

VPAC₂ Receptors Mediate Vasoactive Intestinal Peptide-Induced Neuroprotection against Neonatal Excitotoxic Brain Lesions in Mice

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ABSTRACT

Prepro-vasoactive intestinal peptide (VIP) mRNA codes for two neuropeptides: VIP and peptide histidine isoleucine (PHI). Two VIP receptors, shared with a similar affinity by pituitary adenylate cyclase-activating polypeptide (PACAP), have been cloned: VPAC₁ and VPAC₂. PHI binds to these receptors with a lower affinity. VPAC receptors are classically associated with a cAMP-dependent pathway, although other pathways, including calcium mobilization and protein kinase C activation have been described. We previously showed that intracerebral administration of the glutamate agonist ibotenate to postnatal day 5 mice induces white matter lesions mimicking human periventricular leukomalacia. In this model, coinjection of VIP protects against white matter lesions. This neuroprotection is independent from cAMP and is mediated by protein kinase C. Using this model, this study aimed to determine the receptor involved

in VIP-induced neuroprotection. VIP effects were mimicked with a similar potency by VPAC₂ agonists and PHI but not by VPAC₁ agonists, PACAP 27, or PACAP 38. VIP neuroprotective effects were lost in mice lacking VPAC₂ receptor. In situ hybridization confirmed the presence of VPAC₂ mRNA in the postnatal day 5 white matter. When analyzed between embryonic life and adulthood, VIP-specific binding site density peaked at postnatal day 5. These data suggest that, in this model, VIP-induced neuroprotection is mediated by VPAC₂ receptors. The pharmacology of this VPAC₂ receptor seems unconventional because 1) PACAP does not mimic VIP effects, 2) PHI acts with a comparable potency, and 3) PACAP 27 modestly inhibited the VIP-specific binding, whereas for PHI or VIP, inhibition was complete.

Vasoactive intestinal peptide (VIP) is a central nervous system neurotransmitter and neuromodulator with neurotrophic properties (Rosselin et al., 1982; Moody and Jensen, 2003).

The mouse VIP precursor can produce two peptides: VIP, a 28-amino acid peptide, and peptide histidine isoleucine

(PHI). However, PHI shares moderate amino acid identity with VIP (37%), and its biological significance still remains unclear. VIP is a member of a superfamily of peptides, including pituitary adenylate cyclase-activating polypeptide (PACAP), a 27- (PACAP27) or 38-amino acid (PACAP38) peptide. The biologically active region of PACAP, corresponding to the PACAP27 sequence, shows 68% identity with VIP (for review, see Vaudry et al., 2000).

In transfected cells, VIP, PACAP27, and PACAP38 bind with similar affinities to two receptors common for VIP and PACAP called VPAC₁ and VPAC₂ receptors (Ishihara et al.,

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ABBREVIATIONS: VIP, vasoactive intestinal peptide; PHI, peptide histidine-isoleucine; PACAP, pituitary adenylate cyclase-activating peptide; PKC, protein kinase C; NPR-C, natriuretic peptide clearance receptor; ANP, atrial natriuretic peptide; P, postnatal; E, embryonic; kb, kilobase; PBS, phosphate buffer saline; GRF, growth hormone-releasing factor; BSA, bovine serum albumin; ANOVA, analysis of variance; ADNF, activity-dependent neuroprotective protein.

1992; Lutz et al., 1993). Furthermore, PACAP27 and PACAP38, but not VIP, bind with high affinity to a specific PACAP receptor called the PAC₁ receptor (Vaudry et al., 2000). ¹²⁵I-VIP and ¹²⁵I-PHI binding experiments suggested that a 48-kDa component expressed in chicken liver membranes display the properties of a GTP-insensitive VIP/PHI receptor that can be pharmacologically discriminated from the GTP-sensitive 60-kDa form, through its much higher affinity for PHI. Therefore, GTP-insensitive VIP receptors may correspond to a subclass of high-affinity PHI receptors (Pineau et al., 2001).

VPAC receptors are preferentially coupled to G_{as} protein that stimulates adenylate cyclase activity and induces cAMP increase (Harmar et al., 1998). VPAC receptors can also be coupled to G_{aq} and G_{ai} proteins that stimulate the inositol phosphate/calcium/protein kinase C (PKC) pathways (Olah et al., 1994; Rawlings et al., 1995; Ransjo et al., 2000; Langer et al., 2001). Interestingly, the natriuretic peptide clearance receptor (NPR-C), which binds to atrial natriuretic peptide (ANP), has been proposed to be involved in VIP-induced calcium entry response in gastric smooth muscle (Murthy et al., 1998). Indeed, in binding studies using ¹²⁵I-labeled atrial natriuretic peptide (¹²⁵I-ANP) and ¹²⁵I-VIP as radioligands, VIP, ANP, and the selective NPR-C ligand cANP-(4-23) bound with high affinity to NPR-C. ANP, cANP-(4-23), and VIP initiated identical signaling cascades consisting of Ca²⁺ influx, stimulation of cGMP formation, and muscle relaxation.

