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# Activation of cortical 5-HT<sub>3</sub> receptor-expressing interneurons induces NO mediated vasodilatations and NPY mediated vasoconstrictions

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GABAergic interneurons are local integrators of cortical activity that have been reported to be involved in the control of cerebral blood flow (CBF) through their ability to produce vasoactive molecules and their rich innervation of neighboring blood vessels. They form a highly diverse population among which the serotonin 5-hydroxytryptamine 3A receptor (5-HT<sub>3A</sub>)-expressing interneurons share a common developmental origin, in addition to the responsiveness to serotonergic ascending pathway. We have recently shown that these neurons regroup two distinct subpopulations within the somatosensory cortex: Neuropeptide Y (NPY)-expressing interneurons, displaying morphological properties similar to those of neurogliaform cells and Vasoactive Intestinal Peptide (VIP)-expressing bipolar/bitufted interneurons. The aim of the present study was to determine the role of these neuronal populations in the control of vascular tone by monitoring blood vessels diameter changes, using infrared videomicroscopy in mouse neocortical slices. Bath applications of 1-(3-Chlorophenyl)biguanide hydrochloride (mCPBG), a 5-HT<sub>3R</sub> agonist, induced both constrictions (30%) and dilations (70%) of penetrating arterioles within supragranular layers. All vasoconstrictions were abolished in the presence of the NPY receptor antagonist (BIBP 3226), suggesting that they were elicited by NPY release. Vasodilations persisted in the presence of the VIP receptor antagonist VPAC1 (PG-97-269), whereas they were blocked in the presence of the neuronal Nitric Oxide (NO) Synthase (nNOS) inhibitor, L-NNA. Altogether, these results strongly suggest that activation of neocortical 5-HT<sub>3A</sub>-expressing interneurons by serotonergic input could induces NO mediated vasodilatations and NPY mediated vasoconstrictions.

**Keywords:** neurovascular coupling, mCPBG, serotonin, U46619, Pet1 knock-out mouse, vasoactive intestinal peptide, brain slices, neurogliaform cells

## INTRODUCTION

Within the cerebral cortex, different types of GABAergic inhibitory interneurons have been described according to their distinctive morphological, molecular, and electrophysiological characteristics (Cauli et al., 1997; Markram et al., 2004; Vitalis and Rossier, 2011). Although the final classification scheme of cortical interneurons is still a matter of debate (Ascoli et al., 2008), data from *in vitro* and *in vivo* experiments tend to demonstrate that distinct subpopulations of inhibitory interneurons exert specific functional roles in the integrative processes of the cortical network (Whittington and Traub, 2003; Markram et al., 2004; Fanselow and Connors, 2010; Gentet et al., 2010; Mendez and Bacci, 2011). Furthermore, some GABAergic interneurons have been reported recently to be involved in the control of cerebral blood flow (CBF) through their ability to express and release vasoactive molecules (Cauli et al., 2004; Cauli and Hamel, 2010). However, further characterization of these vasoactive interneurons subpopulations remains to be established.

Interestingly, the robust cortical serotonergic innervation from raphe nuclei (Reinhard et al., 1979; Steinbusch, 1981; Tork,

1990), which modulate cortical activity (Takeuchi and Sano, 1984; Papadopoulos et al., 1987; DeFelipe et al., 1991) and CBF (Rappoport et al., 1948; Cohen et al., 1996; Riad et al., 1998), preferentially targets inhibitory interneurons (DeFelipe et al., 1991; Smiley and Goldman-Rakic, 1996; Paspalas and Papadopoulos, 2001). However, the processes by which 5-hydroxytryptamine (serotonin, 5-HT) acts on the cortical network and CBF are complex and deserve to be further understood. Indeed, responses to 5-HT seem to depend upon the nature of the receptors involved, and the recruited neuronal populations (Underwood et al., 1992; Cohen et al., 1996; Foehring et al., 2002).

Serotonin can notably induce a fast excitation of specific interneuron subpopulations through the activation of the 5-hydroxytryptamine 3A receptor (5-HT<sub>3A</sub>) (Ferezou et al., 2002; Lee et al., 2010) which is the only ionotropic serotonergic receptor (Barnes and Sharp, 1999; Chameau and van Hooft, 2006). In the mouse primary somatosensory cortex, the 5-HT<sub>3A</sub> receptor is expressed by two distinct types of interneurons (Vucurovic et al., 2010). The first one was characterized by a bipolar/bitufted morphology, an adaptative or bursting firing behavior and the

frequent expression of the vasoactive intestinal peptide (VIP), reported to be a vasodilator in the cerebral cortex (McCulloch and Edvinsson, 1980; Yaksh et al., 1987; Dacey et al., 1988), whereas the second population of interneurons includes neurogliaform like regular spiking neurons and therefore frequently expressed the neuropeptide Y (NPY), a potent vasoconstrictor (Dacey et al., 1988; Abounader et al., 1995; Cauli et al., 2004). In rat neocortical slices, it has been shown that electrical stimulation of a single VIP- or NOS/NPY-expressing interneuron was able to induce a dilation of nearby microvessels, probably by releasing vasoactive molecules. Additionally, direct perfusion of VIP or NO donor onto cortical slices dilated blood vessels, whereas perfusion of NPY induced vasoconstrictions (Cauli et al., 2004).

In the present study, we investigated how the pharmacological activation of 5-HT<sub>3A</sub>-expressing interneurons can induce blood vessel diameter changes by means of infrared videomicroscopy on mice cortical slices. We find that activation of 5-HT<sub>3A</sub>-expressing interneurons mostly induced vasodilations mediated by NO release and also, but less frequently, vasoconstrictions through NPY release. Our results show that these interneurons are strategically positioned to transmute incoming neuronal afferent signals into vascular responses.

## MATERIALS AND METHODS

### ANIMALS AND SURGERY

Animal procedures were conducted in strict compliance with approved institutional protocols and in accordance with the provisions for animal care and use described in the *European Communities Council directive of 24 November 1986 (86-16-09/EEC)*.

C57Bl6J mice (14–21 days old; Charles River) were used for vascular reactivity. All animals were housed in a temperature-controlled (21–25°C) room under daylight conditions. They arrived in the laboratory at least 1 week before initiating experiments to acclimate to their new environment.

Immunohistochemistry was performed on a transgenic mouse line expressing the enhanced green fluorescent protein (GFP) under the control of the 5-HT<sub>3A</sub> promoter (5-HT<sub>3A</sub>:GFP). This line, obtained by using modified bacterial artificial chromosomes (BACs) was provided by the GENSAT Consortium (Rockefeller University-GENSAT Consortium; (Heintz, 2004) and maintained under the Swiss genetic background by breeding heterozygous mice. The selective expression of GFP in 5-HT<sub>3</sub> expressing neurons has been previously controlled in the cortex of these mice (Lee et al., 2010; Vucurovic et al., 2010).

The Pet1 knock-out mouse line (gift from Evan Deneris, Case Western Reserve University, Cleveland, OH) was maintained on a C57BL6 genetic background. Heterozygous Pet1<sup>+/-</sup> females were mated with Pet1<sup>+/-</sup> or Pet1<sup>-/-</sup> males to produce mixed litters. Genotyping of the progeny was done by PCR analysis of tail DNA as described previously (Hendricks et al., 2003).

### IMMUNOHISTOCHEMISTRY

Neuronal populations expressing 5-HT<sub>3A</sub>:GFP were analyzed at postnatal day P18–P25 ( $n = 6$ ). Animals were deeply anesthetized with an intraperitoneal (IP) injection of pentobarbital (150 mg/kg body weight) and perfused transcardially with

4% paraformaldehyde (PFA). Brains were cryoprotected in 30% sucrose and cut on a freezing microtome (35 μm). For immunofluorescence, sections were incubated overnight at 4°C with the following antibodies diluted in phosphate buffer (PS) saline (PBS): chicken anti-GFP (1:1000, Molecular Probes) and rabbit anti-NPY (1:500, Amersham), rabbit anti-VIP (1:800, Incstar) or rabbit anti-nNOS (1:400, Santa-Cruz). After washing in PBS, sections were incubated in alexa 568 conjugated goat anti-rabbit and alexa 488 conjugated goat anti-chicken antibodies (1:300; Molecular Probes).

