

**CEREBROVASCULAR EFFECTS OF PITUITARY ADENYLATE CYCLASE  
ACTIVATING POLYPEPTIDE AND VASOACTIVE INTESTINAL PEPTIDE  
IN NEWBORN PIGS**

By

LAURA LENTI

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**LIST OF ABBREVIATIONS**

Ach: acetylcholine  
aCSF: artificial cerebrospinal fluid  
AMPA: alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate  
ANOVA: analysis of variance  
BBB: blood-brain barrier  
cAMP: 3'-5'-cyclic adenosine monophosphate  
ATP: adenosine-5'-triphosphate  
CoBF: cortical blood flow  
COX: cyclooxygenase  
CR: cerebrovascular reactivity  
CSD: cortical spreading depression  
CSF: cerebrospinal fluid  
DC: direct current  
ICP: intracranial pressure  
I/R: ischemia/reperfusion  
K<sub>ATP</sub> channel: ATP-sensitive potassium channel  
K<sub>Ca</sub> channel: calcium-activated potassium channel  
L-NAME: N- $\omega$ -nitro-L-arginine methyl ester  
NMDA: N-methyl-D-aspartate  
NO: nitric oxide  
NOS: nitric oxide synthase  
nNOS: neuronal NO synthase  
PACAP: pituitary adenylate cyclase activating polypeptide  
PLA<sub>2</sub>: phospholipase A<sub>2</sub>  
PU: perfusion unit  
ROS: reactive oxygen species  
SEM: standard error of the mean  
VIP: vasoactive intestinal peptide  
VSM: vascular smooth muscle

## Összefoglalás

A pituiter adenilát cikláz aktiváló polipeptid (PACAP) és a vazóaktív intesztinális peptid (VIP) az emlős szervezet széles, egymással részben átfedő hatásspektrummal rendelkező hírvivő molekulái. Potens értágítók, neuroprotektív hatásuk pedig az agyi infarktus számos modelljében bizonyított. Az agyi iszkémia/reperfúzió (I/R) során endogén neuroprotektív mediátorként PACAP és VIP is felszabadulhat a neurovaszkuláris egység elemeiből. Ezeknek a peptideknek az agyi keringésszabályozásban illetve az agyi iszkémia patofiziológiájában betöltött pontos szerepe azonban egyelőre még nem tisztázott.

Elsődleges célunk az volt, hogy megvizsgáljuk a két PACAP izoformának (PACAP38 és PACAP27), illetve a VIP-nek az újszülött agy vérkeringésére gyakorolt hatását. Munkánkban a pia mater arterioláinak reakcióit elemeztük különböző enzimbénítók, illetve peptid PACAP/VIP-receptor antagonisták segítségével. Kísérleteink második részében arra kerestünk választ, vajon PACAP illetve VIP előkezeléssel mérsékelhetők-e az I/R által súlyosan károsított agyi érreakciók. Az endotélium funkcionális károsodását a hiperkapniára adott, a neuronális–vaszkuláris csatolás zavarát a glutamát-receptor agonista N-metil-D-aszpartáttal (NMDA) kiváltott vazodilatáció csökkenésével jellemeztük.

Kísérleteinket altatott, lélegeztetett újszülött malacokon végeztük. Az érátmérők változásait a zárt koponyaablak/intravitális mikroszkópia módszer segítségével határoztuk meg. A PACAP izoformák és a VIP dóziszfüggő, reprodukálható, nitrogén monoxid independens piális arteriola dilatációt váltottak ki. A PACAP38 és a kis dózisban alkalmazott VIP vazodilatátor hatásában a cikooxigenáz(COX)-1 aktivitása játszott fontos szerepet. A vizsgált peptid antagonisták közül a PACAP6–38 kivédte mindhárom vizsgált peptid értágító hatását, míg a PACAP6–27 csak a két PACAP izoformával szemben bizonyult hatásosnak. A hiperkapnia által indukált vazodilatáció károsodását I/R után mind a PACAP, mind a VIP előkezelés megakadályozta. Ezzel szemben csak a PACAP izoformák jelentettek védelmet az NMDA által kiváltott értágulat I/R-t követő károsodásával szemben.

Összefoglalva, a vizsgált neuropeptidok csak részben átfedő vaszkuláris hatásait egy COX-1 aktivitástól függő és egy attól független mechanizmus mediálhatja. A PACAP izoformák a VIP-hez képest a neurovaszkuláris egység több elemére fejtenek ki védő hatást I/R során, amely magyarázhatja más modellekben megfigyelt nagyobb neuroprotektív potenciáljukat. A PACAP és a VIP általunk tapasztalt tulajdonságai új, neuroprotektív stratégiák alapját képezhetik az újszülöttkori agyi vaszkuláris történések esetében is.

## Summary

Pituitary adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) share a wide range of biological activities in mammals. These peptide transmitters are potent vasodilators, and were found neuroprotective in numerous models of cerebral ischemia. PACAP and VIP may also be released from the various elements of the neurovascular unit during ischemia so as to reduce the extent of neuronal injury. However, the mechanisms of the neurovascular actions of these neuropeptides are poorly known, especially in newborns.

Our first goal was to evaluate the effects of PACAP38, PACAP27, and VIP on pial arterioles, and to characterize these responses using various enzyme inhibitors and PACAP/VIP-receptor antagonist PACAP fragments. In the second set of our experiments, we sought to test if PACAP or VIP pretreatments preserve endothelial and/or neuronal function-dependent I/R-sensitive vascular responsiveness such as hypercapnia- or N-methyl-D-aspartate (NMDA)-induced vasodilation.

Anesthetized, ventilated piglets were equipped with closed cranial windows. Pial arteriolar responses were evaluated via intravital microscopy. PACAP isoforms and VIP evoked dose-dependent, reproducible, nitric oxide independent vasodilation. PACAP38 and low dose VIP-evoked vascular responses were attenuated by cyclooxygenase (COX)-1 inhibition. PACAP6–38 inhibited vasodilation induced by either PACAP38, PACAP27, or VIP. In contrast, PACAP6-27 altered only PACAP38 and PACAP27 but not VIP-induced vasodilation. PACAP38, PACAP27, and VIP all prevented the attenuation of hypercapnia-induced vasodilation caused by I/R; however, PACAP isoforms, but not VIP preserved NMDA-dependent vascular reactivity.

In summary, PACAP38, PACAP27, and VIP are potent vasodilators in the neonatal cerebral circulation with at least two distinct mechanisms of action: a COX-dependent and a COX-independent pathway. After I/R, PACAP isoforms preserve more functions of the neurovascular unit than VIP possibly explaining the more pronounced neuroprotection afforded by PACAP. Our present findings may form the foundation of new neuroprotective therapies in the treatment of neonatal stroke.



## **Introduction**

Stroke is a rapidly developing loss of brain functions due to disturbance in the blood supply to the brain caused either by a hypoxic-ischemic or a hemorrhagic insult. Stroke is known to be one of the leading causes of adult disability and death; however, stroke also affects millions of human babies, especially premature infants, every year worldwide (Zanelli et al. 2008; Lynch 2009). Neonatal stroke may result in death, but more often, in severe, life-long neurological dysfunction that has a considerable impact on both the health care system and the families taking care of the disabled children. Therefore, development of reasonable and effective therapies to reduce neural damage following stroke is of great importance. However, to develop new treatment strategies, a better understanding of stroke related pathophysiology is required.

According to our present knowledge the piglet is the best model for invasively examining the central nervous system of the newborn. At birth, concerning the body size, the developmental stage of the brain, the basic hemodynamic parameters, and cerebrovascular responses, piglets are very similar to human babies (Book and Bustad 1974; DeRoth and Downie 1978; Busija 1994). Therefore, our laboratory chose this model to examine the central nervous system and cerebral circulation, in order to have a broader insight into the stroke pathology of newborns.

Cerebral ischemia/reperfusion (I/R) induces not only neuronal injury, but also, I/R impacts more or less all components of the so-called neurovascular unit. The concept of the neurovascular unit/neurovascular coupling comprises the functional and morphological interactions between neurons, astrocytes, and all cerebrovascular cell types that provide adequate local cerebral blood flow to match tissue metabolic needs (Roy and Sherrington 1890; Girouard and Iadecola 2006; Drake and Iadecola 2007). One of the major manifestations of postischemic neurovascular unit dysfunction is impaired cerebrovascular reactivity (CR) (Leffler et al. 1989b; Busija et al. 1996a; Ben-Haim and Armstead 2000; Kulik et al. 2008). Altered CR appears to play a significant role in stroke pathophysiology since it results in inadequate cerebral blood flow regulation following the original stress and worsens metabolic challenges of the already compromised tissue that may significantly aggravate the brain injury setting the stage for secondary insults (Kogure et al. 1980; Hossmann 1997). Thus, preservation of CR is a promising area of research to develop treatments that may accompany other neuroprotective therapies. Neuroprotective agents such

as pituitary adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) may be released from nerve endings and decrease the extent of ischemic injury (Reglodi et al. 2000; Reglodi et al. 2002; Brenneman 2007; Stumm et al. 2007). Among several putative mechanisms, preservation of normal vascular reactions may be an important contributor to the *in vivo* neuroprotective potential of these peptide transmitters.

#### *PACAP and VIP: structure, distribution, and receptors*

PACAP and VIP are structurally related endogenous peptide members of the VIP/glucagon/secretin superfamily (Sherwood et al. 2000; Edvinsson and Krause 2002). They are important intercellular messengers in both the central and peripheral nervous systems and in a variety of non-neural tissues (Fahrenkrug 1993; Vaudry et al. 2000b). PACAP was originally isolated from the ovine hypothalamus and was described as a factor potently stimulating adenylate cyclase activity in rat pituitary cell culture (Miyata et al. 1989). PACAP has two naturally occurring isoforms; PACAP38, a basic, amidated 38-residue peptide and PACAP27, the N-terminal portion consisting of 27 amino acids (Miyata et al. 1989; Miyata et al. 1990). PACAP38 is the more abundant form that comprises 90% of PACAP in the body (Arimura et al. 1991; Ghatei et al. 1993; Palkovits et al. 1995). VIP was originally isolated from porcine duodenum and characterized as a factor inducing dilation of peripheral vessels (Said and Mutt 1970). VIP contains 28 amino acids and shows 67% sequence-homology with PACAP27 (Mutt and Said 1974; Miyata et al. 1990). The primary structures of PACAP and VIP have been remarkably conserved throughout evolution. The amino acid sequence of PACAP is identical in all mammalian species investigated so far; and furthermore, the sequence of the first 27 amino acid residues necessary for the peptide's biological activity is identical in humans, amphibians, and fish. The primary structure of VIP is also identical in most mammalian species (Sherwood et al. 2000). This extremely preserved structure of PACAP and VIP throughout phylogenesis may indicate the vital importance of these peptides in the living organisms.

PACAP and VIP show widespread and similar tissue-organ distribution and pleiotropic functions. They share at least partially the same receptors, and thus a rich spectrum of biological activities (Harmar et al. 1998; Vaudry et al. 2000b; Laburthe and Couvineau 2002; Laburthe et al. 2002). Among many examples, PACAP and VIP are widely expressed throughout the central and peripheral nervous system, where they act as hypothalamic hormones, neurotransmitters, neuromodulators, and neurotrophic factors

(Vaudry et al. 1999; Vaudry et al. 2000a; Edvinsson and Krause 2002). PACAP and VIP have been identified as regulators of hormone secretion in the pituitary gland (Arimura and Shioda 1995). They have also been detected in other endocrine organs like the adrenal glands and the endocrine pancreas (Vaudry et al. 2000b). PACAP and VIP were discovered in various cell types of the immune system as factors affecting the immune responses (Vaudry et al. 2000b). In the central nervous system, beside their obvious role in neurodevelopment, these neuropeptides are also involved in the homeostatic control of circadian clock and behavioral actions, learning and memory processes (Zhou et al. 2002; Harmar 2003). They are present in nerve endings of the respiratory system, gastrointestinal, and urogenital tracts, where PACAP and VIP are potent relaxants of the smooth musculature (Vaudry et al. 2000b). They also relax smooth muscle cells in the wall of arteries and veins throughout the body, causing vasodilation in different vascular beds (Edvinsson and Ekman 1984; Warren et al. 1992; Henning and Sawmiller 2001; Edvinsson and Krause 2002).

Three main VIP/PACAP receptor types have been identified so far (Harmar et al. 1998). Generally, these receptors belong to the family of G-protein-coupled receptors with seven transmembrane domains and they are known as strong activators of adenylate cyclase. On the other hand, accumulated evidence suggests that they activate a number of additional intracellular signals (Vaudry et al. 2000b; Dickson and Finlayson 2009). Some of these are likely to be downstream of the conventional adenylate cyclase pathway, while others reflect other primary coupling events of the receptors. PAC<sub>1</sub> receptors are more selective to either PACAP<sub>38</sub>, or PACAP<sub>27</sub>, than to VIP, and exist in at least eight splice variants. Some of the splice variants are linked to phosphatidyl inositol turnover and calcium mobilization. VPAC<sub>1</sub> and VPAC<sub>2</sub> receptors exhibit almost equal affinity for PACAP and VIP. However, VPAC<sub>1</sub> receptors have low, while VPAC<sub>2</sub> receptors show high affinity for helodermin, another member of the same peptide family. According to the structural similarities and the partially common receptors of PACAP and VIP, it is reasonable to presume that they exert their actions by activating at least partially overlapping mechanisms. However, based on the literature, it is already evident that these two peptides are not completely identical in their action and each of them activates multiple pathways (McCulloch et al. 2002).

#### *Vasodilation induced by PACAP and VIP*

Considerable data suggest that vasoactive peptides, like PACAP and VIP, are involved in cerebrovascular control mechanisms under physiologic conditions in newborn

and adult animal models as well as in humans (Wei et al. 1980; Edvinsson and Ekman 1984; Tong et al. 1993; Anzai et al. 1995; Gulbenkian et al. 2001). These mediators, among others, probably also play a role in the neurovascular coupling via connections between peptidergic intracortical neurons and blood vessels that may provide a mechanism by which PACAP and VIP participate directly in linking neuronal activity to cerebral blood flow (Benagiano et al. 1996; Edvinsson and Krause 2002; Kulik et al. 2008). On the other hand, glial cells may also possibly convey information from neurons to blood vessels (Girouard and Iadecola 2006). Interestingly, other peptides from the same family with structural similarities (e.g. secretin, glucagon) have no such vasoactive effects in the central nervous system (Edvinsson and Krause 2002).

PACAP and VIP have been detected in nerve endings innervating intracerebral as well as pial vessels (Fahrenkrug et al. 2000; Gulbenkian et al. 2001). PACAP-immunoreactive extrinsic perivascular nerve fibers are either of parasympathetic or trigeminal sensory origin, whereas VIP immunoreactive fibers appear to be exclusively parasympathetic originating from the sphenopalatine and otic ganglia virtually in all species studied including humans (Tajti et al. 1999; Uddman et al. 1999; Edvinsson and Krause 2002). Furthermore, PACAP- and VIP-immunoreactive intrinsic cortical neurons and afferents from the brainstem appear to regulate blood vessel diameters of both intraparenchymal and pial arterioles (Morrison et al. 1984; Benagiano et al. 1996; Tajti et al. 2001; Edvinsson and Krause 2002; Hannibal 2002; Drake and Iadecola 2007). Although recent studies revealed the complexity of perivascular peptidergic innervation, the mechanisms by which neuropeptides participate in regulation of the vessel caliber, the neuronal excitability, and the cerebral metabolism remains unclear.

PACAP and VIP are thought to exert a significant direct effect on the vascular smooth muscle (VSM) of pial and intracerebral resistance vessels. Activation of PACAP/VIP receptors increases intracellular cAMP production that results in cerebrovascular dilation (Edvinsson and Krause 2002). On the other hand, many observations support the idea that other, supplementary pathways play roles in the circulatory effects of PACAP and VIP with the involvement of other cell types of the neurovascular unit. The activation of these indirect processes may result in additional vasodilation.

The release of nitric oxide (NO) from the perivascular nerve-endings is one of the most important neuron-derived effector mechanisms in the regulation of vasodilatory responses of cerebral arteries. Immunohistochemical studies indicate that PACAP and VIP are co-localized with neuronal NO synthase (nNOS) in the perivascular nerves, and in the

parasympathetic ganglia. PACAP also coexist with nNOS in the trigeminal ganglion (Tajti et al. 1999; Uddman et al. 1999; Edvinsson et al. 2001). In vitro experiments suggest that there is not only an anatomical but also a functional connection between the vascular action of PACAP and the release of NO (Sano et al. 2000; Seebeck et al. 2002). VIP-mediated relaxation depends on NO production in isolated bovine, ovine, and rat cerebral arteries (Gaw et al. 1991; Gonzalez et al. 1997; Seebeck et al. 2002). The degree of inhibition of VIP-induced cerebrovascular dilation by NOS blockers, however; is not consistent among studies, and it is not always observed (Edvinsson and Krause 2002). Research studies have not yet clarified if NO participates in mediation of the cerebrovascular effects elicited by PACAP and VIP in newborns.

Accumulating data indicate that cyclooxygenase (COX)-derived arachidonic acid metabolites are major contributors to the regulation of cerebral circulation in the newborn (Peri et al. 1995; Busija et al. 1996b; Degi et al. 1998a; Degi et al. 1998b). COX-1 and COX-2 enzymes are both expressed in the CNS; neurons, glial cells, vascular smooth muscle cells, and the vascular endothelium all produce some kind of prostanoids. Outstandingly, all of the numerous formerly assessed COX-dependent vascular reactions; such as hypotension induced vasodilation, and acetylcholine (Ach) induced vasoconstriction, appeared to depend largely on COX-2 activity (Domoki et al. 2005b) in the newborn pig. Although some preceding studies revealed interaction between the prostanoid synthesis and PACAP action (Kis et al. 1999a; Kis et al. 1999b), our understanding of the possible role of the COX derived metabolites in the vascular effects of the PACAP in piglets is limited. VIP dilates the isolated porcine ophthalmic artery via COX-mediated mechanisms at lower ( $10^{-10}$ - $10^{-9}$  M) VIP concentrations (Vincent 1992); but its action is COX-independent when higher ( $10^{-8}$ - $10^{-7}$  M) doses are applied. VIP-induced pial arteriolar vasodilation is also indomethacin-sensitive in cats (Wei et al. 1980). However, according to our knowledge, VIP-induced vasodilation has not yet been characterized in newborns.

#### *Neuroprotection induced by PACAP and VIP*

Neuroprotection is one among the many functions shared by PACAP and VIP. Antioxidant, anti-apoptotic, neurotrophic, and anti-inflammatory effects have been promoted as putative mechanisms of neuroprotection in numerous *in vitro* and *in vivo* experimental models (Dejda et al. 2005; Brenneman 2007). Additionally, PACAP and VIP-induced

neuroprotection *in vivo* can be, at least partially, based on their cerebrovascular effects (Wei et al. 1980; Tong et al. 1993; Anzai et al. 1995).

PACAP and VIP were demonstrated to protect cultured cerebellar granule cells from naturally occurring apoptosis, neurotoxic effects of ethanol, and hydrogen peroxide-induced oxidative stress. PACAP also protected cerebellar granule cells against C2 ceramide which is a critical mediator of cellular stress response and an important factor involved in apoptosis. PACAP and VIP protected neurons from cytotoxic effects of  $\beta$ -amyloid, aggregation of which is thought to be a key element of Alzheimer's disease, and glycoprotein 120, the envelope protein of the human immunodeficiency virus. Moreover, PACAP showed neuroprotective properties against the toxic effects of glutamate, and human prion protein fragment 106-126. The receptor(s) mediating PACAP/VIP-induced neuroprotection are still under investigation. The neuroprotective effect can be direct, influencing different steps of apoptotic cascade mechanisms within the affected neurons; or indirect, via release of glia-derived trophic substances associated with the protection of developing neurons. On the other hand, PACAP and VIP inhibit the release of proinflammatory mediators which may contribute to neuronal death (Dejda et al. 2005; Brenneman 2007).

These *in vitro* observations were supported by results from *in vivo* experiments. It has been observed that PACAP effectively protected the dopaminergic nigrostriatal neurons from apoptotic death in a model of Parkinson's disease (Reglodi et al. 2004b). PACAP also attenuated neuronal and axonal injuries evoked by brain or spinal cord trauma (Farkas et al. 2004; Chen and Tzeng 2005; Tamas et al. 2006; Kovesdi et al. 2008). In rats, intracerebroventricular administration of PACAP38 before the animals underwent permanent middle cerebral artery occlusion; significantly reduced the infarct size measured 12 and 24 h after the onset of the ischemia (Reglodi et al. 2002). Similarly, in the rat model of transient focal ischemia produced by middle cerebral artery occlusion, PACAP administered intravenously for 48 h beginning 4 h after the insult, significantly reduced the infarct size (Reglodi et al. 2000). The results imply that PACAP might be a promising therapeutic agent in reducing ischemic brain damage even when its administration is delayed (Uchida et al. 1996; Reglodi et al. 2000).

Ideally, any compound for the treatment of stroke should sufficiently cross the blood-brain barrier (BBB) and reach therapeutic levels within the target, injured brain area. The BBB is not a rigid, static barrier but rather a dynamic interface between the circulating blood and the central nervous system (Zlokovic 2008). PACAP27 and VIP are transported into the brain by transmembrane diffusion, a non-saturable mechanism. However, transport of

PACAP38 across BBB by peptide transport system-6 underlies its effectiveness after intravenous administration (Banks et al. 1996). Cerebral ischemia alters the function of the BBB, therefore, in this case, the entry of different neuroprotective substances into the brain depends on the extent of BBB disruption and the physicochemical properties of the substance examined. For example, the passage of PACAP38 was found to selectively increase after unilateral middle cerebral artery occlusion in rats, independently of the generally experienced permeability changes (Somogyvari-Vigh et al. 2000).

Although PACAP and VIP display neuroprotective properties against a wide range of pathological conditions, PACAP is generally more potent than VIP and its function has been more widely investigated (Tamas et al. 2002; Brenneman 2007). Of course, there are exceptions from this generalization. Former studies revealed the unique role for VIP in neuroprotection from neurotoxicity in white matter (Gressens 1999). Thus, there may be a more VIP-specific subtype of PACAP/VIP receptors yet to be described, that could account for such neuroprotective activities.

PACAP and VIP are among of the most promising candidates of endogenous neuroprotective mediators that may also be released from these neuronal-vascular elements during I/R that could abrogate/reduce neuronal injury (Brenneman 2007; Stumm et al. 2007). Cerebrospinal fluid (CSF) PACAP concentration has been determined to be ~2 ng/ml under resting conditions in piglets (Wilderman and Armstead 1997), and its level has been shown to elevate in response to different neural injuries (Somogyvari-Vigh et al. 2000; Stumm et al. 2007). Moreover, it has also been demonstrated that PAC<sub>1</sub> receptor expression increases in pathological states like ischemic insults (Gillardon et al. 1998).

In the piglet, impaired CR in the pial circulation to different stimuli depending on different elements of the neurovascular unit was demonstrated after I/R, asphyxia, or hypoxic stress (Leffler et al. 1989a; Leffler et al. 1989b; Busija and Wei 1993; Bari et al. 1996a; Bari et al. 1996c; Busija et al. 1996a; Ben-Haim and Armstead 2000; Domoki et al. 2005a), and also, after traumatic brain injury (Armstead 1997; Armstead 2004). Specifically, previous studies showed that CO<sub>2</sub>- and NMDA-induced vasodilatations are severely deteriorated by I/R. However, natural neuroprotective mediators such as PACAP or VIP despite their and their receptors' widespread presence in the elements of the neurovascular unit have not been considered previously whether they could possibly counteract the deleterious effect of I/R on CR.

*Hypercapnia-induced pial arteriolar dilation: a sensitive indicator of I/R-induced endothelial dysfunction*

In porcine cerebral vessels, hypercapnia evokes vasodilation not only *in vivo* (Leffler et al. 1989b), but also in isolated, denervated vessels (Kokubun et al. 2009) suggesting that neuronal/glial factors are not essential for the hypercapnia-induced vasodilation. In the piglet, hypercapnia-induced vasodilation requires intact endothelium (Leffler et al. 1994b); more specifically, the endothelium appears to serve as a source of prostanoids for the vascular smooth muscle to permit the relaxation (Leffler et al. 1994a). Prostanoid synthesis increases in newborn pig brain microvascular endothelial cells in response to hypercapnia, but high CO<sub>2</sub> level does not increase prostanoid production by cerebral microvascular smooth muscle or glial cells (Hsu et al. 1993). Hypercapnia-induced vasodilation is vulnerable to I/R; however, supplementation of arachidonic acid restores this vasodilation and hypercapnia-related increases in the CSF 6-keto-prostaglandinF<sub>1α</sub> levels (Leffler et al. 1992). Based on these findings, I/R seems to reduce hypercapnia-induced dilation of pial arterioles through endothelial damage in piglets. Although most data suggest the principal involvement of endothelium, the role of other cell types cannot be excluded, since neuronal/glial components also contribute to hypercapnia-induced cerebrovascular dilation in other experimental models (Wang et al. 1999; Xu et al. 2004).

*NMDA-induced pial arteriolar dilation: a sensitive indicator of I/R-induced neuronal-vascular dysfunction*

Glutamate is one of the most abundant excitatory neurotransmitter in the mammalian brain (Bonvento et al. 2002; Fellin et al. 2006) that activates a number of ionotropic and metabotropic receptors on neurons and astrocytes (Fergus and Lee 1997; Zonta et al. 2003; Busija et al. 2007; Koehler et al. 2009). The linkage between neural activity-based release and actions of glutamate on parenchymal cells, and the associated decrease in cerebral vascular resistance provides one possible functional explanation for the neurovascular unit (Leffler et al. 1989b; Busija et al. 2007; Drake and Iadecola 2007). Three types of ionotropic glutamatergic receptors are characterized by the names of their synthetic agonists: N-methyl-D-aspartate (NMDA), kainate, and alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) (McDonald et al. 1990; Garthwaite 1991; Lerma et al. 1997). Glutamate or its analogues are devoid of any vasoactive effects on isolated arteries from various species,



including humans and piglets, studied *in vitro* (Hardebo et al. 1989; Faraci and Breese 1993; Simandle et al. 2005). However, when applied topically *in vivo*, glutamate agonists elicit apparent dose-dependent vasodilation in the intact cerebral circulation (Busija et al. 2007). Although glutamate has the potential to exert vasoactive influences via activation of any of the three ionotropic glutamate receptors, topical application to the surface of the cerebral cortex appears to dilate pial arterioles predominantly via NMDA receptor activation (Faraci and Breese 1993; Meng et al. 1995).

It has been hypothesized that stimulation of NMDA receptors on neurons and the consequent membrane depolarization/ $\text{Ca}^{++}$  influx result in the activation of subsequent neurons, and production of vasodilator mediators such as NO that ultimately leads to cerebral vascular relaxation, thereby adjusting the cortical blood flow (CoBF) to the local metabolic activity.

Faraci and Breese (Faraci and Breese 1993) first demonstrated that dilation to NMDA was eliminated or reduced by treatment with the general NOS inhibitor, N- $\omega$ -nitro-L-arginine methyl ester (L-NAME) in rabbits, that results were later confirmed in the piglet (Meng et al. 1995). Subsequent studies showed that administration of 7-nitroindazole, the selective inhibitor of the neuronal type of NOS (nNOS), exerted similar, inhibitory effect on NMDA-induced vasodilation in these animal models (Faraci and Brian 1995; Bari et al. 1996b). Furthermore, supporting these pharmacological findings, NMDA application to the cortex resulted in accumulation of NO degradation products in the CSF surrounding the arterioles (Domoki et al. 2002). The presence of a vein underlying the pial arteriole reduced its ability to dilate to NMDA compared to an arteriole lying directly on the cortical surface (Domoki et al. 2002), thereby further reinforcing the concept that diffusion of a vasoactive substance from the cortex reaches and dilates the arteriole in response to NMDA application.

Studies using double immunostaining showed that NMDA receptor positive neurons were more abundant than nNOS immunoreactive neurons in the cerebral cortex, and additionally, NMDA receptors and nNOS seem to occur in separate pool of neurons (Bari et al. 1998). Administration of the voltage-gated sodium channel blocker tetrodotoxin completely eliminates NMDA-induced cerebrovascular dilation *in vivo* in rabbits (Faraci and Breese 1993), and rats (Pelligrino et al. 1995), indicating the involvement of a chain of activated neurons in this process. NMDA or glutamate does not dilate isolated vessels, that finding further strengthens the possible role of neuronal components in the process of dilation. The absence of functional NMDA and AMPA receptors in rat and human cerebrovascular endothelial cells has also been demonstrated (Morley et al. 1998).

Moreover, endothelial damage *in vivo* failed to affect NMDA-induced dilation while responses to other endothelium-dependent dilator stimuli were blocked (Domoki et al. 2002). The response was also unaffected by blockade of prostaglandin synthesis. Piglet cerebrovascular endothelial cells were also insensitive to either glutamate or NMDA-induced toxicity under both *in vitro* and *in vivo* conditions (Domoki et al. 2008).

Briefly, the activation of neuronal NMDA receptors leads to the subsequent activation of a specific population of nNOS positive neurons via local neuronal connections (Faraci and Breese 1993; Bari et al. 1996b). The released NO then diffuses to and acts on the vascular smooth muscle, resulting in dilation of the pial arterioles (Meng et al. 1995; Domoki et al. 2002). In addition to the unequivocal role of NO, the participation of other species-specific factors such as adenosine (Iloff et al. 2003), carbon monoxide (CO) (Leffler et al. 2006), ATP-sensitive potassium ( $K_{ATP}$ ), and/or calcium-activated potassium ( $K_{Ca}$ ) channels (Philip and Armstead 2004) has been proposed.

Dilator responses to NMDA were repeatable several times in the same preparation (Busija and Leffler 1989; Busija et al. 1996a; Philip and Armstead 2004) and even prolonged application to the cortical surface did not appear to damage the cortex *in vivo* or affect pial arteriolar responsiveness to other, non-glutamatergic dilator stimuli (Busija and Leffler 1989). However, NMDA-induced vasodilation has been shown to be vulnerable to even short periods of global cerebral ischemia or asphyxia (Bari et al. 1996a; Busija et al. 1996a). Importantly, the pial arteriolar response to NO itself was unaffected by I/R (Busija et al. 1996a). All available evidence strongly suggests the causative role of reactive oxygen species (ROS) in the attenuation of NMDA-induced vasodilation after I/R (Girouard and Iadecola 2006). In piglets, topical application of ROS scavengers preserves cerebral arteriolar dilator responses to NMDA after I/R (Bari et al. 1996a). The primary site of ROS action appears to be at the level of the NMDA receptor (Choi et al. 2000; Guerguerian et al. 2002). Alternatively, the functional coupling between NMDA receptor and nNOS expressing neuronal populations may be disrupted after I/R.

*Cortical spreading depression (CSD) may be involved in the mediation of NMDA-induced vasodilation in the newborn*

NMDA, when applied to the exposed surface of the piglet cerebral cortex, dilated overlying pial arterioles in a dose-dependent fashion (Busija and Leffler 1989; Armstead 2001; Busija et al. 2007). All of the original studies in piglets have been replicated numerous

times in other species such as the rabbit (Faraci and Breese 1993) and rat (Faraci and Heistad 1994; Iliff et al. 2003; Sun and Mayhan 2005). Thus, the fundamental finding that NMDA elicits cerebrovascular dilation *in vivo* appears to be a universal finding across species. However, Ayata and Moskowitz (Ayata and Moskowitz 2006) suggested an alternative mechanism of NMDA-induced pial arteriolar vasodilation in mice. Using electrophysiological recordings and vessel-diameter measurements, they demonstrated the involvement of cortical spreading depression (CSD) in the complex vascular responses evoked by topical application of NMDA.

The concept of NMDA receptor activation contributing to the generation and propagation of CSD is well established; synthetic antagonists of NMDA receptors are potent inhibitors of CSD development in numerous experimental models (Marrannes et al. 1988; Gill et al. 1992; Lauritzen and Hansen 1992; Richter et al. 2008). CSD is a self-propagating wave of cellular depolarization in the cerebral cortex that is typically evoked by localized trauma, excess neuronal firing, potassium chloride or neurotransmitter application, and is associated with short-lived but dramatic increases in cortical blood flow (CoBF). The underlying mechanisms of this cerebral hyperemia are very complex and probably require the contribution of diverse cell types within the neurovascular unit as well as a wide range of vasoactive factors depending on age, species, and metabolic status of the cerebral cortex (Busija et al. 2008). Because NMDA dilates pial arterioles via species- and age-dependent mechanisms and is implicated in CSD initiation and propagation, it is possible, that under some circumstances NMDA-evoked CSDs contribute to the overall vascular responses. The recent findings of Ayata and Moskowitz (Ayata and Moskowitz 2006) support this concept by providing evidence that in mice NMDA-induced pial arteriolar dilation is at least partially mediated by CSD. These authors also suggest that, in some former studies where electrophysiological analyses were not performed, the virtual dose-dependent nature of the pial arterial responses was due to the increasing prevalence of CSDs in conjunction with the increasing concentration of NMDA. Most data on NMDA-induced cerebrovascular changes have been acquired through studies on pial arteriolar diameters which may or may not fully represent the overall response of the cerebral vasculature (Busija et al. 1981; Busija et al. 1982; Shibata et al. 1991). However, using Laser-Doppler flowmetry, it has been proven that similar CSD occurs in response to NMDA in adult rats (Lenti et al. 2009). In these experiments the CoBF response to NMDA was a combination of a CSD-related hyperemia and a CSD-independent, dose-dependent component. It is unclear, however, whether a

similar complex interaction between CSD and the direct effects of NMDA occurs in other animal models, especially in newborns.

Interestingly, the blood flow response to CSD is rather immune to many moderate to severe disease conditions in different animal models (Busija et al. 2008), while the response to NMDA is very susceptible to even moderate brain insults as it was discussed above. Therefore, it is of high importance to critically examine the effect of NMDA on CoBF using laser-Doppler flowmetry and to test whether CSD-related cortical hyperemia contributes to the overall NMDA-induced cerebrovascular response in the piglet. Because the newborn pig seems to be resistant to KCl-induced CSDs (Domoki et al. 1999), NMDA-induced cerebrovascular changes are likely not confounded by CSDs in this subject. In this case, the use of piglets would allow us to compare the NMDA-evoked CSD-independent cerebral hyperemia across experimental models.

#### *Aims of the present studies*

The purpose of the first set of our experiments was to (1) evaluate the vasoactive potency of PACAP isoforms, as well as short PACAP fragments, and VIP on the newborn pial vasculature; (2) compare the magnitude of vasodilation induced by PACAP38 and PACAP27; (3) explore the mechanism of cerebral arteriolar responses to PACAP38, PACAP27, and VIP using putative receptor antagonists, non-selective NOS, and COX inhibitors. The results emanating from these studies urged us to (4) investigate the roles of COX-1 and COX-2 activity in the mechanism of PACAP38 and VIP-induced vasodilation using selective enzyme inhibitors and to (5) test the effect of the soluble phospholipase A<sub>2</sub> (PLA<sub>2</sub>) blocker PX-18 on PACAP38-evoked vascular caliber changes.

The major goal of our second study was to determine whether (1) PACAP38, PACAP27 and/or VIP preserves CR to hypercapnia and/or NMDA after I/R, and whether preservation of pial arteriolar reactivity to these stimuli can possibly play a role in PACAP- and VIP-induced neuroprotection. We also tested (2) whether the protective effects of these neuropeptides are dependent on their vasoactivity.

Additionally, modifying our original protocol, we sought to test if CSD contributed to NMDA-induced cortical hyperemia in piglets, as it was described in other animal models, by measuring not pial arteriolar diameter but CoBF and DC potential changes.

## Materials and methods

### *Animals*

In our experiments, we employed the newborn pig animal model that most closely reflects the brain developmental stage, metabolism, and basic circulatory parameters of the human term neonate. This model is widely accepted and relevant to study the cerebral circulation as well as the cardiovascular, respiratory, and endocrine systems of newborns (Book and Bustad 1974; DeRoth and Downie 1978; Busija 1994).

1-3 days old piglets of either sex (body weight 1–3 kg, n=305) were used in our experiments. Animals were maintained and used in compliance with the principles set forth by the Institutional Animal Care and Use Committee of the University of Szeged and by the Animal Care and Use Committee of Wake Forest University Health Sciences.

### *Surgery*

Anesthesia was initiated with thiopental sodium (40 mg/kg ip.; Biochemie, Vienna, Austria) and a bolus injection of  $\alpha$ -chloralose (40 mg/kg iv.; Sigma, St. Louis, MO, USA). Additional doses of  $\alpha$ -chloralose (2-7 mg/kg/h iv.) were given to maintain a constant level of anesthesia. A catheter was inserted into the right femoral artery to monitor mean arterial blood pressure (MABP) and to sample blood for determination of blood gas tensions and pH. Fluid and drugs were administered through a second catheter placed in the right femoral vein. The animals were intubated via tracheotomy and mechanically ventilated with a pressure controlled animal respirator (TSE GMBH, Bad Homburg, Germany) using room air. The ventilation rate (~30 breaths/min) and tidal volume (~20 ml) were adjusted to maintain arterial blood gas values and pH in the physiological range. A water-circulating heating pad was used to maintain the body temperature at ~37 °C. Core temperature was monitored with a rectal probe.

At the end of the experiments, the animals were euthanized with an intravenous injection of saturated KCl solution.

### *The closed cranial window technique*

Although several investigators have used variations of the cranial window technique to study the cerebral microcirculation, the first detailed description of the closed cranial window was published by Levasseur and his co-workers (Levasseur et al. 1975). They employed this technique in both acute and chronic experiments using cats and rabbits. According to Busija and his co-workers we used basically the same method adapted to the newborn pig (Busija et al. 1985).

The head of the animal was fixed in a stereotactic frame. The scalp was incised and removed along with the connective tissue over the calvaria. A circular (19 mm diameter) craniotomy was made in the left parietal cranium. The dura was cut and reflected over the skull. The complete window consisted of two parts; a stainless steel ring and a circular glass cover slip through which the pial vessels were visualized. The junction between the ring and the glass was sealed with melted beeswax. Three needle ports were connected to the steel ring serving as inlets and/or outlets to enable drug administration and flushing of the brain surface. The window was placed into the craniotomy, sealed with bone wax, and cemented with cyanoacrylate ester and dental acrylic. Following surgery, the closed window was filled with artificial cerebrospinal fluid (aCSF) which was similar to the endogenous CSF (aCSF composition: KCl 220, MgCl<sub>2</sub> 132, CaCl<sub>2</sub> 221, NaCl 7710, urea 402, dextrose 665, and NaHCO<sub>3</sub> 2066, in mg/l), warmed to 37 °C and equilibrated with a gas mixture containing 6% O<sub>2</sub>, 6.5% CO<sub>2</sub> and 87.5% N<sub>2</sub> to obtain pH 7.33,  $p\text{CO}_2=46$  mm Hg and  $p\text{O}_2=43$  mm Hg.

There are several advantages of this method. First, it allows the investigator to continuously observe the pial circulation. Second, the closed cranial window restores the integrity of the skull, therefore the intracranial pressure (ICP) remains around normal, physiological levels. Also, aCSF provides physiological environment for the pial vessels observed. Finally, the mechanical stability of the preparation allows the induction of induce global cerebral ischemia via elevated ICP.

### *Assessment of cerebrovascular reactivity (CR)*

Pial vessels were observed with an operating microscope (Wild, Switzerland) equipped with a video camera (Sanyo digital color CCD camera, Japan), and a video monitor (Panasonic, Japan). Vascular diameters were measured using a video microscaler. In each experiment, a ~100 µm-diameter (range 74–114 µm) pial arteriole was selected. We chose

this vessel size because this is the level of the first-order pial arterioles and the primary site of vascular resistance. Some authors separate the resistance vessels into two groups corresponding their size (e.g. arterioles and small arteries) to rule out the possibility that regional changes in the reactivity modify the results of the experiments (Armstead 1995). However, in our experiments, arterioles at both end of the range studied displayed statistically identical reactivity to the stimuli used thus data from all observed vessels were combined as shown in the Results.

After the arteriole reached a stable baseline diameter, the window was flushed with aCSF as control. Graded hypercapnia was elicited by ventilation with a gas mixture (5 or 10% CO<sub>2</sub>, 21% O<sub>2</sub>, balance N<sub>2</sub>). Vasodilator drugs were applied to the pial surface through one of the injectable ports of the cranial window. The pial arterioles were exposed to each vasodilator stimulus for 5 min, while arteriolar diameters were observed continuously. After completion of a stimulus, the cranial window was repeatedly flushed with aCSF and the vessel diameter was allowed to return to the baseline level.