In line with its previously reported neurotrophic properties (Gressens et al., 1993, 1994), VIP potently protects the developing brain against an excitotoxic insult in newborn mice (Gressens et al., 1997). In this *in vivo* model, VIP, coinjected with the glutamatergic agonist ibotenate in the brain of 5-day-old (P5) pups, reduces ibotenate-induced white matter lesions by up to 85% compared with controls. Surprisingly, VIP-induced neuroprotection is not mimicked by PACAP 38 but by stearyl norleucine VIP, a specific VIP agonist that does not activate adenylate cyclase. Moreover, treatment with forskolin, an adenylate cyclase activator, fails to provide a VIP-like protection. In contrast, VIP protective effects are abolished by a PKC inhibitor and a mitogen-associated protein kinase inhibitor in a dose-dependent manner (Gressens et al., 1997, 1998).

This atypical pharmacology of VIP-induced neuroprotection in newborn mice raises several hypotheses. 1) Activation of PAC₁ receptors could have a toxic effect on the excitotoxic lesions, whereas activation of VPAC receptors could be neuroprotective, leading to a lack of detectable effect for PACAP38. Interestingly, VIP and PACAP have been shown to have opposite effects on synaptic transmission (Ciranna and Cavallaro, 2003). 2) During some stages of brain development, the binding of VIP or PACAP to VPAC receptors leads to activation of separate transduction pathways. This differential coupling could be secondary to VPAC receptor dimerization (homo- or heterodimers) or to their interaction with larger oligomeric complexes, as demonstrated for other types of G protein-coupled receptors (for review, see Milligan, 2004). 3) VIP acts through a yet to be identified specific VIP receptor, which is not recognized by PACAP. Indeed, Ekblad et al. (2000) characterized a PACAP 27-preferring receptor and a VIP-specific receptor, distinct from those that have

been cloned (VPAC₁, VPAC₂, and PAC₁ receptors), in intestine of rat and PAC₁^{-/-} mice.

Accordingly, in the present study, the neuroprotective properties of several agonists of VIP and PACAP receptors were tested in the above-described neonatal excitotoxic model. Neuroprotective effects of VIP and/or PACAP were evaluated in mutant mice lacking PAC₁ or VPAC₂ receptors, further allowing the characterization of the receptor involved in VIP-induced neuroprotection. In addition, *in situ* hybridization for VPAC₂ receptor mRNA was performed on P5 mouse brains. Finally, binding studies using radiolabeled VIP, PHI, and PACAP27 were performed on brain membranes from embryonic day 17 (E17), P5 and adult animals.

Materials and Methods

Animals. Different types of mice of both sexes were used in this study: Swiss mice (Janvier, Le Genest-Saint-Isle, France), C57BL/6 control PAC₁^{+/+} mice, C57BL/6 PAC₁^{-/-} mice (Jamen et al., 2000), C57BL/6 control VPAC₂^{+/+} mice, C57BL/6 control VPAC₂^{+/-} mice, and C57BL/6 VPAC₂^{-/-} mice (Goetzl et al., 2001). C57BL/6 VPAC₂ mice were kindly provided by Dr. Anthony J. Harmar (Division of Neuroscience, University of Neuroscience, Edinburgh, Scotland).

PAC₁^{-/-} and VPAC₂^{-/-} mice were generated as described previously (Jamen et al., 2000; Goetzl et al., 2001; Harmar et al., 2002). Briefly, for PAC₁^{-/-} knockout mice, a 2.3-kb NcoI fragment containing PAC₁ receptor exons 8 to 11 was replaced by a pPolIII-neo^R cassette. The targeting vector contained 4.2 and 2.8 kb of homologous sequences up- and downstream from the pPolIII-neomycin sequence, respectively (Jamen et al., 2000). The VPAC₂ receptor null mice were generated by replacing 132 base pairs of exon 1, including the translation start site with a lac Z-neo^R cassette. The total construct was 10 kb (Goetzl et al., 2001).

Experimental protocols meet the guidelines of the Institut National de la Santé et de la Recherche Médicale and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Drugs. Ibotenate (Sigma, St-Quentin Fallavier, France) and VIP (Peninsula Laboratories, Belmont, CA) were diluted in phosphate-buffered saline (PBS) containing 0.01% acetic acid. PACAP27 (Bachem, Weil am Rhein, Germany), PACAP38 (Peninsula Laboratories), the selective VPAC₁ receptor agonist derived from VIP and growth hormone-releasing factor (GRF) [Lys¹⁵, Arg¹⁶, Leu²⁷]VIP (1-7)/GRF (8-27) (VIP-GRF) (generous gift from Dr. Patrick Robberecht (Laboratoire de Chimie Biologique et de la Nutrition, University of Brussels Medical School, Brussels, Belgium) (Gourlet et al., 1997a), the selective VPAC₁ receptor agonist [R¹⁶]chicken secretin (generous gift from Dr. Patrick Robberecht) (Gourlet et al., 1997a), the selective VPAC₂ receptor agonist RO25-1553 Ac-[Glu⁸, Lys¹², Nle¹⁷, Ala¹⁹, Asp²⁵, Leu²⁶, Lys^{27,28}, Gly^{29,30}, Thr³¹]-VIP cyclo₂₁₋₂₅ (generous gift from Dr. Patrick Robberecht) (Gourlet et al., 1997b), PHI (Bachem), the selective NPR-C receptor agonist des-[Gln¹⁸, Ser¹⁹, Gly²⁰, Leu²¹, Gly²²] ANP (4-23) amide (des-ANP; Sigma) (Brown and Czarnecki, 1990), and ANP (Sigma) were diluted in PBS.