Sections were rinsed in PB, mounted in Vectashield (Vector) containing 4',6'-diamidino-2-phenylindole (DAPI) and were observed with a fluorescent microscope (Leica, DMR). Images were acquired with a Coolsnap camera (Photometrics). Quantifications of GFP:5-HT<sub>3A</sub><sup>+</sup> and NOS<sup>+</sup>, NPY<sup>+</sup>, or VIP<sup>+</sup> cells were performed at the level of the primary somatosensory cortex, in 500 μm-wide cortical strips (data are expressed as percentages). Three adjacent sections of at least five animals were used.

The estimation of the neuronal density at specific distances from the closest penetrating blood vessel was performed on coronal brain slices (60 μm thick) that were fixed by immersion overnight in PFA. Slices were then rinsed in PBS prior immunolabeling. For immunofluorescence, sections were incubated sequentially overnight at 4°C with the following antibodies diluted in PBS: rabbit anti-GFP (1:1000, Molecular Probes) and goat anti-collagen IV (1:400, Millipore). After washing in PBS, sections were incubated in CY3 conjugated rabbit anti-goat and alexa 488 conjugated chicken anti-rabbit antibodies (1:300; Molecular Probes). Sections were rinsed in PB, mounted in Vectashield (Vector) containing 4',6'-diamidino-2-phenylindole (DAPI) and were observed with a fluorescent microscope (Zeiss). Images were acquired at the level of the somatosensory cortex with a Zeiss microscope equipped with an AxioCam MRm CDC camera (Zeiss) and three adjacent sections of four animals were used. When two penetrating blood vessels were identified the distance of GFP:5-HT<sub>3A</sub><sup>+</sup> cells within the region defined by these two blood vessels was determined. The distance from the closest penetrating blood vessel was calculated using Image J software. The distance from the closest blood vessel and the center of two adjacent penetrating blood vessels was subsequently subdivided in four equal parts. The percentages of 5-HT<sub>3A</sub>:GFP<sup>+</sup> cells located within these four radial bins were then calculated.

Some captions were also taken with a Nikon confocal system and maximal projections were used for illustration.

### PREPARATION OF ACUTE CORTICAL SLICES

Mice were rapidly decapitated and brains were quickly removed and placed into cold (~4°C) slicing solution containing (in mM): 110 choline chloride, 11.6 Na-ascorbate, 7 MgCl<sub>2</sub>, 2.5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, and 0.5 CaCl<sub>2</sub>, continuously bubbled with 95% O<sub>2</sub>–5% CO<sub>2</sub>. Coronal brain slices (300 μm thick) containing the primary somatosensory cortex were cut with a vibratome (VT1200S; Leica), and transferred to a holding chamber enclosing artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>,

26 NaHCO<sub>3</sub>, 20 glucose and 1 kynurenic acid (a nonspecific glutamate receptor antagonist, Sigma), constantly oxygenated (95% O<sub>2</sub>-5% CO<sub>2</sub>), kept 30 min at 35°C and then held at room temperature. Individual slices were next placed in a submerged recording chamber kept at 32°C and perfused (1.5 ml/min) with oxygenated ACSF (in the absence of kynurenic acid). Blood vessels were visualized using infrared videomicroscopy with Dodt gradient contrast optics (Luigs and Neumann) mounted on an upright microscope (Zeiss) equipped with a CDD camera (Hamamatsu).

## DRUGS

The 5-HT<sub>3</sub> receptor selective agonist (1-(3-Chlorophenyl) biguanide hydrochloride, mCPBG, 100 μM, Sigma) was used to stimulate specifically 5-HT<sub>3</sub>-expressing interneurons. To block neuronal synaptic transmission, mCPBG was applied in the presence and after 10 min application of tetrodotoxin (TTX; 1 μM; Latoxan). To block the nNOS, slices were treated for at least 1 h with an irreversible inhibitor of constitutive nitric oxide synthase (nNOS) and a reversible inhibitor of inducible nitric oxide synthase (iNOS), Nω-Nitro-L-arginine (L-NNA; 100 μM; Sigma). NPY Y1 receptors were blocked with the selective antagonist N2-(Diphenylacetyl)-N-[(4-hydroxyphenyl) methyl]-D-arginine amide (BIBP 3226; 100 μM; Sigma). VIP type 1 (VPAC1) receptors of VIP were blocked with the high-affinity, selective antagonist PG-97-269 (100 nM; Biochem, Shanghai). The thromboxane A<sub>2</sub> receptors agonist (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F<sub>2</sub>α, U46619, 5 nM, Sigma) was used to pre-constrict the blood vessels.

## VASCULAR REACTIVITY

Arterioles remaining in focal plane, exhibiting a well-defined luminal diameter (10–20 μm) and located in the supragranular layers of the somatosensory cortex were selected for vascular reactivity. Images of arterioles were acquired every 15 s (Media Cybernetics). The eventual drift of the images along the time of the recording was corrected on-line for the z-drift and off-line for *x* and *y* directions using Image Pro Plus 7.0. Manual replacement of the images to minimize the differences between two consecutive frames was realized by using a subtraction tool from Image Pro Plus and luminal diameters were quantified at different locations along the blood vessel using custom written routines running within IgorPro (WaveMetrics) to determine the location that moved the most.

Control baselines were determined for 5 min at the start of the recording. Unresponsive blood vessels or vessels with unstable baseline were discarded from the analysis. Vessels were considered unstable when their diameter moved more than 5% during the control baseline. Only one vessel per slice was recorded.

As blood vessels in the slice preparation lack intraluminal flow and pressure (Sagher et al., 1993; Cauli et al., 2004), vasomotor movements were detected in vessels pre-constricted for 10 min with the thromboxane agonist U46619 (5 nM), which was applied throughout the experiment. Only blood vessels that presented a diameter reduction of at least 10% were kept for analyses. The vasoconstriction of blood vessels induced by U46619 followed an exponential progression along the time. This exponential drifting

contraction was fitted and subtracted from the recordings using IgorPro.

Maximal vasomotor responses were then expressed as percentages of the mean baseline diameter, which is the averaged diameter measured during the control period of one minute, after correction for the pre-constriction and just before the mCPBG application.

The maximal response diameter was averaged between the fifth and sixth minute after the onset of the mCPBG application for vasodilations and between the fourth and the fifth minute for vasoconstrictions.

## STATISTICAL ANALYSES

Data were expressed as mean ± standard error of the mean (SEM). A paired *t*-test was used to determine the statistical significance of vasomotor responses to mCPBG application under the same conditions and a *t*-test was used to determine the statistical significance of response amplitudes between different set of experiments.