#### *Global cerebral ischemia*

To induce global cerebral ischemia, before inserting the cranial window, a 3-mm hole was made with an electric drill with a toothless bit in the left frontal cranium rostral to the cranial window and the dura was exposed. A hollow brass bolt was inserted and secured in place with bone wax, cyanoacrylate ester, and dental acrylic. Cerebral ischemia was induced by the infusion of aCSF so as to raise ICP above the arterial pressure for 10 minutes. Ischemia was verified by the cessation of blood flow in the vessels observed through the cranial window. Using microspheres, former studies of Beasley and his colleagues (Beasley et al. 1998) provided evidence that cerebral blood flow was virtually zero in all examined brain areas during the ischemic period. Venous blood was withdrawn as necessary to MABP near the normal values. At the end of the ischemic period, the infusion tube was clamped and the intracranial pressure returned to the preischemic level. The withdrawn and heparinized blood was reinfused (Domoki et al. 2005a).

#### *Measurement of cortical blood flow (CoBF)*

The effect of NMDA on pial arteriolar diameters has been demonstrated in several previous studies (Leffler et al. 1989b; Busija and Wei 1993; Faraci and Breese 1993; Faraci

and Breese 1994; Faraci and Heistad 1994; Meng et al. 1995); however, the temporal resolution of diameter measurements may exclude the possibility to record dynamic – short lasting changes in CoBF. Therefore, in 5 male piglets, we minimally modified the surgical procedure and the experimental setup in order to critically examine the integrated CoBF responses and the contribution of possible CSDs to NMDA-induced cortical hyperemia.

Anesthesia was induced with pentobarbital (50-60 mg/kg ip.) and minimally supplemented by  $\alpha$ -chloralose (Sigma, St. Louis, MO; 5-10 mg/kg iv.) only when needed (in 2 animals). These anesthetics were chosen because they are known to have no influence on CSD generation and propagation (Kitahara et al. 2001; Kudo et al. 2008). In addition to the blood gas parameter measurements, end-tidal CO<sub>2</sub> levels were also continuously monitored using a capnometer (Micro-Capnograph CI240, Columbus Instrument, Columbus, OH). The cranial window consisted of the same kind of stainless steel ring with three needle ports as in the previously described experiments; however, it was sealed with Parafilm (American National Can, Greenwich, CT) instead of glass cover slips through which a laser-Doppler probe (referred to as probe 1) and a DC electrode were guided onto the brain surface. Following surgery, this closed window was filled with aCSF. For the placement of Doppler probe 2, a hole was drilled 5-8 mm anterior to the window (distance between the two probes was ~9-13 mm) and the dura was removed. The Doppler probes were mounted on micromanipulators and positioned so that they touched the surface of the brain, avoiding larger pial vessels. CoBF was monitored and recorded using a two-channel laser-Doppler flowmeter (Periflux 4001 master, Perimed, Sweden). Data were analyzed offline. The fibre separation of the Doppler probes used was 0.25 mm. Using this fibre separation in brain tissue, most of the recorded signal is reflected from a ~200  $\mu$ m depth (Jakobsson and Nilsson 1993). Therefore, any interference from the other, ~10 mm distant light source was negligible. For the measurement of DC potential changes in response to topically applied NMDA, an Ag-AgCl electrode (1 mm diameter) was positioned on the brain surface under the cranial window and was connected to a DC amplifier (DAM 50, Differential Amplifier, World Precision Instruments, Sarasota, FL). The reference platinum electrode (Grass, Astro-Med, West Warwick, RI) was fixed in the neck muscles.

### *Drugs*

VIP (Sigma Chemical Co., St. Louis, MI, USA), PACAP38, PACAP27, PACAP6–38, PACAP6–27, PACAP1-15, PACAP6-15, and PACAP20-31 (Department of Medical



Chemistry, University of Szeged, Hungary) were prepared as stock solutions in saline ( $10^{-4}$  M) and, before use, were diluted with aCSF ( $10^{-9}$  to  $10^{-5}$  M). Indomethacin (Merck & Co, Whitehouse Station, NJ) and L-NAME (Sigma Chemical Co.) were dissolved in saline (30 mg/ml and 10 mg/ml, respectively). NS-398 (Sigma Chemical Co.) and SC-560 (Sigma Chemical Co.) were dissolved in dimethyl sulfoxide (DMSO, 5 mg/ml) and further diluted with saline to 1 ml. NMDA (Sigma Chemical Co.) was dissolved in aCSF ( $10^{-5}$  to  $10^3$  M). PX-18 (Dept. of Pharmaceutics, Biopharmaceutics and NutriCosmetics, Freie Universität Berlin, Germany) formulated as drug nanosuspension with an active content of 3% (w/w) applying high pressure homogenization. PX-18 nanosuspension was further diluted in aCSF to a concentration of  $10^{-8}$  to  $10^{-4}$  M. A 1% (w/w) Tween 80 solution isotonized with glycerol 85% served as vehicle control of the experiments where PX-18 was used.

#### *Protocol and experimental groups*

Time control experiments were executed first to obtain arteriolar responses to PACAP isoforms and VIP initially and then 20 min after vehicle (1 ml of intravenous saline, or diluted DMSO) treatment. This procedure allowed us to test PACAP/VIP-induced caliber changes and identify the presence of potential tachyphylaxis or sensitization. In groups 1, 2, and 3; PACAP38 (n=9), PACAP27 (n=7), or VIP (n=8) was applied repeatedly onto the brain surface at  $10^{-8}$ - $10^{-7}$ - $10^{-6}$  M concentrations cumulatively. Moreover, we compared the efficacy of the two PACAP isoforms on the same subjects; in group 4 (n=9) PACAP38 and PACAP27 were applied alternately with a 40-minute delay.

The second set of our experiments was designed similarly, with one modification. Between the first and the second topical application of the respective vasodilator peptide, a COX enzyme blocker was administered intravenously. In groups 5-7, we examined the responses of pial arterioles to PACAP38 (n=9), PACAP27 (n=10), or VIP (n=7) before and after intravenous administration of the non-selective COX inhibitor indomethacin (5 mg/kg). The effect of the selective COX-1 inhibitor SC-560 (1 mg/kg) was tested in groups 8 (n=9) and 9 (n=6) on PACAP38 and VIP induced vasodilation, respectively. In groups 10 (n=9) and 11 (n=7), the effect of selective COX-2-inhibition by NS-398 (1 mg/kg iv.) was analyzed on the above mentioned processes.

In group 12 (n=5), pial arteriolar reactivity was determined for local PX-18 nanosuspension ( $10^{-8}$  to  $10^{-5}$  M) and prior to PX-18, for its vehicle in the corresponding

dilutions. In Group 13 (n=7), vasodilation was determined to  $10^{-6}$  M PACAP38 before and after local treatment with PX-18 nanosuspension ( $10^{-5}$  M, 20 min).

In the next series of experiments, the brain surface was exposed to the vasoactive neuropeptides before and after the inhibition of the NOS enzyme. In groups 14 and 15, vasodilation to PACAP38 (n=13) or PACAP27 (n=7); in group 16, vasodilation in response to VIP (n=8) was tested before and after parenteral L-NAME (15 mg/kg iv.) administration.

In groups 16-20 arteriolar responses to short PACAP fragments were measured (PACAP6-38, PACAP6-27, PACAP1-15, PACAP6-15, and PACAP20-31 with n=4 in each subgroup, respectively). Each of the peptides was applied in ascending concentration manner ( $10^{-8}$ - $10^{-7}$ - $10^{-6}$  M cumulatively). Additionally, we determined the effect of these short fragments on PACAP38 and PACAP27-induced vasodilation. In these experiments we co-applied short PACAP sequences to examine their effects on vascular reactions evoked by the entire PACAP segments. First  $10^{-6}$  M of PACAP38 or PACAP27 was used topically. Following a ~30-minute exposure-free period, when arterioles regained stable baseline diameters, one short segment was applied onto the brain surface in a tenfold ( $10^{-5}$ M) concentration for 10 min. Next, we co-applied PACAP6-38, PACAP6-27, PACAP1-15, PACAP6-15, or PACAP20-31 with PACAP38 (groups 21, 24, 27, 29 and 31) or PACAP27 (groups 22, 25, 28, 30, and 32; n=5 in each subgroups) for a subsequent 5 min. Pial arteriolar dilation to  $10^{-6}$  M VIP was also determined before and after 20 min topical application of PACAP6-38 (group 23, n=6,  $10^{-5}$  M) or PACAP6-27 (group 26, n=7,  $10^{-5}$  M).

In the final experiments, the effect of global cerebral I/R (10 min of ischemia followed by 1 h of reperfusion) was tested on CO<sub>2</sub>- and NMDA-induced vasodilation, with or without PACAP38, PACAP27 or VIP treatment. Prior to the induction of ischemia, the brain surface was incubated for 30 min with vehicle (aCSF, groups 33 and 37, n=6-6),  $10^{-8}$  M PACAP38 (groups 34 and 38, n=6-6),  $10^{-8}$  M PACAP27 (groups 35 and 39, n=6-6), or  $10^{-9}$  M VIP (groups 36 and 40, n=8-6). Pial arteriolar caliber changes to 5 and 10% CO<sub>2</sub> ventilation (groups 33-36), or to topical,  $10^{-4}$  M NMDA (groups 37-40) were measured before the pretreatments and after 1 h of reperfusion. We performed additional experiments on 4-4 animals in groups 40b and 40c to test whether a higher ( $10^{-8}$  M) dose of VIP can protect NMDA-induced vasodilation after I/R. In group 40b we used the NMDA at  $10^{-4}$  M concentration similar to the original experiments, while in group 40c we tested if the vascular response to a lower,  $10^{-5}$  M and  $5 \times 10^{-5}$  M concentration of NMDA can be protected by VIP ( $10^{-8}$  M) after I/R. The effects of the same PACAP38, PACAP27 or VIP pretreatments in the

absence of I/R were also tested on the vasodilation evoked by hypercapnia (groups 41-43, n=6-5-6) or by NMDA (groups 44-46, n=5-7-5), respectively.

In additional experiments (group 47, n=5) we tested, whether NMDA induces CSD in the piglet. The effects of topical NMDA ( $10^{-4}$  and  $10^{-3}$  M) on CoBF were determined. The brain surface was exposed to each NMDA concentration for 10 minutes after which this stimulus was removed with aCSF. DC potential and CoBF recording continued for an additional 20 minutes. At least 30 minutes after the removal of the highest dose of NMDA, we attempted to evoke CSDs using repeated pinpricks (26 G needle, 1-2 mm in depth) as a mechanical stimulation. CoBF and DC potential were recorded in a 30-minute period following each pinprick.

### *Statistical analysis*

Pial arteriolar diameters were determined under baseline conditions and then at each 30 seconds during application of vasodilator stimuli. Results are presented as the maximal vasodilation maintained for at least 1 minute, and expressed as % changes from corresponding baseline diameters. CoBF was recorded in arbitrary perfusion units. The biological zero was subtracted from the absolute values and CoBF data were normalized to the average flow of 5 minutes prior to drug application. Maximal CoBF response was determined occurring over a one minute interval. The pial artery diameter and CoBF data were analyzed with statistical software (SigmaStat, Systat Software Inc., San Jose, CA, USA) using one-way repeated measures ANOVA. For *post hoc* analysis Tukey's test was performed where appropriate. Values of  $p < 0.05$  were considered significant. Data are reported as mean $\pm$ SEM.

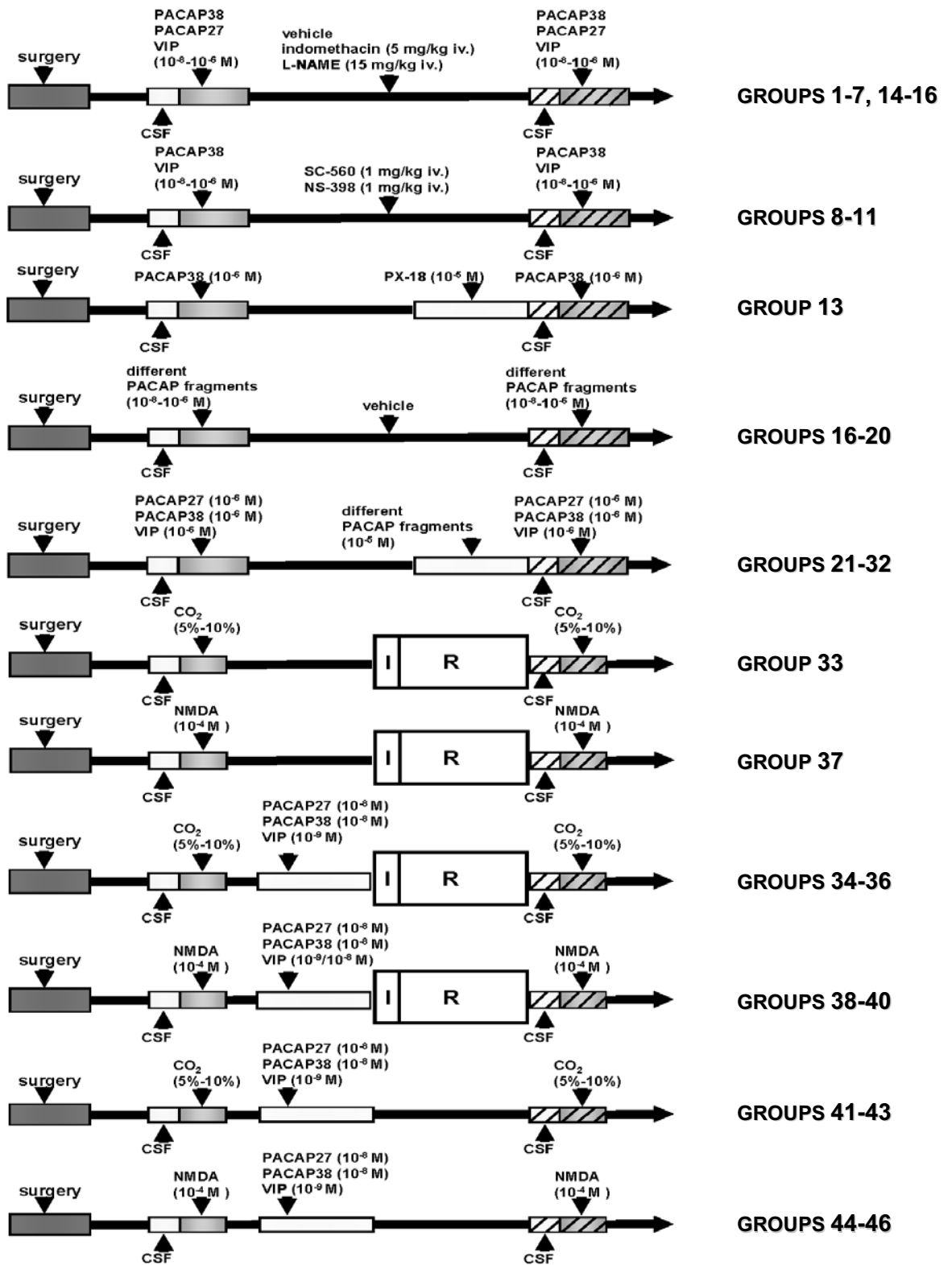


Figure 1. Experimental protocol.

## Results

### *Physiological variables*

MABP values were always within the range characteristic for anesthetized newborn pigs. In each animal, care was taken to avoid any fluctuation in MABP during recordings so as to prevent effects of short-term changes in arterial pressure on vascular diameters. Additionally, the blood pressure of the animals did not differ significantly between groups, for example, in control groups 33 and 37, MABP values were  $70\pm 3$  mm Hg and  $69\pm 3$  mm Hg, respectively. Body temperature, arterial pH, and blood gases were kept in the normal ranges as well and did not vary significantly among the different groups or throughout the experiments, except pH and CO<sub>2</sub> levels in groups where hypercapnia was induced. In group 33, for instance, core temperature was  $37\pm 0.2$  °C, pH was  $7.43\pm 0.03$ , pCO<sub>2</sub> was  $38\pm 2$  mm Hg, and pO<sub>2</sub> was  $91\pm 5$  mm Hg under baseline conditions. In group 41, where ventilation parameters were set to reach the normal end tidal CO<sub>2</sub> value of 5% (40 mm Hg), pH was  $7.41\pm 0.01$ , and arterial oxygen tension was  $88\pm 4$  mm Hg.

Graded (5 and 10%) hypercapnia significantly elevated the arterial pCO<sub>2</sub> levels in all experimental groups where CR to hypercapnia was tested (groups 27-30). The pCO<sub>2</sub> changes were similar before and after I/R and between groups. For instance, in group 36, 5 and 10% CO<sub>2</sub> elevated pCO<sub>2</sub> from  $34\pm 2.5$  to  $52\pm 2.1$  and  $79\pm 5$  mm Hg and from  $37\pm 2.7$  to  $56\pm 2.7$  and  $84\pm 5.9$  mm Hg before and after I/R, respectively. The respective pO<sub>2</sub> levels were  $90\pm 5.9$ - $94\pm 6.4$ - $94\pm 7.4$  and  $90\pm 7.6$ - $89\pm 5.8$ - $88\pm 4.2$  mm Hg in this group and were similar in all groups.

There were no significant differences between the baseline arteriolar diameters during experiments and among experimental groups. For example, in group 3, mean pial arteriolar diameters were  $92\pm 3$  μm and  $95\pm 5$  μm before the first and the second VIP application, respectively. Similarly, in the experiments where CoBF was measured (group 41), raw baseline laser-Doppler flowmetry data (in arbitrary perfusion units; PU) did not shift considerably over the course of experiments. Baseline perfusions under the window were  $163\pm 16$  PU and  $160\pm 28$  PU in group 41, before the first and the second NMDA-application onto the brain surface, respectively. Flushing of the brain surface with aCSF did not affect the pial arteriolar diameter or the CoBF significantly.

*Effects of PACAP38, PACAP27, and VIP on pial arteriolar diameter*

Topical application of PACAP38 (group 1) and PACAP27 (group 2) resulted in similar, concentration-dependent ( $10^{-8}$  to  $10^{-6}$  M), reproducible pial arteriolar dilation (Figure 2). Indeed, PACAP38 and PACAP27 produced equally prominent vascular changes compared in the same animals (group 4,  $43\pm 7\%$  vasodilation to  $10^{-6}$  M PACAP38 and  $36\pm 5\%$  vasodilation in response to  $10^{-6}$  M PACAP27). Topically applied VIP also induced dose-dependent, reproducible pial arteriolar dilation at  $10^{-8}$  to  $10^{-6}$  M concentrations (group 3, Figure 2). However, VIP was significantly more potent than either PACAP38 or PACAP27, since even the lowest,  $10^{-8}$  M concentration of VIP evoked significant,  $16\pm 3\%$  dilation, while PACAP isoforms were vasoactive only at higher,  $10^{-7}$ - $10^{-6}$  M doses (Figure 2).

*Effects of COX enzyme inhibitors on PACAP38-, PACAP27-, and VIP-induced pial arteriolar dilation*

The non-selective COX enzyme inhibitor indomethacin (5 mg/kg iv.) apparently abolished the vasodilator effect of  $10^{-8}$ - $10^{-7}$ - $10^{-6}$  M PACAP38 (group 5). In contrast, PACAP27-induced dilation remained completely intact after indomethacin treatment (group 6) at all concentrations used. Interestingly, VIP-induced vasodilation was only partially COX-dependent, since administration of indomethacin blocked the vasodilation evoked by the lowest ( $10^{-8}$  M) dose of VIP, but COX-inhibition was not effective when  $10^{-7}$ , or  $10^{-6}$  M VIP was applied (Figure 2).

In group 8, the selective COX-1 blocker SC-560 (1 mg/kg iv.) completely eliminated the diameter changes evoked by  $10^{-8}$ - $10^{-7}$ - $10^{-6}$  M PACAP38, similarly to indomethacin. SC-560 also mimicked the effect of indomethacin on VIP-induced vasodilation (group 9). SC-560 treatment abolished the pial arteriolar reactions in response to  $10^{-8}$  M, but not to  $10^{-7}$  and  $10^{-6}$  M VIP (Figure 2.).

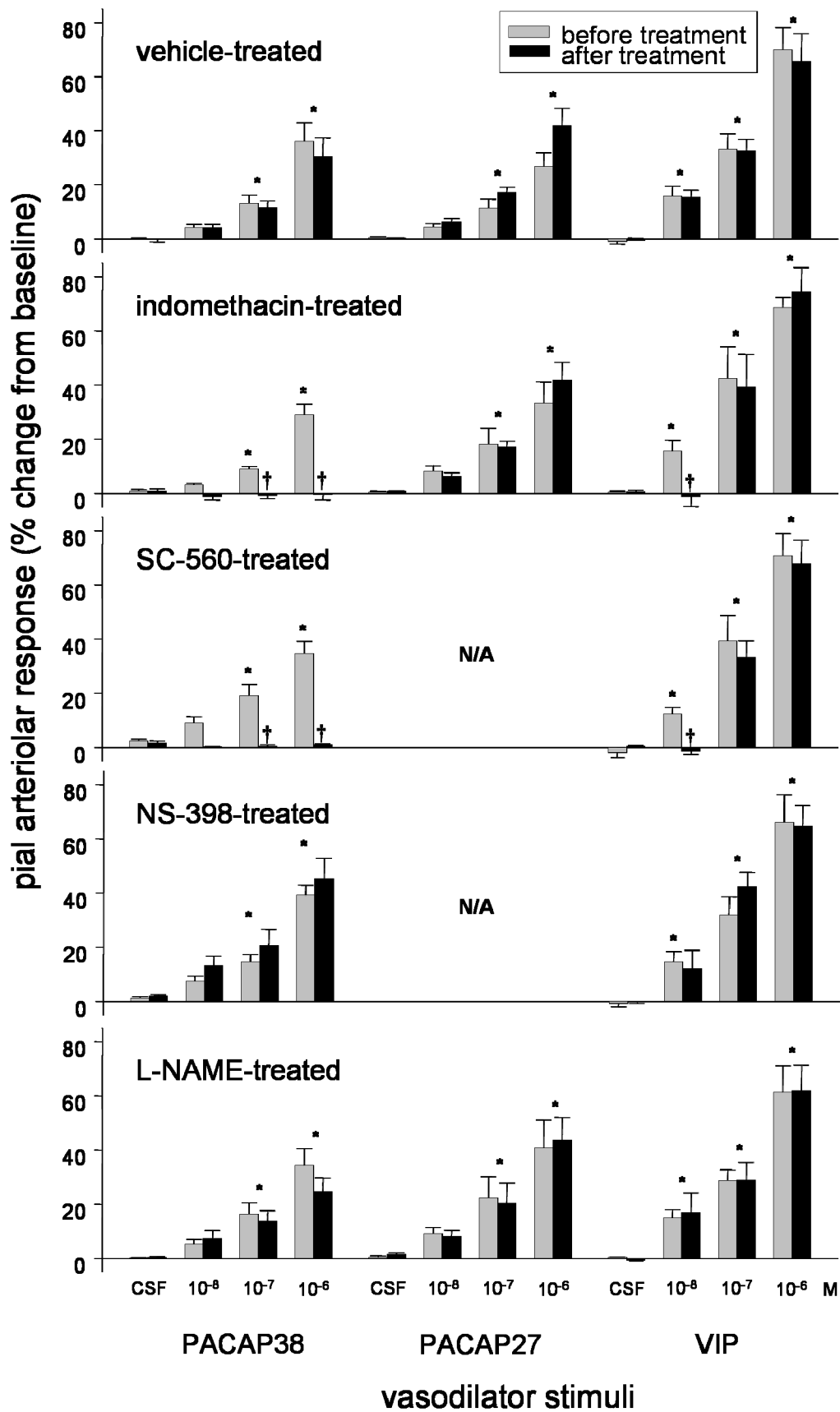
Importantly, the selective COX-2 inhibitor NS-398 (1 mg/kg iv.) failed to exert any inhibitory effect on either PACAP38- or VIP-induced vasodilation (groups 10 and 11, Figure 2).

*Effect of PLA<sub>2</sub> inhibition on PACAP38-induced pial arteriolar dilation*

PX-18 nanosuspension or its vehicle did not induce significant increase in pial arteriolar diameters when applied locally at  $10^{-8}$  to  $10^{-5}$  M concentrations (group 12). Mean arteriolar diameters were  $86 \pm 6$   $\mu\text{m}$  and  $88 \pm 8$   $\mu\text{m}$  before and after  $10^{-5}$  M PX-18 application, and  $86 \pm 6$   $\mu\text{m}$  and  $89 \pm 5$   $\mu\text{m}$  before and after vehicle (at the respective concentration) administration onto the brain surface. PACAP38 ( $10^{-6}$  M) elicited robust pial arteriolar vasodilation in group 13, that was reduced by PX-18 from  $67 \pm 11\%$  to  $24 \pm 6\%^*$ .

*Effects of NOS enzyme inhibition on PACAP38-, PACAP27-, and VIP-induced pial arteriolar dilation*

The inhibition of NOS enzyme by L-NAME (15 mg/kg, iv.) had no significant effect on PACAP/VIP-induced vascular reactions. PACAP38, PACAP27, and VIP elicited similar dilation of pial arterioles before and after L-NAME treatment (Figure 2).





**Figure 2. Effects of different enzyme inhibitors on PACAP38-, PACAP27-, and VIP-induced pial arteriolar dilation.** Topical PACAP38, PACAP27, and VIP elicited dose-dependent, reproducible increases in pial arteriolar diameters. The non-selective cyclooxygenase (COX) inhibitor abolished pial arteriolar dilation to PACAP38, whereas it left vasodilation to PACAP27 intact at all concentrations applied. VIP-induced vasodilation was indomethacin-sensitive at  $10^{-8}$  M, but not at  $10^{-7}$  and  $10^{-6}$  M concentrations. Inhibition of COX-1 enzyme by SC-560-treatment resembled the effect of indomethacin; it abolished both PACAP38 ( $10^{-8}$  to  $10^{-6}$  M) and  $10^{-8}$  M VIP-induced pial arteriolar dilations. In contrast, the COX-2-inhibitor NS-398, or the nitric oxide synthase (NOS)-inhibitor L-NAME did not influence either PACAP- or VIP-evoked vascular caliber changes. (Data are mean±SEM, n=6–10, \*p <0.05 significantly different from vasodilation in response to aCSF, †p <0.05 significantly less than corresponding values before treatment.)

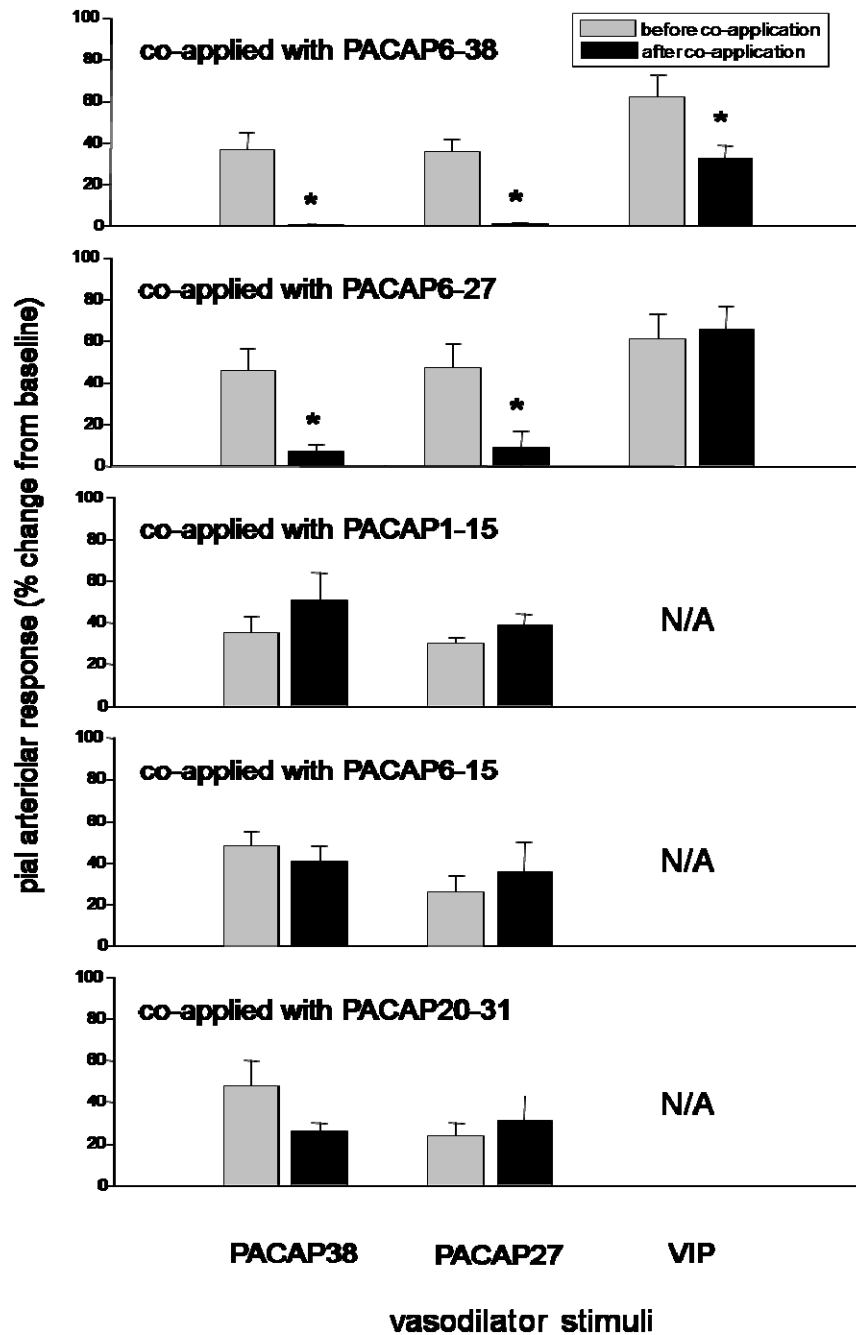
*Effects of various length PACAP fragments on pial arteriolar diameter*

In contrast to PACAP38, PACAP27, and VIP, PACAP fragments did not display any vasoactivity, even when repeatedly applied (groups 16-20). Percent changes in diameters to the highest ( $10^{-6}$  M) concentration used were:  $1\pm 1$  and  $-2\pm 2$  to PACAP6–38 and PACAP6–27;  $2\pm 3$ ,  $0\pm 0$  and  $2\pm 1$  to fragments PACAP1–15, PACAP6–15, and PACAP20–31, respectively, during the first application. The vascular responses were  $1\pm 1\%$  and  $1\pm 1\%$  to PACAP6–38 and PACAP6–27,  $2\pm 2\%$ ,  $0\pm 0\%$  and  $1\pm 1\%$  to fragments PACAP1–15, PACAP6–15, and PACAP20-31, respectively, during the second application.

*Effect of co-application with PACAP fragments on PACAP38, PACAP27, and VIP-induced pial arteriolar dilation*

In comparison to the control values, arteriolar dilations to PACAP38 or PACAP27 showed statistically significant decreases when these peptides were co-applied with PACAP6–38 or PACAP6–27 (groups 21-22 and groups 24-25, respectively). PACAP6-38 ( $10^{-5}$  M) pretreatment effectively diminished VIP-induced vasodilation (group 23,  $62\pm 10\%$  and  $33\pm 6\%$ \* vasodilation in response to  $10^{-6}$  M VIP, before and after PACAP6-38); however, in group 26, PACAP6-27 ( $10^{-5}$  M) failed to exhibit any inhibitory effect on the VIP-related pial arteriolar dilation ( $61\pm 12\%$  and  $66\pm 11\%$  changes in diameter before and after topical

PACAP6-27 treatment). The other applied short segments (PACAP1-15, PACAP6-15, and PACAP20-31) did not affect either PACAP38 or PACAP27-induced vasodilation (Figure 3).



**Figure 3. Effects of PACAP fragments on PACAP38-, PACAP27-, and VIP-induced pial arteriolar dilation.** PACAP6-38 ( $10^{-5}$  M) pretreatment effectively decreased the PACAP38-, PACAP27-, and VIP ( $10^{-6}$  M)-induced vasodilation. PACAP6-27 ( $10^{-5}$  M) also inhibited the

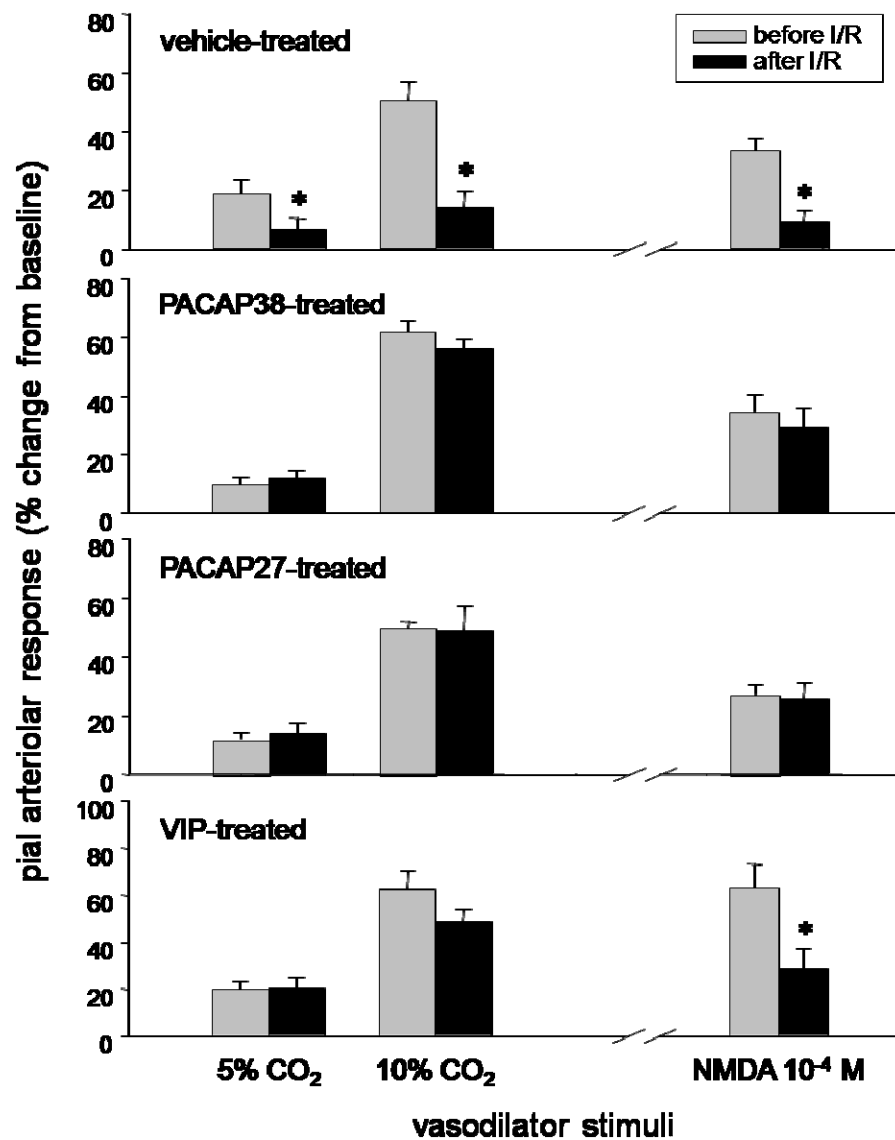
pial arteriolar dilation in response to PACAP38 and PACAP27 ( $10^{-6}$  M); however, it failed to exhibit any inhibitory effect on the VIP ( $10^{-6}$  M)-related vasodilation. PACAP1–15, PACAP6–15, and PACAP20–31 ( $10^{-5}$  M) failed to alter either PACAP38- or PACAP27 ( $10^{-6}$  M)-induced vascular responses. (Data are mean+SEM, n=5-7, \*p <0.05 significantly less than corresponding values before the co-application.)

*Effects of PACAP38, PACAP27, and VIP pretreatment on pial arteriolar responsiveness to CO<sub>2</sub> after I/R*

Graded hypercapnia (5-10% CO<sub>2</sub> ventilation) resulted in large, dose-dependent, reversible increases in pial arteriolar diameters. In vehicle-treated animals (group 33), I/R severely attenuated the hypercapnia-induced arteriolar vasodilation (Figure 4, left). Incubation of the brain surface with PACAP38 ( $10^{-8}$  M), PACAP27 ( $10^{-8}$  M) or VIP ( $10^{-9}$  M) for 30 minutes did not affect the diameter of pial arterioles significantly ( $2\pm 1\%$ ,  $2\pm 1\%$ , and  $3\pm 1\%$  maximal dilation in response to PACAP27, PACAP38, and VIP, respectively); however, they efficiently preserved CR to both levels of hypercapnia after I/R (groups 34, 35, and 36; Figure 4, left).

*Effects of PACAP38, PACAP27, and VIP pretreatment on pial arteriolar responsiveness to NMDA after I/R*

Topical application of NMDA ( $10^{-4}$  M) induced a marked vasodilation that was also sensitive to I/R (group 37, Figure 4, right). PACAP38 ( $10^{-8}$  M) or PACAP27 ( $10^{-8}$  M) pretreatment of the brain surface effectively preserved the NMDA-evoked pial arteriolar dilation after I/R, whereas incubation of the brain surface with  $10^{-9}$  M VIP was not protective (groups 38, 39, and 40; Figure 4, right). Furthermore, a higher dose of VIP ( $10^{-8}$  M, group 40b) was still unable to preserve CR to  $10^{-4}$  M NMDA reduced by I/R. CR values were  $36\pm 12\%$  vs  $51\pm 6\%$  (vehicle vs VIP, CR expressed as a percentage of the preischemic response). In group 40c, lower,  $10^{-5}$  M and  $5\times 10^{-5}$  M concentrations of NMDA also dilated the pial arterioles with  $4.4\pm 0.2\%$  and  $12.6\pm 0.4\%^*$ , respectively. I/R reduced these vascular reactions significantly, and moreover, a tendency towards vasoconstriction was observed after I/R ( $-2\pm 3\%$  and  $-3\pm 4\%$  changes in diameter to  $10^{-5}$  M and  $5\times 10^{-5}$  M NMDA), irrespectively of the VIP ( $10^{-8}$  M) treatment.



**Figure 4. Effects of ischemia-reperfusion (I/R) on hypercapnia- or NMDA-induced pial arteriolar vasodilation.** Arteriolar responses to 5-10% CO<sub>2</sub> ventilation and topical NMDA were recorded before and after 10 min of global cerebral ischemia followed by 1 h of reperfusion. Hypercapnia elicited dose-dependent pial arteriolar dilation, which was markedly attenuated after I/R in vehicle-treated piglets. PACAP isoforms and VIP preserved vascular responsiveness to CO<sub>2</sub>. Vasodilation in response to 10<sup>-4</sup> M NMDA was also deteriorated after I/R. PACAP38 and PACAP27, but not VIP, protected the vasodilator effect of NMDA. (Data are mean±SEM, n=6-8, \*p<0.05.)

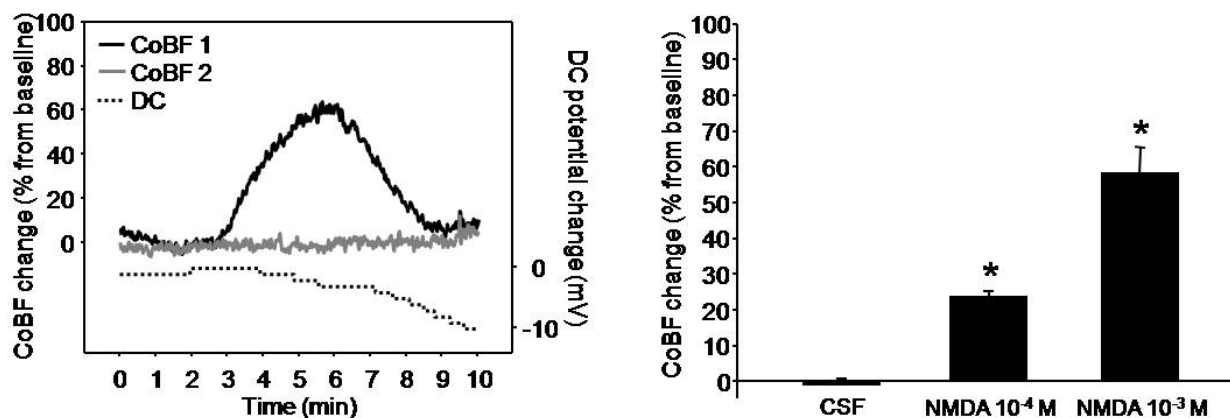
*Effects of PACAP38, PACAP27, and VIP treatment on CO<sub>2</sub>- and NMDA-induced pial arteriolar dilation*

Topical PACAP38 ( $10^{-8}$  M), PACAP27 ( $10^{-8}$  M) or VIP ( $10^{-9}$  M) treatment alone did not affect the vasodilation induced by either hypercapnia or NMDA. Vascular responses to 10% CO<sub>2</sub> were  $63\pm 5\%$  and  $64\pm 6\%$  before and after PACAP38 (group 41), and  $50\pm 2\%$  and  $57\pm 7\%$  before and after PACAP27 (group 42) incubation. Topical NMDA ( $10^{-4}$  M) evoked  $37\pm 5\%$  and  $43\pm 7\%$  vasodilation before and after PACAP38, and  $36\pm 7\%$  and  $33\pm 5\%$  vasodilation before and after PACAP27 application (groups 44 and 45). Responses to 10% CO<sub>2</sub> were  $60\pm 9\%$  and  $56\pm 11\%$  (group 43), and to  $10^{-4}$  M NMDA were  $49\pm 7\%$  and  $48\pm 7\%$  (group 46), before and after VIP treatment. Accordingly, the protective effects of the tested neuropeptides are not caused by direct facilitation of the vascular reactivity to hypercapnia or to NMDA.