Administration of the Excitotoxic Drug. Excitotoxic brain lesions were induced by injecting ibotenate into developing mouse brains, as described previously (Marret et al., 1995; Gressens et al., 1997, 1998). Briefly, mouse pups anesthetized with isoflurane were kept under a warming lamp and were injected intracerebrally (into the neopallial parenchyma) at P5. Intraparenchymal injections were performed with a 25-gauge needle on a 50-μl Hamilton syringe mounted on a calibrated microdispenser. The needle was inserted 2 mm under the external surface of the scalp skin in the frontoparietal area of the right hemisphere, 2 mm from the midline in the lateral-medial plane, and 3 mm anterior to bregma in the rostro-caudal

plane. Histopathology confirmed that the tip of the needle always reached the periventricular white matter. Two 1- μ l boluses of ibotenate were injected at 20-s intervals. The needle was left in place for an additional 20 s; 10 μ g of ibotenate was administered to each pup.

Experimental Groups. Pups from at least two different litters were used in each experimental group, and data were obtained from two or more successive experiments. Mice were injected with ibotenate alone (controls) or in combination with different peptides: 0.01, 0.1, or 1 μ g of VIP; 0.0001, 0.001, 0.01, 0.1, or 1 μ g of PACAP38; 0.001, 0.01, 0.1 or 1 μ g of PACAP27; 0.01, 0.1 or 1 μ g of RO25-1553; 1 μ g of [R16]chicken secretin; 1 μ g of VIP-GRF; 0.01, 0.1, or 1 μ g of PHI; 0.01, 0.1, or 1 μ g of des-ANP; and 0.01, 0.1, or 1 μ g of ANP.

Determination of Lesion Size. Mouse pups were sacrificed by decapitation 5 days after the excitotoxic challenge. Brains were immediately fixed in 4% formalin for 7 days. After embedding in paraffin, we cut 15- μ m-thick coronal or sagittal sections. Every third section was stained with cresyl violet. Previous studies (Marret et al., 1995; Gressens et al., 1997; Husson et al., 2002) have shown an excellent correlation between the maximal size of the lesion in the lateral-medial and fronto-occipital axes of the excitotoxic lesions. To further confirm these observations, we cut serial sections of the entire brain in the coronal plane or in the sagittal plane. This permitted an accurate and reproducible determination of the maximal fronto-occipital (on coronal sections) or lateral-medial (on sagittal sections) diameters of the lesions (which is equal to the number of sections where the lesion was present multiplied by 15 μ m). We used these linear measures as an index of the volume of the lesion.

In Situ Hybridization. P5 Swiss mice were perfused intracardially with a 4% paraformaldehyde solution. The brains were put in 4% paraformaldehyde for 2 h at 4°C and then in a 10% saccharose 0.12 M phosphate buffer for 24 h. Brains were frozen at -80°C, and 10- μ m-thick coronal sections were cut.

VPAC₂ receptor riboprobes were prepared by in vitro transcription of rat VPAC₂ receptor cDNA fragments generated by reverse transcription-polymerase chain reaction, and subcloned into the pGEM-T cloning vector (Promega, Charbonnières, France). The primers used for polymerase chain reaction were designed as follows: forward primer, 5'-GTCAACTTTGCCCTCTCCATCA-3' and reverse primer, 5'-GCCTCTCCACCTTCTTTTCAGT-3' (accession no. Z25885). Antisense and sense riboprobes were generated with T7 or SP6 polymerase in the presence of ³⁵S-UTP (Amersham Biosciences, Inc., Les Ulis, France).

In situ hybridization was performed as described previously (Bellemère et al., 2004). Briefly, sections were acetylated, treated with 0.2% Triton X-100 and covered with prehybridization buffer [50% formamide, 0.6 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin (BSA), 1 mM EDTA, pH 8.0, 550 μ g/ml denatured salmon sperm DNA, and 50 μ g/ml yeast tRNA]. Hybridization was performed overnight at 55°C in the same buffer (except for salmon sperm DNA, the concentration of which was lowered to 60 μ g/ml) supplemented with 10 mM dithiothreitol, 10% dextran sulfate, and 1.5×10^7 cpm/ml heat-denatured riboprobes. Sections were then washed in 2 $\times$ standard saline citrate at 60°C and treated with 50 μ g/ml RNase A for 1 h at 37°C. Five final high-stringency washes were performed in 0.01 $\times$ standard saline citrate containing 14 mM β -mercaptoethanol and 0.05% sodium pyrophosphate. Brain slices were dehydrated in graded alcohols containing ammonium acetate and apposed onto Hyperfilm β -max (Amersham Biosciences, Inc.) for 2 weeks. Slices were subsequently dipped into Kodak NTB2 liquid emulsion at 40°C, exposed for 30 days, and developed. To identify anatomic structures, sections were stained with hematoxylin. Autoradiograms were analyzed by means of a computer-assisted image analysis system (SAMBA Autoradio 4.10; SAMBA Technologies, Meylan, France). Photomicrographs were imported into MERCATOR software (Explora Nova, La Rochelle, France) interfaced with a Nikon Eclipse E600 microscope (Les Ulis, France).