## RESULTS

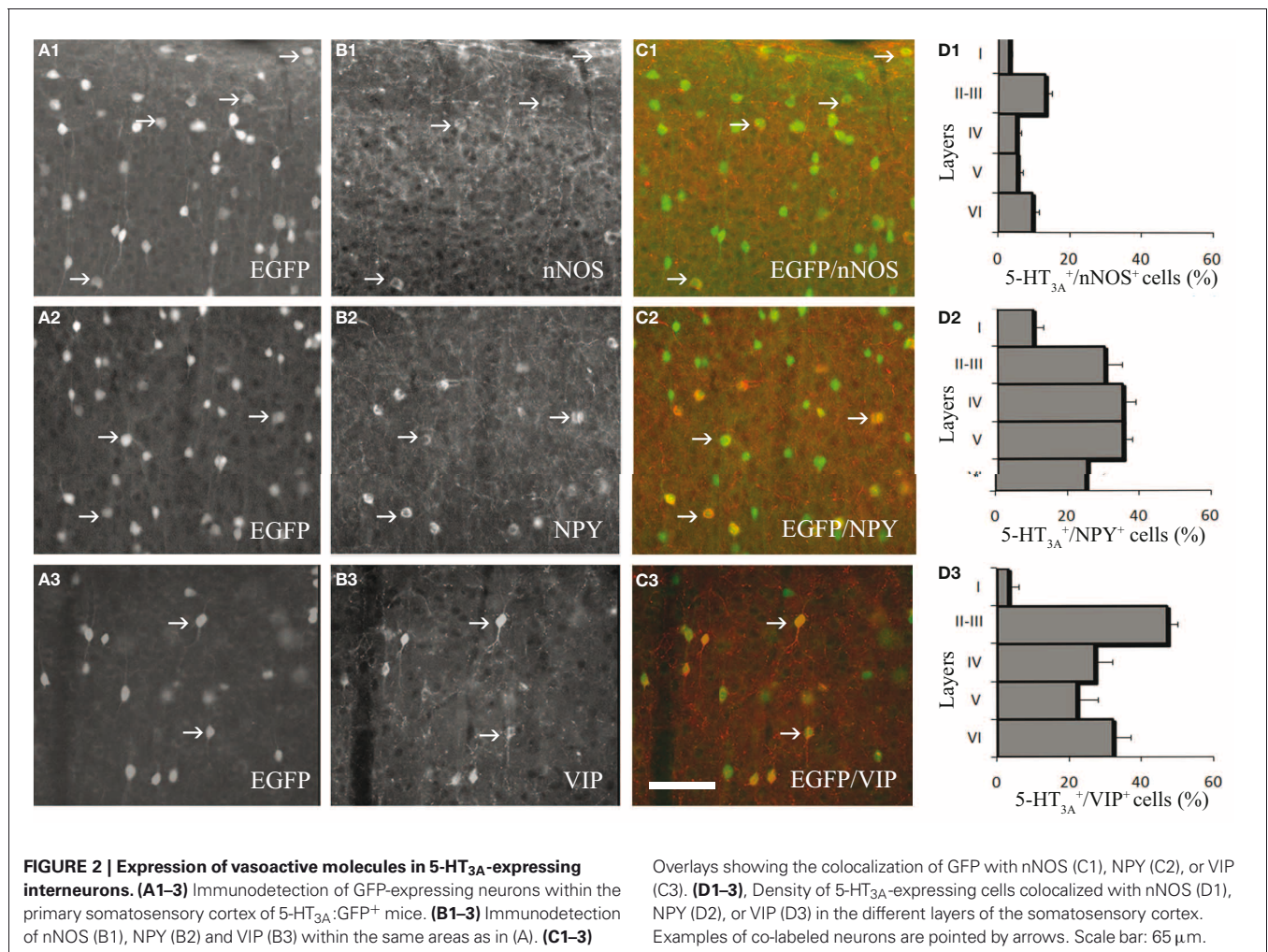
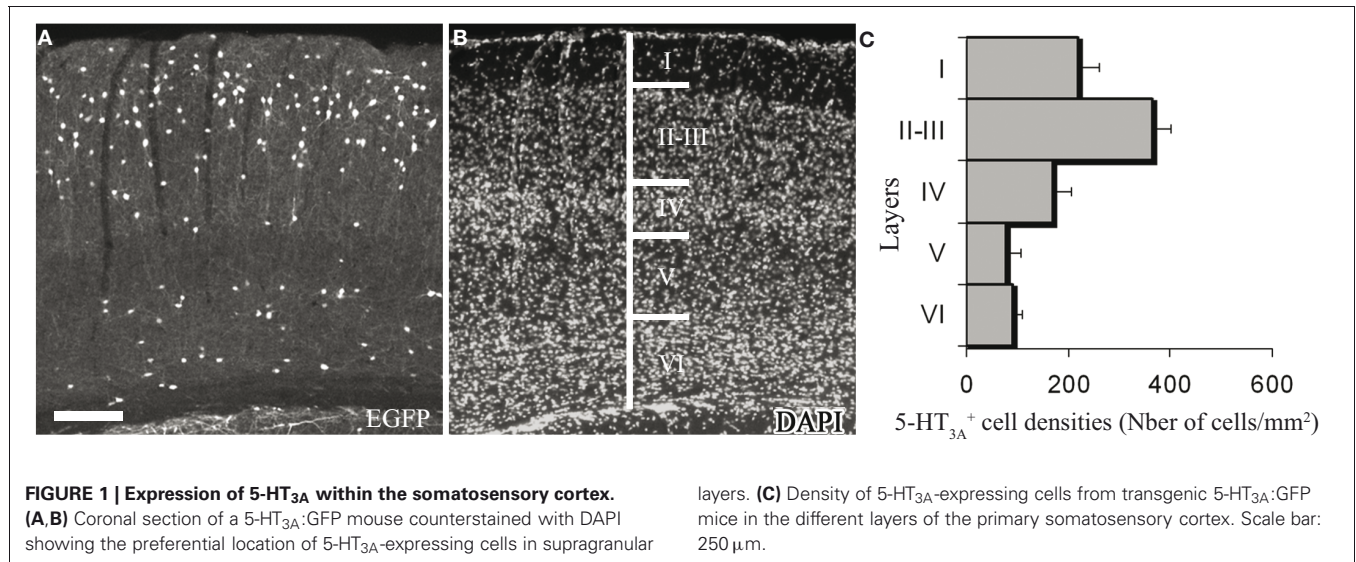
### DISTRIBUTION OF 5-HT<sub>3A</sub>-EXPRESSING NEURONS AND CO-EXPRESSION OF VASOACTIVE MOLECULES IN TRANSGENIC 5-HT<sub>3A</sub>:GFP MICE

In order to determine the laminar distribution of 5-HT<sub>3</sub>-expressing interneurons and the vasoactive molecules that they express, we used a reporter mouse strain in which the enhanced GFP expression is under the control of the 5-HT<sub>3A</sub> promoter (Heintz, 2001). This 5-HT<sub>3A</sub>:GFP mouse line provided by GENSAT shows similar distribution of cortical 5-HT<sub>3A</sub>:GFP<sup>+</sup> neurons, compared to the expression pattern of 5-HT<sub>3A</sub> transcripts observed in wild type animals (Vucurovic et al., 2010). The 5-HT<sub>3</sub>-expressing interneurons are distributed in all cortical layers (**Figure 1**), with a higher density in supragranular layers.

We next performed immunohistochemical analyses to assess the expression of vasoactive molecules in 5-HT<sub>3A</sub>-expressing interneurons. Within the primary somatosensory cortex, 5-HT<sub>3A</sub>:GFP<sup>+</sup> cells frequently co-expressed NPY (31 ± 4.3%), VIP (30.2 ± 5.2% or nNOS (9.8 ± 0.9%; **Figure 2**). These nNOS<sup>+</sup>/GFP<sup>+</sup> cells correspond to 34.4 ± 0.9% of type II nNOS cells (see Perrenoud et al., 2012), whereas no type I nNOS cell were found to express 5-HT<sub>3</sub>.

The proportion of cells in which GFP was co-detected with one of the three markers is layer dependent (**Figure 2D**). Indeed, VIP<sup>+</sup>/GFP<sup>+</sup> interneurons were preferentially located in layers II-III, while nNOS<sup>+</sup>/GFP<sup>+</sup> and NPY<sup>+</sup>/GFP<sup>+</sup> interneurons were distributed in all cortical layers. These distributions correspond to those recently reported by Lee and collaborators for the NPY and VIP (Lee et al., 2010).