*NMDA elicits dose-dependent CoBF increase without generating CSD in the piglet*

Topical application of NMDA (group 47) produced concentration-dependent increases in CoBF as recorded with Laser-Doppler flowmetry under the cranial window (Figure 5). The pattern of the CoBF responses was not consistent with those seen during CSD (Ayata and Moskowitz 2006; Lenti et al. 2009). Also, we did not detect significant CoBF changes at the second, distant registration site outside the window ( $3\pm 2\%$  and  $2\pm 1\%$  to  $10^{-4}$  and  $10^{-3}$  M NMDA) verifying that no CSD propagated from the site of NMDA application. The DC potential decreased gradually (by 5-10 mV) and returned to the baseline level in ~2-3 minutes after removal of the NMDA with aCSF.

Local activation of cortical neurons with “pinpricks” using a sterile needle, although sufficient to cause CSD in other models, also failed to initiate CSD (0/5 animals) or significantly change CoBF in piglets ( $3.2\pm 1.2\%$  in response to the first pinprick).



**Figure 5. NMDA evoked dose-dependent cortical blood flow (CoBF) increases without generating cortical spreading depression (CSD) in newborn pigs.** *Left panel:* NMDA ( $10^{-3}$  M) application onto the cortex elicited a transient CoBF increase (CoBF 1) and a prolonged direct current (DC) potential deflection registered under the window; however, propagation of the CoBF response was not detected with the distant probe (CoBF 2, representative recording). On the time axis the 0 time point indicates the beginning of drug application onto the brain surface. The stimulus was removed 10 minutes later, when the window was flushed with artificial cerebrospinal fluid. *Right panel:* Average CoBF change per minute was calculated during a 10-minute period of drug application. Bars represent the maximal CoBF changes under the window, within the examined time interval. Artificial cerebrospinal fluid did not produce significant CoBF changes, while topical NMDA ( $10^{-4}$  and  $10^{-3}$  M) increased CoBF in a concentration-dependent manner. (Data are mean $\pm$ SEM,  $n=5$ ,  $*p<0.05$ .)

## Discussion

The major findings of the present studies were the following:

1. Both PACAP38 and PACAP27 were similarly potent dilators of pial resistance vessels in newborn pigs. VIP also evoked concentration-dependent, reproducible pial arteriolar dilation; however, it was more potent than either of the natural PACAP isoforms.
2. PACAP38-induced vasodilation was abolished by indomethacin and the selective COX-1 inhibitor SC-560, but not by the selective COX-2 inhibitor NS-398. In contrast, PACAP27 evoked vasodilation independently of COX activation. The effect of VIP on the pial vasculature resembled that of PACAP38 at low, but PACAP27 at higher concentrations; since arteriolar dilation was blocked by indomethacin and SC-560 only when VIP was applied at the lowest,  $10^{-8}$  M dose (Table 1). There was also a significant decrease in PACAP38-induced vasodilation when PX-18, the soluble PLA<sub>2</sub> blocker was administered locally. Interestingly, NOS activity did not appear to play a significant role in the dilatory effect of either PACAP or VIP.
3. The short PACAP sequences were not vasoactive *per se*, but both PACAP6–38 and PACAP6–27 inhibited the vasodilation induced by PACAP38 or PACAP27. VIP-induced vasodilation was attenuated only by PACAP6-38, but not by PACAP6–27.
4. I/R deteriorated CR to both hypercapnia, an endothelial function-dependent, and NMDA, a neuron-dependent stimulus. PACAP38, PACAP27, and VIP prevented the attenuation of hypercapnia-induced vasodilation caused by I/R; however, PACAP38 and PACAP27, but not VIP preserved NMDA-induced vasodilation.
5. NMDA induced dose-dependent CoBF increases in the piglet measured by Laser-Doppler flowmetry. NMDA-evoked CSDs did not contribute to this integrated CoBF response. Moreover, we were unable to evoke CSDs even with mechanical stimulation of the cortex.

One remarkable property of PACAP and VIP is their ability to relax cerebral arteries both *in vitro* and *in vivo* (Suzuki et al. 1984; Seki et al. 1995; Edvinsson and Krause 2002). This effect is probably of physiological relevance since nerve fibers containing PACAP and VIP have been demonstrated in the adventitia and adventitia-media border of cerebral arteries from different species (Uddman et al. 1993; Fahrenkrug et al. 2000). Moreover, PACAP and VIP were detected by immunohistochemistry in cell bodies of autonomic ganglia and also, cortical and brainstem neurons (Edvinsson and Krause 2002; Drake and Iadecola 2007).

Receptors for PACAP and VIP have been identified in practically all cell types of the central nervous system in numerous species including humans. Knutsson and Edvinsson detected mRNA of all three (VPAC<sub>1</sub>, VPAC<sub>2</sub>, and PAC<sub>1</sub>) receptor types in human cerebral ganglia and arteries (Knutsson and Edvinsson 2002). The expression of the receptor mRNA remained unaltered after removal of the endothelium. In rats, VPAC<sub>1</sub> receptor immunoreactivity was found in the smooth muscle cell layer of the superficial cerebral arteries and arterioles (Fahrenkrug et al. 2000); however, VIP/PACAP receptors were also found on neuroglia as well as on neurons (Joo et al. 2004). Previous functional studies in rat cerebral arteries demonstrated that the vasorelaxant effects of PACAP38 and VIP are not impaired in endothelium-denuded arteries (Lee et al. 1984; Gaw et al. 1991; Anzai et al. 1995). In porcine coronary arteries, PACAP38 and PACAP27 were also found to be endothelium-independent relaxing factors (Kastner et al. 1995). On the contrary, beside VSM VPAC<sub>2</sub> receptors, functioning endothelial VPAC<sub>1</sub> receptors were detected in the porcine basilar artery by Grant et al (Grant et al. 2006). Unfortunately, no data exist on the expression of these receptors in the newborn pig.

Previous studies in piglets have shown that PACAP38 and PACAP27 dilate pial arterioles in a dose-dependent manner, and that the dilator capabilities of these peptides are very similar in this animal model (Tong et al. 1993). In piglets, the observed vascular caliber changes to PACAP38 and PACAP27 have been associated with elevated cortical periarachnoid CSF cAMP concentration (Tong et al. 1993; Armstead 1997). Both PACAP38, and PACAP27 increase cAMP level in rat cerebral microvessels (Kobayashi et al. 1994), and also, VIP was found to stimulate cortical cAMP formation in mammals (Dejda et al. 2004). PACAP and VIP are thought to exert a significant direct effect on the VSM of pial resistance vessels: activation of PACAP/VIP receptors on smooth muscle cells increases intracellular cAMP production resulting in cerebrovascular vasodilation (Dickson and Finlayson 2009). Additionally, the pial arteriolar dilation produced by PACAP27 can be blocked by iberiotoxin, a potent antagonist of the K<sub>Ca</sub> channels, but unaltered by the K<sub>ATP</sub> channel inhibitor glibenclamide (Armstead 1997). Other indirect pathways of PACAP-induced dilation are also possible.

Our present results confirm that PACAP38, and PACAP27 are similarly potent pial arteriolar dilators in the newborn pig; however, in our model, VIP proved to be more potent than either of the PACAP isoforms. The caliber changes evoked by all these peptides were apparently dose dependent, and more importantly, these responses were reproducible over time. Although tachyphylaxis to either PACAP or VIP was observed in some experimental



models (Edvinsson and Krause 2002), this desensitization was absent when employing the piglet, using our protocol. This reproducibility allowed us to perform pharmacological experiments concerning the evaluation of mechanisms in the background of vasodilation.

One of the most interesting findings of our work was that PACAP38-induced vasodilation was extremely indomethacin sensitive, in contrast to the effect of PACAP27. Vasodilation to  $10^{-8}$  M VIP resembled the vasodilator effect of PACAP38 characterized by indomethacin-sensitivity. In contrast, at  $10^{-7}$ - $10^{-6}$  M VIP concentrations, the response was unaffected by COX-inhibition, similar to PACAP27 (Table 1). Correspondingly, VIP dilates the isolated porcine ophthalmic artery via COX-mediated mechanisms only at lower ( $10^{-10}$ - $10^{-9}$  M), but not at higher ( $10^{-8}$ - $10^{-7}$  M) VIP concentrations (Vincent 1992). VIP-induced pial arteriolar vasodilation is also indomethacin-sensitive in cats (Wei et al. 1980), whereas we are not aware of studies where PACAP-induced cerebrovascular dilation was reduced by COX-inhibition.

By dissecting the role of the two COX isoforms in the PACAP38- and VIP-induced response using selective COX-1 and COX-2 inhibitors we found that administration of SC-560 virtually abolished the PACAP38-, as well as the low-dose VIP-elicited arteriolar dilation, while these vascular reactions remained unchanged after NS-398 treatment. These data suggest that COX-1 derived prostanoids are substantially involved in PACAP38 dependent, and only partially involved in VIP-dependent vascular caliber changes, while the role of the COX-2 enzyme seems to be ancillary. The importance of COX-derived prostanoids in the cerebrovascular control of newborns is well established, however, there are many open questions related to the functional significance of the two distinct COX isoforms. In the piglet brain, COX-2 is the dominant constitutive enzyme. Unlike COX-1, COX-2 mRNA is expressed abundantly in the cerebral cortex and microvasculature, and this isoform comprises the main part of the total COX protein level here. Moreover, data show that COX-2 contributes approximately 80–90% to the central nervous system prostanoid levels in the newborn; as NS-398, a specific COX-2 inhibitor, produces great decrease in the overall prostanoid synthesis during both basal and certain stimulated conditions. The remnant 10-20% is attributed to the activity of the COX-1 enzyme (Peri et al. 1995; Parfenova et al. 1997). In concert with these observations, other functional studies found that COX-dependent vascular reactions (such as hypotension-induced vasodilation or Ach-induced vasoconstriction) appear to depend largely on COX-2 activity (Domoki et al. 2005b), while to the best of our knowledge our studies are the first to demonstrate COX-1 dependent vasoactive mechanisms in the pial vasculature of piglets.

Surprisingly, the involvement of NOS activity in PACAP- and VIP-induced vasodilation has not been supported by our experiments with L-NAME, despite numerous anatomical and functional studies suggesting otherwise in other species. In the human and rat parasympathetic and trigeminal ganglia high numbers of nerve cell bodies containing both NOS and PACAP were visualized by fluorescent immunohistochemistry (Tajti et al., 1999; Uddman et al., 1999; Edvinsson et al., 2001). Activation of nitrenergic neurons, at least in part, plays a role in the PACAP-induced vasorelaxant mechanism in isolated rat basilar arteries (Seebeck et al., 2002). The inhibition of NO production results in tachyphylaxis to PACAP27 in rat mesenteric and abdominal aortic artery (Whalen et al., 1999). Involvement of NOS activity in VIP-induced vasodilation was also reported in a number of adult animal models (Gaw et al. 1991; Grant et al. 2006).

However, there may be significant differences between the cerebrovascular regulatory processes seen in newborns and in adults. In the piglet, several former studies demonstrated marked age-related changes in the cerebrovascular regulation, predominantly in the NO-mediated responsiveness. The expression and activity of NOS in newborn and adult pigs are different: NOS enzyme level as well as NOS activity increases during maturation, while e.g. the COX expression and activity remain constant (Parfenova et al. 2000). This phenomenon results in the predominance of prostanoids over NO in the newborn period and gives a possible explanation of our results. Since neither COX nor NOS inhibition had any effect on PACAP27 ( $10^{-8}$ - $10^{-6}$  M) and VIP ( $10^{-7}$ - $10^{-6}$  M)-induced vasodilation, PACAP27 and VIP probably exert a robust direct effect on vascular smooth muscle cells at these doses.

In any pharmacological study, the specificity of the used drugs limits the interpretation of the results. Indomethacin at the used dose was found to be a strong inhibitor of the prostanoid synthesis in many experiments in which its ability to block either COX-1 or COX-2 dependent mechanisms was investigated. The dose of NS-398 was chosen for similar reasons since it proved to be a selective and effective inhibitor of the COX-2 enzyme (Domoki et al. 2005b). The COX-1 activity was targeted with SC-560. In piglets, our laboratory was the first (and to the best of our knowledge the only) where SC-560 was used *in vivo* (Domoki et al. 2005b), though in that series of experiments none of the assessed vascular reactions appeared to be COX-1 dependent. On the other hand, other investigators found this dose effective in blocking the COX-1-mediated prostaglandin production in the rat brain, even after COX-1 gene transfer (Lin et al. 2002). However, SC-560 is known to act, at least in some cell types, as an unselective COX enzyme blocker (Brenneis et al. 2006). In our model, PACAP38 may act on a particular cell type in which SC-560 has the potential to

block both COX-1 and COX-2, such as indomethacin. Nonetheless it seems to be more likely that PACAP38 (and VIP at low concentration) uniquely activates COX-1 present in small amounts inducing the synthesis of such vasodilator prostanoids as prostacyclin or PGE<sub>2</sub> that stimulates cAMP formation by VSM resulting in vasodilation (Parfenova et al. 1995). The effectiveness of PLA<sub>2</sub>-inhibition on PACAP38-induced caliber changes seems to support this concept as well. The selected dose of L-NAME (15 mg/kg iv) was also reported to provide appropriate NOS inhibition in former studies (Lacza et al. 2002).

	PACAP38 10 <sup>-8</sup> -10 <sup>-6</sup> M	PACAP27 10 <sup>-8</sup> -10 <sup>-6</sup> M	VIP 10 <sup>-8</sup> M	VIP 10 <sup>-7</sup> -10 <sup>-6</sup> M
COX-1	+	-	+	-
COX-2	-	-	-	-
NOS	-	-	-	-

**Table 1. Summary of the effects of different cyclooxygenase (COX) and nitric oxide synthase (NOS) enzyme inhibitors on PACAP38-, PACAP27-, and VIP-induced vasodilation.** The non-selective COX-inhibitor indomethacin and the selective COX-1-inhibitor SC-560 had remarkable inhibitory effect on PACAP38-induced vasodilation, and partially, on VIP-induced vasodilation. COX-2 inhibition by NS-398 or non-selective NOS-inhibition by L-NAME proved to be ineffective.

The short PACAP sequences used in our experiments provide novel insight into the mechanism of PACAP- and VIP-induced vascular changes since their role has not been investigated in the cerebral circulation before. PACAP6-38 and PACAP6-27 were established as competitive VIP/PACAP receptor antagonists in other experimental models (Harmar et al. 1998; Vaudry et al. 2000b). Our knowledge is limited on the degradation of PACAP *in vivo*, but PACAP6-27 can be an important endogenous inhibitor providing negative feedback since PACAP27 is rapidly degraded by dipeptidylpeptidase IV to produce PACAP6-27 among other metabolites *in vitro* (Green et al. 2006).

The VIP/PACAP receptor antagonist PACAP fragments (PACAP6-38 and PACAP6-27) inhibited both PACAP38- and PACAP27-induced vasodilation, supporting

previous results; however, in our present study, only PACAP6-38 but not PACAP6-27 attenuated VIP-induced vasodilation. PACAP6-38, unlike PACAP6-27, is often reported to be a selective blocker of the PAC1 receptor group (Edvinsson and Krause 2002), while others identify PACAP6-38 as a nonspecific VIP/PACAP antagonist (Harmar et al. 1998). Possibly, PACAP6-38 simply is a more potent inhibitor than PACAP6-27 on the VIP/PACAP receptors involved in VIP-induced vasodilation. Unfortunately, we are unaware of the existence of any widely accepted, subtype-selective VIP/PACAP receptor blockers that would be required to unveil the functional relevance of each receptor subtype in cerebrovascular and neuroprotective effects of these neuropeptides (Edvinsson and Krause 2002). The other short fragments (PACAP1-15, PACAP6-15, and PACAP20-31) were designed only for mapping purposes, given that a short fragment is a much better starting compound for any further pharmacological development. These short PACAP fragments did not show any vasoactivity or inhibitory effects suggesting that they were not capable of sufficient binding to the VPAC/PACAP receptors.

Considering the discrepancy between the probable signal transduction pathways related to PACAP isoforms and VIP, it seems feasible that these related peptides act on different cells or different subtypes of the PACAP/VIP receptor group. Currently this field is hampered by the lack of potent, subtype selective blockers necessary to reveal the functional relevance of VIP/PACAP receptor subtypes in the newborn cerebral circulation.

A remarkable, novel finding of our studies is that PACAP and VIP exert a protective effect on the functional integrity of the neurovascular unit after I/R, adding a new mechanism of action to the diversity of neuroprotective mechanisms, fully consistent with the multiple pathways that are activated or inhibited by these peptides.

In the piglet, hypercapnia evokes cerebral vasodilation not only *in vivo* (Leffler et al. 1989a), but also in isolated, denervated vessels (Kokubun et al. 2009). Hypercapnia-induced vasodilation requires intact endothelium; however, the function of neuronal and glial components in this vascular response seems to be unnecessary. Based on these findings, I/R seems to reduce hypercapnia-induced dilation of pial arterioles through endothelial damage in piglets. Our data show that PACAP38, PACAP27, and VIP pretreatments are all capable to preserve hypercapnia-induced vasodilation after I/R even in non-vasoactive doses, indicating a decreased/shortened postischemic endothelial dysfunction by PACAP or VIP pretreatment. We are not aware of any studies in which similar protective effects of PACAP and VIP have been demonstrated on the cerebrovascular endothelium. Our results are in agreement with the findings of Lange et al., who demonstrated both the synthesis of VIP and the expression of

VIP receptor associated protein in microvascular endothelial cells of pial vessels in piglets (Lange et al. 1999), allowing a direct protective effect of both VIP and PACAP. The function of endothelial VIP production/effects is unclear, but an autocrine growth factor role involved in postnatal endothelial cell differentiation has been suggested. The exact mechanism of endothelial protection by PACAP and VIP is unclear and its exploration demands further experiments.

The mechanism of NMDA-induced vasodilation has been extensively studied in numerous models including the newborn pig. NMDA-induced vasodilation is considered to be a neuronal-triggered multi-step process involving the activation of nNOS and production of NO that diffuses to the vascular smooth muscle, causing dilation of the pial arterioles (Meng et al. 1995; Domoki et al. 2002). The response has been shown to be susceptible to I/R as well as to asphyxia (Bari et al. 1996a; Busija et al. 1996a); however, the pathomechanism of this decreased neuronal-vascular responsiveness is only partly understood. Most probably, the increased ROS-production-induced NMDA-receptor dysfunction is responsible for the altered CR to NMDA. This notion is supported by experiments where ROS scavengers like superoxide dismutase and oxypurinol preserved the pial arteriolar responsiveness to NMDA (Busija and Wei 1993; Bari et al. 1996a). NMDA-induced vasodilation was also unaltered when piglets were pretreated with indomethacin that further supports this presumption, since COX has been revealed as the main source of superoxide anions after I/R in the piglet (Domoki et al. 2001). Our present study clearly demonstrated that PACAP27 and PACAP38, but not VIP preserved CR to NMDA after I/R. This preservation seemed to be independent of the increase in cerebral blood flow mediated by vasodilation to PACAP38 or PACAP27. In fact, PACAP isoforms effectively preserved NMDA-induced vasodilation in non-vasoactive doses, whereas VIP was ineffective in even an equimolar, vasoactive dose. The reported antioxidant property of PACAP can be an important factor in the preservation of the NMDA-receptor function, especially that an analogous antioxidant capacity of VIP is absent (Reglodi et al. 2004a). The PACAP-induced initiation of anti-apoptotic and anti-inflammatory mechanisms may also lead to increased general viability of the neurons. More specifically, PAC1 receptor stimulation leads to activation of Bcl-2/inhibition of Bad resulting in enhanced mitochondrial integrity/decreased release of apoptotic cytochrom c. Conceivably, preservation of mitochondrial function fastens the restoration of cellular ATP levels and also reduces postischemic ROS production. PACAP binding sites in the rat cerebral cortex are ten times more numerous as compared to VIP; this difference may also explain in part the higher general neuroprotective potency of PACAP (Masuo et al. 1991). Alternatively, PACAP

isoforms may act on PAC1 receptors expressed on a specific population of cortical neurons critically involved in NMDA-induced vasodilation. Furthermore, the differences observed between PACAP and VIP can be due to different signal transduction mechanisms coupled to PACAP and/or VIP-stimulated receptors in these cells.

Two recent works have challenged the concept that glutamate- or NMDA-mediated dilator responses involve sequential stimulation of neuronal NMDA receptors, activation of nNOS in associated neurons, and direct relaxation of vascular smooth muscle by NO. First, Ayata and Moskowitz (Ayata and Moskowitz 2006) have reported that NMDA application elicits CSD which complicates the elucidation of mechanism involved in NMDA-induced cerebral vasodilation. Second, our laboratory had very similar observations in rats, although with detailed analysis of the blood flow response to NMDA, we found both CSD-dependent and CSD-independent components (Lenti et al. 2009).

In our present work, we examined the effect of NMDA on CoBF in newborn pigs since NMDA-induced cortical hyperemia had not yet been studied in this species, although NMDA-induced pial arteriolar vasodilation had been extensively studied. The integrated CoBF responses to NMDA confirmed earlier results concerning the dose-dependent nature of microvascular responses to NMDA based upon measurements of pial arteriolar diameters in our model (Busija and Leffler 1989). Our results also provided novel evidence that the hemodynamic changes in response to NMDA were not consequences of CSD-related events in piglets, but rather represented a direct, specific effect on cortical neurons in this important experimental model (Busija et al. 2007). In contrast, cerebral vascular responses to NMDA are confounded by CSD-related hyperemia in mice (Ayata and Moskowitz 2006) and rats (Lenti et al. 2009). While CSD has been shown to occur in the mature brains of all mammalian species studied, the neonatal brain appears to be relatively resistant to generation of CSD. For example, the capacity of the developing cerebral cortex to respond to certain stimuli with CSD appears first between 24 and 30 days after birth in rabbits (Schade 1959) and some days earlier in the rat (Richter et al. 1998). This period is characterized by an increase in the neuron size and by branching of the axons (Schade 1959). However, morphological and structural state of the CNS may not adequately explain the decreased susceptibility of the immature brain to generating CSDs. The development of sodium and chloride channels such as the different expression of the NMDA receptor subunits in newborn and adult animals can be responsible for this phenomenon observed in the examined species (Takahashi 2005). In the piglet, we have not been able to induce CSD even with mechanical means (pinpricks) during the first days of life. Moreover, previous results of our

laboratory showed that locally applied, concentrated KCl solution also failed to evoke CSD (Domoki et al. 1999). The elevated threshold or incapability of the piglet brain to produce CSDs is probably due to this immaturity of the nervous system, rather than to a difference between species or differences between the lissencephalic (mouse, rat, rabbit) and gyrencephalic (piglet, cat) brains, since CSD apparently can be evoked in the adult swine (Bowyer et al. 1999) and cats (Van Harreveld et al. 1956).

## **Conclusions**

In conclusion, PACAP and VIP are powerful vasodilators in the newborn cerebral circulation. The actions of PACAP38, PACAP27, and VIP seem to be independent of NOS activation, but the COX-1 activity appears to be essential for PACAP38-induced vasodilation. The presumed activation of COX-1 by PACAP38 and the low dose of VIP is unique, since COX-2 is known to be the predominant COX isoform in the newborn central nervous system, and SC-560 did not affect the vasodilation in response to any other COX-dependent stimuli studied so far.

PACAP38, PACAP27, and VIP all protected postischemic vascular reactivity to hypercapnia, an ischemia-sensitive indicator of endothelial function, in the newborn pig. However, PACAP isoforms but not VIP preserved the NMDA-induced neuron-dependent vasodilation. Although PACAP27, PACAP38, and VIP all induce dose-dependent pial arteriolar dilation, the protective effects of these neuropeptides were independent of their vasoactivity. This neurovascular protection probably supports the restoration of adequate perfusion of the brain tissue after I/R likely enhancing the direct neuroprotective effects of PACAP and VIP.

NMDA elicited dose-dependent CoBF increases in piglets, similarly to previous data obtained by pial arteriolar diameter measurements. In piglets, the CoBF increase/pial arteriolar dilation seems to be solely the result of the local activation of neuronal NMDA receptors without the confounding effect of CSDs, unlike in adult rodents.

Our results indicate that PACAP and VIP are markedly different in their respective vasodilator and protective actions, indicating that these neuropeptides may activate only partially overlapping mechanisms. Nevertheless, the protection of CR provided by PACAP and VIP may form the foundation of new neuroprotective strategies in the treatment of neonatal stroke.

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## References

- Anzai M, Suzuki Y, Takayasu M, Kajita Y, Mori Y, Seki Y, Saito K, Shibuya M (1995) Vasorelaxant effect of PACAP-27 on canine cerebral arteries and rat intracerebral arterioles. *Eur J Pharmacol* 285: 173-179
- Arimura A, Shioda S (1995) Pituitary adenylate cyclase activating polypeptide (PACAP) and its receptors: neuroendocrine and endocrine interaction. *Front Neuroendocrinol* 16: 53-88
- Arimura A, Somogyvari-Vigh A, Miyata A, Mizuno K, Coy DH, Kitada C (1991) Tissue distribution of PACAP as determined by RIA: highly abundant in the rat brain and testes. *Endocrinology* 129: 2787-2789
- Armstead WM (1995) Relationship between opioids and activation of phospholipase C and protein kinase C in brain injury induced pial artery vasoconstriction. *Brain Res* 689: 183-188
- Armstead WM (1997) Role of impaired cAMP and calcium-sensitive K<sup>+</sup> channel function in altered cerebral hemodynamics following brain injury. *Brain Res* 768: 177-184
- Armstead WM (2001) Age dependent endothelin contribution to NOC/oFQ induced impairment of NMDA cerebrovasodilation after brain injury. *Peptides* 22: 39-46
- Armstead WM (2004) NMDA and age dependent cerebral hemodynamics after traumatic brain injury. *Exp Toxicol Pathol* 56: 75-81
- Ayata C, Moskowitz MA (2006) Cortical spreading depression confounds concentration-dependent pial arteriolar dilation during N-methyl-D-aspartate superfusion. *Am J Physiol Heart Circ Physiol* 290: H1837-1841
- Banks WA, Uchida D, Arimura A, Somogyvari-Vigh A, Shioda S (1996) Transport of pituitary adenylate cyclase-activating polypeptide across the blood-brain barrier and the prevention of ischemia-induced death of hippocampal neurons. *Ann N Y Acad Sci* 805: 270-277; discussion 277-279
- Bari F, Errico RA, Louis TM, Busija DW (1996a) Differential effects of short-term hypoxia and hypercapnia on N-methyl-D-aspartate-induced cerebral vasodilatation in piglets. *Stroke* 27: 1634-1639; discussion 1639-1640
- Bari F, Errico RA, Louis TM, Busija DW (1996b) Interaction between ATP-sensitive K<sup>+</sup> channels and nitric oxide on pial arterioles in piglets. *J Cereb Blood Flow Metab* 16: 1158-1164
- Bari F, Louis TM, Meng W, Busija DW (1996c) Global ischemia impairs ATP-sensitive K<sup>+</sup> channel function in cerebral arterioles in piglets. *Stroke* 27: 1874-1880; discussion 1880-1871
- Bari F, Thore CR, Louis TM, Busija DW (1998) Inhibitory effects of hypoxia and adenosine on N-methyl-D-aspartate-induced pial arteriolar dilation in piglets. *Brain Res* 780: 237-244
- Beasley TC, Bari F, Thore C, Thrikawala N, Louis T, Busija D (1998) Cerebral ischemia/reperfusion increases endothelial nitric oxide synthase levels by an indomethacin-sensitive mechanism. *J Cereb Blood Flow Metab* 18: 88-96
- Ben-Haim G, Armstead WM (2000) Role of cAMP and K<sup>(+)</sup> channel-dependent mechanisms in piglet hypoxic/ischemic impaired nociceptin/orphanin FQ-induced cerebrovasodilation. *Brain Res* 884: 51-58
- Benagiano V, Virgintino D, Maiorano E, Rizzi A, Palombo S, Roncali L, Ambrosi G (1996) VIP-like immunoreactivity within neurons and perivascular neuronal processes of the human cerebral cortex. *Eur J Histochem* 40: 53-56

- Bonvento G, Sibson N, Pellerin L (2002) Does glutamate image your thoughts? *Trends Neurosci* 25: 359-364
- Book SA, Bustad LK (1974) The fetal and neonatal pig in biomedical research. *J Anim Sci* 38: 997-1002
- Bowyer SM, Tepley N, Papuashvili N, Kato S, Barkley GL, Welch KM, Okada YC (1999) Analysis of MEG signals of spreading cortical depression with propagation constrained to a rectangular cortical strip. II. Gyrencephalic swine model. *Brain Res* 843: 79-86
- Brenneis C, Maier TJ, Schmidt R, Hofacker A, Zulauf L, Jakobsson PJ, Scholich K, Geisslinger G (2006) Inhibition of prostaglandin E2 synthesis by SC-560 is independent of cyclooxygenase 1 inhibition. *Faseb J* 20: 1352-1360
- Brenneman DE (2007) Neuroprotection: a comparative view of vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Peptides* 28: 1720-1726
- Busija DW (1994) Cerebral circulation of the fetus and newborn. In: Bevan JA, Bevan RD (eds) *The human brain circulation*. Humana Press, Totowa, New Jersey, pp 259-269
- Busija DW, Bari F, Domoki F, Horiguchi T, Shimizu K (2008) Mechanisms involved in the cerebrovascular dilator effects of cortical spreading depression. *Prog Neurobiol* 86: 379-395
- Busija DW, Bari F, Domoki F, Louis T (2007) Mechanisms involved in the cerebrovascular dilator effects of N-methyl-D-aspartate in cerebral cortex. *Brain Res Rev* 56: 89-100
- Busija DW, Heistad DD, Marcus ML (1981) Continuous measurement of cerebral blood flow in anesthetized cats and dogs. *Am J Physiol* 241: H228-234
- Busija DW, Leffler CW (1989) Dilator effects of amino acid neurotransmitters on piglet pial arterioles. *Am J Physiol* 257: H1200-1203
- Busija DW, Leffler CW, Wagerle LC (1985) Responses of newborn pig pial arteries to sympathetic nervous stimulation and exogenous norepinephrine. *Pediatr Res* 19: 1210-1214
- Busija DW, Marcus ML, Heistad DD (1982) Pial artery diameter and blood flow velocity during sympathetic stimulation in cats. *J Cereb Blood Flow Metab* 2: 363-367
- Busija DW, Meng W, Bari F, McGough PS, Errico RA, Tobin JR, Louis TM (1996a) Effects of ischemia on cerebrovascular responses to N-methyl-D-aspartate in piglets. *Am J Physiol* 270: H1225-1230
- Busija DW, Thore C, Beasley T, Bari F (1996b) Induction of cyclooxygenase-2 following anoxic stress in piglet cerebral arteries. *Microcirculation* 3: 379-386
- Busija DW, Wei M (1993) Altered cerebrovascular responsiveness to N-methyl-D-aspartate after asphyxia in piglets. *Am J Physiol* 265: H389-394
- Chen WH, Tzeng SF (2005) Pituitary adenylate cyclase-activating polypeptide prevents cell death in the spinal cord with traumatic injury. *Neurosci Lett* 384: 117-121
- Choi YB, Tenneti L, Le DA, Ortiz J, Bai G, Chen HS, Lipton SA (2000) Molecular basis of NMDA receptor-coupled ion channel modulation by S-nitrosylation. *Nat Neurosci* 3: 15-21
- Degi R, Bari F, Beasley TC, Thrikawala N, Thore C, Louis TM, Busija DW (1998a) Regional distribution of prostaglandin H synthase-2 and neuronal nitric oxide synthase in piglet brain. *Pediatr Res* 43: 683-689
- Degi R, Bari F, Thrikawala N, Beasley TC, Thore C, Louis TM, Busija DW (1998b) Effects of anoxic stress on prostaglandin H synthase isoforms in piglet brain. *Brain Res Dev Brain Res* 107: 265-276

- Dejda A, Gendek-Kubiak H, Nowak JZ (2004) Effect of different forms of vasoactive intestinal peptide on cAMP formation in the mammalian cerebral cortex. *Pol J Pharmacol* 56: 421-425
- Dejda A, Sokolowska P, Nowak JZ (2005) Neuroprotective potential of three neuropeptides PACAP, VIP and PHI. *Pharmacol Rep* 57: 307-320
- DeRoth L, Downie HG (1978) Basic cardiovascular parameters in the underweight neonatal swine. *Biol Neonate* 34: 155-160
- Dickson L, Finlayson K (2009) VPAC and PAC receptors: From ligands to function. *Pharmacol Ther* 121: 294-316
- Domoki F, Kis B, Gaspar T, Bari F, Busija DW (2008) Cerebromicrovascular endothelial cells are resistant to L-glutamate. *Am J Physiol Regul Integr Comp Physiol* 295: R1099-1108
- Domoki F, Kis B, Nagy K, Farkas E, Busija DW, Bari F (2005a) Diazoxide preserves hypercapnia-induced arteriolar vasodilation after global cerebral ischemia in piglets. *Am J Physiol Heart Circ Physiol* 289: H368-373
- Domoki F, Nagy K, Temesvari P, Bari F (2005b) Selective inhibitors differentially affect cyclooxygenase-dependent pial arteriolar responses in newborn pigs. *Pediatr Res* 57: 853-857
- Domoki F, Perciaccante JV, Puskar M, Bari F, Busija DW (2001) Cyclooxygenase-2 inhibitor NS398 preserves neuronal function after hypoxia/ischemia in piglets. *Neuroreport* 12: 4065-4068
- Domoki F, Perciaccante JV, Shimizu K, Puskar M, Busija DW, Bari F (2002) N-methyl-D-aspartate-induced vasodilation is mediated by endothelium-independent nitric oxide release in piglets. *Am J Physiol Heart Circ Physiol* 282: H1404-1409
- Domoki F, Veltkamp R, Bari F, Louis TM, Busija DW (1999) Cerebrovascular reactivity remains intact after cortical depolarization in newborn piglets. *Pediatr Res* 45: 834-837
- Drake CT, Iadecola C (2007) The role of neuronal signaling in controlling cerebral blood flow. *Brain Lang* 102: 141-152
- Edvinsson L, Ekman R (1984) Distribution and dilatory effect of vasoactive intestinal polypeptide (VIP) in human cerebral arteries. *Peptides* 5: 329-331
- Edvinsson L, Elsas T, Suzuki N, Shimizu T, Lee TJ (2001) Origin and Co-localization of nitric oxide synthase, CGRP, PACAP, and VIP in the cerebral circulation of the rat. *Microsc Res Tech* 53: 221-228
- Edvinsson L, Krause DN (2002) *Cerebral Blood Flow and Metabolism*. . Lippincott Williams and Wilkins, Philadelphia, USA.
- Fahrenkrug J (1993) Transmitter role of vasoactive intestinal peptide. *Pharmacol Toxicol* 72: 354-363
- Fahrenkrug J, Hannibal J, Tams J, Georg B (2000) Immunohistochemical localization of the VIP1 receptor (VPAC1R) in rat cerebral blood vessels: relation to PACAP and VIP containing nerves. *J Cereb Blood Flow Metab* 20: 1205-1214
- Faraci FM, Breese KR (1993) Nitric oxide mediates vasodilatation in response to activation of N-methyl-D-aspartate receptors in brain. *Circ Res* 72: 476-480
- Faraci FM, Breese KR (1994) Dilatation of cerebral arterioles in response to N-methyl-D-aspartate: role of CGRP and acetylcholine. *Brain Res* 640: 93-97

- Faraci FM, Brian JE, Jr. (1995) 7-Nitroindazole inhibits brain nitric oxide synthase and cerebral vasodilatation in response to N-methyl-D-aspartate. *Stroke* 26: 2172-2175; discussion 2176
- Faraci FM, Heistad DD (1994) Responses of cerebral arterioles to N-methyl-D-aspartate and activation of ATP-sensitive potassium channels in old rats. *Brain Res* 654: 349-351
- Farkas O, Tamas A, Zsombok A, Reglodi D, Pal J, Buki A, Lengvari I, Povlishock JT, Doczi T (2004) Effects of pituitary adenylate cyclase activating polypeptide in a rat model of traumatic brain injury. *Regul Pept* 123: 69-75
- Fellin T, Sul JY, D'Ascenzo M, Takano H, Pascual O, Haydon PG (2006) Bidirectional astrocyte-neuron communication: the many roles of glutamate and ATP. *Novartis Found Symp* 276: 208-217; discussion 217-221, 233-207, 275-281
- Fergus A, Lee KS (1997) Regulation of cerebral microvessels by glutamatergic mechanisms. *Brain Res* 754: 35-45
- Garthwaite J (1991) Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neurosci* 14: 60-67
- Gaw AJ, Aberdeen J, Humphrey PP, Wadsworth RM, Burnstock G (1991) Relaxation of sheep cerebral arteries by vasoactive intestinal polypeptide and neurogenic stimulation: inhibition by L-NG-monomethyl arginine in endothelium-denuded vessels. *Br J Pharmacol* 102: 567-572
- Ghatei MA, Takahashi K, Suzuki Y, Gardiner J, Jones PM, Bloom SR (1993) Distribution, molecular characterization of pituitary adenylate cyclase-activating polypeptide and its precursor encoding messenger RNA in human and rat tissues. *J Endocrinol* 136: 159-166
- Gill R, Andine P, Hillered L, Persson L, Hagberg H (1992) The effect of MK-801 on cortical spreading depression in the penumbral zone following focal ischaemia in the rat. *J Cereb Blood Flow Metab* 12: 371-379
- Gillardon F, Hata R, Hossmann KA (1998) Delayed up-regulation of Zac1 and PACAP type I receptor after transient focal cerebral ischemia in mice. *Brain Res Mol Brain Res* 61: 207-210
- Girouard H, Iadecola C (2006) Neurovascular coupling in the normal brain and in hypertension, stroke, and Alzheimer disease. *J Appl Physiol* 100: 328-335
- Gonzalez C, Barroso C, Martin C, Gulbenkian S, Estrada C (1997) Neuronal nitric oxide synthase activation by vasoactive intestinal peptide in bovine cerebral arteries. *J Cereb Blood Flow Metab* 17: 977-984
- Grant S, Lutz EM, McPhaden AR, Wadsworth RM (2006) Location and function of VPAC1, VPAC2 and NPR-C receptors in VIP-induced vasodilation of porcine basilar arteries. *J Cereb Blood Flow Metab* 26: 58-67
- Green BD, Irwin N, Flatt PR (2006) Pituitary adenylate cyclase-activating peptide (PACAP): assessment of dipeptidyl peptidase IV degradation, insulin-releasing activity and antidiabetic potential. *Peptides* 27: 1349-1358
- Gressens P (1999) VIP neuroprotection against excitotoxic lesions of the developing mouse brain. *Ann N Y Acad Sci* 897: 109-124
- Guerguerian AM, Brambrink AM, Traystman RJ, Haganir RL, Martin LJ (2002) Altered expression and phosphorylation of N-methyl-D-aspartate receptors in piglet striatum after hypoxia-ischemia. *Brain Res Mol Brain Res* 104: 66-80