Receptor Binding Studies on Mouse Forebrain Membrane Preparations. Forebrains were freshly removed from E17, P5, and P60 Swiss mice. They were immediately frozen in liquid nitrogen and stored at -80°C until membrane purification. Membranes were prepared as described previously (Pineau et al., 2001). Protein content was determined with a Bradford assay (Bio-Rad protein assay dye reagent concentrate; Bio-Rad, Ivry-sur-Seine, France) and BSA fatty acid-free fraction V (Sigma) as a standard. Membranes aliquots were stored at -80°C until use.

VIP, PHI, and PACAP27 (Neosystem, Strasbourg, France) were radioiodinated with the chloramine-T method as reported previously (Martin et al., 1986) using a modified protocol. Free ¹²⁵I-iodine was removed onto C₁₈ Sep-Pak Cartridges (Waters, St-Quentin-en-Yvelines, France) according to the manufacturer's instructions. Radioiodinated peptidic forms were separated by reverse-phase high-performance liquid chromatography (Spectraphysics; Waters) using a 5- μ m VYDAC C18 column (Interchim, Montluçon, France). Elution was conducted for 37 min at 1 ml/min with a 0 to 85% acetonitrile linear gradient in 0.1% trifluoroacetic acid/H₂O. Fractions containing mono ¹²⁵I-VIP, ¹²⁵I-PHI, or ¹²⁵I-PACAP 27 were pooled, evaporated under nitrogen, and stored at -20°C until use.

Membrane preparations were homogenized in cold binding buffer, pH 7.4, (Dulbecco's modified Eagle's medium supplemented with 15 mM HEPES, 150 μ M phenylmethylsulfonyl fluoride, 0.1% bacitracin, and 1% BSA) containing 50 pM of ¹²⁵I-VIP or ¹²⁵I-PHI or ¹²⁵I-PACAP 27 and unlabeled peptides at specified concentrations. Bind-

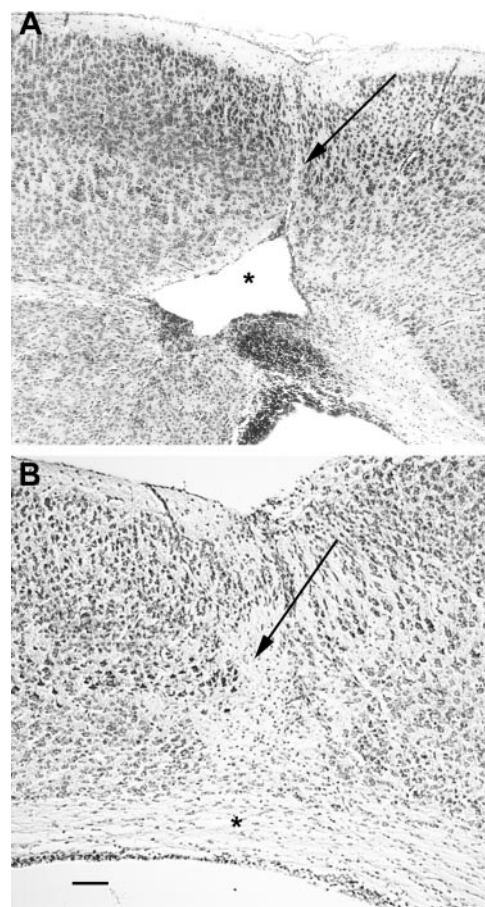


Fig. 1. VIP protects the periventricular white matter against ibotenate-induced lesions in Swiss mice. Cresyl violet-stained sections showing brain lesions induced by ibotenate injected at P5 and studied at the age of P10. A, brain from Swiss pup treated with intracerebral ibotenate and PBS (control), showing the typical neuronal loss in layers II to VI (arrow) and the white matter cystic lesion (*). B, brain from Swiss pup cotreated with intracerebral ibotenate and 1 μ g of VIP. LV, lateral ventricle. Scale bar, 40 μ m.