We then determined the relative distribution of 5-HT<sub>3A</sub>:GFP<sup>+</sup> neurons in relation with the closest large penetrating blood vessel identified on a coronal section at the level of the somatosensory cortex (17–32 μm, **Figure 3**). This was done in layer I and in layer II where subsequent analyses were performed. In layer I the distribution of 5-HT<sub>3A</sub>:GFP<sup>+</sup> cells was homogenous between two penetrating blood vessels (**Figure 3A**). By contrast, in layer II, both multipolar 5-HT<sub>3A</sub>:GFP<sup>+</sup> neurons (**Figure 3B**) and bipolar

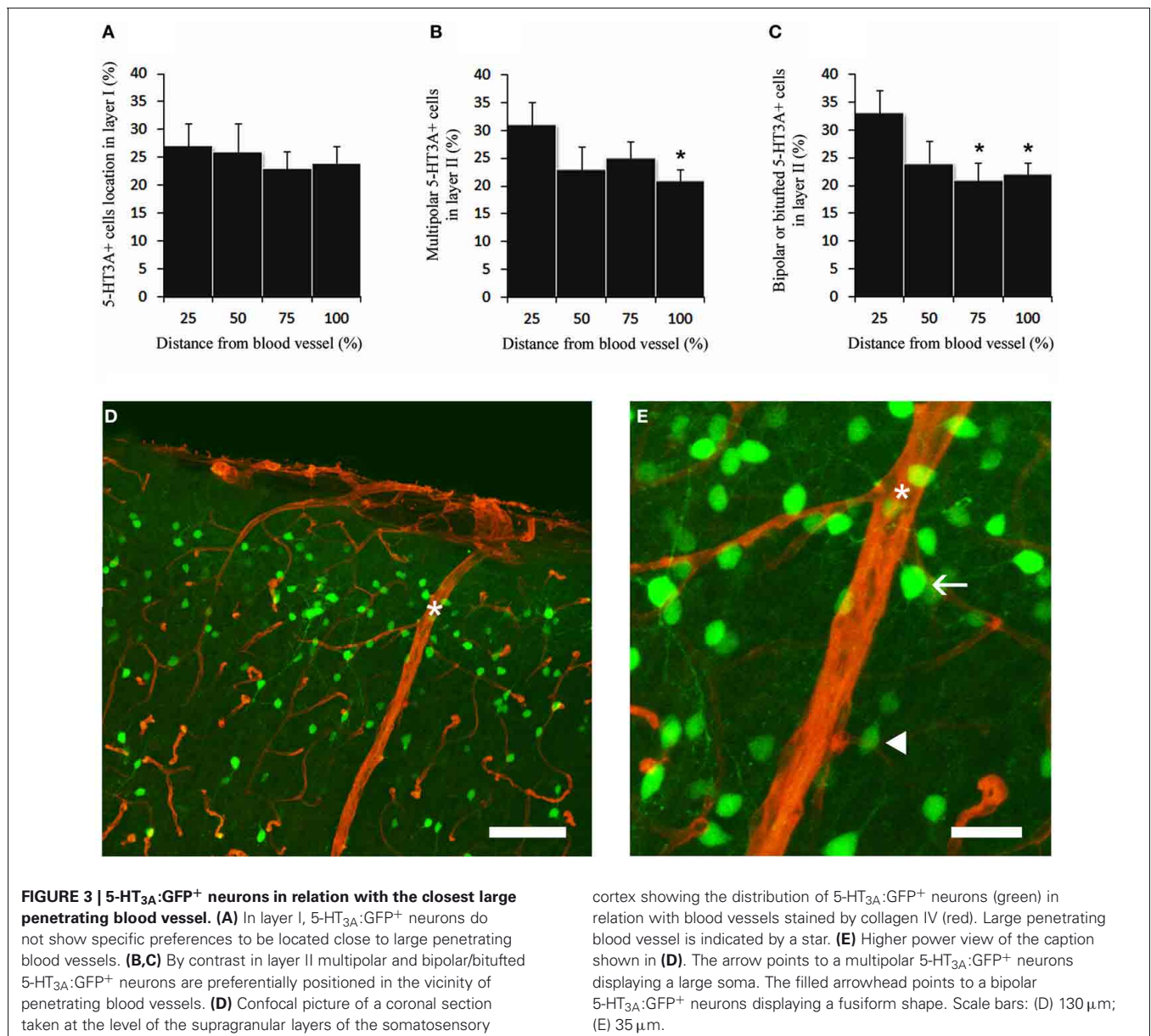


5-HT<sub>3A</sub>:GFP<sup>+</sup> neurons (**Figure 3C**) showed a non-uniform distribution as the densities of 5-HT<sub>3A</sub>:GFP<sup>+</sup> neurons were higher closest to the penetrating blood vessel (0–25% away from the closest blood vessel) then away from it (75–100% for multipolar 5-HT<sub>3A</sub>:GFP<sup>+</sup> neurons,  $P < 0.05$  and 50–100% for bipolar 5-HT<sub>3A</sub>:GFP<sup>+</sup> neurons,  $P < 0.01$ ).

#### PHARMACOLOGICAL STIMULATION OF 5-HT<sub>3A</sub>-EXPRESSING INTERNEURONS IN CORTICAL SLICES INDUCES VASOMOTOR MOVEMENTS RECORDED BY INFRARED VIDEOMICROSCOPY

The stimulation of GABAergic interneurons can induce vasomotricity via the production of vasoactive substances such as VIP, NPY, or nitric oxide (NO), a highly diffusible gas which is known to be a potent vasodilator (Estrada and DeFelipe, 1998; Cauli

et al., 2004; Rancillac et al., 2006). Here we investigated the functional role of 5-HT<sub>3</sub>-expressing interneurons in the control of vascular tone within the supragranular layers of the cortex. In this aim, we applied a 5-HT<sub>3</sub>R selective agonist mCPBG (100 μM) for 6 min, directly onto acute slices while recording associated blood vessel movements using infrared videomicroscopy. We focused our study on penetrating arterioles that are known to be of prime importance in feeding deeply located microvessels and neurons (Nishimura et al., 2007). Well defined arterioles of supragranular layers were therefore selected for quantitative analyses. As blood vessels in the slice preparation lack intraluminal flow and pressure (Sagher et al., 1993; Cauli et al., 2004), vasomotor changes were detected in pre-constricted arterioles using the thromboxane agonist U46619 (5 nM) throughout the experiment. When bath applied, mCPBG (100 μM) induced