- Gulbenkian S, Uddman R, Edvinsson L (2001) Neuronal messengers in the human cerebral circulation. *Peptides* 22: 995-1007
- Hannibal J (2002) Pituitary adenylate cyclase-activating peptide in the rat central nervous system: an immunohistochemical and in situ hybridization study. *J Comp Neurol* 453: 389-417
- Hardebo JE, Wieloch T, Kahrstrom J (1989) Excitatory amino acids and cerebrovascular tone. *Acta Physiol Scand* 136: 483-485
- Harmar AJ (2003) An essential role for peptidergic signalling in the control of circadian rhythms in the suprachiasmatic nuclei. *J Neuroendocrinol* 15: 335-338
- Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR, Rawlings SR, Robberecht P, Said SI, Sreedharan SP, Wank SA, Waschek JA (1998) International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Pharmacol Rev* 50: 265-270
- Henning RJ, Sawmiller DR (2001) Vasoactive intestinal peptide: cardiovascular effects. *Cardiovasc Res* 49: 27-37
- Hossmann KA (1997) Reperfusion of the brain after global ischemia: hemodynamic disturbances. *Shock* 8: 95-101; discussion 102-103
- Hsu P, Shibata M, Leffler CW (1993) Prostanoid synthesis in response to high CO<sub>2</sub> in newborn pig brain microvascular endothelial cells. *Am J Physiol* 264: H1485-1492
- Illiff JJ, D'Ambrosio R, Ngai AC, Winn HR (2003) Adenosine receptors mediate glutamate-evoked arteriolar dilation in the rat cerebral cortex. *Am J Physiol Heart Circ Physiol* 284: H1631-1637
- Jakobsson A, Nilsson GE (1993) Prediction of sampling depth and photon pathlength in laser Doppler flowmetry. *Med Biol Eng Comput* 31: 301-307
- Joo KM, Chung YH, Kim MK, Nam RH, Lee BL, Lee KH, Cha CI (2004) Distribution of vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide receptors (VPAC1, VPAC2, and PAC1 receptor) in the rat brain. *J Comp Neurol* 476: 388-413
- Kastner A, Bruch L, Will-Shahab L, Modersohn D, Baumann G (1995) Pituitary adenylate cyclase activating peptides are endothelium-independent dilators of human and porcine coronary arteries. *Agents Actions Suppl* 45: 283-289
- Kis B, Gaspar T, Mezei Z, Gecse A, Telegdy G (1999a) Pituitary adenylate cyclase-activating polypeptide inhibits the cyclooxygenase pathway of rat cerebral microvessels. *Acta Physiol Scand* 167: 43-47
- Kis B, Mezei Z, Dancso G, Pataricza J, Gecse A, Papp JG, Telegdy G (1999b) Effects of pituitary adenylate cyclase-activating polypeptide on the cyclooxygenase pathway of rat platelets and on platelet aggregation. *Prostaglandins Other Lipid Mediat* 58: 103-112
- Kitahara Y, Taga K, Abe H, Shimoji K (2001) The effects of anesthetics on cortical spreading depression elicitation and c-fos expression in rats. *J Neurosurg Anesthesiol* 13: 26-32
- Knutsson M, Edvinsson L (2002) Distribution of mRNA for VIP and PACAP receptors in human cerebral arteries and cranial ganglia. *Neuroreport* 13: 507-509
- Kobayashi H, Uezono Y, Ueno S, Izumi F (1994) Pituitary adenylate cyclase-activating polypeptides (PACAPs) increase cAMP in rat cerebral microvessels. *Brain Res* 647: 145-147

- Koehler RC, Roman RJ, Harder DR (2009) Astrocytes and the regulation of cerebral blood flow. *Trends Neurosci* 32: 160-169
- Kogure K, Busto R, Schwartzman RJ, Scheinberg P (1980) The dissociation of cerebral blood flow, metabolism, and function in the early stages of developing cerebral infarction. *Ann Neurol* 8: 278-290
- Kokubun S, Fukuda S, Shimoji K, Sakamoto H, Gamou S, Ogura M, Yunokawa S, Morita S (2009) Differential responses of porcine anterior spinal and middle cerebral arteries to carbon dioxide and pH. *Crit Care Med* 37: 987-992
- Kovesdi E, Tamas A, Reglodi D, Farkas O, Pal J, Toth G, Bukovics P, Doczi T, Buki A (2008) Posttraumatic administration of pituitary adenylate cyclase activating polypeptide in central fluid percussion injury in rats. *Neurotox Res* 13: 71-78
- Kudo C, Nozari A, Moskowitz MA, Ayata C (2008) The impact of anesthetics and hyperoxia on cortical spreading depression. *Exp Neurol* 212: 201-206
- Kulik T, Kusano Y, Aronhime S, Sandler AL, Winn HR (2008) Regulation of cerebral vasculature in normal and ischemic brain. *Neuropharmacology* 55: 281-288
- Laburthe M, Couvineau A (2002) Molecular pharmacology and structure of VPAC Receptors for VIP and PACAP. *Regul Pept* 108: 165-173
- Laburthe M, Couvineau A, Marie JC (2002) VPAC receptors for VIP and PACAP. *Receptors Channels* 8: 137-153
- Lacza Z, Puskar M, Kis B, Perciaccante JV, Miller AW, Busija DW (2002) Hydrogen peroxide acts as an EDHF in the piglet pial vasculature in response to bradykinin. *Am J Physiol Heart Circ Physiol* 283: H406-411
- Lange D, Funa K, Ishisaki A, Bauer R, Wollina U (1999) Autocrine endothelial regulation in brain stem vessels of newborn piglets. *Histol Histopathol* 14: 821-825
- Lauritzen M, Hansen AJ (1992) The effect of glutamate receptor blockade on anoxic depolarization and cortical spreading depression. *J Cereb Blood Flow Metab* 12: 223-229
- Lee TJ, Saito A, Berezin I (1984) Vasoactive intestinal polypeptide-like substance: the potential transmitter for cerebral vasodilation. *Science* 224: 898-901
- Leffler CW, Beasley DG, Busija DW (1989a) Cerebral ischemia alters cerebral microvascular reactivity in newborn pigs. *Am J Physiol* 257: H266-271
- Leffler CW, Busija DW, Armstead WM, Mirro R, Beasley DG (1989b) Ischemia alters cerebral vascular responses to hypercapnia and acetylcholine in piglets. *Pediatr Res* 25: 180-183
- Leffler CW, Mirro R, Armstead WM, Shibata M (1992) Topical arachidonic acid restores pial arteriolar dilation to hypercapnia of postischemic newborn pig brain. *Am J Physiol* 263: H746-751
- Leffler CW, Mirro R, Pharris LJ, Shibata M (1994a) Permissive role of prostacyclin in cerebral vasodilation to hypercapnia in newborn pigs. *Am J Physiol* 267: H285-291
- Leffler CW, Mirro R, Shanklin DR, Armstead WM, Shibata M (1994b) Light/dye microvascular injury selectively eliminates hypercapnia-induced pial arteriolar dilation in newborn pigs. *Am J Physiol* 266: H623-630

- Leffler CW, Parfenova H, Fedinec AL, Basuroy S, Tcheranova D (2006) Contributions of astrocytes and CO to pial arteriolar dilation to glutamate in newborn pigs. *Am J Physiol Heart Circ Physiol* 291: H2897-2904
- Lenti L, Domoki F, Gaspar T, Snipes JA, Bari F, Busija DW (2009) N-Methyl-D-Aspartate Induces Cortical Hyperemia through Cortical Spreading Depression-Dependent and -Independent Mechanisms in Rats. *Microcirculation*: 1-11
- Lerma J, Morales M, Vicente MA, Herreras O (1997) Glutamate receptors of the kainate type and synaptic transmission. *Trends Neurosci* 20: 9-12
- Levasseur JE, Wei EP, Raper AJ, Kontos AA, Patterson JL (1975) Detailed description of a cranial window technique for acute and chronic experiments. *Stroke* 6: 308-317
- Lin H, Lin TN, Cheung WM, Nian GM, Tseng PH, Chen SF, Chen JJ, Shyue SK, Liou JY, Wu CW, Wu KK (2002) Cyclooxygenase-1 and bicistronic cyclooxygenase-1/prostacyclin synthase gene transfer protect against ischemic cerebral infarction. *Circulation* 105: 1962-1969
- Lynch JK (2009) Epidemiology and classification of perinatal stroke. *Semin Fetal Neonatal Med*
- Marrannes R, Willems R, De Prins E, Wauquier A (1988) Evidence for a role of the N-methyl-D-aspartate (NMDA) receptor in cortical spreading depression in the rat. *Brain Res* 457: 226-240
- Masuo Y, Ohtaki T, Masuda Y, Nagai Y, Suno M, Tsuda M, Fujino M (1991) Autoradiographic distribution of pituitary adenylate cyclase activating polypeptide (PACAP) binding sites in the rat brain. *Neurosci Lett* 126: 103-106
- McCulloch DA, MacKenzie CJ, Johnson MS, Robertson DN, Holland PJ, Ronaldson E, Lutz EM, Mitchell R (2002) Additional signals from VPAC/PAC family receptors. *Biochem Soc Trans* 30: 441-446
- McDonald JW, Johnston MV, Young AB (1990) Differential ontogenic development of three receptors comprising the NMDA receptor/channel complex in the rat hippocampus. *Exp Neurol* 110: 237-247
- Meng W, Tobin JR, Busija DW (1995) Glutamate-induced cerebral vasodilation is mediated by nitric oxide through N-methyl-D-aspartate receptors. *Stroke* 26: 857-862; discussion 863
- Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, Culler MD, Coy DH (1989) Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochem Biophys Res Commun* 164: 567-574
- Miyata A, Jiang L, Dahl RD, Kitada C, Kubo K, Fujino M, Minamino N, Arimura A (1990) Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP38). *Biochem Biophys Res Commun* 170: 643-648
- Morley P, Small DL, Murray CL, Mealing GA, Poulter MO, Durkin JP, Stanimirovic DB (1998) Evidence that functional glutamate receptors are not expressed on rat or human cerebrovascular endothelial cells. *J Cereb Blood Flow Metab* 18: 396-406
- Morrison JH, Magistretti PJ, Benoit R, Bloom FE (1984) The distribution and morphological characteristics of the intracortical VIP-positive cell: an immunohistochemical analysis. *Brain Res* 292: 269-282
- Mutt V, Said SI (1974) Structure of the porcine vasoactive intestinal octacosapeptide. The amino-acid sequence. Use of kallikrein in its determination. *Eur J Biochem* 42: 581-589
- Palkovits M, Somogyvari-Vigh A, Arimura A (1995) Concentrations of pituitary adenylate cyclase activating polypeptide (PACAP) in human brain nuclei. *Brain Res* 699: 116-120

- Parfenova H, Eidson TH, Leffler CW (1997) Upregulation of COX-2 in cerebral microvascular endothelial cells by smooth muscle cell signals. *Am J Physiol* 273: C277-288
- Parfenova H, Massie V, Leffler CW (2000) Developmental changes in endothelium-derived vasorelaxant factors in cerebral circulation. *Am J Physiol Heart Circ Physiol* 278: H780-788
- Parfenova H, Zuckerman S, Leffler CW (1995) Inhibitory effect of indomethacin on prostacyclin receptor-mediated cerebral vascular responses. *Am J Physiol* 268: H1884-1890
- Pelligrino DA, Wang Q, Koenig HM, Albrecht RF (1995) Role of nitric oxide, adenosine, N-methyl-D-aspartate receptors, and neuronal activation in hypoxia-induced pial arteriolar dilation in rats. *Brain Res* 704: 61-70
- Peri KG, Hardy P, Li DY, Varma DR, Chemtob S (1995) Prostaglandin G/H synthase-2 is a major contributor of brain prostaglandins in the newborn. *J Biol Chem* 270: 24615-24620
- Philip S, Armstead WM (2004) NMDA dilates pial arteries by KATP and Kca channel activation. *Brain Res Bull* 63: 127-131
- Reglodi D, Fabian Z, Tamas A, Lubics A, Szeberenyi J, Alexy T, Toth K, Marton Z, Borsiczky B, Roth E, Szalontay L, Lengvari I (2004a) Effects of PACAP on in vitro and in vivo neuronal cell death, platelet aggregation, and production of reactive oxygen radicals. *Regul Pept* 123: 51-59
- Reglodi D, Lubics A, Tamas A, Szalontay L, Lengvari I (2004b) Pituitary adenylate cyclase activating polypeptide protects dopaminergic neurons and improves behavioral deficits in a rat model of Parkinson's disease. *Behav Brain Res* 151: 303-312
- Reglodi D, Somogyvari-Vigh A, Vigh S, Kozicz T, Arimura A (2000) Delayed systemic administration of PACAP38 is neuroprotective in transient middle cerebral artery occlusion in the rat. *Stroke* 31: 1411-1417
- Reglodi D, Tamas A, Somogyvari-Vigh A, Szanto Z, Kertes E, Lenard L, Arimura A, Lengvari I (2002) Effects of pretreatment with PACAP on the infarct size and functional outcome in rat permanent focal cerebral ischemia. *Peptides* 23: 2227-2234
- Richter F, Bauer R, Lehmenkuhler A, Schaible HG (2008) Spreading depression in the brainstem of the adult rat: electrophysiological parameters and influences on regional brainstem blood flow. *J Cereb Blood Flow Metab* 28: 984-994
- Richter F, Lehmenkuhler A, Fechner R, Manveljan L, Haschke W (1998) Postnatal conditioning for spreading cortical depression in the rat brain. *Brain Res Dev Brain Res* 106: 217-221
- Roy CS, Sherrington CS (1890) On the Regulation of the Blood-supply of the Brain. *J Physiol* 11: 85-158 117
- Said SI, Mutt V (1970) Polypeptide with broad biological activity: isolation from small intestine. *Science* 169: 1217-1218
- Sano H, Miyata A, Sato K, Kaiya H, Matsuo H, Kangawa K (2000) PACAP augments nitric oxide synthesis in rat vascular smooth muscle cells stimulated with IL-1 alpha. *Ann N Y Acad Sci* 921: 415-419
- Schade JP (1959) Maturational aspects of EEG and of spreading depression in rabbit. *J Neurophysiol* 22: 245-257
- Seebeck J, Lowe M, Kruse ML, Schmidt WE, Mehdorn HM, Ziegler A, Hempelmann RG (2002) The vasorelaxant effect of pituitary adenylate cyclase activating polypeptide and vasoactive intestinal polypeptide in isolated rat basilar arteries is partially mediated by activation of nitrergic neurons. *Regul Pept* 107: 115-123



- Seki Y, Suzuki Y, Baskaya MK, Kano T, Saito K, Takayasu M, Shibuya M, Sugita K (1995) The effects of pituitary adenylate cyclase-activating polypeptide on cerebral arteries and vertebral artery blood flow in anesthetized dogs. *Eur J Pharmacol* 275: 259-266
- Sherwood NM, Krueckl SL, McRory JE (2000) The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. *Endocr Rev* 21: 619-670
- Shibata M, Leffler CW, Busija DW (1991) Prostanoids attenuate pial arteriolar dilation induced by cortical spreading depression in rabbits. *Am J Physiol* 261: R828-834
- Simandle SA, Kerr BA, Lacza Z, Eckman DM, Busija DW, Bari F (2005) Piglet pial arteries respond to N-methyl-D-aspartate in vivo but not in vitro. *Microvasc Res* 70: 76-83
- Somogyvari-Vigh A, Pan W, Reglodi D, Kastin AJ, Arimura A (2000) Effect of middle cerebral artery occlusion on the passage of pituitary adenylate cyclase activating polypeptide across the blood-brain barrier in the rat. *Regul Pept* 91: 89-95
- Stumm R, Kolodziej A, Prinz V, Endres M, Wu DF, Holtt V (2007) Pituitary adenylate cyclase-activating polypeptide is up-regulated in cortical pyramidal cells after focal ischemia and protects neurons from mild hypoxic/ischemic damage. *J Neurochem* 103: 1666-1681
- Sun H, Mayhan WG (2005) Sex difference in nitric oxide synthase-dependent dilatation of cerebral arterioles during long-term alcohol consumption. *Alcohol Clin Exp Res* 29: 430-436
- Suzuki Y, McMaster D, Lederis K, Rorstad OP (1984) Characterization of the relaxant effects of vasoactive intestinal peptide (VIP) and PHI on isolated brain arteries. *Brain Res* 322: 9-16
- Tajti J, Uddman R, Edvinsson L (2001) Neuropeptide localization in the "migraine generator" region of the human brainstem. *Cephalalgia* 21: 96-101
- Tajti J, Uddman R, Moller S, Sundler F, Edvinsson L (1999) Messenger molecules and receptor mRNA in the human trigeminal ganglion. *J Auton Nerv Syst* 76: 176-183
- Takahashi T (2005) Postsynaptic receptor mechanisms underlying developmental speeding of synaptic transmission. *Neurosci Res* 53: 229-240
- Tamas A, Reglodi D, Szanto Z, Borsiczky B, Nemeth J, Lengvari I (2002) Comparative neuroprotective effects of preischemic PACAP and VIP administration in permanent occlusion of the middle cerebral artery in rats. *Neuro Endocrinol Lett* 23: 249-254
- Tamas A, Zsombok A, Farkas O, Reglodi D, Pal J, Buki A, Lengvari I, Povlishock JT, Doczi T (2006) Postinjury administration of pituitary adenylate cyclase activating polypeptide (PACAP) attenuates traumatically induced axonal injury in rats. *J Neurotrauma* 23: 686-695
- Tong S, Parfenova H, Shibata M, Zuckerman S, Armstead WM, Leffler CW (1993) Pituitary adenylate cyclase-activating polypeptide dilates cerebral arterioles of newborn pigs. *Proc Soc Exp Biol Med* 203: 343-347
- Uchida D, Arimura A, Somogyvari-Vigh A, Shioda S, Banks WA (1996) Prevention of ischemia-induced death of hippocampal neurons by pituitary adenylate cyclase activating polypeptide. *Brain Res* 736: 280-286
- Uddman R, Goadsby PJ, Jansen I, Edvinsson L (1993) PACAP, a VIP-like peptide: immunohistochemical localization and effect upon cat pial arteries and cerebral blood flow. *J Cereb Blood Flow Metab* 13: 291-297
- Uddman R, Tajti J, Moller S, Sundler F, Edvinsson L (1999) Neuronal messengers and peptide receptors in the human sphenopalatine and otic ganglia. *Brain Res* 826: 193-199

- Van Harreveld A, Stamm JS, Christensen E (1956) Spreading depression in rabbit, cat and monkey. *Am J Physiol* 184: 312-320
- Vaudry D, Gonzalez BJ, Basille M, Fournier A, Vaudry H (1999) Neurotrophic activity of pituitary adenylate cyclase-activating polypeptide on rat cerebellar cortex during development. *Proc Natl Acad Sci U S A* 96: 9415-9420
- Vaudry D, Gonzalez BJ, Basille M, Pamantung TF, Fournier A, Vaudry H (2000a) PACAP acts as a neurotrophic factor during histogenesis of the rat cerebellar cortex. *Ann N Y Acad Sci* 921: 293-299
- Vaudry D, Gonzalez BJ, Basille M, Yon L, Fournier A, Vaudry H (2000b) Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. *Pharmacol Rev* 52: 269-324
- Vincent MB (1992) Cyclooxygenase inhibitors modify the relaxant effect of vasoactive intestinal polypeptide and substance P in isolated porcine ophthalmic artery. *Cephalalgia* 12: 15-19
- Wang Q, Bryowsky J, Minshall RD, Pelligrino DA (1999) Possible obligatory functions of cyclic nucleotides in hypercapnia-induced cerebral vasodilation in adult rats. *Am J Physiol* 276: H480-487
- Warren JB, Cockcroft JR, Larkin SW, Kajekar R, Macrae A, Ghatei MA, Bloom SR (1992) Pituitary adenylate cyclase activating polypeptide is a potent vasodilator in humans. *J Cardiovasc Pharmacol* 20: 83-87
- Wei EP, Kontos HA, Said SI (1980) Mechanism of action of vasoactive intestinal polypeptide on cerebral arterioles. *Am J Physiol* 239: H765-768
- Wilderman MJ, Armstead WM (1997) Role of PACAP in the relationship between cAMP and opioids in hypoxia-induced pial artery vasodilation. *Am J Physiol* 272: H1350-1358
- Xu HL, Koenig HM, Ye S, Feinstein DL, Pelligrino DA (2004) Influence of the glia limitans on pial arteriolar relaxation in the rat. *Am J Physiol Heart Circ Physiol* 287: H331-339
- Zanelli SA, Stanley DP, Kaufman D (2008) Hypoxic-Ischemic Encephalopathy. *Emedicine*: <http://emedicine.medscape.com/article/973501-overview>
- Zhou CJ, Shioda S, Yada T, Inagaki N, Pleasure SJ, Kikuyama S (2002) PACAP and its receptors exert pleiotropic effects in the nervous system by activating multiple signaling pathways. *Curr Protein Pept Sci* 3: 423-439
- Zlokovic BV (2008) The blood-brain barrier in health and chronic neurodegenerative disorders. *Neuron* 57: 178-201
- Zonta M, Angulo MC, Gobbo S, Rosengarten B, Hossmann KA, Pozzan T, Carmignoto G (2003) Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. *Nat Neurosci* 6: 43-50



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## Research Report

# Pituitary adenylate cyclase-activating polypeptide induces pial arteriolar vasodilation through cyclooxygenase-dependent and independent mechanisms in newborn pigs

Laura Lenti<sup>a</sup>, Ferenc Domoki<sup>a,b</sup>, David Kis<sup>a</sup>, Orsolya Hegyi<sup>c</sup>, Gabor K. Toth<sup>c</sup>,  
David W. Busija<sup>b</sup>, Ferenc Bari<sup>a,\*</sup>

<sup>a</sup>Department of Physiology, Faculty of Medicine, University of Szeged, 6720 Szeged, Dom ter 10, Hungary

<sup>b</sup>Department of Physiology and Pharmacology, Wake Forest University of Health Sciences, Winston-Salem, NC, USA

<sup>c</sup>Department of Medical Chemistry, Faculty of Medicine, University of Szeged, Hungary

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### ABSTRACT

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a cerebrovascular dilator and was found neuroprotective in numerous in vitro and in vivo models of cerebral ischemia. However, the mechanism of its cerebrovascular action is poorly known, especially in newborns. Therefore, we tested pial arteriolar responses to the two naturally occurring forms PACAP27 and 38 as well as to shorter sequences (PACAP6–27, 6–38, 1–15, 6–15, 20–31). We also investigated the involvement of nitric oxide synthase (NOS), cyclooxygenase-1 and -2 (COX-1 and -2) activity in PACAP-induced pial arteriolar responses using the NOS inhibitor *N*- $\omega$ -nitro-L-arginine methyl ester (L-NAME 15 mg/kg iv), the non-selective COX inhibitor indomethacin (5 mg/kg iv), and the selective COX-1 and COX-2 inhibitors SC-560 (1 mg/kg iv) and NS-398 (1 mg/kg iv), respectively. Anesthetized, ventilated piglets ( $n=127$ ) were equipped with closed cranial windows, and pial arteriolar diameters were determined via intravital microscopy. Topical application of both natural PACAPs, but none of the PACAP segments, resulted in prominent, repeatable, dose-dependent vasodilation. Percentage changes ranged  $5 \pm 1-29 \pm 6$  ( $n=7$ ) and  $4 \pm 1-36 \pm 7$  ( $n=9$ ) to  $10^{-8}$  to  $10^{-6}$  M PACAP27 and 38 (mean  $\pm$  SEM), respectively. Vasodilation to both natural PACAPs was significantly reduced by co-application with PACAP6–27 or 6–38, but not by L-NAME. Indomethacin abolished PACAP38 but not PACAP27-induced vasodilation. Arteriolar responses to PACAP38 were also sensitive to SC-560 but not to NS-398 suggesting the unique involvement of COX-1 activity in this response. In summary, PACAP27 and 38 are potent vasodilators in the neonatal cerebral circulation with at least two distinct mechanisms of action: a COX-dependent and a COX-independent pathway.

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\* Corresponding author. Fax: +36 62 544 978.

E-mail address: [bari@phys.szote.u-szeged.hu](mailto:bari@phys.szote.u-szeged.hu) (F. Bari).

Abbreviations: PACAP, pituitary adenylate cyclase-activating polypeptide; COX, cyclooxygenase; NOS, nitric oxide synthase; L-NAME, *N*- $\omega$ -nitro-L-arginine methyl ester; CSF, cerebrospinal fluid; VSM, vascular smooth muscle; ANOVA, analysis of variance; SEM, standard error of the mean

## 1. Introduction

Cerebrovascular control mechanisms play an essential role in providing adequate blood supply to nervous tissue in order to meet metabolic demands. Hypoxic/ischemic (H/I) brain injury which frequently occurs in the perinatal period (Berger and Garnier, 2000) has been shown to deteriorate cerebrovascular control by impairing the “ischemia-sensitive” mechanisms of the neurovascular axis (Leffler et al., 1989). Thus, impaired cerebrovascular regulation probably contributes to neuronal cell death following H/I brain injury. However, neuroprotective mediators may also be released from these neuronal-vascular elements during H/I stress that could abrogate neuronal injury. These neuroprotective mechanisms are attractive targets for developing new strategies in the treatment of neonatal stroke.

Pituitary adenylate cyclase-activating peptide (PACAP) is one of the most promising of these neuroprotective mediators. PACAP has displayed marked protective effects in numerous *in vivo* and *in vitro* models of cerebral injury, including ischemia (Uchida et al., 1996; Reglodi et al., 2000; Suk et al., 2004; Vaudry et al., 2004; Chen et al., 2006). PACAP-induced neuroprotection *in vivo* can be, at least partially, based on its cerebrovascular effects (Tong et al., 1993; Anzai et al., 1995), therefore the characterization of PACAP-induced vascular changes and the mechanism of the vascular actions are of high importance.

PACAP belongs to the vasoactive intestinal polypeptide (VIP)/glucagon/secretin superfamily that includes structurally related neuroendocrine peptides. It exists in two molecular forms: PACAP38, a basic, amidated 38-residue peptide; and PACAP27, the N-terminal portion consisting of 27 amino acids (Miyata et al., 1989, 1990). PACAP has been detected in the neural tissue and cerebrospinal fluid (CSF) as well, and under resting conditions its concentration in the CSF is ~2 ng/ml (Wilderman and Armstead, 1997). The peptide has been shown to act as a neurotransmitter, neuromodulator, and a neurotrophic factor. Although recent studies revealed the complexity of perivascular peptidergic innervation, the mechanisms by which neuropeptides participate in regulation of the vessel caliber, the neuronal excitability, and the cerebral metabolism remains unclear. Considerable data suggest that vasoactive peptides, including PACAP, are involved in cerebrovascular control (Edvinsson and Krause, 2002).

In this study, we employed the newborn pig animal model that most closely reflects the brain developmental stage, metabolism, and basic circulatory parameters of human babies. We studied the effects of PACAP on the neonatal cortical pial microcirculation that significantly (~50%) contributes to the total cerebrovascular resistance.

Previous studies in piglets have shown that the dilator capabilities of PACAP27 and 38 are very similar and that they are potent dilators of pial arterioles (Tong et al., 1993). Immunohistochemical studies indicate that PACAP is co-localized with neuronal nitric oxide (NO) synthase (NOS) in the perivascular nerves and the sensory and parasympathetic ganglia (Tajti et al., 1999; Uddman et al., 1999; Edvinsson et al., 2001). *In vitro* experiments suggest that

there is not only an anatomical but also a functional connection between PACAP and NO (Seebeck et al., 2002). Currently, research studies have not clarified if NO does indeed participate in mediation of the cerebrovascular effects elicited by PACAP. Accumulating data indicate that cyclooxygenase (COX)-derived arachidonic acid metabolites are major contributors to the regulation of cerebral circulation in the newborn (Peri et al., 1995; Busija et al., 1996; Parfenova et al., 1997; Degi et al., 1998). Although numerous preceding *in vitro* and *in vivo* studies revealed tight interaction between the prostanoid synthesis and PACAP action (Kis et al., 1999), our understanding of the possible role of the COX derived metabolites in the vascular effects of the PACAP in piglets is still limited.

The purpose of this study was to (1) compare the vasoactivity of the natural PACAP forms as well as short PACAP sequences; (2) explore the mechanism of cerebral arteriolar responses to PACAP using supposed PACAP receptor antagonists, NOS and COX inhibitors. The results emanating from these studies urged us to (3) investigate the roles of COX-1 and COX-2 activity in the mechanism of PACAP38-induced vasodilation using selective COX-1 and -2 inhibitors.

## 2. Results

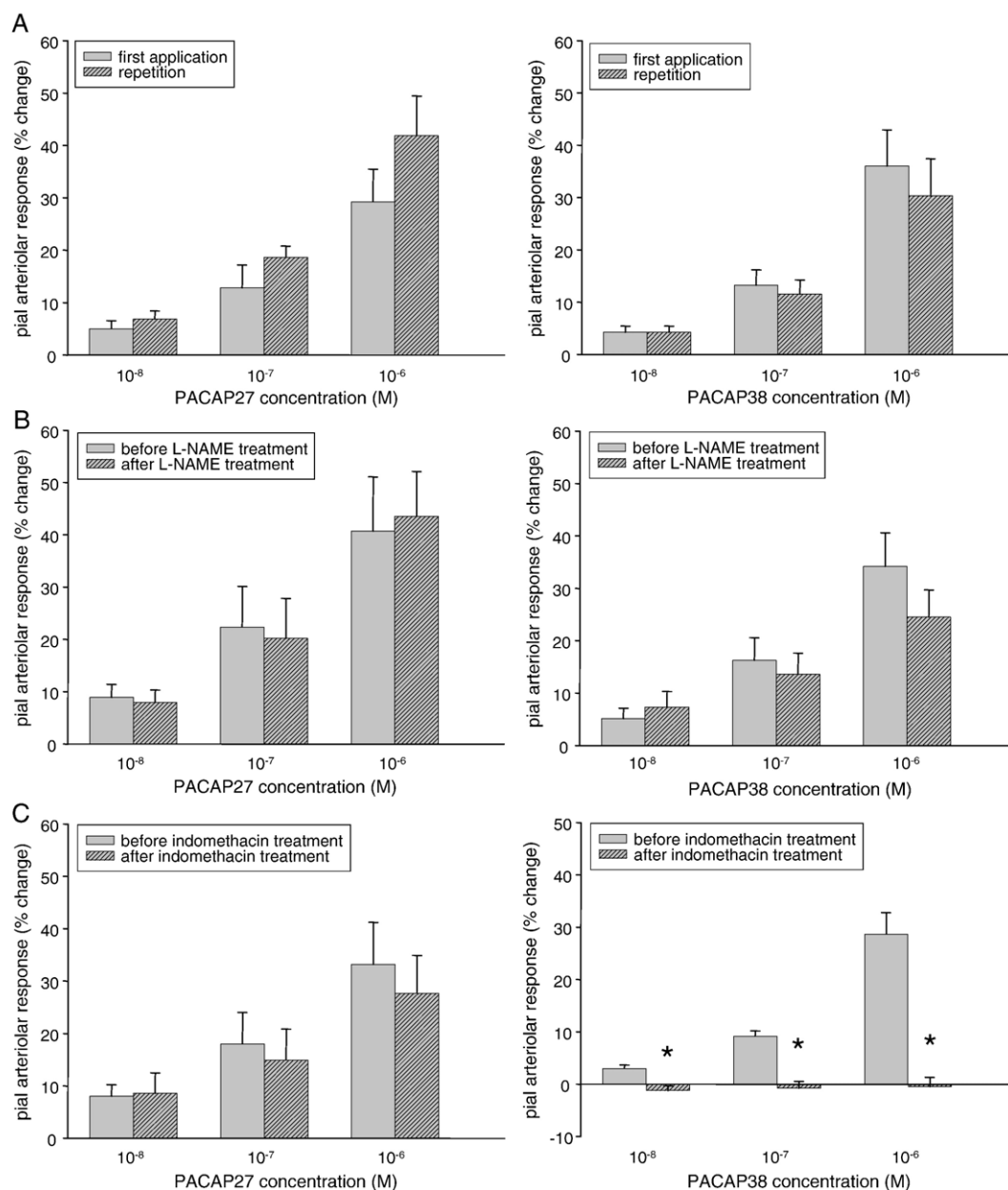
### 2.1. Physiological variables

Body temperature, arterial pH, and blood gases were kept in the respective normal ranges and did not vary significantly among different groups throughout the experiments. For instance, in *group 1a* the values were the following: core temperature = 36.9 ± 0.1 °C, pH = 7.4 ± 0.02,  $p\text{CO}_2$  = 33.9 ± 1.9 mm Hg,  $p\text{O}_2$  = 93.7 ± 3.6 mm Hg. Similarly, MABP values were within normal range and were not significantly different between groups (e.g. 73 ± 3 mm Hg and 71 ± 2 mm Hg in *group 1a* and *group 1b* respectively). Furthermore, in each animal care was taken to avoid any fluctuation in MABP during recordings to prevent effects of short-term changes in arterial pressure on vascular diameters. There were no significant differences between the baseline diameters in any group, for example, in *group 11* the values were 92 ± 6 and 91 ± 6 μm before the first and second PACAP application, respectively.

### 2.2. Effect of various length PACAP sequences on pial arteriolar diameter

Topical application of PACAP27 (*group 1a*) and PACAP38 (*group 1b*) resulted in similar, concentration-dependent ( $10^{-8}$  to  $10^{-6}$  M), repeatable pial arteriolar dilation (Fig. 1). Indeed, PACAP27 and PACAP38 produced equally prominent vascular changes compared in the same animals (*group 1c*, 36 ± 5% to  $10^{-6}$  M PACAP27 and 43 ± 7% to  $10^{-6}$  M PACAP38).

In contrast, in *groups 2a–e*, even repeated application of shorter PACAP peptide segments did not display any vasoactivity. Percent changes in diameters at the highest concentration used were: -2 ± 2 and 1 ± 1 to PACAP6–27 and 6–38; 2 ± 3, 0 ± 0 and 2 ± 1 to fragment 1–15, 6–15 and 20–31,



**Fig. 1** – Characterization of PACAP27 and PACAP38-induced pial arteriolar vasodilation. PACAP27 (A, left) and PACAP38 (A, right) elicited dose-dependent, repeatable increases in pial arteriolar diameters. Inhibition of NOS by L-NAME did not alter vascular responses to either PACAP27 or PACAP38 (B). In contrast, the COX inhibitor indomethacin virtually abolished pial arteriolar dilation to PACAP38 (C, right), whereas it left vasodilation to PACAP27 essentially intact (C, left). Data are mean  $\pm$  SEM,  $n=7-13$ , \* $p<0.05$  significantly less than corresponding values at first application.

respectively, during the first application. The vascular responses were  $1\pm 1\%$  and  $1\pm 1\%$  to PACAP6–27 and 6–38,  $2\pm 2\%$ ,  $0\pm 0\%$  and  $1\pm 1\%$  to fragment 1–15, 6–15 and 20–31, respectively, during the second application.

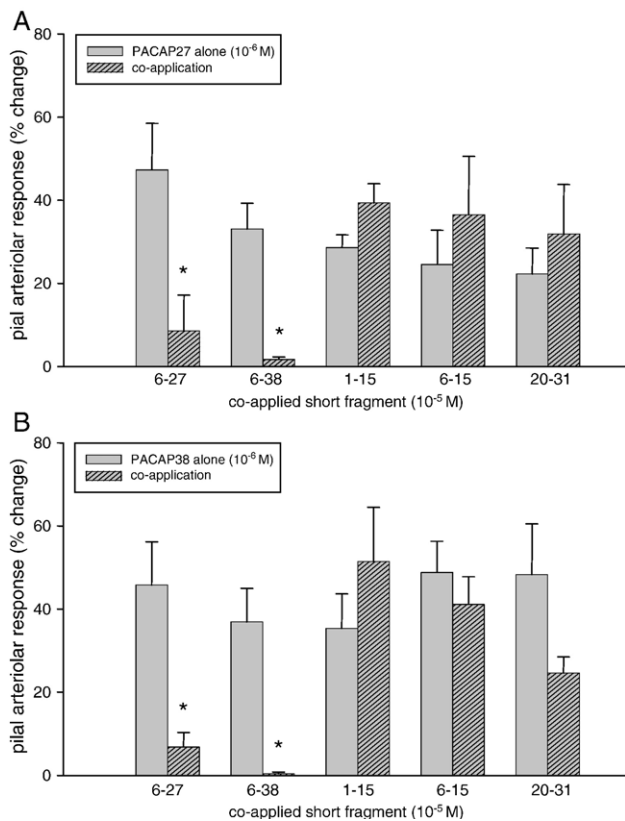
### 2.3. Effect of co-application with short segments on PACAP27 and 38 elicited vasodilation

In comparison to the control values, arteriolar dilations to PACAP27 or PACAP38 showed a statistically significant decrease when these peptides were co-applied with PACAP6–27 or

PACAP6–38. The other applied short segments (PACAP1–15, 6–15, and 20–31) did not affect either PACAP27 or PACAP38-induced vasodilation (Fig. 2).

### 2.4. Effect of NOS and COX inhibitors on PACAP-induced vascular reactions

The inhibition of NOS enzyme had no significant effect on the PACAP-induced vascular reactions (Fig. 1). Both PACAP27 and PACAP38 elicited similar vasodilation on pial arterioles before and after L-NAME treatment. The non-selective COX enzyme



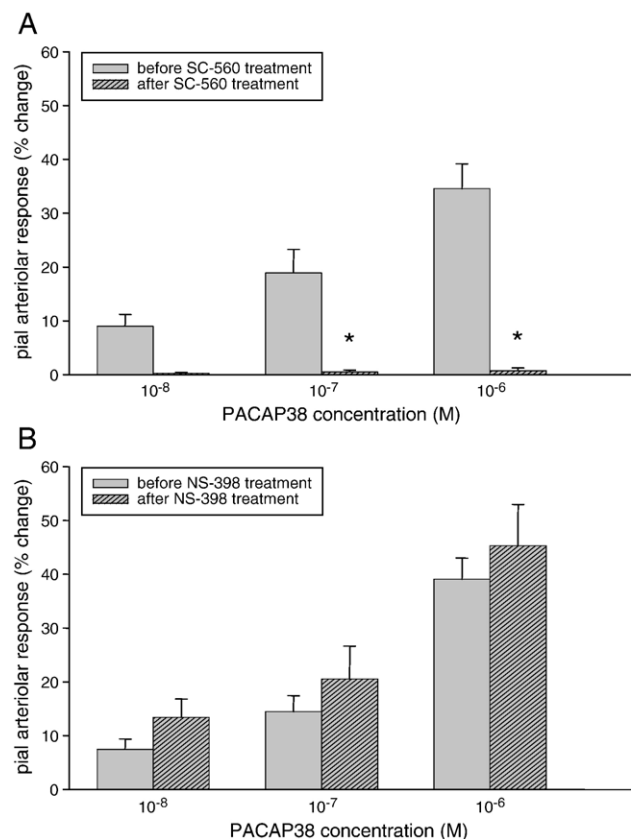
**Fig. 2 – Co-application of specific PACAP segments modifies pial arteriolar dilations to PACAP27 (A) and PACAP38 (B). Co-application of PACAP6–27 and 6–38 resulted in significant reduction of PACAP27 and PACAP38-induced vasodilation as compared with the control vascular reactivity determined earlier. In contrast, PACAP1–15, 6–15, and 20–31 failed to alter these responses. Data are mean + SEM,  $n=5-5$ , \* $p<0.05$  significantly less than corresponding values before the co-application.**

inhibitor indomethacin reduced the vasorelaxant effect of PACAP38 (group 9b), however in group 9a (when PACAP27 was applied), this prominent influence was absent (Fig. 1). Interestingly, the selective COX-2 inhibitor NS-398 had no inhibitory effect on PACAP38-induced vasodilation (group 10), whereas in group 11 the COX-1 blocker SC-560 was able to abolish these diameter changes in the used dosages (Fig. 3).

### 3. Discussion

The major findings of the present studies are as follows: (1) both PACAP27 and PACAP38 are similarly potent dilators of pial resistance vessels in newborn pigs confirming previous work (Tong et al., 1993). (2) The short PACAP sequences are not vasoactive per se, but PACAP6–27 and 6–38 can inhibit PACAP27 and 38-induced vasodilation. (3) NOS activity does not appear to play a significant role in the dilatory effect of PACAP. (4) Finally, PACAP38 (but not PACAP27) induced vasodilation was abolished by indomethacin and the selective COX-1 inhibitor SC-560 but not by the selective COX-2 inhibitor NS-398.

Three main types of PACAP/VIP-recognizing receptors have been reported so far to mediate the various effects of PACAP (Harmar et al., 1998; Vaudry et al., 2000). VPAC receptors are closely related proteins binding VIP and PACAP with almost the same efficacy, while PAC1 receptors (at least eight variants exist) exhibit a high affinity for PACAP and a much lower affinity for VIP. Most of these subtypes are G-protein-coupled receptors that principally stimulate adenylate cyclase. VIP/PACAP receptors have been identified in many brain/cerebrovascular cell types studied in many species including humans. Knutsson and Edvinsson detected mRNA of all three receptor types in human cerebral ganglia and arteries (Knutsson and Edvinsson, 2002). The expression of the receptor mRNA remained unaltered after removal of the endothelium. In rats, VPAC1 receptor immunoreactivity was attached to the smooth muscle cell layer of the superficial cerebral arteries and arterioles (Fahrenkrug et al., 2000), and VIP/PACAP receptors were also found on neuroglia as well as on neurons (Joo et al., 2004). Unfortunately, no data exist on the expression of these receptors in the newborn piglet, however, endothelial VPAC1 and VSM VPAC2 receptors have been described in the basilar artery of adult pigs (Grant et al., 2006). Moreover, PACAP-immunoreactive nerve fibers originating from either sensory or parasympathetic ganglia do innervate the pial



**Fig. 3 – Pial arteriolar vasodilation to PACAP38 is differentially affected by selective COX inhibitors. The COX-1 inhibitor SC-560 (A) abolished arteriolar responses to PACAP38, whereas the COX-2 inhibitor NS-398 (B) did not alter the vasodilation. Data are the mean + SEM,  $n=9-9$ , \* $p<0.05$  significantly less than corresponding control values.**

blood vessels in piglets (our unpublished observations), and these nerves are the most important source of PACAP to the pial arterioles (Uddman et al., 1993).