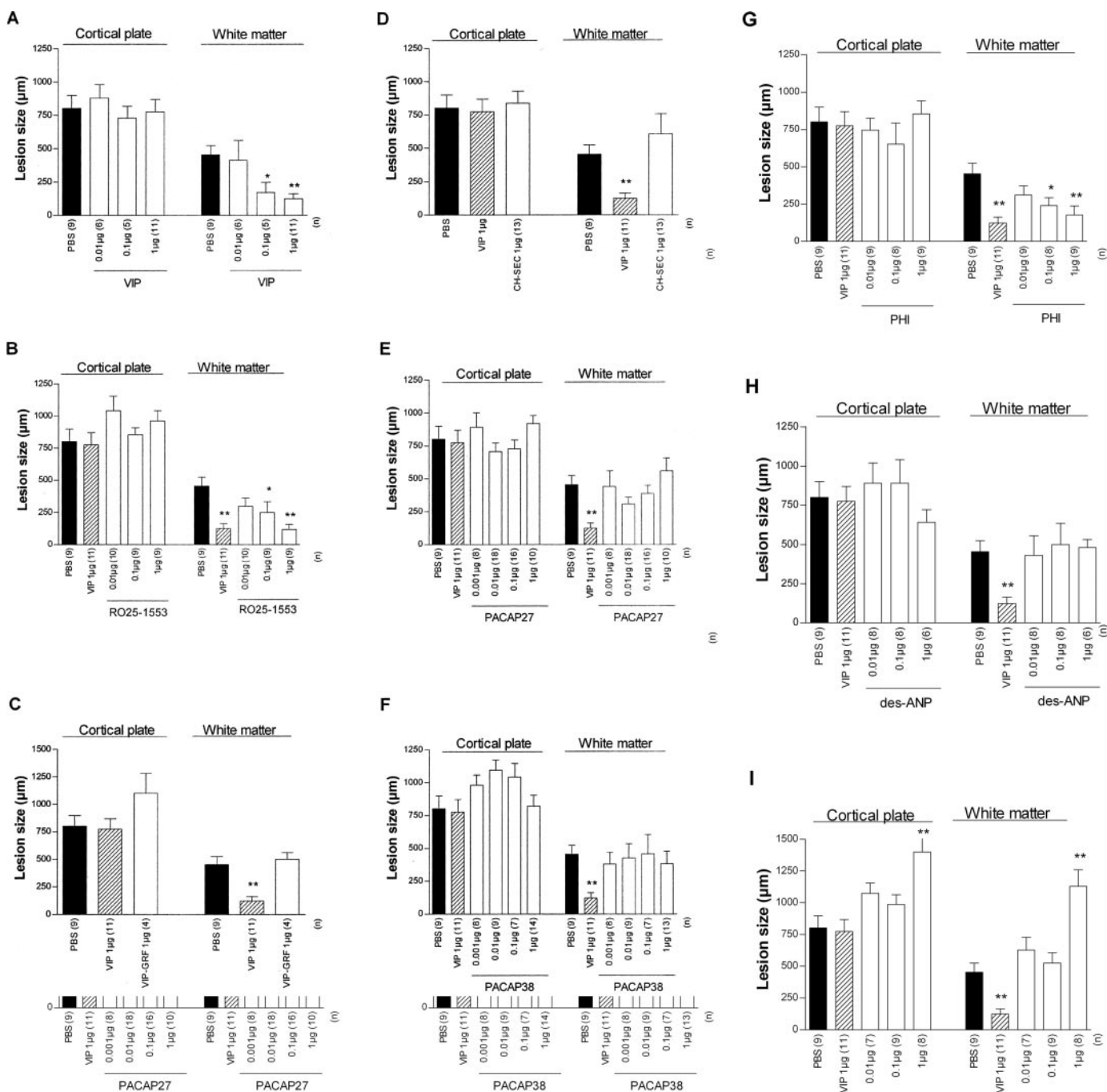


Fig. 2. Effects of VIP (A), RO25-1553 (B), VIP-GRF (C), [R16]chicken secretin (CH-SEC) (D), PACAP27 (E), PACAP38 (F), PHI (G), des-ANP (H), and ANP (I) on brain lesions induced by ibotenate injected to P5 Swiss mice. Bar represents mean length of the lesions $\pm$ S.E.M. Asterisks indicate difference from PBS control group (black column); *, $p < 0.05$; **, $p < 0.01$ in ANOVA with Dunnett's multiple comparison test. Numbers in parentheses (n) on the x -axis indicate the number of analyzed animals in each experimental group.

ing experiments were performed with shaking for 50 min at 20°C and stopped at 4°C . Samples were filtered through Whatman GF/C glass microfiber filters (Fisher Scientific LABOSI, Illkirch, France), pre-treated for 30 min with a 0.5% polyethyleneimine and 0.5% BSA solution, using a samples collector (Millipore Corporation, Billerica, MA). Filters were washed with 1 ml and 3 ml of an ice-cold 150 mM Tris with 5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, pH 7.5, solution, dried under vacuum. The retained radioactivity was measured in a Cobra II Packard gamma-counter.

Preliminary dose-dependent experiments (data not shown) were performed on the mouse brain membrane preparations to determine protein quantities (350 μg) necessary to maximize specific binding.

Specific binding represents the difference between values obtained in the absence or in presence of 10^{-6} M unlabeled neuropeptides.

Statistical Analysis. Quantitative data were expressed as the means $\pm$ S.E.M.s for each treatment group. Means were compared using Student's t test or ANOVA with Dunnett's or Newman-Keul's multiple comparison of means test (GraphPad Prism version 3.03 for Windows; GraphPad Software Inc., San Diego, CA).