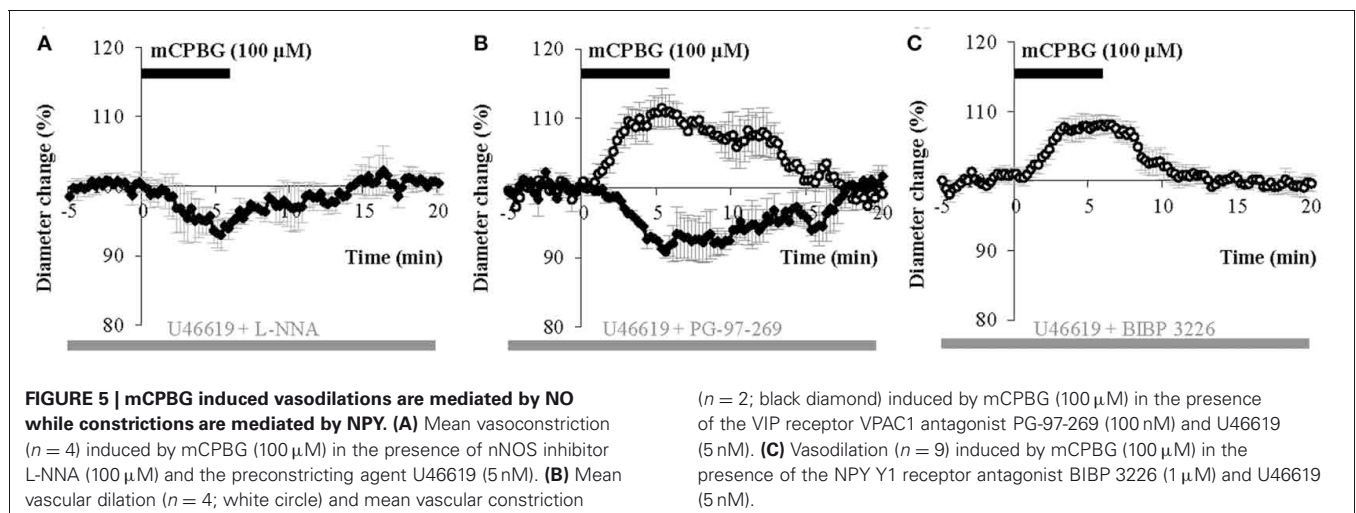
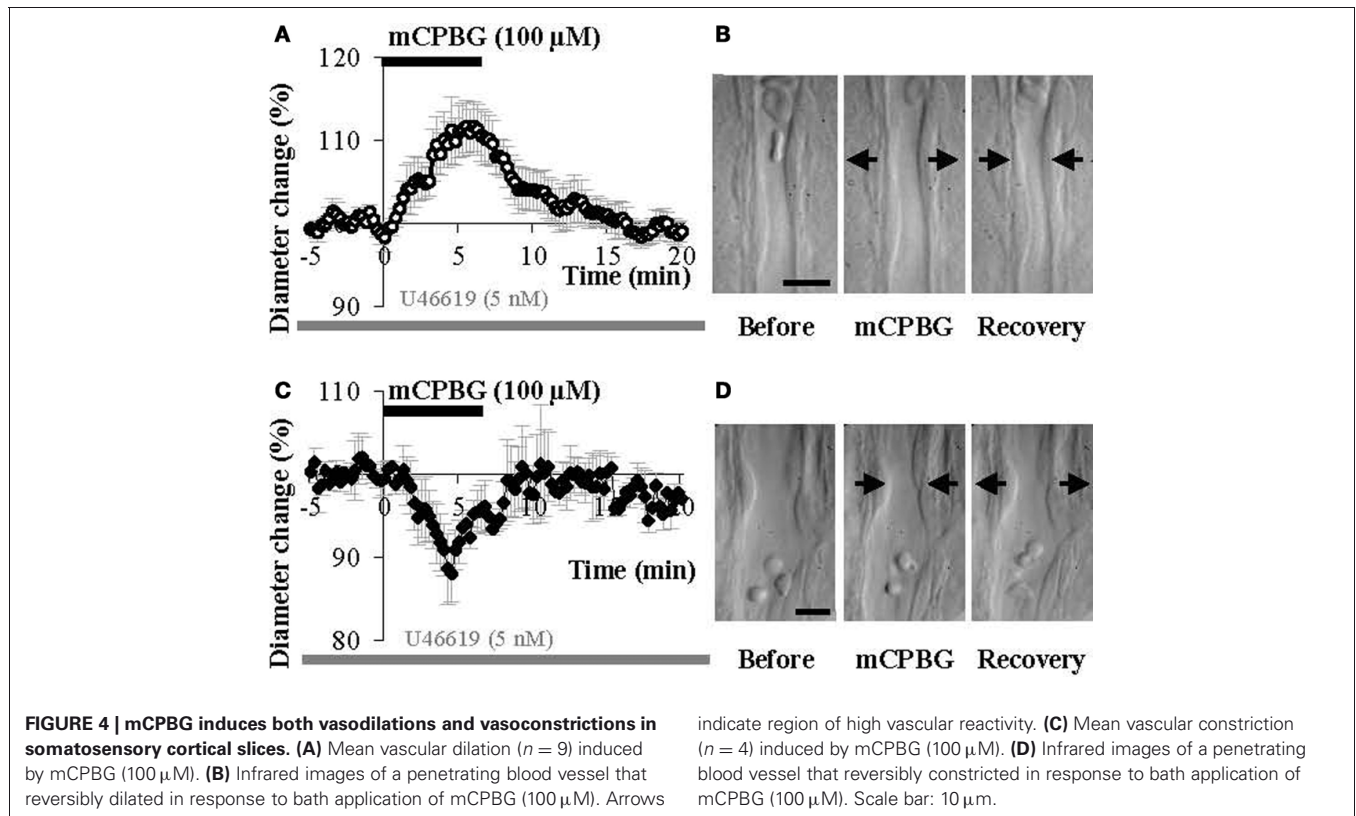


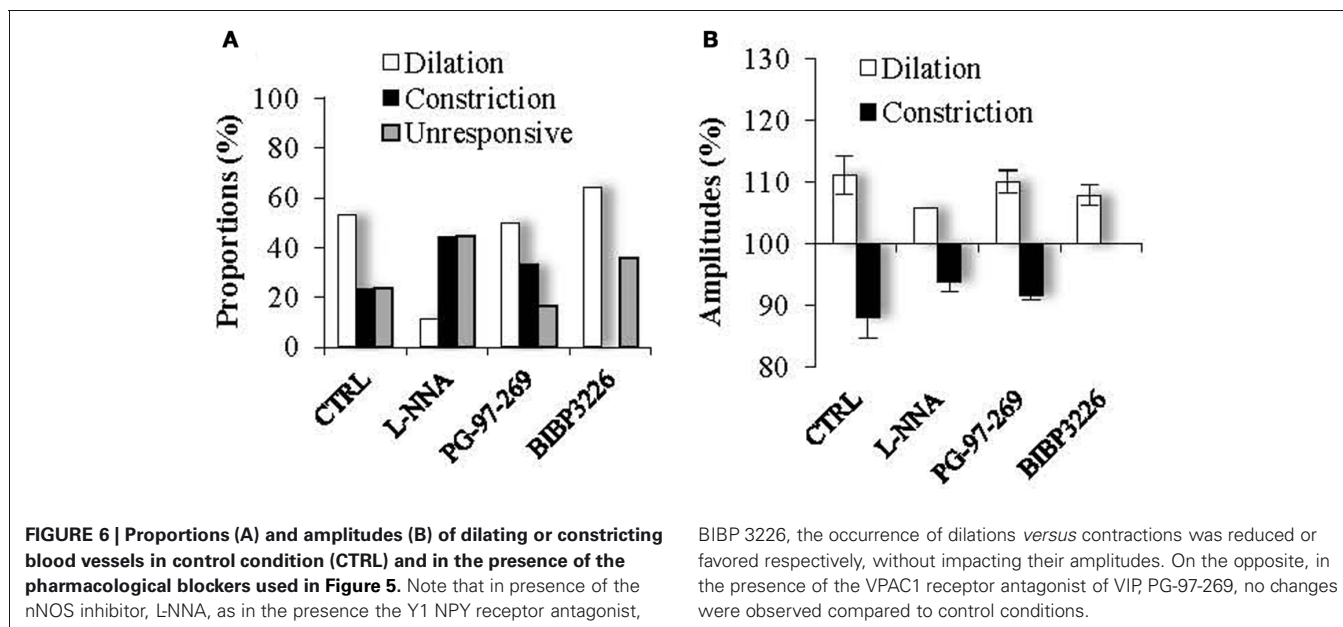
either reversible vasodilations ( $111.11 \pm 3.03\%$  over baseline,  $n = 9/17$ ;  $P < 0.01$ ; **Figures 4A and B**) or reversible vasoconstrictions ( $90.04 \pm 3.15\%$  over baseline,  $n = 4/17$ ;  $P < 0.05$ ; **Figures 4C and D**).

#### VASODILATIONS ARE MAINLY MEDIATED BY NO WHEREAS VASOCONSTRICTIONS ARE DUE TO NPY RELEASE

We hypothesized that mCPBG induced vasodilation could be due to NO and/or VIP release by 5-HT<sub>3A</sub>R-interneurons, whereas

vasoconstriction could be caused by NPY release. To determine the molecular events underlying vasomotor changes, we successively blocked different possible mechanisms. Lowering basal NO levels by treatment with the constitutive nNOS inhibitor L-NNA ( $100 \mu\text{M}$ ) strongly reduced the proportion of vasodilations observed in response to mCPBG applications from 69% in control conditions to 11% ( $n = 1/9$ ) and favored vasoconstrictions from 31% in control condition to 80% ( $93.83 \pm 1.60\%$  of baseline diameter;  $n = 4/9$ ,  $P < 0.01$ ) (**Figures 5A and 6**).