The short PACAP sequences used in the present study provide novel insight into the mechanism of PACAP-induced vascular changes since their role has not been investigated in the cerebral circulation before. PACAP6–27 and PACAP6–38 are supposed competitive PACAP antagonists established in other experimental models (Harmar et al., 1998; Vaudry et al., 2000), and our results concur since they abolish the vascular effect of both PACAP27 and 38. Our knowledge is limited on the degradation of PACAP in vivo, but PACAP 6–27 can be an important endogenous inhibitor providing negative feedback since PACAP27 is rapidly degraded by dipeptidylpeptidase IV (DPP IV) to produce PACAP6–27 among other metabolites in vitro (Green et al., 2006). The other short fragments (PACAP1–15, 6–15, and 20–31) were designed only for mapping purposes, given that a short fragment is a much better starting compound for any mutual pharmacological development. These short PACAPs do not show any vasoactivity or inhibitory effects suggesting that they are not capable of sufficient binding to the VPAC/PACAP receptors.

PACAP is thought to exert a significant direct effect on the vascular smooth muscle (VSM) of pial resistance vessels. PACAP increases intracellular cAMP production resulting in cerebrovascular vasodilation via subsequent activation of plasmolemmal K<sup>+</sup> channels in rats (Kobayashi et al., 1994). In piglets, the observed vascular caliber changes to both PACAP27 and 38 have been associated with elevated cortical periarachnoid CSF cAMP concentration (Tong et al., 1993; Armstead, 1997). Furthermore, the pial arteriolar dilation produced by PACAP27 can be blocked by iberiotoxin, a potent antagonist of the Ca<sup>2+</sup>-sensitive K<sup>+</sup> channels, but unaltered by the ATP-sensitive K<sup>+</sup> channel inhibitor glibenclamide (Armstead, 1997). PACAP was found to be an endothelium-independent relaxing factor in several experiments (Kastner et al., 1995), but other indirect pathways of PACAP-induced dilation are possible.

Surprisingly, the involvement of NOS activity in PACAP-induced vasodilation has not been supported by our experiments with L-NAME despite numerous anatomical and functional studies suggesting otherwise in other species. The PACAP-containing perivascular nerve fibers originate from several sources, e.g. from the trigeminal, sphenopalatine, and otic ganglia. In the human and rat parasympathetic and trigeminal ganglia high numbers of nerve cell bodies containing both NOS and PACAP were visualized by fluorescent immunohistochemistry (Tajti et al., 1999; Uddman et al., 1999; Edvinsson et al., 2001). Activation of nitrergic neurons, at least in part, plays a role in the PACAP-induced vasorelaxant mechanism in isolated rat basilar arteries (Seebeck et al., 2002). The inhibition of NO production results in tachyphylaxis to PACAP27 in rat mesenteric and abdominal aortic artery (Whalen et al., 1999). However, in the piglet, several former studies demonstrate age-related changes in the cerebrovascular regulation, predominantly in the NO-mediated responsiveness. The expression and activity of NOS in newborn and adult pigs are different: NOS enzyme is up-regulated during maturation as well as the NOS activity increases, while e.g. the COX expression and activity remain

constant (Parfenova et al., 2000). This phenomenon results in the predominance of COX-derived products over NO in the newborn period and gives a possible explanation of our negative result. The selected dose of L-NAME (15 mg/kg iv), however, was reported to provide appropriate NOS inhibition in former studies (Lacza et al., 2002).

The major finding of our work is that, in contrast to the effect of PACAP27, PACAP38-induced vasodilation is extremely indomethacin sensitive similarly to VIP-induced pial arteriolar vasodilation in cats (Wei et al., 1980). By dissecting the role of the two COX isoforms in the PACAP38-induced response using selective COX-1 and COX-2 inhibitors we find that administration of SC-560 virtually abolishes the PACAP38 elicited arteriolar dilation, while these vascular reactions remain unchanged after NS-398. These data suggest that COX-1 derived prostanoids are substantially involved in PACAP38 dependent vascular caliber changes while the role of the COX-2 enzyme seems to be ancillary.

The importance of cyclooxygenase (COX)-derived prostanoids in the cerebrovascular control of newborns is well established, however, there are many open questions related to the functional significance of the two distinct COX isoforms. In the piglet brain, COX-2 is the dominant constitutive enzyme: unlike COX-1, COX-2 mRNA is expressed in large quantities in the cerebral cortex and microvasculature, and this isoform comprises the main part of the total COX protein level here. Moreover, data show that COX-2 gives the major, approximately 80–90% contribution to the central nervous system prostanoid level in the newborns as NS-398, a specific COX-2 inhibitor, produces great decrease in the overall prostanoid synthesis during both basal and certain stimulated conditions. The remnant 10–20% is expected to be the contribution of the COX-1 enzyme (Peri et al., 1995; Parfenova et al., 1997). In concert with these observations, other functional studies found that COX-dependent vascular reactions (such as hypotension-induced vasodilation or Ach-induced vasoconstriction) appear to depend largely on COX-2 activity (Domoki et al., 2005), while to the best of our knowledge this study is the first to show a COX-1 dependent vasoactive mechanism observed on the pial vasculature of piglets.

In any pharmacological study, the specificity of the used drugs limits the interpretation of the results. Indomethacin at the used dose was found to be a strong inhibitor of the prostanoid synthesis in many experiments in which its ability to block either COX-1 or COX-2 dependent mechanisms was investigated. The dose of NS-398 was chosen for similar reasons since it proved to be a selective and effective inhibitor of the COX-2 enzyme (Domoki et al., 2005). The COX-1 activity was targeted with SC-560. In piglets, our laboratory was the first (and to the best of our knowledge the only) where SC-560 was used in vivo (Domoki et al., 2005), though in that series of experiments none of the assessed vascular reactions appeared to be COX-1 dependent. On the other hand, other investigators found this dose effective in blocking the COX-1 mediated prostaglandin production in the rat brain, even after COX-1 gene transfer (Lin et al., 2002). However, SC-560 is known to act, at least in some cell types, as an unselective COX enzyme blocker (Brenneis et al., 2006). In our model, PACAP38 may act on a particular cell type in which SC-560 has the potential to

block both COX-1 and COX-2. Nonetheless it seems to be more likely that PACAP38 uniquely activates COX-1 present in small amounts inducing the synthesis of such vasodilator prostanooids as prostacyclin or PGE<sub>2</sub> that stimulates cAMP formation by VSM resulting in vasodilation (Parfenova et al., 1995).

In conclusion, PACAP is a powerful vasodilator in the newborn cerebral circulation. The action of PACAP27 and PACAP38 seems to be independent of NOS activation, but the COX-1 activity appears to be essential only for PACAP38 elicited vascular effects. Considering the discrepancy between the probable signal transduction courses related to the distinct entire PACAP sequences, it seems feasible that PACAP27 and PACAP38 act on different cells or different subtypes of the PACAP receptor group. Currently our field is hampered by the lack of potent, subtype selective blockers necessary to reveal the functional relevance of VIP/PACAP receptor subtypes in the newborn cerebral circulation. As a perspective, the findings in the present paper may give crucial information for the future investigations on PACAP-induced neuroprotection and are critical for understanding the mechanism of neonatal cerebrovascular disorders and endogenous protective processes.

## 4. Experimental procedures

### 4.1. Animals

Newborn piglets of either sex (~1 day old, body weight 1.2–2.5 kg, n=127) were used. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Szeged.

### 4.2. Surgery

Anesthesia was initiated with thiopental sodium (40 mg/kg ip.; Biochemie, Vienna, Austria) and a bolus injection of  $\alpha$ -chloralose (40 mg/kg iv.; Sigma, St. Louis, MO, USA). Supplemental doses of  $\alpha$ -chloralose were given to maintain a constant level of anesthesia. A catheter was inserted in the right femoral artery to monitor blood pressure and to sample blood for determination of blood gas tensions and pH. Drugs and fluids were administered through a second catheter placed in the right femoral vein. The animals were intubated via tracheotomy and mechanically ventilated with room air. The ventilation rate (~30 breaths/min) and tidal volume (~20 ml) were adjusted to maintain arterial blood gas values and pH in the physiological range. A water-circulating heating pad was used to maintain the body temperature at ~37 °C (core temperature was monitored with a rectal probe).

The animals were equipped with a closed cranial window as previously described (Domoki et al., 2005). Following surgery, the closed window was filled with artificial CSF (aCSF) which was similar to endogenous CSF (aCSF composition: KCl 220, MgCl<sub>2</sub> 132, CaCl<sub>2</sub> 221, NaCl 7710, urea 402, dextrose 665, and NaHCO 2066 in mg/l), warmed to 37 °C and equilibrated with a gas mixture containing 6% O<sub>2</sub>, 6.5% CO<sub>2</sub> and 87.5% N<sub>2</sub> to obtain pH 7.33, pCO<sub>2</sub>=46 mm Hg and pO<sub>2</sub>=43 mm Hg. Different PACAP sequences were dissolved in aCSF and applied to the pial surface through one of the

injectable ports of the cranial window. Pial arterial vessels were observed with an operating microscope (Wild, Switzerland), equipped with a video camera (Sanyo digital color CCD camera, Japan), and a video monitor (Panasonic, Japan). Vascular diameter was measured using a video microscaler. In each experiment, a ~100- $\mu$ m-diameter (range 85–115  $\mu$ m) pial arteriole was selected. We chose this vessel size because this is the level of the first order pial arterioles and the primary site of vascular resistance. Some authors separate the resistance vessels into two groups corresponding their size (e.g. arterioles and small arteries) to rule out the possibility that regional changes in the reactivity modify the results of the experiments (Armstead, 1995). However, in the present work, arterioles at both end of the range studied displayed virtually identical reactivity to the stimuli employed (data are not shown).

At the end of the experiments the animals were euthanized with an iv injection of saturated KCl solution.

### 4.3. Drugs

NS-398 (Sigma Chemical Co.) and SC-560 (Sigma Chemical Co.) were dissolved in DMSO (5 mg/ml). L-NAME (Sigma Chemical Co.) and indomethacin (Merck & Co, Whitehouse Station, NJ) were dissolved in saline (30 mg/ml and 10 mg/ml respectively). PACAP1–27, 1–38, 6–27, 6–38, 1–15, 6–15 and 20–31 (Department of Medical Chemistry, University of Szeged, Hungary) were prepared as stock solutions in saline (10<sup>-4</sup>) and then were diluted with aCSF (10<sup>-8</sup>, 10<sup>-7</sup>, 10<sup>-6</sup> or 10<sup>-5</sup> M).

### 4.4. Assessment of cerebrovascular reactivity

#### 4.4.1. Protocol I. Determination of PACAP-induced vasodilation and repeatability

Time control experiments were executed first in separate series of animals to obtain arteriolar responses to different PACAP sequences initially and then 40 min later. In group 1 PACAP27 (subgroup 1a, n=7) or PACAP38 (subgroup 1b, n=9) was applied repeatedly. Additionally, we compared the efficacy of the two natural peptides; in subgroup 1c (n=9) PACAP27 and 38 were applied alternately with 40-minute delay. In group 2 arteriolar responses to short PACAP segments were measured (PACAP6–27, 6–38, 1–15, 6–15 and 20–31 in subgroups 2a, b, c, d and e with n=4 in each subgroup, respectively). Each of the peptides was applied in ascending concentration manner (10<sup>-8</sup>, 10<sup>-7</sup> and 10<sup>-6</sup> M cumulatively). Arteriolar diameters were measured every minute during a 5-minute exposure period for each PACAP concentration. After the highest concentration of the series was applied the area under the window was gently flushed with aCSF to eliminate the previous stimulus, and the arteriolar diameters were allowed to return to the baseline value. Then the stimulus was repeated. In this manner we were able to detect the PACAP-induced caliber changes and identify the presence of potential tachyphylaxis or sensitization.

#### 4.4.2. Protocol II. Determination of the effect of short sequences on PACAP-induced vasodilation

In these experiments we co-applied short PACAP sequences to examine their effects on vascular reactions evoked by the



entire PACAP segments. Co-application of PACAP6–27, 6–38, 1–15, 6–15, and 20–31 with PACAP27 or PACAP38 occurred in groups 3–7a and b ( $n=5$  in each subgroup) respectively. First PACAP27 or 38 in  $10^{-6}$  M concentration was used topically for 5 min. Following a 30-minute exposure-free period, when arterioles regained stable baseline diameters, one short segment was applied in a tenfold concentration for 10 min. Next we co-applied the entire peptide sequence with the short PACAP used previously for a subsequent 5 min. Vascular diameters were measured continuously during the stimuli.

#### 4.4.3. Protocol III. Determination the effect of enzyme inhibitors on PACAP-induced vasodilation

This experiment was designed the same as protocol I with one addition. Between the first and the second local application of the distinct entire PACAP sequence, a COX or NOS enzyme blocker was administered intravenously. After obtaining stable arteriolar baseline diameters, we examined the responses of pial arterioles to PACAP27 or PACAP38 before and 20 min after iv administration of the NOS inhibitor L-NAME in group 8, on PACAP27 and 38 elicited vasodilation (subgroup 8a,  $n=7$  and 8b,  $n=13$ ), respectively. In group 9 the effect of the non-selective COX inhibitor indomethacin (5 mg/kg iv) on PACAP-induced arteriolar dilation was detected. We used PACAP27 in subgroup 9a ( $n=10$ ) and PACAP38 in subgroup 9b ( $n=9$ ) topically before and after the parenteral indomethacin administration. In group 10 ( $n=9$ ) the effect of the selective COX-2-inhibitor NS-398 (1 mg/kg) was tested. The effect of the selective COX-1 inhibitor SC-560 (1 mg/kg) was analyzed in group 11 ( $n=9$ ).

#### 4.5. Statistical analysis

The measured pial artery diameter values (absolute diameters or percentage change from control values) were analyzed by ANOVA for repeated measures. For post hoc analysis Holm–Sidak's exact test was performed where appropriate. A value of  $p < 0.05$  was considered significant. Data are represented as mean  $\pm$  SEM. The  $n$  number reflects data for one vessel in each animal.

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## REFERENCES

- Anzai, M., Suzuki, Y., Takayasu, M., Kajita, Y., Mori, Y., Seki, Y., Saito, K., Shibuya, M., 1995. Vasorelaxant effect of PACAP-27 on canine cerebral arteries and rat intracerebral arterioles. *Eur. J. Pharmacol.* 285, 173–179.
- Armstead, W.M., 1995. Relationship between opioids and activation of phospholipase C and protein kinase C in brain injury induced pial artery vasoconstriction. *Brain Res.* 689, 183–188.
- Armstead, W.M., 1997. Role of impaired cAMP and calcium-sensitive  $K^+$  channel function in altered cerebral hemodynamics following brain injury. *Brain Res.* 768, 177–184.
- Berger, R., Garmier, Y., 2000. Perinatal brain injury. *J. Perinat. Med.* 28, 261–285.
- Brenneis, C., Maier, T.J., Schmidt, R., Hofacker, A., Zulauf, L., Jakobsson, P.J., Scholich, K., Geisslinger, G., 2006. Inhibition of prostaglandin E2 synthesis by SC-560 is independent of cyclooxygenase 1 inhibition. *FASEB J.* 20, 1352–1360.
- Busija, D.W., Thore, C., Beasley, T., Bari, F., 1996. Induction of cyclooxygenase-2 following anoxic stress in piglet cerebral arteries. *Microcirculation* 3, 379–386.
- Chen, Y., Samal, B., Hamelink, C.R., Xiang, C.C., Chen, M., Vaudry, D., Brownstein, M.J., Hallenbeck, J.M., Eiden, L.E., 2006. Neuroprotection by endogenous and exogenous PACAP following stroke. *Regul. Pept.* 137, 4–19.
- Degi, R., Bari, F., Thrikawala, N., Beasley, T.C., Thore, C., Louis, T.M., Busija, D.W., 1998. Effects of anoxic stress on prostaglandin H synthase isoforms in piglet brain. *Brain Res. Dev. Brain Res.* 107, 265–276.
- Domoki, F., Nagy, K., Temesvari, P., Bari, F., 2005. Selective inhibitors differentially affect cyclooxygenase-dependent pial arteriolar responses in newborn pigs. *Pediatr. Res.* 57, 853–857.
- Edvinsson, L., Krause, D.N., 2002. *Cerebral Blood Flow and Metabolism*. Lippincott Williams and Wilkins, Philadelphia, USA.
- Edvinsson, L., Elsas, T., Suzuki, N., Shimizu, T., Lee, T.J., 2001. Origin and co-localization of nitric oxide synthase, CGRP, PACAP, and VIP in the cerebral circulation of the rat. *Microsc. Res. Tech.* 53, 221–228.
- Fahrenkrug, J., Hannibal, J., Tams, J., Georg, B., 2000. Immunohistochemical localization of the VIP1 receptor (VPAC1R) in rat cerebral blood vessels: relation to PACAP and VIP containing nerves. *J. Cereb. Blood Flow Metab.* 20, 1205–1214.
- Grant, S., Lutz, E.M., McPhaden, A.R., Wadsworth, R.M., 2006. Location and function of VPAC1, VPAC2 and NPR-C receptors in VIP-induced vasodilation of porcine basilar arteries. *J. Cereb. Blood Flow Metab.* 26, 58–67.
- Green, B.D., Irwin, N., Flatt, P.R., 2006. Pituitary adenylate cyclase-activating peptide (PACAP): assessment of dipeptidyl peptidase IV degradation, insulin-releasing activity and antidiabetic potential. *Peptides* 27, 1349–1358.
- Harmar, A.J., Arimura, A., Gozes, I., Journot, L., Laburthe, M., Pisegna, J.R., Rawlings, S.R., Robberecht, P., Said, S.I., Sreedharan, S.P., Wank, S.A., Waschek, J.A., 1998. International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Pharmacol. Rev.* 50, 265–270.
- Joo, K.M., Chung, Y.H., Kim, M.K., Nam, R.H., Lee, B.L., Lee, K.H., Cha, C.I., 2004. Distribution of vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide receptors (VPAC1, VPAC2, and PAC1 receptor) in the rat brain. *J. Comp. Neurol.* 476, 388–413.
- Kastner, A., Bruch, L., Will-Shahab, L., Modersohn, D., Baumann, G., 1995. Pituitary adenylate cyclase activating peptides are endothelium-independent dilators of human and porcine coronary arteries. *Agents Actions Suppl.* 45, 283–289.
- Kis, B., Gaspar, T., Mezei, Z., Gecse, A., Telegdy, G., 1999. Pituitary adenylate cyclase-activating polypeptide inhibits the cyclooxygenase pathway of rat cerebral microvessels. *Acta Physiol. Scand.* 167, 43–47.
- Knutsson, M., Edvinsson, L., 2002. Distribution of mRNA for VIP and PACAP receptors in human cerebral arteries and cranial ganglia. *NeuroReport* 13, 507–509.

- Kobayashi, H., Uezono, Y., Ueno, S., Izumi, F., 1994. Pituitary adenylate cyclase-activating polypeptides (PACAPs) increase cAMP in rat cerebral microvessels. *Brain Res.* 647, 145–147.
- Lacza, Z., Puskar, M., Kis, B., Perciaccante, J.V., Miller, A.W., Busija, D.W., 2002. Hydrogen peroxide acts as an EDHF in the piglet pial vasculature in response to bradykinin. *Am. J. Physiol.: Heart Circ. Physiol.* 283, H406–H411.
- Leffler, C.W., Beasley, D.G., Busija, D.W., 1989. Cerebral ischemia alters cerebral microvascular reactivity in newborn pigs. *Am. J. Physiol.* 257, H266–H271.
- Lin, H., Lin, T.N., Cheung, W.M., Nian, G.M., Tseng, P.H., Chen, S.F., Chen, J.J., Shyue, S.K., Liou, J.Y., Wu, C.W., Wu, K.K., 2002. Cyclooxygenase-1 and bicistronic cyclooxygenase-1/prostacyclin synthase gene transfer protect against ischemic cerebral infarction. *Circulation* 105, 1962–1969.
- Miyata, A., Arimura, A., Dahl, R.R., Minamino, N., Uehara, A., Jiang, L., Culler, M.D., Coy, D.H., 1989. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochem. Biophys. Res. Commun.* 164, 567–574.
- Miyata, A., Jiang, L., Dahl, R.D., Kitada, C., Kubo, K., Fujino, M., Minamino, N., Arimura, A., 1990. Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP38). *Biochem. Biophys. Res. Commun.* 170, 643–648.
- Parfenova, H., Hsu, P., Leffler, C.W., 1995. Dilator prostanoid-induced cyclic AMP formation and release by cerebral microvascular smooth muscle cells: inhibition by indomethacin. *J. Pharmacol. Exp. Ther.* 272, 44–52.
- Parfenova, H., Eidson, T.H., Leffler, C.W., 1997. Upregulation of COX-2 in cerebral microvascular endothelial cells by smooth muscle cell signals. *Am. J. Physiol.* 273, C277–C288.
- Parfenova, H., Massie, V., Leffler, C.W., 2000. Developmental changes in endothelium-derived vasorelaxant factors in cerebral circulation. *Am. J. Physiol.: Heart Circ. Physiol.* 278, H780–H788.
- Peri, K.G., Hardy, P., Li, D.Y., Varma, D.R., Chemtob, S., 1995. Prostaglandin G/H synthase-2 is a major contributor of brain prostaglandins in the newborn. *J. Biol. Chem.* 270, 24615–24620.
- Reglodi, D., Somogyvari-Vigh, A., Vigh, S., Kozicz, T., Arimura, A., 2000. Delayed systemic administration of PACAP38 is neuroprotective in transient middle cerebral artery occlusion in the rat. *Stroke* 31, 1411–1417.
- Seebeck, J., Lowe, M., Kruse, M.L., Schmidt, W.E., Mehdorn, H.M., Ziegler, A., Hempelmann, R.G., 2002. The vasorelaxant effect of pituitary adenylate cyclase activating polypeptide and vasoactive intestinal polypeptide in isolated rat basilar arteries is partially mediated by activation of nitrenergic neurons. *Regul. Pept.* 107, 115–123.
- Suk, K., Park, J.H., Lee, W.H., 2004. Neuropeptide PACAP inhibits hypoxic activation of brain microglia: a protective mechanism against microglial neurotoxicity in ischemia. *Brain Res.* 1026, 151–156.
- Tajti, J., Uddman, R., Moller, S., Sundler, F., Edvinsson, L., 1999. Messenger molecules and receptor mRNA in the human trigeminal ganglion. *J. Auton. Nerv. Syst.* 76, 176–183.
- Tong, S., Parfenova, H., Shibata, M., Zuckerman, S., Armstead, W.M., Leffler, C.W., 1993. Pituitary adenylate cyclase-activating polypeptide dilates cerebral arterioles of newborn pigs. *Proc. Soc. Exp. Biol. Med.* 203, 343–347.
- Uchida, D., Arimura, A., Somogyvari-Vigh, A., Shioda, S., Banks, W.A., 1996. Prevention of ischemia-induced death of hippocampal neurons by pituitary adenylate cyclase activating polypeptide. *Brain Res.* 736, 280–286.
- Uddman, R., Goadsby, P.J., Jansen, I., Edvinsson, L., 1993. PACAP, a VIP-like peptide: immunohistochemical localization and effect upon cat pial arteries and cerebral blood flow. *J. Cereb. Blood Flow Metab.* 13, 291–297.
- Uddman, R., Tajti, J., Moller, S., Sundler, F., Edvinsson, L., 1999. Neuronal messengers and peptide receptors in the human sphenopalatine and otic ganglia. *Brain Res.* 826, 193–199.
- Vaudry, D., Gonzalez, B.J., Basille, M., Yon, L., Fournier, A., Vaudry, H., 2000. Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. *Pharmacol. Rev.* 52, 269–324.
- Vaudry, D., Cottet-Rousselle, C., Basille, M., Falluel-Morel, A., Fournier, A., Vaudry, H., Gonzalez, B.J., 2004. Pituitary adenylate cyclase-activating polypeptide inhibits caspase-3 activity but does not protect cerebellar granule neurons against beta-amyloid (25–35)-induced apoptosis. *Regul. Pept.* 123, 43–49.
- Wei, E.P., Kontos, H.A., Said, S.I., 1980. Mechanism of action of vasoactive intestinal polypeptide on cerebral arterioles. *Am. J. Physiol.* 239, H765–H768.
- Whalen, E.J., Johnson, A.K., Lewis, S.J., 1999. Tachyphylaxis to PACAP-27 after inhibition of NO synthesis: a loss of adenylate cyclase activation. *Am. J. Physiol.* 277, R1453–R1461.
- Wilderman, M.J., Armstead, W.M., 1997. Role of PACAP in the relationship between cAMP and opioids in hypoxia-induced pial artery vasodilation. *Am. J. Physiol.* 272, H1350–H1358.

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# PACAP and VIP differentially preserve neurovascular reactivity after global cerebral ischemia in newborn pigs

Laura Lenti<sup>a,b,\*,1</sup>, Aliz Zimmermann<sup>a,1</sup>, Dávid Kis<sup>a</sup>, Orsolya Oláh<sup>a</sup>, Gábor K. Tóth<sup>c</sup>, Orsolya Hegyi<sup>c</sup>, David W. Busija<sup>b</sup>, Ferenc Bari<sup>c</sup>, Ferenc Domoki<sup>a</sup>

<sup>a</sup>Department of Physiology, Faculty of Medicine, University of Szeged, Szeged, Hungary

<sup>b</sup>Department of Physiology and Pharmacology, Wake Forest University Health Sciences, Winston-Salem, NC, USA

<sup>c</sup>Department of Medical Chemistry, Faculty of Medicine, University of Szeged, Szeged, Hungary

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**ABSTRACT**

Pituitary adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are neuroprotective in numerous models. Impairment of cerebrovascular reactivity (CR) contributes to ischemia/reperfusion (I/R)-induced neuronal damage. We tested whether PACAP and/or VIP preserve CR to I/R-sensitive dilator responses dependent on endothelial and/or neuronal function. Accordingly, changes in pial arteriolar diameters in response to hypercapnia (5–10% CO<sub>2</sub> ventilation) or topical N-methyl-D-aspartate (NMDA, 10<sup>-4</sup> M) were determined before and after I/R via intravital microscopy in anesthetized/ventilated piglets. Local pretreatment with non-vasoactive doses of PACAP (10<sup>-8</sup> M) and VIP (10<sup>-9</sup> M) prevented the attenuation of postischemic CR to hypercapnia; to 10% CO<sub>2</sub>, the CR values were 27 ± 8% vs 92 ± 5%\* vs 88 ± 13%\* (vehicle vs PACAP38 vs VIP, CR expressed as a percentage of the response before I/R, mean ± SEM, n = 8–8, \*p < 0.05). PACAP, but not VIP, preserved CR to NMDA after I/R, with CR values of 31 ± 10% vs 87 ± 8%\* vs 35 ± 12% (vehicle vs PACAP38 vs VIP, n = 6–6). Unlike PACAP, VIP-induced vasodilation has not yet been investigated in the piglet. We tested whether VIP-induced arteriolar dilation was sensitive to inhibitors of cyclooxygenase (COX)-1 (SC-560, 1 mg/kg), COX-2 (NS-398, 1 mg/kg), indomethacin (5 mg/kg), and nitric oxide synthase (L-NAME, 15 mg/kg). VIP (10<sup>-8</sup>–10<sup>-7</sup>–10<sup>-6</sup> M, n = 8) induced reproducible, dose-dependent vasodilation of 16 ± 3%, 33 ± 6%\*, and 70 ± 8%\*. The response was unaffected by all drugs, except that the vasodilation to 10<sup>-8</sup> M VIP was abolished by SC-560 and indomethacin. In conclusion, PACAP and VIP differentially preserve postischemic CR; independent of their vasodilatory effect.

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\* Corresponding author. Department of Physiology, Faculty of Medicine, University of Szeged, Szeged, Dóm tér 10, 6720, Hungary. Fax: +36 62 544978.

E-mail address: [lentilaura@gmail.com](mailto:lentilaura@gmail.com) (L. Lenti).

Abbreviations: PACAP, pituitary adenylate cyclase activating polypeptide; VIP, vasoactive intestinal peptide; NMDA, N-methyl-D-aspartate; COX, cyclooxygenase; NOS, nitric oxide synthase; NO, nitric oxide; L-NAME, N-ω-nitro-L-arginine methyl ester; DMSO, dimethyl sulfoxide; aCSF, artificial cerebrospinal fluid; ROS, reactive oxygen species; CR, cerebrovascular reactivity; I/R, ischemia/reperfusion; MABP, mean arterial blood pressure; ANOVA, analysis of variance; SEM, standard error of the mean

<sup>1</sup> These authors contributed equally to this manuscript.

## 1. Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are structurally related neuropeptides. PACAP has two naturally occurring isoforms; PACAP38, a basic, amidated 38-residue peptide, and PACAP27, the N-terminal portion consisting of 27 amino acids (Miyata et al. 1989; Miyata et al. 1990). PACAP possesses specific receptors (8 subtypes of PAC1), but also shares common receptors with VIP (VPAC1, VPAC2) (Harmar et al. 1998; Vaudry et al. 2000). Thus, despite the high sequence homology, these peptides are expected to activate multiple pathways resulting in distinct biological actions.

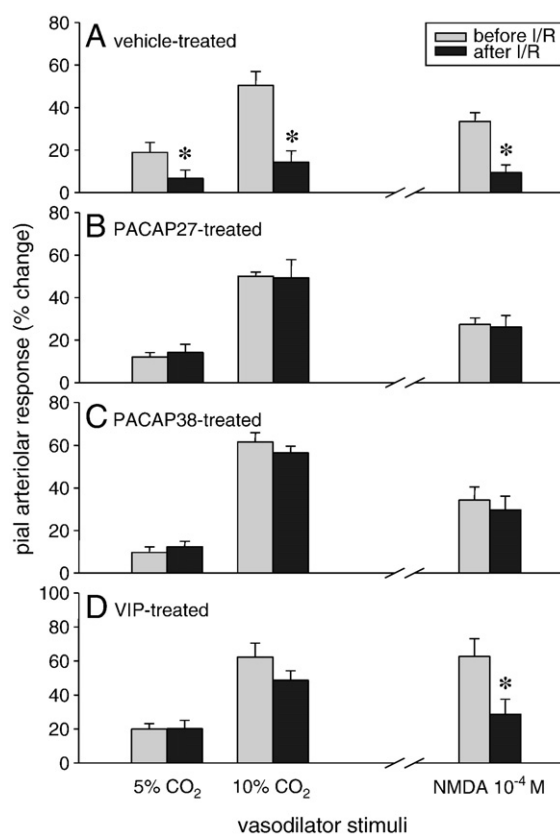
In the cerebral circulation of the newborn pig, PACAP27 and PACAP38 have been reported to induce prominent pial arteriolar dilation (Tong et al. 1993; Lenti et al. 2007). We found that PACAP38-induced vasodilation was abolished by a cyclooxygenase (COX)-1-inhibitor, while PACAP27-induced vasodilation was insensitive to either COX-1 or COX-2 blockade (Lenti et al. 2007). Ischemia/reperfusion (I/R), which frequently occurs in the perinatal period (Berger et al. 2002), has been shown to impair several cerebrovascular regulatory mechanisms of the neurovascular axis in piglets (Leffler et al. 1989a; Leffler et al. 1989b; Busija et al. 1996). Impaired cerebrovascular reactivity (CR) probably contributes to and aggravates neuronal cell death following hypoxic/ischemic brain injury. Previous studies identified N-methyl-D-aspartate (NMDA)- and CO<sub>2</sub>-induced vasodilatory responses as prototypes of such I/R-sensitive mechanisms. However, these stimuli dilate cerebral arterioles by completely different mechanisms. NMDA-induced pial arteriolar dilation is a neuronal-triggered multi-step process involving the activation of neuronal nitric oxide (NO) synthase (NOS) (Meng et al. 1995; Domoki et al. 2002), while CO<sub>2</sub> induces vasodilation via an endothelium-dependent, indomethacin-sensitive process in the newborn pig (Leffler et al. 1993; Leffler et al. 1994b).

PACAP and VIP are considered as endogenous neuroprotective mediators that may be released from neural and vascular cells during I/R and could possibly reduce neuronal injury (Brenneman 2007; Stumm et al. 2007). Conceivably, these neuroprotective peptides may protect CR after I/R as well. Therefore, the major goal of our study was to determine whether PACAP27, PACAP38 and/or VIP preserves CR to NMDA and/or hypercapnia after I/R, and whether the protective effects of the neuropeptides are dependent on their vasoactivity. Since the pial arteriolar response to VIP has not yet been described in the piglet, we also characterized VIP-induced vasodilation using peptide antagonists (PACAP6-38 and PACAP6-27), COX- and NOS inhibitors.

## 2. Results

### 2.1. Effects of PACAP/VIP pretreatment on pial arteriolar responsiveness to CO<sub>2</sub> after I/R

Graded hypercapnia (5–10% CO<sub>2</sub> ventilation) resulted in large, dose-dependent, reversible increases in pial arteriolar diameters. In the vehicle-treated animals (group 1), I/R (10 min of ischemia and 1 h of reperfusion) severely attenuated the



**Fig. 1 – Effects of ischemia/reperfusion (I/R) on hypercapnia- or NMDA-induced pial arteriolar vasodilation. Arteriolar responses to 5–10% CO<sub>2</sub> ventilation and topical NMDA were recorded before and after 10 min of global cerebral ischemia followed by 1 h of reperfusion. Hypercapnia elicited concentration-dependent pial arteriolar vasodilation, which was markedly attenuated after I/R in vehicle-treated piglets (A, left). PACAP isoforms and VIP preserved the vascular responsiveness to CO<sub>2</sub> (B–D, left). Vasodilation in response to 10<sup>-4</sup> M NMDA was also deteriorated after I/R (A, right). PACAP27 and PACAP38 (B–C, right), but not VIP (D, right), protected the vasodilator effect of NMDA. (Data are mean ± SEM, n = 6–8, \*p < 0.05.)**

hypercapnia-induced arteriolar vasodilation (Fig. 1A, left). Incubation of the brain surface with PACAP27 (10<sup>-8</sup> M), PACAP38 (10<sup>-8</sup> M) or VIP (10<sup>-9</sup> M) for 30 min did not affect the pial arterioles significantly (2 ± 1%, 2 ± 1%, and 3 ± 1% maximal dilation in response to PACAP27, PACAP38, and VIP, respectively); however, they efficiently preserved CR to both levels of hypercapnia after I/R (groups 3, 5, and 7; Figs. 1B–D, left).

### 2.2. Effects of PACAP/VIP pretreatment on pial arteriolar responsiveness to NMDA after I/R

Topical application of NMDA (10<sup>-4</sup> M) induced a marked vasodilation that was also sensitive to I/R (group 2, Fig. 1A, right). PACAP27 (10<sup>-8</sup> M) or PACAP38 (10<sup>-8</sup> M) pretreatment of the brain surface protected the NMDA-evoked pial arteriolar dilation after I/R, whereas incubation of the brain surface with 10<sup>-9</sup> M VIP was not effective (groups 4, 6, and 8; Figs. 1B–D,

right). Furthermore, a higher dose of VIP ( $10^{-8}$  M, group 8b) was still unable to preserve CR to  $10^{-4}$  M NMDA reduced by I/R. CR values were  $36 \pm 12\%$  vs  $51 \pm 6\%$  (vehicle vs VIP, CR expressed as a percentage of the preischemic response). In group 8c, lower,  $10^{-5}$  M and  $5 \times 10^{-5}$  M concentrations of NMDA also dilated the pial arterioles with  $4.4 \pm 0.2\%$  and  $12.6 \pm 0.4\%$ , respectively ( $*p < 0.05$ ). I/R reduced these vascular reactions significantly, and moreover, a tendency towards vasoconstriction was observed after I/R ( $-2 \pm 3\%$  and  $-3 \pm 4\%$  changes in diameter to  $10^{-5}$  M and  $5 \times 10^{-5}$  M NMDA), irrespectively of the VIP ( $10^{-8}$  M) treatment.

### 2.3. Effects of PACAP/VIP treatment on CO<sub>2</sub>- and NMDA-induced pial arteriolar dilation

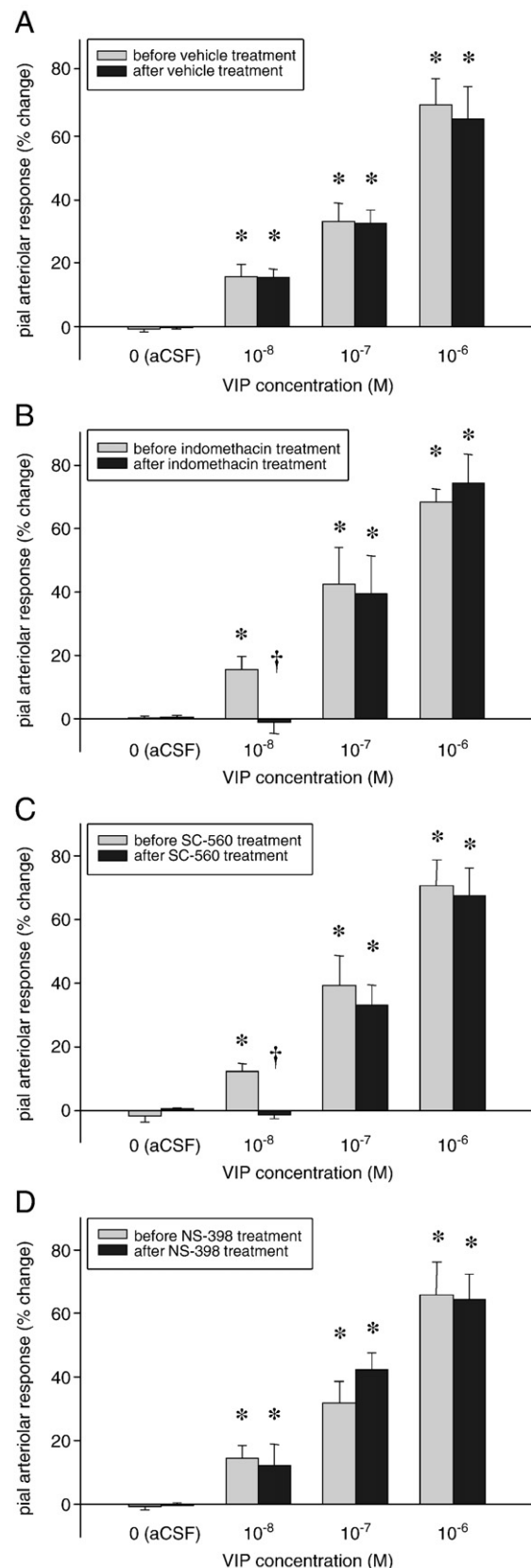
Topical PACAP27 ( $10^{-8}$  M), PACAP38 ( $10^{-8}$  M) or VIP ( $10^{-9}$  M) treatment alone did not affect the vasodilation induced by either hypercapnia or NMDA. Vascular responses to 10% CO<sub>2</sub> were  $50 \pm 2\%$  and  $57 \pm 7\%$  before and after PACAP27 (group 9), and  $63 \pm 5\%$  and  $64 \pm 6\%$  before and after PACAP38 incubation (group 11). Topical NMDA ( $10^{-4}$  M) evoked  $36 \pm 7\%$  and  $33 \pm 5\%$  vasodilation before and after PACAP27, and  $37 \pm 5\%$  and  $43 \pm 7\%$  vasodilation before and after PACAP38 application (groups 10 and 12). Responses to 10% CO<sub>2</sub> were  $60 \pm 9\%$  and  $56 \pm 11\%$  (group 13), and to  $10^{-4}$  M NMDA were  $49 \pm 7\%$  and  $48 \pm 7\%$  (group 14), before and after VIP treatment. Accordingly, the protective effects of these neuropeptides are not caused by direct facilitation of the vascular reactivity to hypercapnia or to NMDA.

### 2.4. Effects of different enzyme inhibitors on VIP-induced pial arteriolar vasodilation

VIP induced dose-dependent, reproducible pial arteriolar vasodilation at  $10^{-8}$ – $10^{-6}$  M concentrations (Fig. 2A). The VIP-induced vasodilation was partially indomethacin-sensitive (Fig. 2B), since administration (5 mg/kg iv) of this non-selective COX inhibitor significantly blocked the vasodilation evoked by the lowest ( $10^{-8}$  M) dose of VIP, whereas it left the vasodilation to higher concentrations ( $10^{-7}$ – $10^{-6}$  M) of VIP essentially intact (B). The pial arteriolar response to VIP was differentially affected by selective COX inhibitors; the COX-1 inhibitor SC-560 abolished the arteriolar responses to  $10^{-8}$  M VIP (C), whereas the COX-2 inhibitor NS-398 was ineffective (D). (Data are mean  $\pm$  SEM,  $n = 6$ – $8$ ,  $*p < 0.05$ : significantly higher than vasodilation to all smaller VIP concentrations,  $\dagger p < 0.05$ : significantly less than the corresponding value before treatment.)