Results

Excitotoxic Brain Lesions. All animals injected with ibotenate at P5 and sacrificed 5 days later displayed large

periventricular white matter cysts in the injected right hemisphere (Fig. 1A). Cortical lesions characterized by neuronal loss affecting all cortical layers (Fig. 1A) were also observed in the injected right hemisphere these animals.

In Swiss mice, as described previously (Gressens et al., 1997, 1998), VIP significantly protected the white matter against ibotenate but had no significant effect on cortical plate lesion (Figs. 1B and 2A). RO25-1553, a VPAC₂ agonist, mimicked the neuroprotective effects of VIP (Fig. 2B), whereas VIP-GRF and [R16]chicken secretin, two VPAC₁ agonists, had no detectable effects of white matter and cortical plate lesions (Fig. 2, C and D). A large range of doses (0.001–1 μ g) of PACAP27 or PACAP38 did not protect or significantly exacerbate the white matter or the gray matter against excitotoxic lesions (Fig. 2, E and F). In contrast, PHI mimicked VIP-induced neuroprotection of white matter with a potency similar to VIP (Fig. 2G). As for VIP, PHI did not protect the cortical plate against ibotenate (Fig. 2G). Finally, des-ANP or ANP did not mimic neuroprotective effects of VIP on excitotoxic white matter damage (Fig. 2, H and I) and the highest dose of ANP even exacerbated excitotoxic cortical plate and white matter (Fig. 2I).

In control C57BL/6 control PAC₁^{+/+} mice, VIP significantly protected the white matter against ibotenate (Fig. 3A). As in Swiss mice, PACAP38 failed to mimic VIP neuroprotective effects (Fig. 3A). In C57BL/6 PAC₁^{-/-} mice, VIP-induced neuroprotection was maintained, whereas PACAP38 had no detectable effect on ibotenate-induced lesions (Fig. 3A). VIP-induced neuroprotection of the white matter was observed in C57BL/6 control VPAC₂^{+/+} and VPAC₂^{+/-} mice but was totally abrogated in C57BL/6 VPAC₂^{-/-} mice (Fig. 3B).

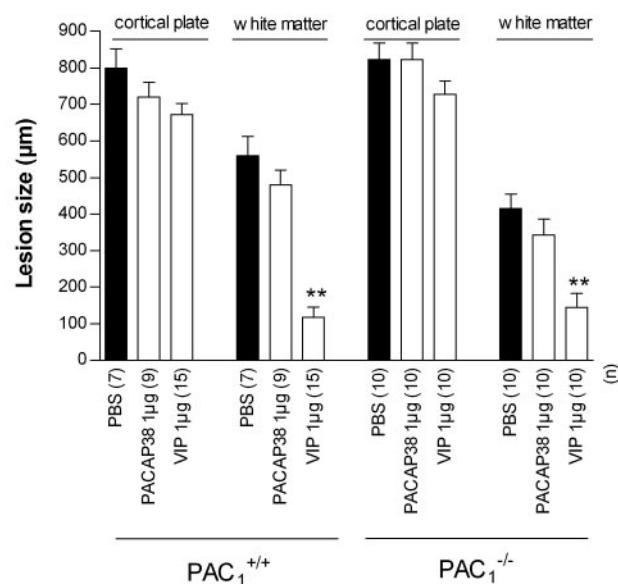
In Situ Hybridization for VPAC₂ mRNA in Untreated Swiss Mice. VPAC₂ mRNA was detected by in situ hybridization in P5 mouse brain (Fig. 4). Specific signal was present in the neocortex and also in the underlying periventricular white matter. This VPAC₂ mRNA expression was sustained along the rostro-caudal axis, including the frontoparietal area where ibotenate was injected in experimental groups described above. When the sense probe was used, no hybridization was observed (Fig. 4).

Neuropeptide Binding Site Studies in Untreated Swiss Mice. ¹²⁵I-VIP and -PHI specific binding site densities changed over time (between E17 and P60), whereas ¹²⁵I-PACAP 27-specific binding sites density remained stable (Fig. 5; Table 1). Of particular interest, the density of ¹²⁵I-VIP-specific binding sites peaked at P5.

In E17 brains, ¹²⁵I-VIP (Fig. 6A), ¹²⁵I-PHI (Fig. 6B), or ¹²⁵I-PACAP 27 (Fig. 6C) binding were similarly inhibited by unlabeled VIP, PHI, or PACAP 27 with kinetics consistent with the presence of a single binding site (Hill slopes varied from 0.96 $\pm$ 0.35 to 0.98 $\pm$ 0.28). Specific ¹²⁵I-PHI or ¹²⁵I-PACAP 27 binding were inhibited with a high affinity (IC₅₀ values were measured between 0.04 $\pm$ 0.009 and 0.82 $\pm$ 0.16 nM), whereas neuropeptide affinities for ¹²⁵I-VIP binding sites were at least 10-fold lower (IC₅₀ from 2.25 $\pm$ 0.67 to 9.10 $\pm$ 3.04 nM).