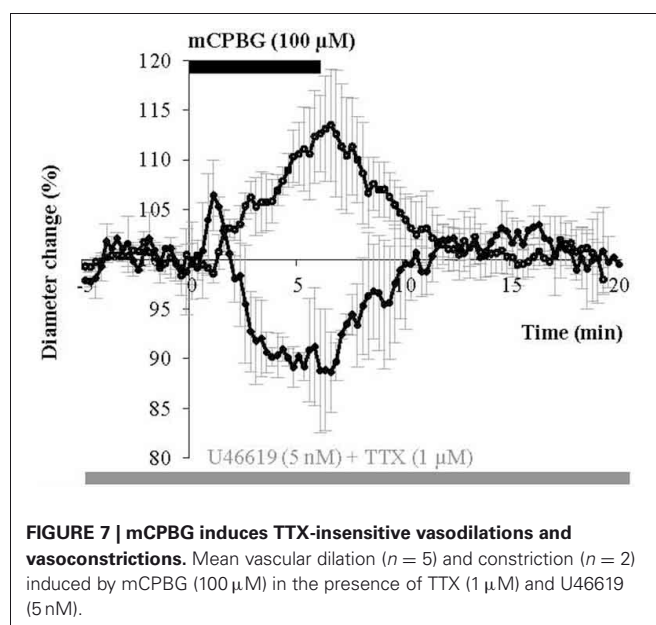




These results suggest that mCPBG-induced vasodilations are mostly mediated by NO release. The remaining vasodilation observed in the presence of L-NNA could be mediated by VIP release. However, in the presence of the VIP receptor VPAC1 antagonist, PG-97-269 (100 nM), mCPBG application induced 60% of vasodilations ( $110.01 \pm 1.87\%$ ;  $n = 3/6$ ;  $P < 0.05$ ) and 40% of vasoconstrictions ( $92.04 \pm 0.61$ ;  $n = 2/6$ ;  $P < 0.05$ ) of similar proportions and amplitudes compared to control conditions (Figures 5B and 6). Then, to determine the molecular pathway underlying vasoconstrictions, treatment of mCPBG was reproduced in the presence of NPY Y1 receptor antagonist (BIBP 3226, 1  $\mu$ M). Indeed, vasoconstrictions mediated by NPY are known to be mediated by smooth muscle NPY Y1 vascular receptor (Abounader et al., 1999). Under BIBP 3226, constrictions were blocked and only dilations ( $108.28 \pm 1.62\%$ ;  $n = 9/14$ ;  $P < 0.01$ ) could be recorded (Figures 5C and 6). Amplitudes of vasomotor responses under these different conditions were not statistically different from control condition (Figure 6B). Altogether, these data strongly suggest that pharmacological stimulations of 5-HT<sub>3A</sub>-expressing interneurons mainly induce vasodilations through NO release, whereas they induce to a less extensive vasoconstrictions through NPY release and activation of its Y1 receptor.

#### TTX-INSENSITIVE PEPTIDERGIC AND NO RELEASE

It is assumed that peptidergic release requires repetitive action potentials at high frequencies (Zupanc, 1996; Ludwig and Pittman, 2003; Baraban and Tallent, 2004) and that nNOS activation depends on Ca<sup>2+</sup> entry (Garthwaite, 2008). Therefore, we tested mCPBG stimulation in the presence of tetrodotoxin (TTX, 1  $\mu$ M) to block action potential generation and propagation. However, this treatment failed to prevent mCPBG-induced vasodilations ( $111.45 \pm 4.81\%$ ;  $n = 5/10$ ,  $P < 0.05$ ) or vasoconstrictions ( $90.04 \pm 2.1\%$ ;  $n = 2/10$ ,  $P < 0.05$ ), (Figure 7). This suggests that the vasomotor changes observed in response to

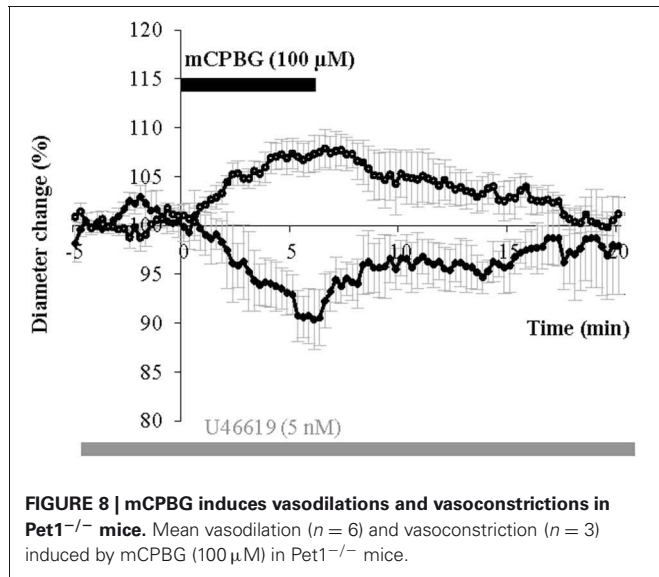


5-HT<sub>3</sub>R activation may depend on the Ca<sup>2+</sup> influx through 5-HT<sub>3</sub>R rather than on action potential generation.

#### ORIGIN OF 5-HT<sub>3</sub>R INDUCED NO RELEASE

In the neocortex the 5-HT<sub>3A</sub>R is exclusively located on GABAergic interneurons (Morales and Bloom, 1997; Ferezou et al., 2002; Chameau and van Hooft, 2006; Vucurovic et al., 2010). However, 5-HT inputs from the raphe also express presynaptic 5-HT<sub>3A</sub>Rs regulating neurotransmitter release (Jackson and Yakel, 1995; Roerig et al., 1997; Nayak et al., 1999). As the presence of NOS in 5-HT-containing axons from dorsal raphe has been already reported in the somatosensory cortex (Simpson et al., 2003) the pharmacological stimulation of 5-HT<sub>3</sub>R realized





in this study could also have induced NO release from the NOS-containing fibers originating from raphe nuclei. In order to evaluate the contribution of such a NO release in our experiments, we used *Pet1*<sup>-/-</sup> (Pheochromocytoma 12 ETS factor-1) knock-out mice, in which raphe projections to the somatosensory cortex were found to be strongly reduced (Hendricks et al., 1999; Liu et al., 2010). In this mutant mice, we found that pharmacological stimulation of 5-HT<sub>3</sub>R could still induce vasodilations ( $107.07 \pm 1.67\%$ ;  $n = 6/11$ ,  $P < 0.01$ ) or vasoconstrictions ( $93.45 \pm 2.60\%$ ;  $n = 3/11$ ,  $P < 0.05$ ), (Figure 8). These vasomotor changes were not significantly different than those induced in control condition neither in amplitude nor in proportion (from 69 to 67% for vasodilations and from 31 to 33% for vasoconstrictions), suggesting a postsynaptic origin of NO release.