**Fig. 2 – Characterization of VIP-induced pial arteriolar vasodilation. VIP elicited significant, dose-dependent, reproducible increases in pial arteriolar diameters, while artificial cerebrospinal fluid (aCSF) did not evoke vasodilation (A). The non-selective cyclooxygenase (COX) inhibitor indomethacin abolished the pial arteriolar dilation in response to  $10^{-8}$  M VIP, whereas it left the vasodilation to higher concentrations ( $10^{-7}$ – $10^{-6}$  M) of VIP essentially intact (B). The pial arteriolar response to VIP was differentially affected by selective COX inhibitors; the COX-1 inhibitor SC-560 abolished the arteriolar responses to  $10^{-8}$  M VIP (C), whereas the COX-2 inhibitor NS-398 was ineffective (D). (Data are mean  $\pm$  SEM,  $n = 6$ – $8$ ,  $*p < 0.05$ : significantly higher than vasodilation to all smaller VIP concentrations,  $\dagger p < 0.05$ : significantly less than the corresponding value before treatment.)**

oxide synthase (NOS) blockade had also no influence on the VIP-induced vasodilation; application of  $10^{-8}$ – $10^{-6}$  M VIP onto the brain surface resulted in significant,  $15 \pm 3$ – $61 \pm 10\%$  and



17±7–62±9% caliber changes before and after intravenous N- $\omega$ -nitro-L-arginine methyl ester (L-NAME, 15 mg/kg) administration.

### 2.5. Effects of PACAP receptor antagonists on VIP-induced pial arteriolar vasodilation

PACAP6-38 ( $10^{-5}$  M, group 20) pretreatment effectively diminished the VIP-induced vasodilation (62±10% and 33±6%\* vasodilation in response to  $10^{-6}$  M VIP, before and after PACAP6-38, \* $p$ <0.05); however, in group 21, PACAP6-27 ( $10^{-5}$  M) failed to exhibit any inhibitory effect on the VIP-related pial arteriolar dilation (61±12% and 66±11% changes in diameter before and after topical PACAP6-27 treatment).

### 2.6. Physiological variables

Graded (5 and 10%) hypercapnia significantly elevated the arterial pCO<sub>2</sub> levels in all experimental groups where CR to hypercapnia was tested. The pCO<sub>2</sub> changes were similar before and after I/R and between groups. For instance, in group 7, 5 and 10% CO<sub>2</sub> elevated pCO<sub>2</sub> from 34±2.5 to 52±2.1 and 79±5 mm Hg and from 37±2.7 to 56±2.7 and 84±5.9 mm Hg before and after I/R, respectively. The respective pO<sub>2</sub> levels were 90±5.9–94±6.4–94±7.4 and 90±7.6–89±5.8–88±4.2 mm Hg in this group and were similar in all groups. Otherwise, body temperature, arterial pH, and blood gases were kept in the normal ranges and did not vary significantly among the different groups throughout the experiments. In group 1, for instance, the baseline values were as follows: core temperature=37±0.2 °C, pH=7.43±0.03, pCO<sub>2</sub>=38±2 mm Hg, pO<sub>2</sub>=91±5 mm Hg. Similarly, the mean arterial blood pressure (MABP) values were always within the range characteristic for anesthetized newborn pigs and did not differ significantly between groups (e.g. 70±3 mm Hg and 69±3 mm Hg in groups 1 and 2, respectively). Furthermore, in each animal, care was taken to avoid any fluctuation in MABP during recordings so as to prevent effects of short-term changes in arterial pressure on vascular diameters. There were no significant differences between the baseline diameters in the groups; for example, in group 15 the values were 92±3  $\mu$ m and 95±5  $\mu$ m before the first and the second VIP application, respectively.

## 3. Discussion

The major findings of the present study: (1) PACAP27, PACAP38, and VIP prevent the attenuation of hypercapnia-induced vasodilation caused by I/R; (2) PACAP27 and PACAP38, but not VIP preserves NMDA-induced vasodilation; (3) VIP evokes concentration-dependent, reproducible pial arteriolar dilation that is independent of NOS activity, and essentially unaffected by COX inhibitors. Finally, (4) VIP-induced vasodilation is attenuated by PACAP6-38, but not by PACAP6-27.

PACAP and VIP are widely distributed throughout the central and peripheral nervous system, and they have been identified in nerve endings innervating pial and intraparenchymal vessels of the cerebral cortex (Fahrenkrug et al. 2000). These perivascular nerve fibers are of either parasympathetic,

trigeminal sensory or cortical neuronal origin (Edvinsson and Krause 2002). Our results indicate that PACAP and VIP are markedly different in their respective protective and vasodilator actions also indicating that these neuropeptides may activate only partially overlapping mechanisms.

In porcine cerebral vessels, hypercapnia evokes vasodilation not only *in vivo* (Leffler et al. 1989a), but also in isolated, denervated vessels (Kokubun et al. 2009) suggesting that neuronal/glial factors are not essential for the hypercapnia-induced vasodilation. In the piglet, hypercapnia-induced vasodilation requires intact endothelium (Leffler et al. 1994b); more specifically, the endothelium appears to serve as a source of prostanoids for the vascular smooth muscle to permit the relaxation (Leffler et al. 1994a). Prostanoid synthesis increases in newborn pig brain microvascular endothelial cells in response to hypercapnia, but high CO<sub>2</sub> level does not increase prostanoid production by cerebral microvascular smooth muscle or glial cells (Hsu et al. 1993). Hypercapnia-induced vasodilation is vulnerable to I/R; however, supplementation of arachidonic acid restores this vasodilation and hypercapnia-related increases in the cerebrospinal fluid 6-keto-prostaglandinF<sub>1 $\alpha$</sub>  levels (Leffler et al. 1992). Based on these findings, I/R seems to reduce hypercapnia-induced dilation of pial arterioles through endothelial damage in piglets. Therefore, the present data indicate decreased/shortened postischemic endothelial dysfunction by PACAP or VIP pretreatment, as suggested by the preserved hypercapnia-induced vasodilation. We are not aware of any studies in which similar protective effects of PACAP and VIP have been demonstrated on the cerebrovascular endothelium. Our findings are in agreement with the findings of Lange et al., who demonstrated both the synthesis of VIP and the expression of VIP receptor associated protein in microvascular endothelial cells of pial vessels in piglets (Lange et al. 1999), allowing a direct protective effect of both VIP and PACAP. The function of endothelial VIP production/effects is unclear, but an autocrine growth factor role involved in postnatal endothelial cell differentiation has been suggested. The exact mechanism of endothelial protection by these neuropeptides is unclear and its exploration demands further experiments. Although most data suggest the principal involvement of endothelium, the role of other cell types cannot be excluded, since neuronal/glial components also contribute to hypercapnia-induced cerebrovascular dilation in other experimental models (Wang et al. 1999; Xu et al. 2004).

Our present study clearly demonstrates that PACAP27 and PACAP38, but not VIP preserves CR to NMDA after I/R. The mechanisms of NMDA-induced pial arteriolar dilation and the attenuation of this response after hypoxic/ischemic stress in piglets has been recently reviewed (Busija et al. 2007). Briefly, the activation of neuronal NMDA receptors leads to the subsequent activation of a specific population of neuronal NOS positive neurons via local neuronal connections (Faraci and Breese 1993; Bari et al. 1996b). The released NO then diffuses to and acts on the vascular smooth muscle, resulting in dilation of the pial arterioles (Meng et al. 1995; Domoki et al. 2002). The response is unaffected by damage to the vascular endothelium (Domoki et al. 2002), but have been shown to be vulnerable to even short periods of hypoxic stress (Bari et al. 1996a; Busija et al. 1996). In contrast, the pial

arteriolar response to NO itself is unaffected by I/R (Busija et al. 1996). All available evidence strongly suggests the causative role of reactive oxygen species (ROS) in the attenuation of NMDA-induced vasodilation after I/R. In piglets, topical application of ROS scavengers preserves cerebral arteriolar dilator responses to NMDA after I/R (Bari et al. 1996a). The primary site of ROS action appears to be at the level of the NMDA receptor (Choi et al. 2000; Guerguerian et al. 2002). Alternatively, the functional coupling between NMDA receptor and nNOS expressing neuronal populations may be disrupted after I/R.

Although PACAP and VIP display neuroprotective properties against a wide range of pathological conditions, PACAP is generally more potent than VIP and its function has been more widely investigated (Tamas et al. 2002). Vasodilatory, antioxidant, anti-apoptotic, neurotrophic, and anti-inflammatory effects have been promoted as the putative mechanisms of neuroprotection in various experimental models. Our present results suggest that preservation of CR to NMDA after I/R is independent of the increase in cerebral blood flow mediated by vasodilation to PACAP. In fact, PACAP effectively preserved NMDA-induced vasodilation in a non-vasoactive dose, whereas VIP was ineffective in an equimolar, vasoactive dose. However, the reported antioxidant property of PACAP can be an important factor in the preservation of the NMDA receptor function, especially that an analogous antioxidant capacity of VIP is absent (Reglodi et al. 2004). The PACAP-induced initiation of anti-apoptotic and anti-inflammatory mechanisms may also lead to increased general viability of the neurons. More specifically, PAC1 receptor stimulation leads to activation of Bcl-2/inhibition of Bad resulting in enhanced mitochondrial integrity/decreased release of apoptotic cytochrome c. Conceivably, preservation of mitochondrial function fastens the restoration of cellular ATP levels and also reduces postischemic ROS production. PACAP binding sites in the rat cerebral cortex are ten times more numerous as compared to VIP; this difference may also explain the higher general neuroprotective potency of PACAP (Masuo et al. 1991). Alternatively, PACAP isoforms may act on PAC1 receptors expressed on a specific population of cortical neurons critically involved in NMDA-induced vasodilation. Furthermore, the differences observed between PACAP and VIP can be due to different signal transduction mechanisms coupled to PACAP and/or VIP-stimulated receptors in these cells.

Vasodilator neuropeptides such as PACAP and VIP have substantial roles in cerebrovascular control mechanisms (Edvinsson and Krause 2002). In our previous study indomethacin and the selective COX-1 inhibitor SC-560 abolished PACAP38-induced vasodilation to all concentrations used ( $10^{-8}$ – $10^{-6}$  M); however, the vascular action of PACAP27 was completely independent of COX activity (Lenti et al. 2007). Our present results show that vasodilation to  $10^{-8}$  M VIP resembles the vasodilator effect of PACAP38 characterized by indomethacin- and SC-560-sensitivity. In contrast, at  $10^{-7}$ – $10^{-6}$  M VIP concentrations the response is unaffected by COX-1 inhibition similar to PACAP27. Since NOS inhibition has also no effect on VIP-induced vasodilation, VIP probably exerts a robust direct effect on vascular smooth muscle cells at these doses. Similarly, VIP dilates the isolated porcine

ophthalmic artery via COX-mediated mechanisms only at lower ( $10^{-10}$ – $10^{-9}$  M) VIP concentrations (Vincent 1992). VIP-induced pial arteriolar vasodilation is also indomethacin-insensitive in cats (Wei et al. 1980), whereas involvement of NOS activity in VIP-induced vasodilation was also reported in other, adult animal models (Gaw et al. 1991; Grant et al. 2006). The presumed activation of COX-1 by VIP and PACAP38 is unique, since COX-2 is known to be the predominant COX isoform in the newborn central nervous system (Peri et al. 1995; Parfenova et al. 1997), and SC-560 did not affect the vasodilation in response to any other COX-dependent stimuli studied (Domoki et al. 2005b).

The VIP/PACAP receptor antagonist PACAP fragments (PACAP6-38 and PACAP6-27) inhibited both PACAP27- and PACAP38-induced vasodilation in our previous study (Lenti et al. 2007). PACAP6-38, unlike PACAP6-27, is often reported to be a selective blocker of the PAC1 receptor group (Edvinsson and Krause 2002); however, in the present study, only PACAP6-38 but not PACAP6-27 attenuates VIP-induced vasodilation. This finding is in accordance with studies identifying PACAP6-38 as a nonspecific VIP/PACAP antagonist (Harmar et al. 1998). Possibly, PACAP6-38 simply is a more potent inhibitor than PACAP6-27 on the VIP/PACAP receptors involved in VIP-induced vasodilation. Unfortunately, we are unaware of the existence of any widely accepted, subtype-selective VIP/PACAP receptor blockers that would be required to unveil the functional relevance of each receptor subtype in cerebrovascular and neuroprotective effects of these neuropeptides (Edvinsson and Krause 2002).

In conclusion, PACAP27, PACAP38, and VIP all protect postischemic vascular reactivity to CO<sub>2</sub>, an ischemia-sensitive indicator of endothelial function in the newborn pig. However, PACAP isoforms but not VIP preserves the NMDA-induced neuron-dependent vasodilation. Although PACAP27, PACAP38, and VIP all induce dose-dependent pial arteriolar dilation by activating partially overlapping mechanisms, the protective effect of these neuropeptides is independent of their vasoactivity. This neurovascular protection probably supports the restoration of adequate perfusion of the brain tissue after I/R likely enhancing the direct neuroprotective effects of VIP and PACAP.

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## 4. Experimental procedures

### 4.1. Animals

Newborn piglets of either sex (1–2 days old, body weight 1–3 kg,  $n=138$ ) were used. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Szeged.

Anesthesia was initiated with thiopental sodium (40 mg/kg ip.; Biochemie, Vienna, Austria) and a bolus injection of  $\alpha$ -chloralose (40 mg/kg iv.; Sigma, St. Louis, MO, USA). Additional doses of  $\alpha$ -chloralose (3–7 mg/kg/h iv.) were given to maintain a constant level of anesthesia. A catheter was inserted into the right femoral artery to monitor blood pressure and to sample blood for determination of blood gas tensions and pH. Fluid and drugs were administered through a second catheter placed in the right femoral vein.

The animals were intubated via tracheotomy and mechanically ventilated with room air. The ventilation rate (~30 breaths/min) and tidal volume (~20 ml) were adjusted to maintain arterial blood gas values and pH in the physiological range. A water-circulating heating pad was used to maintain the body temperature at ~37°C. Core temperature was monitored with a rectal probe. The animals were equipped with a closed cranial window as previously described (Domoki et al. 2005b).

Following surgery, the closed window was filled with artificial cerebrospinal fluid (aCSF) which was similar to the endogenous CSF (aCSF composition: KCl 220, MgCl<sub>2</sub> 132, CaCl<sub>2</sub> 221, NaCl 7710, urea 402, dextrose 665, and NaHCO<sub>3</sub> 2066, in mg/l), warmed to 37 °C and equilibrated with a gas mixture containing 6% O<sub>2</sub>, 6.5% CO<sub>2</sub> and 87.5% N<sub>2</sub> to obtain pH 7.33, pCO<sub>2</sub>=46 mm Hg and pO<sub>2</sub>=43 mm Hg. Pial arterial vessels were observed with an operating microscope (Wild, Switzerland) equipped with a video camera (Sanyo digital color CCD camera, Japan), and a video monitor (Panasonic, Japan). Vascular diameters were measured with a video microscaler. In each experiment, a ~100- $\mu$ m-diameter (range 76–114  $\mu$ m) pial arteriole was selected. We chose this vessel size because this is the level of the first-order pial arterioles and the primary site of vascular resistance. After a stable baseline diameter was reached, the window was flushed with aCSF as control. Topical stimuli were applied to the pial surface through one of the injectable ports of the cranial window. The pial arterioles were exposed to each vasodilator stimulus for 5 min, while arteriolar diameters were measured continuously. After completion of a stimulus, the cranial window was flushed with aCSF and the vessel diameter was allowed to return to the baseline level.

To induce global cerebral ischemia, a 3-mm hole was made with an electric drill in the left frontal cranium rostral to the cranial window and the dura was exposed. A hollow brass bolt was inserted and secured in place with dental acrylic. Cerebral ischemia was induced by the infusion of aCSF so as to raise the intracranial pressure above the arterial pressure. Ischemia was verified by the cessation of blood flow in the vessels observed through the cranial window. Venous blood was withdrawn as necessary to keep MABP near the normal values. At the end of the ischemic period, the infusion tube was clamped and the intracranial pressure returned to the preischemic levels. The withdrawn and heparinized blood was reinfused (Domoki et al. 2005a).

At the end of the experiments, the animals were euthanized with an iv. injection of saturated KCl solution.

#### 4.2. Drugs

VIP (Sigma Chemical Co.), PACAP38, PACAP27, PACAP6-27, and PACAP6-38 (Department of Medical Chemistry, University of Szeged, Hungary) were prepared as stock solutions in saline (10<sup>-4</sup> M) and, before use, were diluted with aCSF (10<sup>-9</sup>–10<sup>-5</sup> M). Indomethacin (Merck & Co, Whitehouse Station, NJ) and L-NAME (Sigma Chemical Co.) were dissolved in saline (30 mg/ml and 10 mg/ml, respectively). NS-398 (Sigma Chemical Co.) and SC-560 (Sigma Chemical Co.) were dissolved in dimethyl sulfoxide (DMSO, 5 mg/ml) and further diluted with saline to 1 ml. NMDA (Sigma Chemical Co.) was dissolved in aCSF. Selection of enzyme inhibitor doses was based on

previous results in this experimental model (Bari et al. 1996b; Domoki et al. 2005b; Lenti et al. 2007).

#### 4.3. Protocol

In the first series of experiments, the effect of global cerebral I/R (10 min of ischemia and 1 h of reperfusion) was tested on CO<sub>2</sub>- and NMDA-induced vasodilation, with or without PACAP or VIP treatment. Prior to the induction of ischemia, the brain surface was incubated for 30 min with vehicle (aCSF, groups 1–2, n=6–6), 10<sup>-8</sup> M PACAP27 (groups 3–4, n=6–6), 10<sup>-8</sup> M PACAP38 (groups 5–6, n=6–6), or 10<sup>-9</sup> M VIP (groups 7–8, n=8–6). Pial arteriolar caliber changes to 5 and 10% CO<sub>2</sub> ventilation (groups 1, 3, 5, and 7), or to topical, 10<sup>-4</sup> M NMDA (groups 2, 4, 6, and 8) were measured before the pretreatments and after 1 h of reperfusion. We performed additional experiments on 4–4 animals in groups 8b and 8c to test whether a higher (10<sup>-8</sup> M) dose of VIP can protect NMDA-induced vasodilation after I/R. In group 8b we used the NMDA at 10<sup>-4</sup> M concentration similar to the original experiments, while in group 8c we tested if the vascular response to a lower, 10<sup>-5</sup> M and 5 × 10<sup>-5</sup> M concentration of NMDA can be protected by VIP (10<sup>-8</sup> M) after I/R. The effects of the same PACAP27, PACAP38 or VIP pretreatments in the absence of I/R were also tested on the vasodilation evoked by hypercapnia (groups 9, 11, and 13, n=6–5–6) or by NMDA (groups 10, 12, and 14, n=5–7–5), respectively.

In the second series of experiments, the brain surface was exposed to VIP before and after the intravenous administration of different enzyme inhibitors or topical application of different PACAP fragments. VIP application (10<sup>-8</sup>–10<sup>-6</sup> M cumulatively) was repeated 20 min after the intravenous administration of vehicle (group 15, saline in 5, and diluted DMSO in 3 animals), indomethacin (group 16, n=7, 5 mg/kg), SC-560 (group 17, n=6, 1 mg/kg), NS-398 (group 18, n=7, 1 mg/kg) or L-NAME (group 19, n=8, 15 mg/kg). Pial arteriolar dilation to 10<sup>-6</sup> M VIP was determined before and after 20 min topical application of PACAP6-38 (group 20, n=6, 10<sup>-5</sup> M) or PACAP6-27 (group 21, n=7, 10<sup>-5</sup> M).

#### 4.4. Statistical analysis

The pial artery diameter data (absolute diameters or maximal percentage changes from the baseline values) were analyzed by one-way repeated measures ANOVA. For post hoc analysis, Tukey's test was performed where appropriate. Values of *p*<0.05 were considered significant. Data are reported as mean ± SEM.

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## REFERENCES

- Bari, F., Errico, R.A., Louis, T.M., Busija, D.W., 1996a. Differential effects of short-term hypoxia and hypercapnia on N-methyl-D-aspartate-induced cerebral vasodilation in piglets. *Stroke* 27, 1634–1639 discussion 1639–1640.
- Bari, F., Errico, R.A., Louis, T.M., Busija, D.W., 1996b. Interaction between ATP-sensitive K<sup>+</sup> channels and nitric oxide on pial arterioles in piglets. *J. Cereb. Blood Flow Metab.* 16, 1158–1164.
- Berger, R., Garnier, Y., Jensen, A., 2002. Perinatal brain damage: underlying mechanisms and neuroprotective strategies. *J. Soc. Gynecol. Investig.* 9, 319–328.
- Brenneman, D.E., 2007. Neuroprotection: a comparative view of vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Peptides* 28, 1720–1726.
- Busija, D.W., Meng, W., Bari, F., McGough, P.S., Errico, R.A., Tobin, J.R., Louis, T.M., 1996. Effects of ischemia on cerebrovascular responses to N-methyl-D-aspartate in piglets. *Am. J. Physiol.* 270, H1225–H1230.
- Busija, D.W., Bari, F., Domoki, F., Louis, T., 2007. Mechanisms involved in the cerebrovascular dilator effects of N-methyl-D-aspartate in cerebral cortex. *Brain Res. Rev.* 56, 89–100.
- Choi, Y.B., Tenneti, L., Le, D.A., Ortiz, J., Bai, G., Chen, H.S., Lipton, S.A., 2000. Molecular basis of NMDA receptor-coupled ion channel modulation by S-nitrosylation. *Nat. Neurosci.* 3, 15–21.
- Domoki, F., Perciaccante, J.V., Shimizu, K., Puskar, M., Busija, D.W., Bari, F., 2002. N-methyl-D-aspartate-induced vasodilation is mediated by endothelium-independent nitric oxide release in piglets. *Am. J. Physiol. Heart Circ. Physiol.* 282, H1404–H1409.
- Domoki, F., Kis, B., Nagy, K., Farkas, E., Busija, D.W., Bari, F., 2005a. Diazoxide preserves hypercapnia-induced arteriolar vasodilation after global cerebral ischemia in piglets. *Am. J. Physiol. Heart Circ. Physiol.* 289, H368–H373.
- Domoki, F., Nagy, K., Temesvari, P., Bari, F., 2005b. Selective inhibitors differentially affect cyclooxygenase-dependent pial arteriolar responses in newborn pigs. *Pediatr. Res.* 57, 853–857.
- Edvinsson, L., Krause, D.N., 2002. *Cerebral Blood Flow and Metabolism*. Lippincott Williams and Wilkins, Philadelphia, USA.
- Fahrenkrug, J., Hannibal, J., Tams, J., Georg, B., 2000. Immunohistochemical localization of the VIP1 receptor (VPAC1R) in rat cerebral blood vessels: relation to PACAP and VIP containing nerves. *J. Cereb. Blood Flow Metab.* 20, 1205–1214.
- Faraci, F.M., Breese, K.R., 1993. Nitric oxide mediates vasodilatation in response to activation of N-methyl-D-aspartate receptors in brain. *Circ. Res.* 72, 476–480.
- Gaw, A.J., Aberdeen, J., Humphrey, P.P., Wadsworth, R.M., Burnstock, G., 1991. Relaxation of sheep cerebral arteries by vasoactive intestinal polypeptide and neurogenic stimulation: inhibition by L-NG-monomethyl arginine in endothelium-denuded vessels. *Br. J. Pharmacol.* 102, 567–572.
- Grant, S., Lutz, E.M., McPhaden, A.R., Wadsworth, R.M., 2006. Location and function of VPAC1, VPAC2 and NPR-C receptors in VIP-induced vasodilation of porcine basilar arteries. *J. Cereb. Blood Flow Metab.* 26, 58–67.
- Guerguerian, A.M., Brambrink, A.M., Traystman, R.J., Huganir, R.L., Martin, L.J., 2002. Altered expression and phosphorylation of N-methyl-D-aspartate receptors in piglet striatum after hypoxia-ischemia. *Brain Res. Mol. Brain Res.* 104, 66–80.
- Harmar, A.J., Arimura, A., Gozes, I., Journot, L., Laburthe, M., Pisegna, J.R., Rawlings, S.R., Robberecht, P., Said, S.I., Sreedharan, S.P., Wank, S.A., Waschek, J.A., 1998. International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Pharmacol. Rev.* 50, 265–270.
- Hsu, P., Shibata, M., Leffler, C.W., 1993. Prostanoid synthesis in response to high CO<sub>2</sub> in newborn pig brain microvascular endothelial cells. *Am. J. Physiol.* 264, H1485–H1492.
- Kokubun, S., Fukuda, S., Shimoji, K., Sakamoto, H., Gamou, S., Ogura, M., Yunokawa, S., Morita, S., 2009. Differential responses of porcine anterior spinal and middle cerebral arteries to carbon dioxide and pH. *Crit. Care Med.* 37, 987–992.
- Lange, D., Funa, K., Ishisaki, A., Bauer, R., Wollina, U., 1999. Autocrine endothelial regulation in brain stem vessels of newborn piglets. *Histol. Histopathol.* 14, 821–825.
- Leffler, C.W., Beasley, D.G., Busija, D.W., 1989a. Cerebral ischemia alters cerebral microvascular reactivity in newborn pigs. *Am. J. Physiol.* 257, H266–H271.
- Leffler, C.W., Busija, D.W., Armstead, W.M., Mirro, R., Beasley, D.G., 1989b. Ischemia alters cerebral vascular responses to hypercapnia and acetylcholine in piglets. *Pediatr. Res.* 25, 180–183.
- Leffler, C.W., Mirro, R., Armstead, W.M., Shibata, M., 1992. Topical arachidonic acid restores pial arteriolar dilation to hypercapnia of postischemic newborn pig brain. *Am. J. Physiol.* 263, H746–H751.
- Leffler, C.W., Mirro, R., Shibata, M., Parfenova, H., Armstead, W.M., Zuckerman, S., 1993. Effects of indomethacin on cerebral vasodilator responses to arachidonic acid and hypercapnia in newborn pigs. *Pediatr. Res.* 33, 609–614.
- Leffler, C.W., Mirro, R., Pharris, L.J., Shibata, M., 1994a. Permissive role of prostacyclin in cerebral vasodilation to hypercapnia in newborn pigs. *Am. J. Physiol.* 267, H285–H291.
- Leffler, C.W., Mirro, R., Shanklin, D.R., Armstead, W.M., Shibata, M., 1994b. Light/dye microvascular injury selectively eliminates hypercapnia-induced pial arteriolar dilation in newborn pigs. *Am. J. Physiol.* 266, H623–H630.
- Lenti, L., Domoki, F., Kis, D., Hegyi, O., Toth, G.K., Busija, D.W., Bari, F., 2007. Pituitary adenylate cyclase-activating polypeptide induces pial arteriolar vasodilation through cyclooxygenase-dependent and independent mechanisms in newborn pigs. *Brain Res.* 1165, 81–88.
- Masuo, Y., Ohtaki, T., Masuda, Y., Nagai, Y., Suno, M., Tsuda, M., Fujino, M., 1991. Autoradiographic distribution of pituitary adenylate cyclase activating polypeptide (PACAP) binding sites in the rat brain. *Neurosci. Lett.* 126, 103–106.
- Meng, W., Tobin, J.R., Busija, D.W., 1995. Glutamate-induced cerebral vasodilation is mediated by nitric oxide through N-methyl-D-aspartate receptors. *Stroke* 26, 857–862 discussion 863.
- Miyata, A., Arimura, A., Dahl, R.R., Minamino, N., Uehara, A., Jiang, L., Culler, M.D., Coy, D.H., 1989. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochem. Biophys. Res. Commun.* 164, 567–574.
- Miyata, A., Jiang, L., Dahl, R.D., Kitada, C., Kubo, K., Fujino, M., Minamino, N., Arimura, A., 1990. Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP38). *Biochem. Biophys. Res. Commun.* 170, 643–648.
- Parfenova, H., Eidson, T.H., Leffler, C.W., 1997. Upregulation of COX-2 in cerebral microvascular endothelial cells by smooth muscle cell signals. *Am. J. Physiol.* 273, C277–C288.
- Peri, K.G., Hardy, P., Li, D.Y., Varma, D.R., Chemtob, S., 1995. Prostaglandin G/H synthase-2 is a major contributor of brain prostaglandins in the newborn. *J. Biol. Chem.* 270, 24615–24620.
- Reglodi, D., Fabian, Z., Tamas, A., Lubics, A., Szeberenyi, J., Alexy, T., Toth, K., Marton, Z., Borsiczky, B., Roth, E., Szalontay, L., Lengvari, I., 2004. Effects of PACAP on in vitro and in vivo neuronal cell death, platelet aggregation, and production of reactive oxygen radicals. *Regul. Pept.* 123, 51–59.

- Stumm, R., Kolodziej, A., Prinz, V., Endres, M., Wu, D.F., Holtt, V., 2007. Pituitary adenylate cyclase-activating polypeptide is up-regulated in cortical pyramidal cells after focal ischemia and protects neurons from mild hypoxic/ischemic damage. *J. Neurochem.* 103, 1666–1681.
- Tamas, A., Reglodi, D., Szanto, Z., Borsiczky, B., Nemeth, J., Lengvari, I., 2002. Comparative neuroprotective effects of preischemic PACAP and VIP administration in permanent occlusion of the middle cerebral artery in rats. *Neuro. Endocrinol. Lett.* 23, 249–254.
- Tong, S., Parfenova, H., Shibata, M., Zuckerman, S., Armstead, W.M., Leffler, C.W., 1993. Pituitary adenylate cyclase-activating polypeptide dilates cerebral arterioles of newborn pigs. *Proc. Soc. Exp. Biol. Med.* 203, 343–347.
- Vaudry, D., Gonzalez, B.J., Basille, M., Yon, L., Fournier, A., Vaudry, H., 2000. Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. *Pharmacol. Rev.* 52, 269–324.
- Vincent, M.B., 1992. Cyclooxygenase inhibitors modify the relaxant effect of vasoactive intestinal polypeptide and substance P in isolated porcine ophthalmic artery. *Cephalalgia* 12, 15–19.
- Wang, Q., Bryowsky, J., Minshall, R.D., Pelligrino, D.A., 1999. Possible obligatory functions of cyclic nucleotides in hypercapnia-induced cerebral vasodilation in adult rats. *Am. J. Physiol.* 276, H480–H487.
- Wei, E.P., Kontos, H.A., Said, S.I., 1980. Mechanism of action of vasoactive intestinal polypeptide on cerebral arterioles. *Am. J. Physiol.* 239, H765–H768.
- Xu, H.L., Koenig, H.M., Ye, S., Feinstein, D.L., Pelligrino, D.A., 2004. Influence of the glia limitans on pial arteriolar relaxation in the rat. *Am. J. Physiol. Heart Circ. Physiol.* 287, H331–H339.

## N-Methyl-D-Aspartate Induces Cortical Hyperemia through Cortical Spreading Depression-Dependent and -Independent Mechanisms in Rats

LAURA LENTI,<sup>\*\*†</sup> FERENC DOMOKI,<sup>\*\*†</sup> TAMÁS GÁSPÁR,<sup>\*</sup> JAMES A. SNIPES,<sup>\*</sup>  
FERENC BARI,<sup>†</sup> AND DAVID W. BUSIJA<sup>\*</sup>

<sup>\*</sup>Department of Physiology and Pharmacology, Wake Forest University Health Sciences, Winston-Salem, North Carolina; <sup>†</sup>Department of Physiology, Faculty of Medicine, University of Szeged, Hungary

### ABSTRACT

**Objective:** N-methyl-D-aspartate (NMDA) is a powerful cerebrovascular dilator *in vivo*. Cortical spreading depression (CSD) has recently been shown to contribute to the pial arteriolar dilation in mice. Our main aim was to examine the participation of CSD in the overall cerebrovascular response to NMDA in the rat.

**Methods:** Anesthetized Wistar rats (eight weeks old) were equipped with a closed cranial window to allow topical application of NMDA ( $10^{-5}$ – $10^{-3}$  M) to the parietal cortex. Cortical blood flow (CoBF) under and outside the cranial window was simultaneously monitored by using a two-channel laser-Doppler flowmeter. CSDs were detected by recording the changes in the cortical DC potential.

**Results:** Concentrations of  $10^{-4}$  and  $10^{-3}$  M of NMDA evoked single CSDs associated with rapid, transient hyperemia, followed by a sustained, but reduced, increase in CoBF. The latency and magnitude of the CoBF responses were dose dependent. The higher dose resulted in shorter latency ( $100 \pm 5^*$  vs.  $146 \pm 11$  seconds,  $*P < 0.05$ ; mean  $\pm$  standard error of the mean) and larger overall flow response ( $77 \pm 12^*$  vs.  $28 \pm 3\%$  from baseline) under, but not outside, the cranial window.

**Conclusions:** NMDA elicits dose-dependent increases in CoBF that are composed of CSD-dependent and -independent components in rats.

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KEY WORDS: glutamate, AMPA, kainate, cerebral blood flow, laser-Doppler flowmetry, piglet

### INTRODUCTION

Glutamatergic neurotransmission is ubiquitous in the brain and plays a key role in neurovascular coupling via activation of ionotropic [N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), and kainate] and metabotropic receptors [8,21,27,45]. Activation of NMDA receptors has been shown to elicit dose-dependent cerebrovascular dilation *in vivo* in numerous species, including rats, rabbits, and newborn pigs [10,16,19,32]. It has been hypothesized that the stimulation of NMDA receptors on neurons and the consequent membrane depolarization/ $\text{Ca}^{++}$  influx

result in the production of vasodilator mediators, such as nitric oxide (NO), and ultimately leads to cerebral vascular relaxation, thereby adjusting the cortical blood flow (CoBF) to the local metabolic activity [4,14,16,21].

The concept of NMDA-receptor activation contributing to the generation and propagation of cortical spreading depression (CSD) is well established; synthetic antagonists of NMDA receptors are potent inhibitors of CSD development in numerous experimental models [22,29,31,37]. CSD is a self-propagating wave of cellular depolarization in the cerebral cortex that is typically evoked by localized trauma, excess neuronal firing, potassium chloride, or neurotransmitter application and is associated with short-lived, but dramatic, increases in CoBF. The underlying mechanisms of this cerebral hyperemia are very complex and probably require the contribution of diverse cell types within the neurovascular unit as well as a wide range of vasoactive

Address correspondence to Laura Lenti, Department of Physiology, Faculty of Medicine, University of Szeged, Dóm tér 10, Szeged 6720, Hungary. E-mail: lentilaura@gmail.com

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factors, depending on age, species, and metabolic status of the cerebral cortex [8].

Because NMDA dilates pial arterioles via species- and age-dependent mechanisms and is implicated in CSD initiation and propagation, it is possible that, under some circumstances, NMDA-evoked CSDs contribute to the overall vascular responses. The recent findings of Ayata and Moskowitz [2] support this concept by providing evidence that, in mice, NMDA-induced pial arteriolar dilation is, at least partially, mediated by CSD. These researchers also suggest that, in some former studies where electrophysiological analyses were not performed, the apparent dose-dependent nature of the pial arterial responses was due to the increasing prevalence of CSDs in conjunction with the increasing concentration of NMDA. It is unclear, however, whether a similar complex interaction between CSD and the direct effects of NMDA occurs in other animal models.

Most data on NMDA-induced cerebrovascular changes have been acquired through studies on pial arteriolar diameters, which may or may not fully represent the overall response of the cerebral vasculature. We have shown previously that pial arterial responses most likely underestimate CoBF responses, and that in some situations the changes in diameter of pial arterioles may not accurately reflect alterations in resistance occurring in parenchymal or large arteries [9,11,40]. The aim of our study was to critically examine the effect of NMDA on CoBF, using laser-Doppler flowmetry, and to test whether CSD-related cortical hyperemia contributes to the overall NMDA-induced cerebrovascular response in the rat. To further clarify the glutamatergic cerebrovascular responses, we also determined if glutamate, AMPA, or kainate induced cortical hyperemia/CSD similar to NMDA, and if coapplication of glutamate or AMPA with NMDA would modify the NMDA-induced CoBF response by affecting CSD. Additionally, we studied the effect of NMDA on CoBF in newborn pigs, since NMDA-induced cortical hyperemia has not been reported in this species, although dose-dependent NMDA-induced pial arteriolar vasodilation has been extensively studied. Because the newborn pig is resistant to KCl-induced CSDs [15], NMDA-induced cerebrovascular changes are likely not confounded by CSDs in this subject. Therefore, use of piglets will allow us to compare the NMDA-evoked CSD-independent cerebral hyperemia across experimental models.

## MATERIALS AND METHODS

### Animals

Adult Wistar rats (male, eight weeks old, body weight 270–320 g,  $n = 57$ ) and newborn pigs (male, one to three days old, body weight 1.6–2.4 kg,  $n = 9$ ) were used in our experiments. Animals were maintained and used in compliance with the principles set forth by the Animal Care and Use Committee of Wake Forest University Health Sciences.

### Surgical Procedures

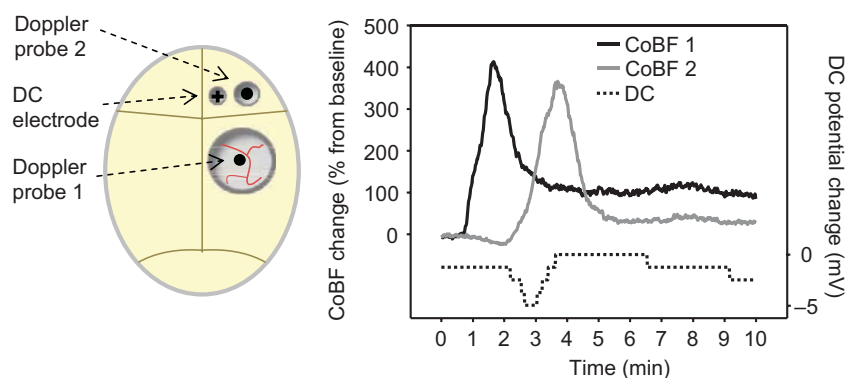
Rats were anesthetized with pentobarbital [Nembutal; Hospira Inc., Lake Forest, Illinois, USA; 60–70 mg/kg intraperitoneally (i.p.) for induction and 25 mg/kg intravenously (i.v.) for maintenance]. This anesthetic is known to have no influence on CSD generation and propagation [26,28]. The surgical sites were treated with lidocaine (2%; Abbott Laboratories, North Chicago, Illinois, USA) locally for analgesia. Rats were intubated via tracheotomy and mechanically ventilated with room air and supplemental oxygen. Ventilation parameters were adjusted to maintain normal end-tidal CO<sub>2</sub> levels (~40 mmHg), which were continuously monitored by a capnometer (Micro-Capnometer; Columbus Instruments, Columbus, Ohio, USA). The right femoral artery and vein were cannulated to measure arterial blood pressure and to administer drugs and fluids. A water-circulating heating mat was used to maintain the body temperature at ~37°C, which was monitored with a rectal probe. Arterial blood-gas tensions and pH were determined to verify the end-tidal pCO<sub>2</sub> levels.

The heads of the rats were fixed in a stereotaxic frame, and a closed cranial window was inserted in the following manner. An incision was made on the scalp, and the soft tissues were retracted from the calvaria. A circular craniotomy was made over the right parietal cortex (~4 mm in diameter; the center of the window was 3 mm lateral and 4 mm caudal to the bregma), and then the dura was carefully removed. A ~2-mm-high rim was formed around the craniotomy, using bone wax, and was stabilized with cyanoacrylate. Two plastic tubes were inserted into the rim as input and output ports to enable drug administration and flushing of the brain surface. The window was sealed with Parafilm (American National Can, Greenwich, Connecticut, USA), through which a laser-Doppler probe

(referred to as probe 1) was guided onto the surface (Figure 1). The window was filled with artificial cerebrospinal fluid (aCSF; composition in mg/L: 220 KCl, 7714 NaCl, 665 dextrose, 251.4 CaCl<sub>2</sub>, 61.9 MgCl<sub>2</sub>, and 2066.6 NaHCO<sub>3</sub>, bubbled with 5% CO<sub>2</sub> in N<sub>2</sub> and maintained at 37–38°C). Two additional holes were drilled under saline cooling over the right frontal cortex close to each other, one for a second laser-Doppler probe (probe 2) and one for a DC electrode, to verify the presence of possible CSDs. The distance between the two Doppler probes (7–8 mm) was measured to calculate CSD velocity. The dura was also removed under probe 2. The Doppler probes were mounted on micromanipulators and positioned so that they touched the surface of the brain, avoiding larger pial vessels. CoBF was monitored by using a two-channel laser-Doppler flowmeter (Periflux 4001 master; Perimed, Stockholm, Sweden). An Ag-AgCl electrode (1 mm in diameter) was connected to a DC amplifier (DAM 50, Differential Amplifier; World Precision Instruments, Sarasota, Florida, USA) for the measurement of DC potentials. A reference platinum electrode (Grass; Astro-Med, West Warwick, Rhode Island, USA) was fixed in the neck muscles.