In P5 brains, unlabeled VIP, PHI, or PACAP 27 inhibited in the same manner the ¹²⁵I-PHI (Fig. 6E) or ¹²⁵I-PACAP 27 (Fig. 6F) binding, on one site (Hill slopes from 0.93 $\pm$ 0.13 to 0.98 $\pm$ 0.06) with a high affinity (IC₅₀ from 0.20 $\pm$ 0.06 to 0.66 $\pm$ 0.06 nM). PACAP27 only modestly inhibited the specific high affinity ¹²⁵I-VIP binding (33%), whereas VIP and PHI abolished the specific radiotracer interaction (Fig. 6D).

A



B

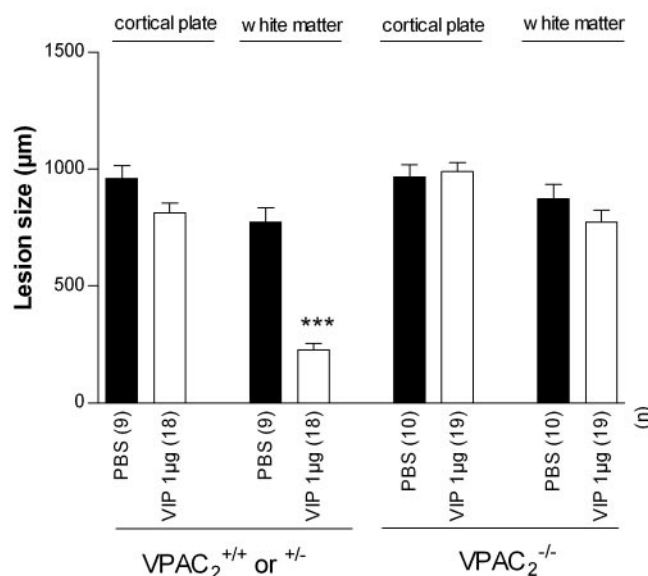


Fig. 3. Effects of VIP or PACAP 38 on brain lesions induced by ibotenate injection to P5 C57BL/6 PAC₁^{-/-} (A) or VPAC₂^{-/-} (B) mice. Bar represents mean length of the lesions $\pm$ S.E.M. Asterisks indicate difference from PBS control group (black column); **, $p < 0.01$; ***, $p < 0.001$ in ANOVA with Dunnett's multiple comparison test. Numbers in parentheses (n) on the x-axis indicate the number of analyzed animals in each experimental group.

In adult brain, the specific high-affinity ¹²⁵I-VIP (Fig. 6G) and -PHI (Fig. 6H) binding, abolished by unlabeled PACAP27, was partially inhibited by, respectively, unlabeled PHI (57%) and VIP (53%). When ¹²⁵I-PACAP 27 was used, two binding site subsets (Hill slopes from 0.74 $\pm$ 0.07 to 0.77 $\pm$ 0.01) were distinguished by the neuropeptides (Fig. 6I), one with a high affinity (IC₅₀ from 0.03 $\pm$ 0.01 to 0.23 $\pm$ 0.02 nM) and the other with a low affinity (IC₅₀ from 41.19 $\pm$ 5.28 to 55.47 $\pm$ 2.09 nM).