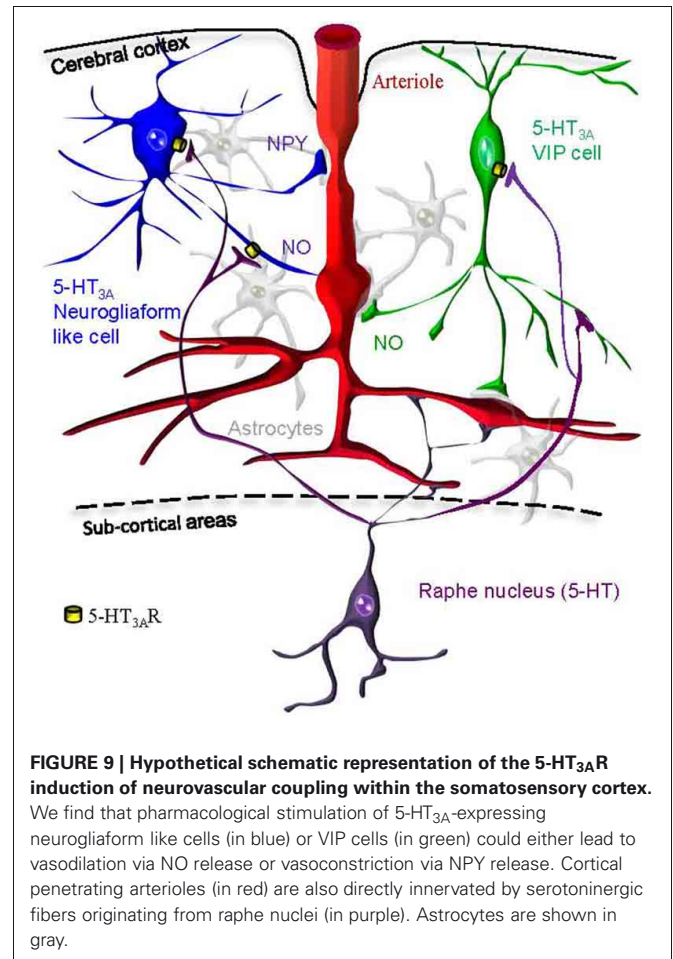
## DISCUSSION

5-HT<sub>3A</sub>-expressing interneurons are composed of two different populations: neurogliaform like interneurons expressing NPY and bipolar/bitufted interneurons expressing VIP that are likely candidates to control cortical blood vessels tonus via neuronally derived vasoactive messengers. In this article we show that in supragranular layers of the mouse somatosensory cortex, pharmacological activation of 5-HT<sub>3</sub>-expressing interneurons can release of NO to dilate, or NPY to constrict arterioles (Figure 9).

### 5-HT<sub>3A</sub>R ACTIVATION INDUCES VASCULAR RESPONSES THROUGH THE ACTIVATION OF SPECIFIC GABAergic INTERNEURONS SUBPOPULATIONS

The mechanisms underlying the vasoactive action of serotonin in the cerebral cortex are not completely understood at present time. Our results demonstrate that the selective activation of the ionotropic 5-HT receptor, 5-HT<sub>3</sub>R, induces a complex vascular response including both local vasoconstrictions and vasodilations.

These vasomotor changes are not mediated by the presynaptic 5-HT<sub>3</sub>R activation of serotonergic axons originating from



raphe nuclei that could potentially release vasoactive substances. Indeed, they were also observed in *Pet1*<sup>-/-</sup> knock-out mice, which display a drastic depletion of cortical serotonergic fibers (Hendricks et al., 1999; Liu et al., 2010; Kiyasova et al., 2011). Conversely, our hypothesis is that a specific activation of local 5-HT<sub>3A</sub>-expressing GABAergic interneurons is responsible for the observed vasomotor responses.

Indeed, it has been reported that 5-HT<sub>3A</sub> expression is confined to GABAergic interneurons in both rat and primate cerebral cortex (Morales and Bloom, 1997; Jakab and Goldman-Rakic, 2000; Ferezou et al., 2002). This has been recently confirmed in the mouse somatosensory cortex since neither the pyramidal cell marker *Satb2* nor the oligodendrocyte marker *Olig2* could be detected in 5-HT<sub>3A</sub>-expressing cells (Lee et al., 2010).

5-HT<sub>3A</sub>-expressing interneurons constitutes nearly 30% of the total interneuronal population (Rudy et al., 2011), which accounts for ~15% of the overall cortical neurons in rodents (Beaulieu, 1993; Gabbott et al., 1997). Using an unsupervised cluster analysis based on 28 electrophysiological parameters, we have shown in a previous study (Vucurovic et al., 2010) that 5-HT<sub>3A</sub>-expressing interneurons are segregated into two distinct subpopulations within the somatosensory cortex. The first one was characterized by the frequent expression of the NPY (87%)

and a multipolar morphology, while the second one expressed frequently the VIP (73%), and presented a bipolar somatodendritic shape. Subsequently, in an article published in 2010, Lee et al. have suggested, based on immunohistochemical analyses and electrophysiological recordings, a much broader diversity of the 5-HT<sub>3A</sub>-expressing neuronal population. Indeed, analyzing the intrinsic membrane properties and the morphology of these neurons, they distinguished seven types of 5-HT<sub>3A</sub>-expressing interneurons. Although our separation of these neurons in two subpopulations could therefore appear restrictive; we consider it is meaningful since it relies on a solid statistical classification analysis (unsupervised clustering). However, we do not exclude the existence of some diversity within these two groups.

In the present study, we analyzed the laminar distribution of these neuronal populations. We observed that the cell bodies of more than two-thirds of these interneurons were positioned within superficial layers (L1,  $13 \pm 0.9\%$ ; L2/3,  $51 \pm 1.2\%$ ) in agreement with what was recently described (Lee et al., 2010; Vucurovic et al., 2010). We further revealed that the nNOS is also expressed by a subset of 5-HT<sub>3A</sub>-expressing interneurons. These neurons corresponded to type II NOS<sup>+</sup> cells, since they were smaller than type I and weakly immunohistochemically-stained for nNOS (Yan et al., 1996; Cho et al., 2010; Perrenoud et al., 2012). Thus, the distribution and the expression of vasoactive substances within 5-HT<sub>3A</sub>-expressing interneurons put them in a unique position to induce a fast and effective modulation of vascular tone in response to 5-HT.

#### DUAL ROLE OF 5-HT<sub>3A</sub>-EXPRESSING INTERNEURONS ON VASOMOTOR CONTROL

In line with our finding that 5-HT<sub>3A</sub>-expressing interneurons produce several vasoactive substances, our results indicate that their activation induces a complex vascular response. Indeed the selective 5-HT<sub>3R</sub> agonist mCPBG induced either constrictions (30%) or dilations (70%) of penetrating arterioles within supragranular layers. All vasoconstrictions were abolished in the presence of the NPY receptor antagonist (BIBP 3226), suggesting that they were elicited by NPY release. Conversely, mCPBG-induced dilations were blocked in the presence of a nNOS inhibitor suggesting that NO is the predominant messenger inducing the vasodilation, in line previous studies performed in the cerebellum (Yang et al., 1999, 2000; Rancillac et al., 2006) and in the cortex (Estrada and DeFelipe, 1998; Lovick et al., 1999; Brown et al., 2000; Tong and Hamel, 2000; Cauli et al., 2004; Liu et al., 2008; Rancillac et al., 2012). Interestingly, a recent study on epileptic seizures has revealed that 5-HT<sub>3</sub> activation can stimulate NO synthesis (Gholipour et al., 2010).

The NPY-mediated constrictions confirm and extend prior studies implicating NPY in vasoconstrictions (Dacey et al., 1988; Abounader et al., 1995; Cauli et al., 2004) and are likely to be caused by the activation of NPY/5-HT<sub>3A</sub>-expressing neurogliaform-like cells (Lee et al., 2010; Vucurovic et al., 2010).

On the other hand, it has been shown that neurogliaform cells substantially express nNOS (Karagiannis et al., 2009; Kubota

et al., 2011; Perrenoud et al., 2012) and are thus well positioned to mediate NO-induced vasodilations. In addition, we have recently found that a significant proportion of nNOS type II cells express VIP (Perrenoud et al., 2012). Therefore, a subset of VIP/5-HT<sub>3</sub>-expressing cells could also release NO following 5-HT<sub>3A</sub>R stimulation (**Figure 9**).