Piglets were anesthetized and underwent surgery, as previously described [10], with some modifications. Anesthesia was induced with pentobarbital

(50–60 mg/kg, i.p.) and minimally supplemented by  $\alpha$ -chloralose only when needed (in four of nine animals; Sigma, St. Louis, Missouri, USA; 5–10 mg/kg, i.v.).  $\alpha$ -chloralose is known to have no effect on CSD, similar to barbiturates [28]. A catheter was inserted in the right femoral artery to monitor blood pressure and blood chemistry. Drugs and fluids were administered through a second catheter placed in the right femoral vein. The animals were intubated via tracheotomy and mechanically ventilated with room air. The ventilation rate (~30 breaths/min) and tidal volume (~20 mL) were adjusted to maintain the end-tidal CO<sub>2</sub> level and arterial blood-gas parameters in the physiological range. The end-tidal CO<sub>2</sub> level was monitored continuously, using a capnometer (Micro-Capnograph CI240; Columbus Instruments). A water-circulating heating pad was used to maintain the body temperature at ~37°C; core temperature was monitored with a rectal probe. The cranial window consisted of a stainless steel ring with three needle ports for drug administration, and aCSF flushing and was covered with Parafilm to facilitate the placement of Doppler probe 1 and the DC electrode onto the brain surface. For DC potential measurement, the same types of recording and reference electrodes were used as in the rat experiments, with appropriate changes in placement



**Figure 1.** Experimental design and representative recordings of cortical blood flow (CoBF) and direct current (DC) potential in the rat. On the left side, schematic figures show the position of the two laser-Doppler probes and the DC electrode during the experiments. The cranial window, where the drugs were applied, was placed over the parietal cortex, and additional holes were drilled anteriorly to the window. The first Doppler probe was placed under the window. The second probe and the DC electrode were positioned at a distant site, to detect the spread of depolarization and related cortical blood flow (CoBF) changes. In the presented experiment, NMDA ( $10^{-3}$  M) applied under the window evoked characteristic CoBF changes at the site of application (CoBF 1). Similar CoBF change was detected after a short delay outside the window (CoBF 2), accompanied by a negative DC-potential shift. The calculated rate of propagation between the two sites was consistent with that of cortical spreading depression. On the time axis, the 0 time point indicates the beginning of drug application onto the brain surface. The stimulus was removed 10 minutes later, when the window was flushed with artificial cerebrospinal fluid.

according to the morphology of the piglet. Following surgery, the closed window was filled with aCSF [10]. For the placement of Doppler probe 2, a hole was drilled 5–8 mm anterior to the window (so that the maximal distance was 10 mm between the two probes) and the dura was removed.

### Protocol

After reaching a stable baseline CoBF, the vehicle (aCSF) was superfused through the window to provide an experimental control for drug application in all animals. In rats, NMDA (group 1A;  $n=8$ ) or glutamate (group 1B;  $n=10$ ) (both drugs were purchased from Sigma, dissolved in aCSF) were administered topically at  $10^{-5}$ ,  $10^{-4}$ , and  $10^{-3}$  M concentrations for 10 minutes, interrupted by 10-minute-long drug-free periods when the surface was flushed with aCSF. To examine reproducibility,  $10^{-4}$  or  $10^{-3}$  M ( $n=6$ ) of NMDA was applied on the surface twice (group 2). In group 3 ( $n=11$ ), glutamate ( $10^{-3}$  M) was applied for 10 minutes prior to the coapplication of  $10^{-4}$  and  $10^{-3}$  M of NMDA. In group 4 ( $n=8$ ), the CoBF responses to AMPA ( $10^{-5}$  and  $10^{-4}$  M, dissolved in 0.1 M of NaOH to  $2 \times 10^{-2}$  M and further diluted with aCSF; Acros Organics, Morris Plains, New Jersey) were characterized. In group 5 ( $n=8$ ), NMDA ( $10^{-4}$  and  $10^{-3}$  M) was coapplied with AMPA ( $10^{-4}$  M) after a 10-minute incubation period with this latter agonist. In group 6 ( $n=6$ ), the brain surface was treated with  $10^{-5}$  and  $10^{-4}$  M of kainate (Sigma, dissolved in aCSF) for 10 minutes.

In piglets, the effects of topical NMDA ( $10^{-4}$  and  $10^{-3}$  M, group 7A;  $n=5$ ) on CoBF were determined. The brain surface was exposed to NMDA for 10 minutes, after which this stimulus was removed with aCSF. DC potential and CoBF recording continued for an additional 20 minutes. In group 7B, CoBF responses to glutamate ( $10^{-4}$  and  $10^{-3}$  M,  $n=4$ ) were evaluated similarly. In five animals, at least 30 minutes after the removal of the highest dose of NMDA/glutamate, we attempted to evoke CSDs by using repeated pinpricks (26-G needle, 1–2 mm in depth) as a mechanical stimulation. CoBF and DC potential were recorded in a 30-minute period following each pinprick.

CoBF, DC potential, blood pressure, and end-tidal  $\text{CO}_2$  were recorded continuously by PC-based software (IOX; EMKA Technologies, Falls Church, Virginia, USA). At the end of the experiments, the

anesthetized animals were euthanized with an i.v. injection of saturated KCl solution. At this time, a biological zero for the laser-Doppler signal was determined. Data were analyzed offline.

### Data Analysis

CoBF was recorded in arbitrary perfusion units. The biological zero was subtracted from the absolute values, and CoBF data were normalized to the average flow of five minutes prior to drug application. We evaluated CoBF responses in two ways, depending upon the experimental protocol and whether a CSD occurred during the application of drugs. In situations in which a CSD was absent, we determined the maximal CoBF response occurring over a one-minute interval. In situations in which a CSD occurred, we determined the maximal increase or peak CoBF response, the latency between the beginning of drug application and peak CoBF, and integrated CoBF responses within different time intervals.

Data were analyzed by one- or two-way ANOVA for repeated measures or by one-way ANOVA, followed by Tukey's exact test, where appropriate. Reproducibility of NMDA-induced CoBF responses (group 2) was tested with the one-sample *t*-test. To compare the frequency of CSD occurrence between groups, Fisher's exact test was used. A value of  $P < 0.05$  was considered significant. Data are represented as mean  $\pm$  standard error of the mean;  $n$  reflects the number of animals.

### RESULTS

Body temperature, arterial blood pressure, end-tidal  $\text{CO}_2$  level, and blood gases were maintained within the normal range and did not vary significantly among different groups or during experiments. For example, in group 1A, rectal temperature was  $37.1 \pm 0.1^\circ\text{C}$ , mean arterial blood pressure was  $106 \pm 5$  mmHg, and while ventilation parameters were set to reach the normal end-tidal  $\text{CO}_2$ , which was approximately 5%, arterial oxygen tension was  $147 \pm 5$  mmHg, arterial carbon dioxide tension was  $40 \pm 1$  mmHg, and pH was  $7.39 \pm 0.01$ . Also, raw baseline laser-Doppler flowmetry data (in arbitrary perfusion units; PUs) were similar among groups and did not shift considerably over the course of experiments. Baseline perfusions under the window were  $163 \pm 16$  and  $147 \pm 14$  PU in group 1A and 1B,

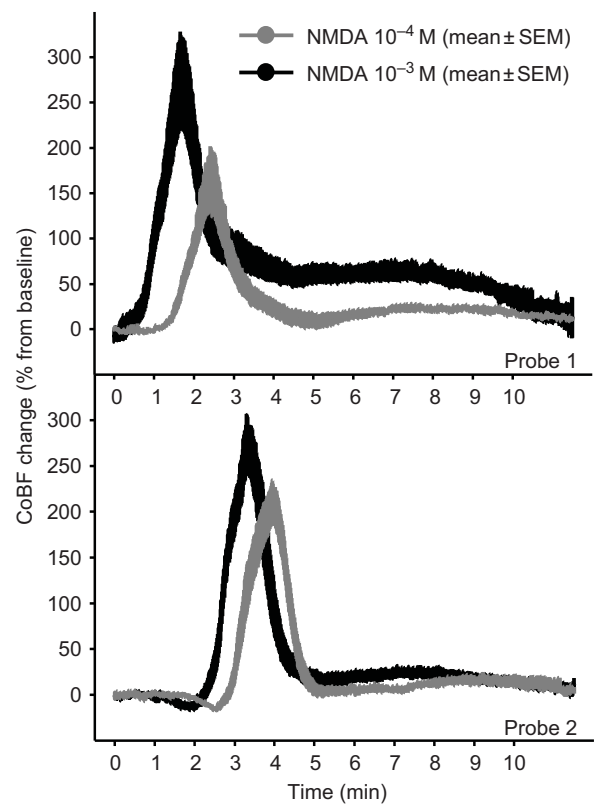
respectively. Flushing of the brain surface with aCSF did not affect the CoBF significantly.

### NMDA Induces CSD and Dose-Dependent CoBF Increase in the Rat

Topical NMDA at  $10^{-5}$  M did not change the local CoBF significantly ( $5 \pm 3\%$ ) and did not evoke any CSDs (0/8 trials). However, after the application of higher concentrations of NMDA ( $10^{-4}$ – $10^{-3}$  M), a rapid onset, but transient hyperemia, was observed, followed by a reduced, but sustained, elevation of CoBF under the cranial window (recorded by probe 1). Subsequently, a negative direct current (DC) potential shift (5–10 mV) and a characteristic CoBF increase were detected at the distant registration site (probe 2), confirming the induction of a CSD by NMDA (Figure 1).

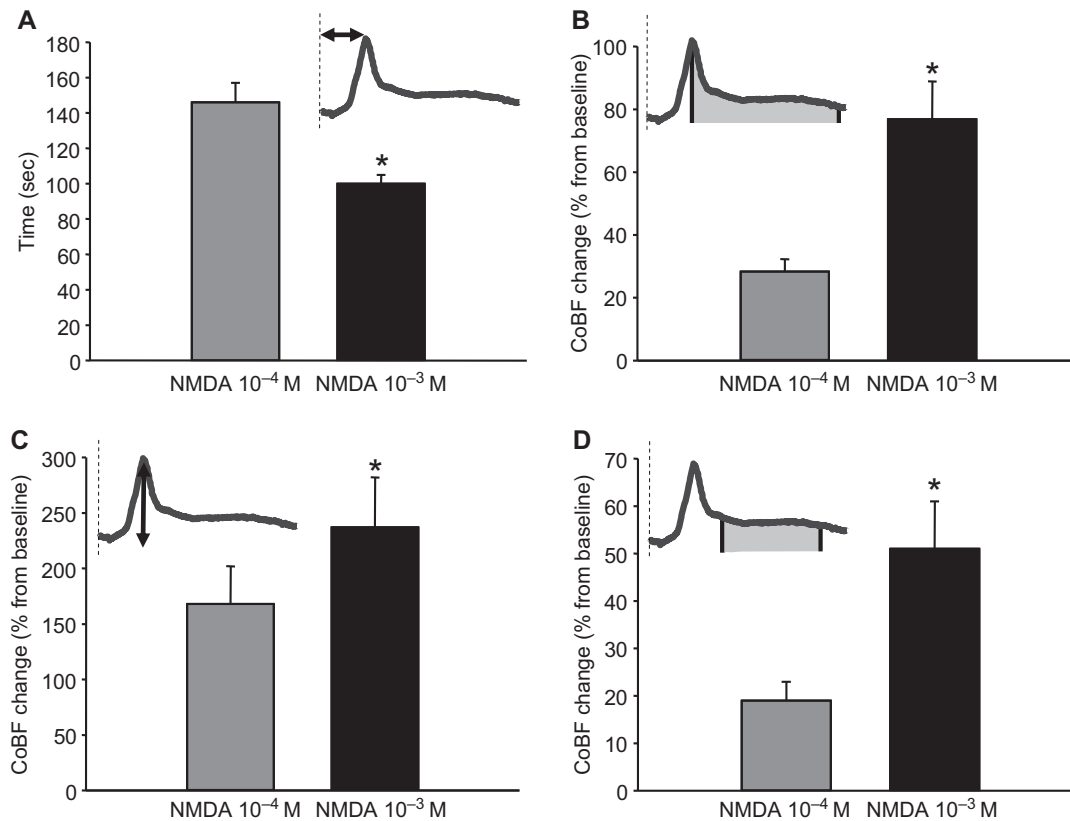
The CoBF responses to  $10^{-4}$  and  $10^{-3}$  M of NMDA were significantly different (Figure 2, upper panel). The higher dose of NMDA evoked CSD-related hyperemia with a significantly shorter latency (i.e., the time from the beginning of drug application to the maximal elevation in the flow) than the lower concentration (Figure 3A). The total CoBF increase (expressed as the integrated average of eight minutes after the flow maximum), the amplitude of the first, rapid CoBF increase (referred to as a peak), and the second, sustained CoBF increase or wave (defined as a five-minute period starting one minute after the peak) all showed prominent dose dependency (Figure 3B–3D) at the site of NMDA application. However, at the distant recording site, features of the CoBF responses were not concentration dependent (Figure 2, lower panel). The total CoBF responses detected by probe 2 were  $21 \pm 5$  and  $36 \pm 8\%$  ( $P = 0.094$ ), the peak CoBF increases were  $213 \pm 21$  and  $278 \pm 28\%$  ( $P = 0.058$ ), and the magnitude of the waves were  $10 \pm 3$  and  $21 \pm 6\%$  ( $P = 0.102$ ) after the  $10^{-4}$  and  $10^{-3}$  M NMDA doses, respectively.

The time intervals between the two peaks detected at the two sites were similar:  $91 \pm 6$  and  $98 \pm 7$  seconds when administering NMDA at  $10^{-4}$  and  $10^{-3}$  M concentrations, respectively ( $P = 0.24$ ). The calculated velocities of  $5.1 \pm 0.4$  and  $4.8 \pm 0.4$  mm/min for  $10^{-4}$  and  $10^{-3}$  M, respectively, are consistent with published rates of CSD propagation [20], and confirmed that the detected CSD spread from the initiation site within the cranial window.



**Figure 2.** The effect of topical NMDA on cortical blood flow (CoBF) in rats. CoBF was recorded under and away from the cranial window (marked as probes 1 and 2, respectively). NMDA evoked a rapid, cortical spreading depression (CSD)-related hyperemia (peak) followed by a sustained, but reduced, increase in CoBF (wave) that was concentration dependent only under the cranial window and not at the distant site. The CoBF data are represented as mean  $\pm$  standard error of the mean (SEM) (SEM plotted as curve thickness;  $n = 8$ ). The intervals between the 0 time point and the peaks represent the average latency (without error) from the beginning of drug application in both cases.

We demonstrated that repeated application of the same concentration of NMDA (group 2) induced remarkably similar CoBF elevations, which verified that dose-dependent responses seen at the site of the drug application were not due to hypersensitivity caused by the previously induced CSD. The ratios of second to first CoBF responses with NMDA application were  $0.96 \pm 0.09$ ,  $1.04 \pm 0.11$ , and  $1.05 \pm 0.12$  for the peak, total, and wave values, respectively, which were not significantly different from the expected value of 1.



**Figure 3.** NMDA evoked dose-dependent cortical blood flow (CoBF) increases in rats. The higher dose of NMDA ( $10^{-3}$  M) induced cortical spreading depression-related hyperemia with a significantly shorter latency than the lower ( $10^{-4}$  M) concentration (A). The magnitude of the total CoBF response (calculated as the average CoBF change of eight minutes after the maximal or peak value) was also dependent on the dose of NMDA applied (B). A similar difference was evident when analyzing its components; the peak (C), and the following, sustained CoBF increase or wave (measured during a five-minute period starting one minute after the peak; D). The dashed lines on the schematic icons show the beginning of NMDA application, whereas the arrows and filled areas determine the represented features of the flow response. Data are mean  $\pm$  standard error of the mean ( $n = 8$ ;  $*P < 0.05$ ) via one-way repeated measures analysis of variance and Tukey's test.

#### Glutamate Does Not Elicit CSD or CoBF Increase

In contrast to NMDA application, administration of glutamate ( $10^{-5}$ – $10^{-3}$  M) failed to increase CoBF significantly (maximal change was  $9 \pm 2\%$  at  $10^{-3}$  M;  $P = 0.25$ ) or to evoke a CSD (0/10 trials at each concentration). The CoBF and the DC potential were constant at the distant registration site following glutamate application.

#### AMPA Does Not Elicit CSD but Increases CoBF

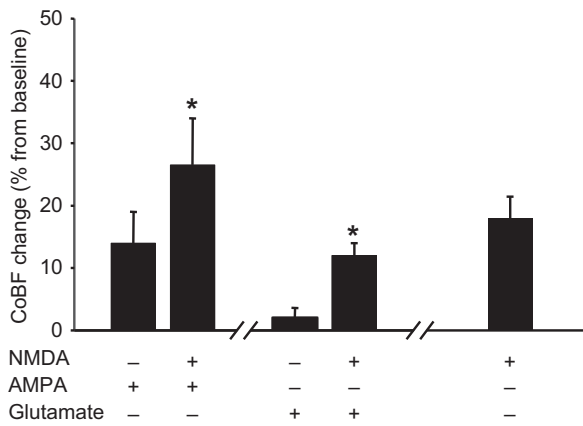
AMPA did not evoke CSD but increased CoBF by  $6.8 \pm 1.7$  and  $17 \pm 5\%*$  (expressed as average CoBF change of one minute at maximal rate of hyperemia;

$*P < 0.05$ ) for  $10^{-5}$  and  $10^{-4}$  M on AMPA, respectively (group 4). The CoBF increase was gradual, reaching the maximum from the fourth to fifth minutes of application. However, CoBF did not change at the distant registration site and the DC potential remained constant.

#### Glutamate and AMPA Reduce the Frequency of NMDA-induced CSDs but Do Not Affect the CSD-Independent Component of NMDA-induced CoBF Increases

Glutamate was able to limit NMDA-induced CSD development (group 3) when it was coapplied with  $10^{-4}$  M (CSD failed to occur in 5 of 11 animals;





**Figure 4.** Cortical spreading depression (CSD)-independent NMDA-evoked cortical blood flow (CoBF) increases in rats. Topical glutamate ( $10^{-3}$  M) and AMPA ( $10^{-4}$  M) administration prevented the NMDA ( $10^{-4}$  M)-induced CSD generation in 5 of 11 and 8 of 8 cases, respectively. Despite blocked CSD formation, coapplication of  $10^{-4}$  M NMDA still resulted in a significant increase in CoBF, as compared with AMPA or glutamate application alone (expressed as average CoBF change of one minute at maximal rate of hyperemia). The CoBF increase was consistent with the post-CSD wave when only NMDA was applied (represented as the average increase of CoBF over a one-minute interval, three minutes after the peak, time-matched with CSD-independent CoBF increases). Data are mean  $\pm$  standard error of the mean; ( $n=5-8$ ;  $*P < 0.05$ ) via one-way repeated measures analysis of variance and Tukey's test.

$*P < 0.05$ ) but not with  $10^{-3}$  M of NMDA (CSD occurred in all 11 animals). Despite the suppression of CSD expression with glutamate coapplication in the five animals, NMDA at  $10^{-4}$  M still increased the CoBF significantly (Figure 4) to a level consistent with the post-CSD wave when only NMDA was applied (Figures 2–4).

Coapplication of AMPA also interfered with CSD induction by NMDA (group 5). AMPA ( $10^{-4}$  M) prevented the initiation of CSDs in all eight animals when coapplied with  $10^{-4}$  M ( $*P < 0.05$ ), and prevented occurrence of CSD only in one of eight animals when coapplied with  $10^{-3}$  M of NMDA. Despite the absence of CSD, coapplication of  $10^{-4}$  M of NMDA still resulted in a significant increase in CoBF, as compared with  $10^{-4}$  M of AMPA alone (Figure 4).

### Kainate Does Not Elicit CSD but Increases CoBF

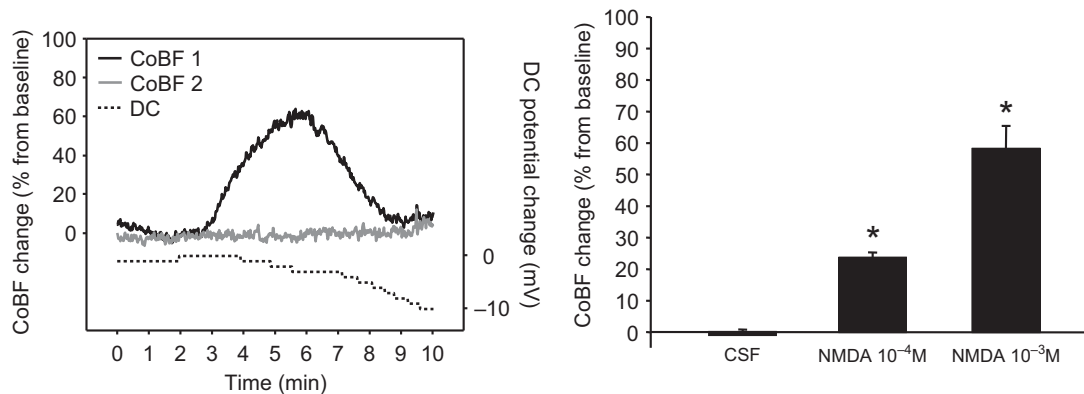
Kainate (group 6) increased CoBF by  $36 \pm 6^*$  and  $114 \pm 16\%^*$  ( $10^{-5}$  and  $10^{-4}$  M, respectively;  $*P < 0.05$ ) but did not induce CSD in rats. However, in contrast to AMPA and NMDA, CoBF did not return to baseline, even after repeated aCSF flushing. Consequently, due to the magnitude and the prolonged nature of the CoBF response, we did not coapply kainate with NMDA.

### NMDA Elicits Dose-Dependent CoBF Increase without CSD in the Piglet

Topical application of NMDA (group 7A) produced concentration-dependent increases in CoBF, as recorded under the cranial window (Figure 5). The pattern of the CoBF responses was not consistent with those seen in the rats during NMDA application (Figure 5), and we did not detect significant CoBF changes at the second, distant registration site outside the window ( $3 \pm 2$  and  $2 \pm 0.5\%$  to  $10^{-4}$  and  $10^{-3}$  M NMDA;  $P = 0.7$  and  $0.9$ , respectively). The DC potential decreased gradually (by 5–10 mV) and returned to the baseline level in approximately two to three minutes after removal of the NMDA with aCSF. Local activation of cortical neurons with “pinpricks,” using a sterile needle, although sufficient to cause CSD in other models, also failed to initiate CSD (0/5 animals) or significantly change CoBF in piglets ( $3.2 \pm 1.2\%$  in response to the first pinprick;  $P = 0.5$ ). In group 7B, we did not detect the presence of CSDs during glutamate application, and the increase in CoBF ( $8.3 \pm 1.4$  and  $9.5 \pm 1.1\%$  to  $10^{-4}$  and  $10^{-3}$  M of glutamate) was not significant ( $P = 0.35$  and  $0.3$ ).

## DISCUSSION

The major findings of our experiments were: 1) NMDA induced dose-dependent CoBF increases in both species studied; 2) NMDA-evoked CSDs contributed to the CoBF response in adult rats; 3) stimulation of cortical AMPA- and kainate-receptors also increased CoBF in rats without generating CSD; 4) glutamate failed to generate CSD-related CoBF changes; 5) glutamate and AMPA reduced the occurrence of NMDA-induced CSDs, thus unveiling CSD-independent vasoactivity of NMDA; and finally, 6) NMDA-induced hyperemia was independent of CSDs in piglets. After the elimination of the



**Figure 5.** A representative recording of cortical blood flow (CoBF) and direct current (DC) potential and NMDA-evoked dose-dependent cortical blood flow (CoBF) increases in newborn pigs. Right panel: NMDA ( $10^{-3}$  M) application onto the cortex elicited a transient CoBF increase (CoBF 1) and a prolonged DC potential deflection; however, propagation of the CoBF response was not detected with the distant probe (CoBF 2). On the time axis, the 0 time point indicates the beginning of drug application onto the brain surface. The stimulus was removed 10 minutes later, when the window was flushed with artificial cerebrospinal fluid. Left panel: Average CoBF change per minute was calculated during a 10-minute period of drug application. Bars represent the maximal CoBF changes within the examined time interval. Artificial cerebrospinal fluid did not produce significant CoBF changes, while topical NMDA ( $10^{-4}$  and  $10^{-3}$  M) increased CoBF in a concentration-dependent manner. Data are mean  $\pm$  standard error of the mean ( $n=5$ ;  $*P<0.05$ ) via one-way repeated measures analysis of variance and Tukey's test.

CSD-dependent component of the CoBF response to NMDA in rats, the degree of cortical hyperemia to NMDA was similar in rats and piglets.

NMDA evokes well-described dose-dependent pial arteriolar dilation *in vivo* [10,12,32,16,17,19]; however, many studies failed to show NMDA-induced vasodilation in isolated cerebral resistance vessels [16,24,41]. NMDA-induced vasodilation is abolished by NMDA-receptor antagonist MK-801 and attenuated by inhibitors of neuronal nitric-oxide synthase (nNOS) [4,16,18,32]. These findings suggest that neuronal NMDA-receptor stimulation and subsequent  $Ca^{++}$  influx/membrane depolarization would activate nNOS, leading to vascular smooth muscle relaxation by NO [8,14,21,35,44]. In addition to the unequivocal role of NO, the participation of other species-specific factors, such as adenosine [25], carbon monoxide (CO) [30], ATP-sensitive potassium ( $K_{ATP}$ ), and/or calcium-activated potassium ( $K_{Ca}$ ) channels [36] has been proposed.

Ayata and Moskowitz [2] suggested an alternative mechanism of NMDA-induced pial arteriolar vasodilation in mice. Using electrophysiological recordings and vessel-diameter measurements, they demonstrated the involvement of CSD in the complex vascular responses evoked by topical application of as little as 50  $\mu$ M of NMDA. However, there

are some limitations of that study. First, responses of small arterioles (approximately 25  $\mu$ m in diameter) were tested that may not represent responses of other resistance blood vessels during CSD. As recently described, different vascular segments of the cerebral circulation are exposed to varying influences from dilator and constrictor stimuli during CSD [7]. Second, pial arteriolar diameter may not accurately reflect the overall response of the cerebral vasculature to CSD [9,11,40]. And, third, the CSD component of the overall responses to NMDA was not removed, so that the direct effects of NMDA could not be conclusively determined.

Our study provides novel evidence that NMDA-evoked CSD is a major component of the overall cerebral vascular response to higher (100  $\mu$ M to 1 mM) doses of NMDA in the rat model; however, detailed analysis of the response to NMDA revealed non-CSD-dependent characteristics as well. CoBF increases were dose dependent, which might be explained by a faster, deeper penetration of the drug at the higher dose and/or by a more extensive receptor occupation. In the present study, we also demonstrated the CSD-independent cerebrovascular effect of AMPA and the inhibitory potential of AMPA-receptor activation on NMDA-induced CSDs. Importantly, even in the absence of CSDs, the CSD-independent increase in CoBF upon NMDA

application remained intact. Similar to AMPA-receptor activation, kainate application also increased CoBF without producing CSDs. The dramatic, sustained CoBF effects of topical kainate prevented the exploration of any potential modulatory influence of kainate on NMDA-induced CoBF and CSD responses in the rat. We previously described the pronounced, prolonged effects of kainate on pial arterioles in piglets [5].

Unlike NMDA, AMPA, or kainate, the application of the natural neurotransmitter, glutamate, failed to significantly increase CoBF or to induce CSDs in the rat. This apparent discrepancy is likely caused by experimental conditions. The peak concentration of glutamate in the synaptic cleft during neurotransmission is unknown, but is estimated at about 1.1 mM in hippocampal glutamatergic synapses [13]. In our study, glutamate and its analogs were applied with a single application in a small volume ( $\sim 15\text{--}20\ \mu\text{L}$ ) under the cranial window to minimally interfere with the laser-Doppler flowmetry signal that is very sensitive to movement. The discrepancy between the cerebrovascular effects of glutamate and NMDA/AMPA/kainate may be due to the well-known difference in the clearance of these drugs from the extracellular space [34]. In our experiments, glutamate concentration may not have stayed high enough or reached the appropriate cell layers in the stimulated cortex because of degradation and neuronal/glial uptake to reproduce the cerebrovascular effects of the selective analogues. Further, the difference between glutamate and NMDA in facilitating CSDs has been described by other researchers [29,33]. These results further indicate that the effect of glutamate is complex, resulting not only from NMDA receptor stimulation, but from the summation of different, and sometimes opposing, actions via multiple receptors and vasoactive substances [8,27]. The complex action of glutamate is further suggested by its efficacy to reduce the incidence of NMDA-induced CSDs. Glutamate has two more ionotropic (AMPA and kainate) and numerous metabotropic receptors. It is possible that the activation of one or more of them acts antagonistically in terms of CSD initiation. The AMPA receptor can be an important site of this effect, since AMPA produced a similar inhibition in the present study. AMPA is able to antagonize NMDA-induced changes in rat cortical networks [1]. Conceivably, AMPA-receptor activation may have antagonized CSD initiation by NMDA, as well. Importantly, glutamate or AMPA did not affect the CSD-independent

component of the CoBF response to NMDA, suggesting that not the direct interaction with the NMDA receptor, but the subsequent electrophysiological sequence triggering a CSD, was impaired by glutamate/AMPA.

Our findings in the piglet provide novel data on the integrated CoBF responses to the activation of cortical neuronal NMDA receptors in this important experimental model and confirm our earlier results concerning the dose-dependent nature of responses to NMDA, based upon measurements of pial arteriolar diameters in this species [3,6,10,12,15,36]. The hemodynamic changes are not the consequence of CSD-related events, but rather represent a direct, specific effect on cortical neurons [8,14]. While CSD has been shown to occur in the mature brains of all mammalian species studied, the neonatal brain appears to be relatively resistant to generation of CSD [39]. For example, in the rat, it is difficult to elicit CSD-like events before postnatal days 9–10 [38], and in the piglet, we have not been able to induce CSD, even with mechanical means (i.e., pinpricks), during the first days of life. While the inability of the neonatal brain to produce CSDs is not completely understood, it seems likely that factors such as lack of maturity of individual neuronal and glial components and their incomplete integration with each other, as well as different expression of ion channels and neurotransmitter receptors, are responsible for this phenomenon [42]. In a recent study, Girouard et al. [23] found that the cortical application of  $40\ \mu\text{M}$  of NMDA increased CoBF in mice by 20–25% without inducing CSD. Additionally, they confirmed earlier reports that cerebral vascular dilation to NMDA is dependent on NO derived from neuronal NOS [4,18]. The amount of the increase in CoBF observed in mice, following NMDA application by Girouard et al. [23], is similar to the non-CSD component of the CoBF response that we observe in rats and to the CoBF responses we see in piglets.

## CONCLUSIONS

In summary, NMDA elicits dose-dependent CoBF increases in rats and piglets. Although previous data obtained by pial arteriolar diameter measurements suggest many superficial similarities in the cerebrovascular responses to NMDA, our results show that the underlying mechanisms are quite different between these two models. In rats, NMDA-evoked

CSD has a substantial, but not exclusive, contribution to the cerebrovascular response. In piglets, however, the CoBF increase seems to be solely the result of the local activation of neuronal NMDA receptors without the confounding effect of CSDs.

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#### REFERENCES

- Addae JI, Evans SM, Ali N, Stone TW. (2000). NMDA-induced changes in a cortical network in vivo are prevented by AMPA. *Brain Res* 869: 211–215.
- Ayata C, Moskowitz MA. (2006). Cortical spreading depression confounds concentration-dependent pial arteriolar dilation during N-methyl-D-aspartate superfusion. *Am J Physiol Heart Circ Physiol* 290:H1837–H1841.
- Bari F, Errico RA, Louis TM, Busija DW. (1996). Differential effects of short-term hypoxia and hypercapnia on N-methyl-D-aspartate-induced cerebral vasodilatation in piglets. *Stroke* 27:1634–1639; discussion, 1639–1640.
- Bari F, Errico RA, Louis TM, Busija DW. (1996). Interaction between ATP-sensitive  $K^+$  channels and nitric oxide on pial arterioles in piglets. *J Cereb Blood Flow Metab* 16:1158–1164.
- Bari F, Louis TM, Busija DW. (1997). Kainate-induced cerebrovascular dilation is resistant to ischemia in piglets. *Stroke* 28:1272–1276; discussion, 1277.
- Bari F, Nagy K, Guidetti P, Schwarcz R, Busija DW, Domoki F. (2006). Kynurenic acid attenuates NMDA-induced pial arteriolar dilation in newborn pigs. *Brain Res* 1069:39–46.
- Busija DW, Bari F, Domoki F, Horiguchi T, Shimizu K. (2008). Mechanisms involved in the cerebrovascular dilator effects of cortical spreading depression. *Prog Neurobiol* 86:379–395.
- Busija DW, Bari F, Domoki F, Louis T. (2007). Mechanisms involved in the cerebrovascular dilator effects of N-methyl-D-aspartate in cerebral cortex. *Brain Res Rev* 56:89–100.
- Busija DW, Heistad DD, Marcus ML. (1981). Continuous measurement of cerebral blood flow in anesthetized cats and dogs. *Am J Physiol* 241:H228–H234.
- Busija DW, Leffler CW. (1989). Dilator effects of amino-acid neurotransmitters on piglet pial arterioles. *Am J Physiol* 257:H1200–H1203.
- Busija DW, Marcus ML, Heistad DD. (1982). Pial artery diameter and blood-flow velocity during sympathetic stimulation in cats. *J Cereb Blood Flow Metab* 2:363–367.
- Busija DW, Wei M. (1993). Altered cerebrovascular responsiveness to N-methyl-D-aspartate after asphyxia in piglets. *Am J Physiol* 265:H389–H394.
- Clements JD, Lester RA, Tong G, Jahr CE, Westbrook GL. (1992). The time course of glutamate in the synaptic cleft. *Science* 258:1498–1501.
- Domoki F, Perciaccante JV, Shimizu K, Puskar M, Busija DW, Bari F. (2002). N-methyl-D-aspartate-induced vasodilation is mediated by endothelium-independent nitric oxide release in piglets. *Am J Physiol Heart Circ Physiol* 282:H1404–H1409.
- Domoki F, Veltkamp R, Bari F, Louis TM, Busija DW. (1999). Cerebrovascular reactivity remains intact after cortical depolarization in newborn piglets. *Pediatr Res* 45:834–837.
- Faraci FM, Breese KR. (1993). Nitric oxide mediates vasodilatation in response to activation of N-methyl-D-aspartate receptors in brain. *Circ Res* 72:476–480.
- Faraci FM, Breese KR. (1994). Dilatation of cerebral arterioles in response to N-methyl-D-aspartate: role of CGRP and acetylcholine. *Brain Res* 640: 93–97.
- Faraci FM, Brian JE, Jr. (1995) 7-nitroindazole inhibits brain nitric oxide synthase and cerebral vasodilatation in response to N-methyl-D-aspartate. *Stroke* 26:2172–2175; discussion, 2176.
- Faraci FM, Heistad DD. (1994). Responses of cerebral arterioles to N-methyl-D-aspartate and activation of ATP-sensitive potassium channels in old rats. *Brain Res* 654:349–351.
- Farkas E, Pratt R, Sengpiel F, Obrenovitch TP. (2008). Direct, live imaging of cortical spreading depression and anoxic depolarisation using a fluorescent, voltage-sensitive dye. *J Cereb Blood Flow Metab* 28:251–262.

21. Fergus A, Lee KS. (1997). Regulation of cerebral microvessels by glutamatergic mechanisms. *Brain Res* 754:35–45.
22. Gill R, Andine P, Hillered L, Persson L, Hagberg H. (1992). The effect of MK-801 on cortical spreading depression in the penumbral zone following focal ischaemia in the rat. *J Cereb Blood Flow Metab* 12:371–379.
23. Girouard H, Wang G, Gallo EF, Anrather J, Zhou P, Pickel VM, et al. (2009). NMDA-receptor activation increases free radical production through nitric oxide and NOX2. *J Neurosci* 29:2545–2552.
24. Hardebo JE, Wieloch T, Kahrstrom J. (1989). Excitatory amino acids and cerebrovascular tone. *Acta Physiol Scand* 136:483–485.
25. Iloff JJ, D'Ambrosio R, Ngai AC, Winn HR. (2003). Adenosine receptors mediate glutamate-evoked arteriolar dilation in the rat cerebral cortex. *Am J Physiol Heart Circ Physiol* 284:H1631–H1637.
26. Kitahara Y, Taga K, Abe H, Shimoji K. (2001). The effects of anesthetics on cortical spreading depression elicitation and c-fos expression in rats. *J Neurosurg Anesthesiol* 13:26–32.
27. Koehler RC, Roman RJ, Harder DR. (2009). Astrocytes and the regulation of cerebral blood flow. *Trends Neurosci* 32:160–169.
28. Kudo C, Nozari A, Moskowitz MA, Ayata C. (2008). The impact of anesthetics and hyperoxia on cortical spreading depression. *Exp Neurol* 212:201–206.
29. Lauritzen M, Hansen AJ. (1992). The effect of glutamate receptor blockade on anoxic depolarization and cortical spreading depression. *J Cereb Blood Flow Metab* 12:223–229.
30. Leffler CW, Parfenova H, Fedinec AL, Basuroy S, Tcheranova D. (2006). Contributions of astrocytes and CO to pial arteriolar dilation to glutamate in newborn pigs. *Am J Physiol Heart Circ Physiol* 291:H2897–H2904.
31. Marrannes R, Willems R, De Prins E, Wauquier A. (1988). Evidence for a role of the N-methyl-D-aspartate (NMDA) receptor in cortical spreading depression in the rat. *Brain Res* 457:226–240.
32. Meng W, Tobin JR, Busija DW. (1995). Glutamate-induced cerebral vasodilation is mediated by nitric oxide through N-methyl-D-aspartate receptors. *Stroke* 26:857–862; discussion, 863.
33. Obrenovitch TP, Zilkha E, Urenjak J. (1996). Evidence against high extracellular glutamate promoting the elicitation of spreading depression by potassium. *J Cereb Blood Flow Metab* 16:923–931.
34. Pellerin L, Magistretti PJ. (1994). Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proc Natl Acad Sci U S A* 91:10625–10629.
35. Pelligrino DA, Gay RL, 3rd, Baughman VL, Wang Q. (1996). NO synthase inhibition modulates NMDA-induced changes in cerebral blood flow and EEG activity. *Am J Physiol* 271:H990–H995.
36. Philip S, Armstead WM. (2004). NMDA dilates pial arteries by KATP and Kca channel activation. *Brain Res Bull* 63:127–131.
37. Richter F, Bauer R, Lehmenkuhler A, Schaible HG. (2008). Spreading depression in the brainstem of the adult rat: electrophysiological parameters and influences on regional brainstem blood flow. *J Cereb Blood Flow Metab* 28:984–994.
38. Richter F, Lehmenkuhler A, Fechner R, Manveljan L, Haschke W. (1998). Postnatal conditioning for spreading cortical depression in the rat brain. *Brain Res Dev Brain Res* 106:217–221.
39. Schade JP. (1959). Maturational aspects of EEG and of spreading depression in rabbit. *J Neurophysiol* 22:245–257.
40. Shibata M, Leffler CW, Busija DW. (1991). Prostanoids attenuate pial arteriolar dilation induced by cortical spreading depression in rabbits. *Am J Physiol* 261:R828–R834.
41. Simandle SA, Kerr BA, Lacza Z, Eckman DM, Busija DW, Bari F. (2005). Piglet pial arteries respond to N-methyl-D-aspartate in vivo but not in vitro. *Microvasc Res* 70:76–83.
42. Takahashi T. (2005). Postsynaptic receptor mechanisms underlying developmental speeding of synaptic transmission. *Neurosci Res* 53:229–240.
43. Van Harreveld A, Stamm JS, Christensen E. (1956). Spreading depression in rabbit, cat, and monkey. *Am J Physiol* 184:312–320.
44. Yang ST, Chang HH. (1998). Nitric oxide of neuronal origin mediates NMDA-induced cerebral hyperemia in rats. *Neuroreport* 9:415–418.
45. Zonta M, Angulo MC, Gobbo S, Rosengarten B, Hossmann KA, Pozzan T, et al. (2003). Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. *Nat Neurosci* 6:43–50.



