOM interneurons suggests facilitation of these specific thalamocortical-activated cell networks, perhaps by effects on firing rate or evoked potentials, compatible with the previously observed increase in functional barrel representation following ACh agonism (*Penschuck et al., 2002*).

8. FUTURE DIRECTIONS

Several avenues of further study suggest themselves. As our investigation of ACh receptor subtype contributions to the CBF response facilitation were conducted using a different method of ACh increase for each subtype (mAChR blockade with BF stimulation, and nicotinic blockade with linopirdine treatment), it would be of interest to confirm these results using scopolamine with linopirdine for mAChRs, and chlorisondamine with BF stimulation for nicotinic receptors. Our data suggest effects of ACh on neuron electrical activity, thus measuring cortical activity such as local field potentials or neuronal firing rates under conditions of increased and decreased cholinergic tone could provide more direct evidence of its mode of action.

Additional studies could be aimed at investigating the BF potentiating effect on the somatosensory-evoked CBF response in pathological conditions. For example. in mouse models of Alzheimer's disease with known cholinergic deficits and decreased hyperemic response to whisker stimulation, this could be a marker of cholinergic hypofunction and, potentially, pharmacologic intervention.

9. CONCLUSION

We show, for the first time, that cholinergic modulation of thalamocortical sensory processing is reflected in the CBF response to whisker stimulation. Increasing ACh promotes a larger CBF response to whisker stimulation; this enhancement involves mAChRs, and does not alter activated cell types or the extent of the c-Fos barrel. In contrast, without a basal level of cholinergic tone, the whisker-evoked CBF response is decreased and a smaller area of c-Fos barrel activation is observed. We suggest that these ACh effects are due to alterations in the electrical activity of networked neurons rather than to a change in the populations of cells involved in processing the afferent signals.

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