5-HT<sub>3A</sub>-nNOS-expressing interneurons could also form a small third class of 5-HT<sub>3A</sub>-expressing interneurons that express neither NPY nor VIP. Although this population has not been isolated with the cluster analysis of these interneurons (Vucurovic et al., 2010), further triple immunolabeling would be necessary to assess this possibility.

Unexpectedly, although VIP is present in 5-HT<sub>3A</sub>-expressing interneurons, the mCPBG-induced dilations persisted in the presence of the VIP receptor VPAC1 antagonist, suggesting that VIP release acting on VPAC1 receptors is not involved in this response. This was surprising since the VPAC1 receptor is a postsynaptic receptor predominantly and uniformly expressed by smooth muscle cells in cerebral arteries and arterioles (Fahrenkrug et al., 2000) and that direct perfusion of VIP as well as electrical stimulation of a single VIP-expressing interneuron have been reported to dilate microvessels onto rat slices (Cauli et al., 2004). Moreover, both light and electron microscopy have revealed that some VIP<sup>+</sup> neurons are closely associated with blood vessels, providing a neuroanatomic substrate for the role of VIP in the regulation of cerebral blood circulation in the rat cerebral cortex (Eckenstein and Baughman, 1984; Chédotal et al., 1994a,b; Fahrenkrug et al., 2000).

In the presence of a nNOS inhibitor, one dilation out of nine tested blood vessels was still observed (**Figure 6A**). This dilation could have been mediated by an activation of perivascular astrocytes resulting from the evoked action potential discharge of 5-HT<sub>3</sub> interneurons. Indeed, astrocytes are known as cellular intermediaries that couple neuronal activity to local blood flow changes through the phospholipase A<sub>2</sub> (PLA<sub>2</sub>)-mediated synthesis of arachidonic acid, which leads to production of prostaglandins and epoxyeicosatrienoic acids (Petzold and Murthy, 2011). However, Kitaura et al. (2007) have shown that hindpaw stimulation-induced cortical vasodilations were suppressed in mice lacking nNOS, while they were unchanged in mice lacking cytosolic phospholipase A<sub>2</sub> alpha (cPLA<sub>2</sub>α).

Together, our observations that in the somatosensory cortex a selective 5-HT<sub>3R</sub> agonist induce NO mediated vasodilations confirms and extends prior studies reinforcing the central role of NO in the neurovascular coupling.

#### TTX-INSENSITIVE VASOMOTOR CHANGES

The cellular mechanisms underlying the release of vasoactive compounds within the cortex are still poorly considered. Here we observed that the vascular responses induced by 5-HT<sub>3A</sub>R activation were unaffected by the bath application of TTX. This result indicates that the release of NO, NPY, and VIP by 5-HT<sub>3</sub>-expressing interneurons is independent of subthreshold activity, raising the question of the mode of secretion of these molecules in the neutrophil.

Studies of hypothalamic neuroendocrine cells indicate that neuropeptides are confined to large dense-core vesicles and

released from dendrites and axons (Ludwig and Pittman, 2003) following high intracellular Ca<sup>2+</sup> concentrations (Kits and Mansvelder, 2000). Action potentials originating at the neuronal soma trigger neuropeptide release from terminals, whereas Ca<sup>2+</sup> release from intracellular stores signals dendritic peptide release (Ludwig et al., 2002). Similarly, the nNOS enzyme, which immunoreactivity spreads throughout the cytoplasm of neuronal cell bodies and their processes (Batista et al., 2001), is well known to be activated by Ca<sup>2+</sup> associated with calmodulin (Bredt et al., 1991).

Therefore, if these mechanisms are conserved within the cortex, NPY and NO release should also be dependent on high intracellular Ca<sup>2+</sup> concentrations influx. Since 5-HT<sub>3A</sub>Rs are predominantly expressed at the somatodendritic level in the cortex (Morales et al., 1996; Jakab and Goldman-Rakic, 2000), our study indicates that the application of mCPBG is likely to evoke enough intracellular Ca<sup>2+</sup> influx through 5-HT<sub>3A</sub>R to induce Ca<sup>2+</sup> release from intracellular stores and trigger NPY and NO release.

Several lines of evidence support this hypothesis. In heterologous expression systems, the Ca<sup>2+</sup> permeability of the 5-HT<sub>3</sub>R has been reported to be enhanced by co-assembly of the 5-HT<sub>3A</sub> subunit with the  $\alpha$ -4 subunit of the nicotinic acetylcholine receptor (van Hooft et al., 1998; Sudweeks et al., 2002). The existence of functional heteromeric 5-HT<sub>3A</sub>/nACh $\alpha$ 4 receptors has been described in CA1 hippocampal interneurons (Sudweeks et al., 2002). Lee et al. (2010), recently reported that in the mouse somatosensory cortex, like in the rat somatosensory cortex (Ferezou et al., 2002), 5-HT<sub>3A</sub>-expressing neurons respond to both 5-HT<sub>3</sub>R and nAChR agonists. Hence, heteromeric 5-HT<sub>3</sub>/nACh $\alpha$ 4 receptors are probably present on the membrane of cortical 5-HT<sub>3A</sub>-expressing interneurons and potentiate the Ca<sup>2+</sup> influx evoked by the mCPBG. Altogether, these results suggests that NPY and NO release were not triggered by action potential generation, but rather by direct Ca<sup>2+</sup> entry through homomeric or heteromeric 5-HT<sub>3A</sub>R channels in response to mCPBG application, supporting the fact that TTX blocked neither NO nor neuropeptides induced vasomotor responses.

#### IMPACT OF 5-HT<sub>3A</sub>-EXPRESSING INTERNEURONS ACTIVATION ON THE CORTICAL NETWORK

*In vitro* electrophysiological studies have shown that ionotropic serotonergic receptor agonists induce a fast excitation of 5-HT<sub>3A</sub>-expressing interneurons through the activation of post-synaptic somatodendritic receptors (Porter et al., 1999; Zhou

and Hablitz, 1999; Ferezou et al., 2002; Lee et al., 2010). This activation is likely to have inhibitory impact on the cortical network. Indeed, because supragranular interneurons innervate pyramidal cells, their activation will induce inhibitory post-synaptic currents in pyramidal neurons and thus inhibits their firing activity (Zhou and Hablitz, 1999; Moreau et al., 2010).

Nonetheless, the physiological release of 5-HT by the fibers originating from the raphe nuclei activate not only 5-HT<sub>3A</sub>Rs, but also metabotropic serotonin receptors subtypes that are divided into six classes (5-HT<sub>1</sub>R, 5-HT<sub>2</sub>R, and 5-HT<sub>4</sub>R–5-HT<sub>7</sub>R) (Hoyer et al., 1994; Walstab et al., 2010; Pytliak et al., 2011). The consequences of intracerebrally released 5-HT, both on the cortical network activity and on the CBF will be based on its various actions through different 5-HTRs (Cohen et al., 1996; Andrade, 2011). Indeed, 5-HT has been reported to have both inhibitory and excitatory effects on the cortical network (Krnjevic and Phillis, 1963; Reader, 1978; Waterhouse et al., 1990; Zhou and Hablitz, 1999; Puig et al., 2005), while it has a negative impact on the CBF in the neocortex through a major vasoconstrictor role of 5-HT through 5-HT<sub>1B</sub> receptors (Cohen et al., 1996; Riad et al., 1998).

#### CONCLUSION

Altogether, this study indicates that 5-HT<sub>3A</sub>-expressing interneurons occupy a strategic position in superficial layers to convey fast effects of serotonergic modulation, thus modulating cortical network activity and blood supply. The brainstem 5-HT pathway, in addition to its direct projections and vasomotor effects on cortical blood vessels, can use 5-HT<sub>3A</sub>-expressing interneurons to control and adapt CBF. These results suggest that blood flow could be enhanced prior to the onset of any metabolic deficit, reinforcing the “neurogenic” hypothesis of the neurovascular coupling versus the “metabolic” one (Rossier, 2009).

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