## Regular Article

Secretory phospholipase A<sub>2</sub> inhibitor PX-18 preserves microvascular reactivity after cerebral ischemia in pigletsFerenc Domoki<sup>a,\*</sup>, Alíz Zimmermann<sup>a</sup>, Laura Lenti<sup>a</sup>, Valéria Tóth-Szúki<sup>a</sup>, Jana Pardeike<sup>b</sup>, Rainer H. Müller<sup>b</sup>, Ferenc Bari<sup>a</sup><sup>a</sup> Department of Physiology, Faculty of Medicine, University of Szeged, H-6720 Szeged, Dóm tér 10, Hungary<sup>b</sup> Department of Pharmaceutics, Biopharmaceutics and NutriCosmetics, Freie Universität Berlin, Berlin, Germany

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## ABSTRACT

Cerebral ischemia/reperfusion (I/R) results in cellular energy failure and dysfunction of the neurovascular unit that contribute to subsequent neuronal cell death in the neonate. PX-18 is a putative neuroprotective inhibitor of secretory phospholipase A<sub>2</sub> (sPLA<sub>2</sub>) but its *in vivo* testing has been limited by its poor solubility. Our purpose was to assess whether PX-18 preserved neuronal–vascular reactivity to I/R-sensitive endothelium-dependent (hypercapnia, bradykinin) and/or neuron-dependent (N-methyl-D-aspartate; NMDA) stimuli. To make the drug available for *in vivo* studies, PX-18 was formulated as a 3% nanosuspension applying high pressure homogenization. Newborn piglets (1-day old,  $n = 40$ ) were anesthetized and ventilated, and cerebrovascular reactivity to the above stimuli was determined by measuring changes in pial arteriolar diameters using the closed cranial window/intravital videomicroscopy technique. Intravenous infusion of PX-18 nanosuspension (6 mg/kg, 20 min) did not affect baseline arteriolar diameters, or hypercapnia-, bradykinin-, or NMDA-induced pial arteriolar vasodilation under normoxic conditions. Global cerebral ischemia (10 min) followed by 1 h of reperfusion significantly attenuated hypercapnia-, bradykinin-, and NMDA-induced vasodilation in untreated or vehicle-treated controls. However, PX-18 resulted in nearly full preservation of cerebrovascular reactivity to all these stimuli. In conclusion, inhibition of sPLA<sub>2</sub> by PX-18 improves neurovascular function both at the neuronal and the microvascular level following I/R. This effect of PX-18 likely contributes to its neuroprotective effect.

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## Introduction

In the central nervous system, the metabolic homeostasis of neurons critically depends on the continuous maintenance of adequate blood supply and bidirectional transport of nutrients and metabolites across the blood–brain barrier. This goal is achieved by the functional and morphological interaction of parenchymal neurons, astrocytes, cerebrovascular endothelial cells, pericytes, vascular smooth muscle cells, and perivascular nerves. To identify this complex system the term “neurovascular unit” has recently been coined (Lo et al., 2003). Hypoxic/ischemic injury that occurs in the perinatal period commonly results in a dysfunction of the neurovascular unit as indicated by decreased responsiveness to important vasodilator stimuli. The newborn pig is a widely accepted model of the human term neonate to study hypoxic/ischemic injury, based on the similarities in brain structure, developmental stage, glucose metabolism and cardiovascular regulation (Book and Bustad, 1974; Flecknell et al., 1980; Gootman et al., 1972). Global cerebral ischemia/reperfusion (I/R) in

newborn pigs has been previously demonstrated to attenuate pial arteriolar vasodilation to N-methyl-D-aspartate (NMDA) and hypercapnia, indicating injury to both neuronal and cerebrovascular members of the neurovascular unit (Busija et al., 1996; Leffler et al., 1989).

Cyclooxygenase (COX) activity plays a special Janus-faced role in the cerebral circulation of the newborn. Both COX-1 and COX-2 expressed in the piglet brain convert arachidonic acid to prostaglandin H<sub>2</sub>, the substrate of further prostanoid biosynthesis (Peri et al., 1995). Prostanoids are involved in the vasodilatory responses to hypercapnia, hypotension, endogenous opiates, histamine, and the 38 amino acid form of pituitary adenylyl cyclase activating polypeptide (PACAP) based on their sensitivity to COX inhibition by indomethacin (Busija, 1994; Lenti et al., 2007). However, after I/R, COX-2 derived reactive oxygen species (ROS) and inflammatory prostanoids are believed to substantially contribute to reperfusion injury (Armstead, 2003; Armstead et al., 1988; Candelario-Jalil and Fiebich, 2008). Indomethacin and the COX-2 inhibitor NS-398 were shown to prevent the attenuation of the I/R-sensitive, COX-independent vasodilatory response to NMDA (Domoki et al., 2001). The use of COX inhibitors, however, necessarily impairs COX-dependent cerebrovascular mechanisms and other physiological functions.

Arachidonic acid availability for COX is regulated by phospholipase A<sub>2</sub> (PLA<sub>2</sub>) activity, a diverse group of enzymes including a group of

\* Corresponding author. Fax: +36 62 545842.

E-mail addresses: [domoki@phys.szote.u-szeged.hu](mailto:domoki@phys.szote.u-szeged.hu), [fdomoki@gmail.com](mailto:fdomoki@gmail.com) (F. Domoki).

secretory (sPLA<sub>2</sub>) and Ca<sup>2+</sup>-dependent as well as independent groups of cytoplasmic PLA<sub>2</sub>s. The physiological function of sPLA<sub>2</sub>s in the brain appears to involve the fission and fusion of synaptic vesicles controlling neurotransmitter release, and also neurite outgrowth (Farooqui and Horrocks, 2006). sPLA<sub>2</sub>s hydrolyze the acyl ester bond at the sn-2 position of glycerol in membrane phospholipids displaying no specificity for the fatty acid at this position. However, addition of ceramide, a metabolite of sphingomyelin makes sPLA<sub>2</sub> highly specific for arachidonic acid (Koumanov et al., 2002). During ischemia, uncontrolled activation of brain PLA<sub>2</sub>s results in a tremendous (>50-fold) increase in free arachidonic acid concentrations in the cerebral cortex eliciting neuronal injury (Bazan, 1970). The contribution of sPLA<sub>2</sub>s in the amplification phase of this “Bazan effect” is unknown. However, sPLA<sub>2</sub>s likely participate in this process being released from the neurons undergoing anoxic depolarization. Neuronal sPLA<sub>2</sub> protein levels are increased 1–6 h after forebrain ischemia in rats suggesting a role for these enzymes in I/R injury (Lauritzen et al., 1994). sPLA<sub>2</sub> activity was shown previously to induce or aggravate neuronal injury (Kolko et al., 2002) perhaps via uncontrolled ROS production by COX. Selective inhibition of PLA<sub>2</sub> isoenzymes may offer an appealing pharmacological target to reduce the injurious effects of COX activation during I/R while preserving its physiological function in the neurovascular unit.

PX-18 is a novel sPLA<sub>2</sub> inhibitor (Rastogi et al., 2007), however, its *in vivo* testing is hindered by poor solubility in aqueous media. Formulating poorly soluble drugs as nanocrystals is a promising approach to overcome this problem. Drug nanocrystals are pure solid drug particles with a mean particle size below 1 μm. Dispersions of drug nanocrystals in liquid dispersion media stabilized with stabilizing agents, typically surfactants or polymeric stabilizers, are called nanosuspensions (Müller and Akkar, 2004). Due to the reduction of the particle size into the nanometer range, nanosuspensions possess several advantages, e.g. increased saturation solubility and consequently an increased dissolution velocity, and an increased adhesion to surfaces, which contribute to an enhanced oral bioavailability of active compounds (Müller et al., 2000; Rabinow, 2004; Van Erdenbrugh et al., 2008). The small size allows also injecting nanosuspensions intravenously. After injection, the nanoparticles dissolve relatively fast in the large blood volume.

Therefore, the specific aims of our present study were to test (1) whether PX-18 can be successfully formulated and used as a nanosuspension in anesthetized newborn pigs, (2) whether PX-18 nanosuspension affects COX-dependent or -independent cerebrovascular dilatory responses, and (3) whether PX-18 nanosuspension preserves cerebrovascular reactivity to I/R-sensitive stimuli such as hypercapnia, bradykinin, and NMDA.

## Materials and methods

### Materials

The drug and reagent sources were the following: Na-thiopental (Biochemie, Vienna, Austria); α-chloralose, NMDA, bradykinin, Tween 80, and glycerol 85% (Sigma Aldrich, St. Louis, MO, USA). PX-18 and PACAP-38 were gifts from Richard Berney Associates (LLC, Bethesda, MD, USA), and from the Department of Medical Chemistry, University of Szeged, Hungary, respectively.

### Preparation of a 3% (w/w) PX-18 nanosuspension

A Micron LAB 40 (APV Deutschland GmbH, Unna, Germany) equipped with a water jacket for temperature control was used for the production of 3% (w/w) PX-18 nanosuspensions. The nanosuspension was stabilized with 1% (w/w) Tween 80. Glycerol 85% was used for isotonicity of the nanosuspension. The PX-18 bulk material was dispersed in the isotonic aqueous surfactant solution using a mortar and pestle. The dispersion was pre-homogenized running 2 cycles at

150 bar followed by 2 cycles at 500 bar and 2 cycles at 1000 bar. The obtained pre-dispersions were subjected to a high pressure homogenization applying 20 cycles at 1500 bar at 5 °C. A 1% (w/w) Tween 80 solution isotonicized with glycerol 85% served as vehicle control during the experiments.

### Photon correlation spectroscopy

The particle size of the nanosuspension was investigated by photon correlation spectroscopy using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Photon correlation spectroscopy yields the mean particle size and the polydispersity index as a measure of the width of the particle size distribution. The polydispersity index ranges from 0 (= monodisperse) to 1 (= very broad size distribution).

### Laser diffractometry (LD)

The particle size of the PX-18 bulk material and of the 3% (w/w) PX-18 nanosuspension was determined by laser diffractometry (LD) using a Coulter LS 230 (Beckman-Coulter, Krefeld, Germany). For data evaluation water with a refractive index (RI) of 1.33 was used as a measurement medium. The real RI of PX-18 is 1.496, the imaginary RI of this compound is zero. The LD data were used to calculate the diameters 50% (LD 50), 90% (LD 90) and 95% (LD 95), which means that either 50%, 90% or 95% (volume distribution) of the measured particles are below this size.

### Stability study

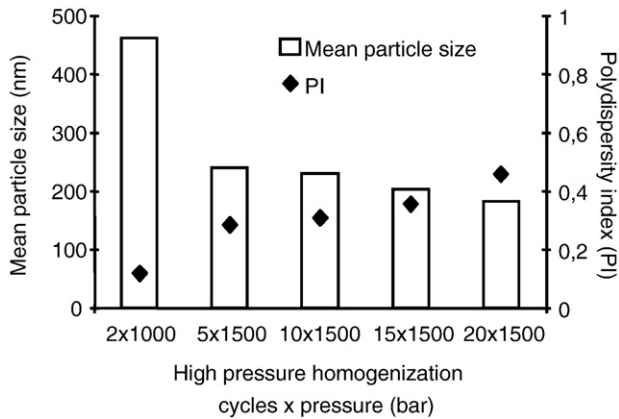
To ensure the physical stability of the 3% (w/w) PX-18 nanosuspension over the period of the experiments, it was stored at 4 °C for three months and the particle size was investigated on days 0, 1, 7, 14, 30, and 90 by photon correlation spectroscopy and LD measurements.

### Intravital videomicroscopy of pial arterioles in piglets

All procedures were approved by the Animal Care and Use Committee of the University of Szeged. Newborn pigs (~1-day old, 1.2–2.5 kg, *n* = 40) were initially anesthetized with Na-thiopental (40 mg/kg ip) followed by α-chloralose (40 mg/kg, supplemented with 2–5 mg kg<sup>-1</sup> h<sup>-1</sup> iv). Animals were intubated via tracheotomy and artificially ventilated with a pressure-controlled ventilator using room air. The ventilation rate (~30 breaths/min) and tidal volume (~20 ml) were adjusted to maintain arterial blood gas values and pH in the physiological range. The right femoral artery and vein were catheterized to monitor arterial blood pressure and for administration of drugs and fluids, respectively. Body temperature was maintained at 37–38 °C with a water-circulating heating pad. The piglets were equipped with a stainless steel closed cranial window with 3 injectible ports to follow the diameter changes of a selected pial arteriole (baseline diameter ~80–100 μm) using intravital videomicroscopy, as described previously (Domoki et al., 2005). The subarachnoid space under the cranial window (~3 ml chamber volume) was filled with artificial cerebrospinal fluid (aCSF): KCl 220, MgCl<sub>2</sub> 132, CaCl<sub>2</sub> 221, NaCl 7710, urea 402, dextrose 665, and NaHCO<sub>3</sub> 2066 in mg/l, warmed to 37 °C and equilibrated with a gas mixture containing 6% O<sub>2</sub>, 6.5% CO<sub>2</sub> and 87.5% N<sub>2</sub>. All drugs were diluted/dissolved in aCSF, and were applied with a single injection through the ports of the cranial window (5 ml of aCSF superfusion in 1 min) onto the parietal cortex.

### Global cerebral ischemia

Cerebral ischemia was produced by infusion of aCSF to raise the intracranial pressure (ICP) above the arterial pressure for 10 min. Ischemia was verified by cessation of blood flow in the vessels observed through the cranial window. Venous blood was withdrawn as



**Fig. 1.** Mean particle size and polydispersity index (PI) measured by photon correlation spectroscopy of a 3% (w/w) PX-18 nanosuspension after 2 homogenization cycles at 1000 bar, and 5, 10, 15 and 20 homogenization cycles at 1500 bar at 5 °C. A continuous decrease in particles size with an increasing number of applied homogenization cycles was observed, while PI did not change considerably. The mean particle size of the 3% (w/w) PX-18 nanosuspension was 183 nm after 20 homogenization cycles at 1500 bar that was used for the *in vivo* experiments.

necessary to keep mean arterial blood pressure (MABP) near normal values. At the end of the ischemic period, the infusion tube was clamped and the ICP was allowed to return to the preischemic level. The withdrawn and heparinized blood was reinfused.

#### Assessment of cerebrovascular reactivity (CR)

Pial arteriolar diameters were determined under baseline conditions and then at each minute during application of vasodilatory stimuli. Hypercapnia was elicited by ventilation with a gas mixture (10% CO<sub>2</sub>, 21% O<sub>2</sub>, balance N<sub>2</sub>) for 7–10 min to allow maximal arteriolar dilation. PX-18 nanosuspension diluted in aCSF to a concentration of 10<sup>-8</sup> M–10<sup>-4</sup> M, corresponding dilutions of its vehicle, PACAP-38 (10<sup>-6</sup> M), NMDA (10<sup>-4</sup> M), or bradykinin (10<sup>-6</sup> M) were applied locally onto the parietal cortex under the cranial window for 5 min each. PX-18 nanosuspension (6 mg/kg) or its vehicle was also

given as a bolus intravenous injection or a continuous infusion for 20 min. Results are presented as the maximal vasodilation maintained for at least 2 min, and expressed as % changes from corresponding baseline diameters. After completion of a stimulus, the cranial window was flushed repeatedly with aCSF and the arteriolar diameter was allowed to return to baseline levels for 15 min before the application of the next stimulus.

#### Experimental protocol

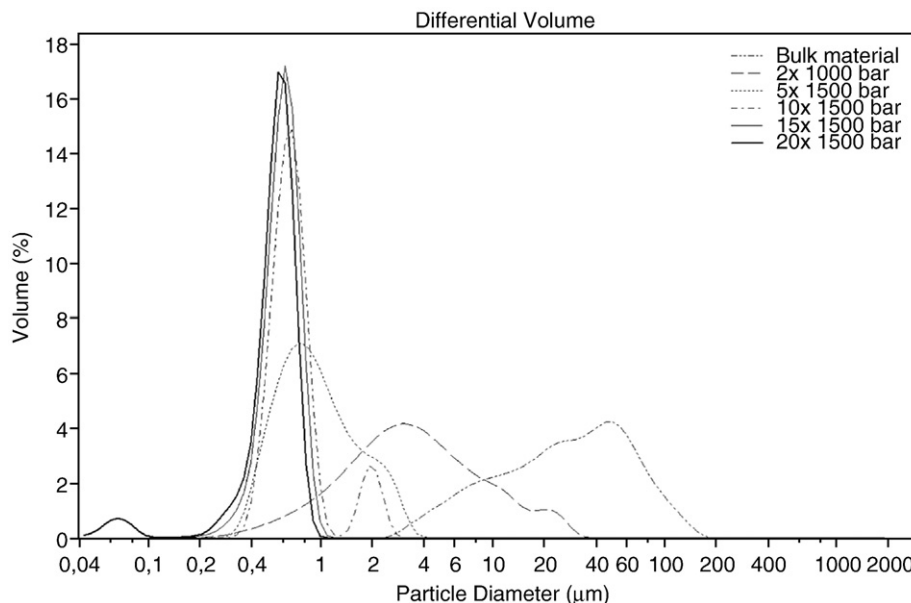
Instrumented piglets were divided into seven experimental groups. In Group 1 (*n* = 5), CR was determined for local vehicle, local PX-18 nanosuspension, systemic vehicle (iv bolus), and systemic PX-18 nanosuspension (iv bolus) administration, consecutively. In Group 2 (*n* = 7), CR was determined to hypercapnia and PACAP38 before and after local treatment with PX-18 nanosuspension (10<sup>-5</sup> M, 20 min). In Group 3 (*n* = 5), the protocol was the same as in Group 2, however, PX-18 nanosuspension was given as an infusion (6 mg/kg, 20 min, iv). In Group 4 (*n* = 5), CR was determined to NMDA and bradykinin before and after local PX-18 treatment, then after systemic PX-18 nanosuspension infusion. In Groups 5, 6 and 7 (*n* = 8, 8, and 7, respectively), CR was determined to hypercapnia, NMDA, and bradykinin before and 1 h after 10 min of global cerebral ischemia. Prior to the induction of ischemia, animals in Group 5, 6, or 7 received no treatment, vehicle infusion, or PX-18 nanosuspension infusion, respectively.

#### Statistics

Data are expressed as mean ± SEM. Data were analyzed with a statistical software (SigmaStat, Systat Software Inc., San Jose, CA, USA) using one-way or two-way repeated measures ANOVA, where appropriate. For *post hoc* analysis the Student–Newman–Keuls test was employed. *p* values of <0.05 were considered as statistically significant.

#### Results

High pressure homogenization produced a PX-18 nanosuspension reducing the mean particle size and simultaneously narrowing the width of the size distribution (Figs. 1–2). 95% of the particles based on



**Fig. 2.** Volume–size distribution curves measured by laser diffractometry (LD) of PX-18 bulk material and a 3% (w/w) PX-18 nanosuspension after 2 homogenization cycles at 1000 bar, as well as after 5, 10, 15 and 20 homogenization cycles at 1500 bar at 5 °C. The bulk material had a particle size in the micrometer range and an inhomogeneous particle size distribution. After 5 homogenization cycles at 1500 bar no particles bigger than 5 μm were detected in the formulation. Continuing the homogenization process the particle size could be further decreased.



**Table 1**

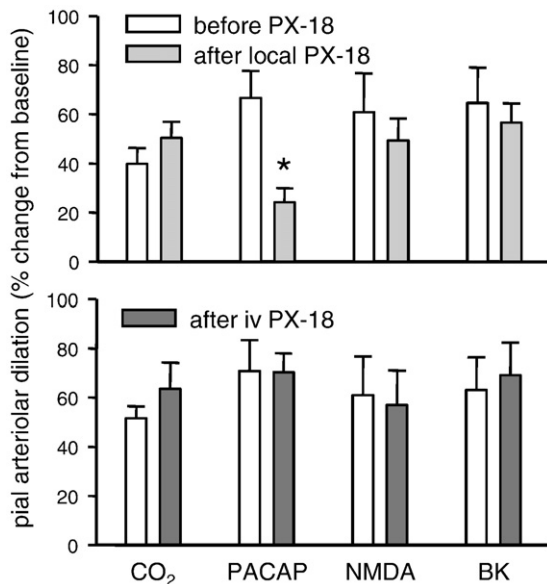
Particle size of a 3% (w/w) PX-18 nanosuspension stored at 4 °C over three months determined by photon correlation spectroscopy and laser diffractometry (LD).

Parameter	Day 0	Day 1	Day 7	Day 14	Day 30	Day 90
MPS [nm]	183	172	171	165	166	167
PI	0.450	0.469	0.491	0.514	0.477	0.513
LD 50 [nm]	566	587	571	586	573	577
LD 90 [nm]	725	765	742	782	751	757
LD 95 [nm]	775	812	786	815	795	805

MPS: mean particle size determined by PCS, PI: polydispersity index, LD 50–90–95: 50–90–95% of the PX-18 volume distribution is below the indicated particle size determined by LD, respectively.

a volume distribution measured by LD had a size smaller than 775 nm in the final nanosuspension used for the *in vivo* experiments (Fig. 2). Table 1 provides an overview of the particle sizes measured over an observation period of three months. The particle size of the 3% (w/w) PX-18 nanosuspension stayed constant when stored at 4 °C. No particle growth was observed under the storage conditions over the observation period.

PX-18 nanosuspension or its vehicle did not induce significant increase in pial arteriolar diameters when applied locally up to 10<sup>-5</sup> M concentration. This dose was used for local PX-18 pretreatments, where pial arteriolar diameters were essentially unchanged by PX-18: the values were 92 ± 4 versus 92 ± 3 μm before versus during 10<sup>-5</sup> M PX-18 application (data combined from Groups 1, 2, and 4, n = 14). In Group 1, rapid *iv* injection of PX-18 nanosuspension, but not its vehicle, resulted in a transient drop in MABP with a simultaneous increase in pial arteriolar diameters, which both returned to baseline levels in 4–6 min. However, heart rate was not significantly affected by PX-18 nanosuspension injection. The maximal changes in MABP, pial arteriolar diameters, and heart rate before versus 2 min after PX-18 nanosuspension injection were 62 ± 7 versus 40 ± 5\* mm Hg, 92 ± 6 versus 116 ± 9\* μm, and 159 ± 19 versus 155 ± 17 1/min, respectively (\*p < 0.05, n = 5). These transient changes in cerebral



**Fig. 3.** PX-18 minimally affects cerebrovascular reactivity. Hypercapnia induced by ventilation with 10% CO<sub>2</sub> (CO<sub>2</sub>), or topical application of the 38 amino acid isoform of pituitary adenyl cyclase activating peptide (10<sup>-6</sup> M; PACAP), N-methyl-D-aspartate (10<sup>-4</sup> M, NMDA), and bradykinin (10<sup>-6</sup> M, BK) all resulted in robust increases in pial arteriolar diameters. Local pretreatment with PX-18 (10<sup>-5</sup> M, upper panel) significantly reduced PACAP-induced vasodilation, but did not affect cerebrovascular reactivity to hypercapnia, NMDA, or BK. Intravenous infusion of PX-18 (6 mg/kg, lower panel) did not affect cerebrovascular reactivity to any stimuli; p < 0.05, \* versus corresponding response before treatment, n = 5–7 for each group.

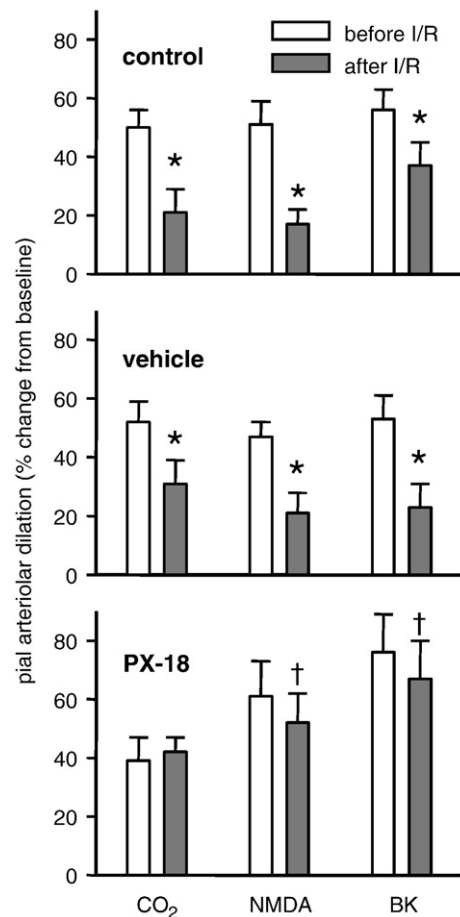
hemodynamics were prevented by administering PX-18 nanosuspension as an infusion (20 min) in Groups 3, 4, and 7 (data not shown).

Hypercapnia, PACAP38, bradykinin, and NMDA all elicited robust pial arteriolar vasodilation (Groups 2–4, Fig. 3) that was unaffected by both local and systemic administrations of PX-18; except vasodilation to PACAP-38 which was significantly reduced only by topical PX-18 (Fig. 3).

In piglets subjected to I/R, MABP and pial arteriolar diameters returned to preischemic values by 1 h of reperfusion. MABP and baseline arteriolar diameters, before versus 1 h after ischemia were: 60 ± 3 versus 57 ± 2 mm Hg and 106 ± 7 versus 107 ± 8 μm (Group 5), 70 ± 4 versus 65 ± 2 mm Hg and 95 ± 6 versus 101 ± 8 μm (Group 6), and 66 ± 4 versus 64 ± 4 mm Hg, and 94 ± 9 versus 92 ± 9 μm (Group 7). I/R significantly attenuated the pial arteriolar dilation to hypercapnia, NMDA, and bradykinin in the control (untreated) and the vehicle-treated animals (Groups 5 and 6, respectively, Fig. 4). However, in Group 7, hypercapnia-, NMDA-, and bradykinin-induced pial arteriolar vasodilation was similar before and after cerebral ischemia, thus PX-18 preserved cerebrovascular reactivity to these stimuli (Fig. 4).

**Discussion**

The major novel finding of our study is that the sPLA<sub>2</sub> inhibitor PX-18 preserves neurovascular reactivity after I/R in the newborn



**Fig. 4.** PX-18 preserves cerebrovascular reactivity after global ischemia/reperfusion (I/R). Pial arteriolar dilations to hypercapnia induced by ventilation with 10% CO<sub>2</sub> (CO<sub>2</sub>), topical application of N-methyl-D-aspartate (10<sup>-4</sup> M, NMDA), and bradykinin (10<sup>-6</sup> M, BK) were all significantly reduced after I/R injury in time control animals (control, upper panel, n = 8). Attenuation of cerebrovascular reactivity to all these stimuli was similar in animals treated with the vehicle of PX-18 (vehicle, middle panel, n = 8). In contrast, pial arteriolar responses to CO<sub>2</sub>, NMDA, and BK remained intact after I/R in the PX-18-treated group (PX-18, lower panel, n = 7); p < 0.05, \* versus corresponding response before I/R, † versus corresponding response after I/R in the control and vehicle-treated groups.

piglet. Importantly, the protective dose of PX-18 does not interfere with indomethacin-sensitive (i.e., COX-dependent) cerebrovascular responses. Our data suggest that preservation of microvascular function can contribute to the cardio- and neuroprotective effects of PX-18 reported in a rabbit and gerbil I/R model, respectively (Ockaili et al., 2005; Wang et al., 2007).

The sPLA<sub>2</sub> inhibitor PX-18 (Rastogi et al., 2007) was formulated as drug nanosuspension suitable for iv injection with an active content of 3% (w/w) applying high pressure homogenization. Similar to o/w emulsions for parenteral nutrition, the number of large particles (above 1 μm) should be limited and no significant number of particles larger than 5 μm should be present in the formulation. The 3% (w/w) PX-18 nanosuspension fulfills these requirements; ≥99% of the particles present in this formulation based on a volume distribution have a size below 0.860 μm (Fig. 2). Therefore, formulating the poorly soluble sPLA<sub>2</sub> inhibitor PX-18 as nanosuspension makes it available for pharmacological testing. Stability investigations of the PX-18 nanosuspension with regard to the particle size showed, that under the storage condition no changes in the particle size distribution occurred (Table 1). Neither particle agglomeration or aggregation nor Ostwald ripening (= crystal growth) took place in the formulation. The lack of Ostwald ripening in the 3% (w/w) PX-18 nanosuspension produced by high pressure homogenization is caused by the uniform particle size and the absence of particles in the micrometer range (Moschwitzter et al., 2004; Muller et al., 2001). This ensures a constant particle size during the performance of the *in vivo* experiments.

In the newborn piglet, hypercapnia-induced vasodilation is an endothelium-dependent and I/R-sensitive cerebrovascular response since it is impaired by light/dye or I/R injury to the microvascular endothelium (Leffler et al., 1989; Leffler et al., 1994). Hypercapnia-induced vasodilation is also COX-dependent since inhibition of PLA<sub>2</sub> activity with quinacrine or p-bromophenacyl bromide or inhibition of COX with indomethacin abolishes pial arteriolar vasodilation to hypercapnia (Wagerle and Mishra, 1988). Attenuation of hypercapnia-induced vasodilation after I/R is considered to be due to decreased availability of arachidonic acid since exogenous supply of arachidonic acid restores normal cerebrovascular reactivity to hypercapnia (Leffler et al., 1992). In the present study, PX-18 does not affect hypercapnia-induced vasodilation, but prevents the postischemic attenuation of this response suggesting that sPLA<sub>2</sub>s may not be involved in the PLA<sub>2</sub> activity required for this response but may be involved in the critical depletion of releasable arachidonic acid during I/R. In addition to intact hypercapnia-induced vasodilation after PX-18, pial arteriolar dilation to PACAP was also essentially unchanged. We have previously demonstrated that pial arteriolar dilation to the 38 amino acid isoform of PACAP is virtually abolished after indomethacin or the selective COX-1 antagonist SC-560, but unaffected by the COX-2 inhibitor NS-398 (Lenti et al., 2007). Although there was a significant decrease in PACAP-induced vasodilation when PX-18 was administered locally (Fig. 3), the remaining response was still robust as compared to the absent response after COX inhibitors in the previous study (Lenti et al., 2007). Importantly, the systemic dose used to assess the protective effect of PX-18 did not affect this COX-dependent response (Fig. 3).

Bradykinin dilates pial arterioles in the newborn piglet via an endothelium-dependent but a COX-independent mechanism (Lacza et al., 2002; Willis and Leffler, 2001). A novel observation of the present study is that pial arteriolar dilation to bradykinin is sensitive to I/R (Fig. 4). Hypertension-induced dysfunction of cerebrovascular endothelium had been known to reduce cerebrovascular reactivity to bradykinin in rats (Yang et al., 1991a; Yang et al., 1991b), but our present results demonstrate for the first time that I/R-induced endothelial injury is reflected in impaired cerebrovascular reactivity to bradykinin in piglets. PX-18 preserved bradykinin-induced vasodilation after I/R similar to hypercapnia-induced vasodilation, confirming that preservation of endothelial function after I/R may be a mechanism of the neuroprotection of this drug (Fig. 4).

NMDA-induced pial arteriolar vasodilation in the piglet is a very well-characterized I/R-sensitive neuronal-vascular response that is critically dependent on activation of neuronal NMDA receptors with subsequent release of nitric oxide produced by neuronal nitric oxide synthase (Busija et al., 2007). In addition to the indirect neuronal-vascular mechanism of NMDA-induced vasodilation, direct cerebrovascular effects of glutamate and the NMDA-receptor agonist 1-aminocyclopentane-cis-1,3-dicarboxylic acid (but not NMDA itself) have been reported in the piglet (Fiumana et al., 2003; Parfenova et al., 2003). However, we found that NMDA did not dilate pial arterioles *ex vivo* and cerebral microvessels lacked functional NMDA receptors in piglets (Domoki et al., 2008; Simandle et al., 2005). NMDA receptor activation-induced increases in arteriolar diameters likely contribute to the coupling of local cortical blood flow to the metabolism of neurons stimulated by the activation of NMDA receptors, and the impairment of this response by I/R may be used as a sensitive bioassay to assess the postischemic dysfunction of the neurovascular unit (Busija et al., 2007). NMDA-induced vasodilation after I/R is impaired by the postischemic generation of COX-derived ROS activity; ROS scavengers, indomethacin, and the selective COX-2 inhibitor NS-398 were shown to preserve neurovascular reactivity to NMDA (Bari et al., 1998; Busija et al., 2007; Domoki et al., 2001; Philip and Armstead, 2003). In the present study, PX-18 provided excellent, virtually total preservation of NMDA-induced vasodilation (Fig. 4), in agreement with the notion that inhibition of sPLA<sub>2</sub> by PX-18 interferes with the release of arachidonic acid during ischemia resulting in significantly less substrate for COX in the reperfusion.

In conclusion, the novel PLA<sub>2</sub> inhibitor PX-18 could be successfully formulated as nanosuspension suitable for iv injection. PX-18 preserves the physiological neurovascular responsiveness to both endothelium- and neuron-dependent dilator stimuli after I/R. The protective effects of PX-18 are likely mediated by inhibition of arachidonic acid depletion and COX-dependent ROS production during I/R. Thus, PX-18 improves the function of the neurovascular unit after I/R contributing to its previously reported neuroprotective effect (Wang et al., 2007). Selective modulation of brain PLA<sub>2</sub> may identify new pharmacological agents to reduce neuronal damage after hypoxic/ischemic injury in the newborn.

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## References

- Armstead, W.M., 2003. Cyclooxygenase-2-dependent superoxide generation contributes to age-dependent impairment of G protein-mediated cerebrovasodilation. *Anesthesiology* 98, 1378–1383.
- Armstead, W.M., et al., 1988. Postischemic generation of superoxide anion by newborn pig brain. *Am. J. Physiol.* 255, H401–H403.
- Bari, F., et al., 1998. Inhibitory effects of hypoxia and adenosine on N-methyl-D-aspartate-induced pial arteriolar dilation in piglets. *Brain Res.* 780, 237–244.
- Bazan Jr, N.G., 1970. Effects of ischemia and electroconvulsive shock on free fatty acid pool in the brain. *Biochim. Biophys. Acta* 218, 1–10.
- Book, S.A., Bustad, L.K., 1974. The fetal and neonatal pig in biomedical research. *J. Anim. Sci.* 38, 997–1002.
- Busija, D.W., 1994. Cerebral Circulation of the Fetus and Newborn. In: Bevan, R.D.B. (Ed.), *The Human Brain Circulation*. Humana Press, Totowa, pp. 259–269.
- Busija, D.W., et al., 1996. Effects of ischemia on cerebrovascular responses to N-methyl-D-aspartate in piglets. *Am. J. Physiol.* 270, H1225–H1230.
- Busija, D.W., et al., 2007. Mechanisms involved in the cerebrovascular dilator effects of N-methyl-D-aspartate in cerebral cortex. *Brain Res. Rev.* 56, 89–100.
- Candelario-Jalil, E., Fiebich, B.L., 2008. Cyclooxygenase inhibition in ischemic brain injury. *Curr. Pharm. Des.* 14, 1401–1418.
- Domoki, F., et al., 2001. Cyclooxygenase-2 inhibitor NS398 preserves neuronal function after hypoxia/ischemia in piglets. *NeuroReport* 12, 4065–4068.

- Domoki, F., et al., 2005. Diazoxide preserves hypercapnia-induced arteriolar vasodilation after global cerebral ischemia in piglets. *Am. J. Physiol. Heart Circ. Physiol.* 289, H368–H373.
- Domoki, F., et al., 2008. Cerebromicrovascular endothelial cells are resistant to L-glutamate. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 295, R1099–R1108.
- Farooqui, A.A., Horrocks, L.A., 2006. Phospholipase A2-generated lipid mediators in the brain: the good, the bad, and the ugly. *Neuroscientist* 12, 245–260.
- Fiumana, E., et al., 2003. Carbon monoxide mediates vasodilator effects of glutamate in isolated pressurized cerebral arterioles of newborn pigs. *Am. J. Physiol. Heart Circ. Physiol.* 284, H1073–H1079.
- Flecknell, P.A., et al., 1980. Total body glucose metabolism in the conscious, unrestrained piglet and its relation to body- and organ weight. *Br. J. Nutr.* 44, 193–203.
- Gootman, N., et al., 1972. Central vasomotor regulation in the newborn piglet *Sus scrofa*. *Am. J. Physiol.* 222, 994–999.
- Kolko, M., et al., 2002. Secretory phospholipase A2-mediated neuronal cell death involves glutamate ionotropic receptors. *NeuroReport* 13, 1963–1966.
- Koumanov, K.S., et al., 2002. Ceramides increase the activity of the secretory phospholipase A2 and alter its fatty acid specificity. *Biochem. J.* 363, 45–51.
- Lacza, Z., et al., 2002. Hydrogen peroxide acts as an EDHF in the piglet pial vasculature in response to bradykinin. *Am. J. Physiol. Heart Circ. Physiol.* 283, H406–H411.
- Lauritzen, I., et al., 1994. Expression of group II phospholipase A2 in rat brain after severe forebrain ischemia and in endotoxic shock. *Brain Res.* 651, 353–356.
- Leffler, C.W., et al., 1989. Cerebral ischemia alters cerebral microvascular reactivity in newborn pigs. *Am. J. Physiol.* 257, H266–H271.
- Leffler, C.W., et al., 1992. Topical arachidonic acid restores pial arteriolar dilation to hypercapnia of postischemic newborn pig brain. *Am. J. Physiol.* 263, H746–H751.
- Leffler, C.W., et al., 1994. Light/dye microvascular injury selectively eliminates hypercapnia-induced pial arteriolar dilation in newborn pigs. *Am. J. Physiol.* 266, H623–H630.
- Lenti, L., et al., 2007. Pituitary adenylate cyclase-activating polypeptide induces pial arteriolar vasodilation through cyclooxygenase-dependent and independent mechanisms in newborn pigs. *Brain Res.* 1165, 81–88.
- Lo, E.H., et al., 2003. Mechanisms, challenges and opportunities in stroke. *Nat. Rev. Neurosci.* 4, 399–415.
- Moschwitzer, J., et al., 2004. Development of an intravenously injectable chemically stable aqueous omeprazole formulation using nanosuspension technology. *Eur. J. Pharm. Biopharm.* 58, 615–619.
- Müller, R.H., Akkar, A., 2004. Drug nanocrystals of poorly soluble drugs. In: Nalwa, H.S. (Ed.), *Encyclopedia of Nanoscience and Nanotechnology*. American Scientific Publishers, New York, pp. 627–638.
- Müller, R.H., et al., 2000. Nanosuspensions for the formulation of poorly soluble drugs. In: Nielloud, F., Marti-Mestres, G. (Eds.), *Pharmaceutical Emulsions and Suspensions*. Marcel Dekker, New York.
- Muller, R.H., et al., 2001. Nanosuspensions as particulate drug formulations in therapy. Rationale for development and what we can expect for the future. *Adv. Drug Deliv. Rev.* 47, 3–19.
- Ockaili, R., et al., 2005. PX-18 — a novel inhibitor of phospholipase A(2) induces 'pharmacological preconditioning' in the rabbit heart through opening of mitoK (ATP) channels. *FASEB J.* 19, A691.
- Parfenova, H., et al., 2003. Ionotropic glutamate receptors in cerebral microvascular endothelium are functionally linked to heme oxygenase. *J. Cereb. Blood Flow Metab.* 23, 190–197.
- Peri, K.G., et al., 1995. Prostaglandin G/H synthase-2 is a major contributor of brain prostaglandins in the newborn. *J. Biol. Chem.* 270, 24615–24620.
- Philip, S., Armstead, W.M., 2003. Differential role of PTK, ERK and p38 MAPK in superoxide impairment of NMDA cerebrovasodilation. *Brain Res.* 979, 98–103.
- Rabinow, B.E., 2004. Nanosuspensions in drug delivery. *Nat. Rev. Drug Discov.* 3, 785–796.
- Rastogi, P., et al., 2007. Prostaglandin production in human coronary artery endothelial cells is modulated differentially by selective phospholipase A(2) inhibitors. *Prostaglandins Leukot. Essent. Fat. Acids* 76, 205–212.
- Simandle, S.A., et al., 2005. Piglet pial arteries respond to N-methyl-D-aspartate in vivo but not in vitro. *Microvasc. Res.* 70, 76–83.
- Van Eerdenbrugh, B., et al., 2008. Top-down production of drug nanocrystals: nanosuspension stabilization, miniaturization and transformation into solid products. *Int. J. Pharm.* 364, 64–75.
- Wagerle, L.C., Mishra, O.P., 1988. Mechanism of CO<sub>2</sub> response in cerebral arteries of the newborn pig: role of phospholipase, cyclooxygenase, and lipoxygenase pathways. *Circ. Res.* 62, 1019–1026.
- Wang, Q., et al., 2007. Neuroprotective effects of a nanoparticulate formulation of sPLA<sub>2</sub> inhibitor PX-18 in cerebral ischemia/reperfusion. *J. Neurochem.* 102, 127.
- Willis, A.P., Leffler, C.W., 2001. Endothelial NO and prostanoid involvement in newborn and juvenile pig pial arteriolar vasomotor responses. *Am. J. Physiol. Heart Circ. Physiol.* 281, H2366–H2377.
- Yang, S.T., et al., 1991a. Endothelium-dependent responses of cerebral blood vessels during chronic hypertension. *Hypertension* 17, 612–618.
- Yang, S.T., et al., 1991b. Mechanisms of impaired endothelium-dependent cerebral vasodilatation in response to bradykinin in hypertensive rats. *Stroke* 22, 1177–1182.