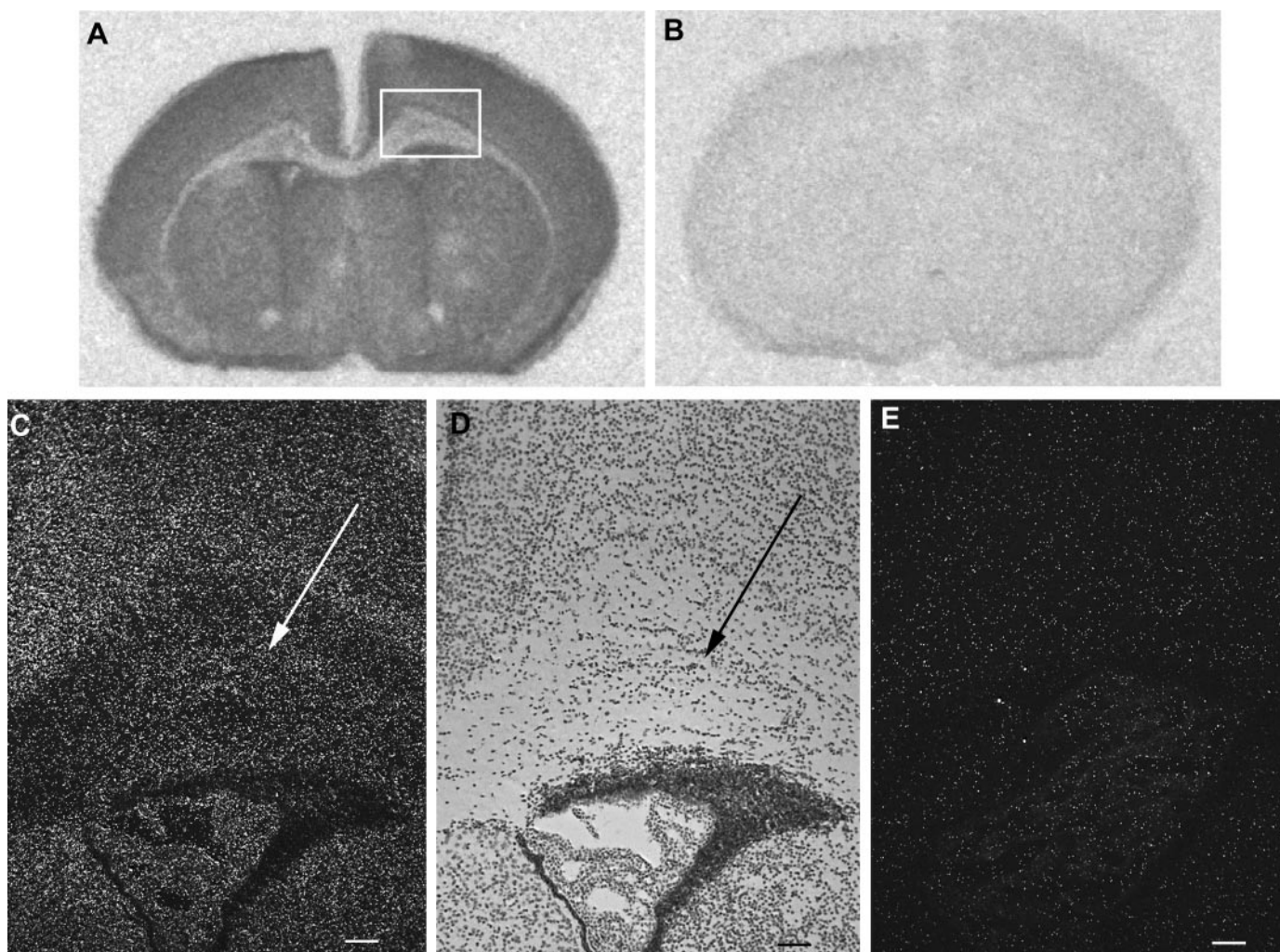


Fig. 4. In situ hybridization for VPAC₂ receptor mRNA with antisense (A and C) or sense (B and E) ³⁵S-labeled-probe on P5 Swiss brain sections. C and E represent higher magnifications of the periventricular white matter area shown in A (boxed area) and B, respectively, and visualized under dark field. Arrows point to white matter cells. D, hematoxylin stained section corresponding to C. Scale bar, 100 μm.

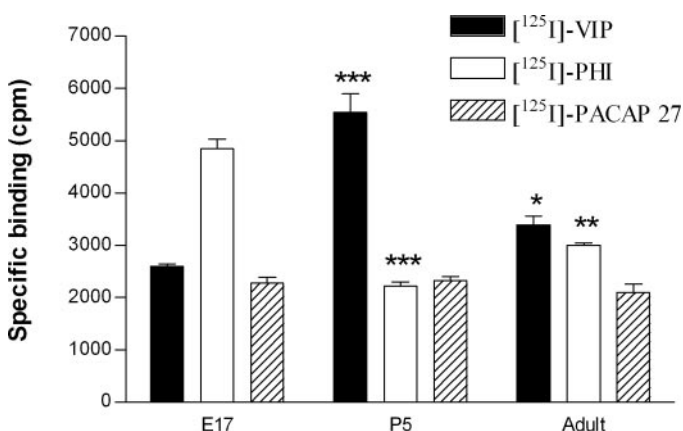


Fig. 5. Specific ¹²⁵I-VIP, -PHI, or -PACAP 27 binding detected at the different ages: E17, P5, and adult in Swiss mouse brain membranes preparations. Specific binding was measured in cpm and represented the mean ± S.E.M. Bar represents mean specific binding ± S.E.M. Asterisks indicate difference from corresponding E17 group; *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001 in ANOVA with Newman-Keuls's multiple comparison test. Three independent experiments were performed in triplicate.

Discussion

The present study extends the evidence on neuroprotective properties of VIP against excitotoxic white matter lesions in the developing mouse brain (Gressens et al., 1997, 1998; Gressens, 1999) and identifies VPAC₂ receptors as critical mediators of VIP effects. The role of VPAC₂ receptors is supported by the neuroprotective effects of VPAC₂ receptor

TABLE 1
Specific maximal binding capacities of ¹²⁵I-VIP, ¹²⁵I-PHI, and ¹²⁵I-PACAP 27

	<i>B</i> _{max} ± S.E.M. ^a		
	E17	P5	Adult
	<i>f</i> mol/mg		
¹²⁵ I-VIP	317.24 ± 9.43	762.46 ± 21.03*** ^b	369.46 ± 1.25*
¹²⁵ I-PHI	609.37 ± 3.97	290.53 ± 7.46***	336.38 ± 5.35**
¹²⁵ I-PACAP27	297.36 ± 12.98	301.66 ± 12.07	264.59 ± 4.36

^a *B*_{max} values ± S.E.M. deduced, after Scatchard analysis (GraphPad Prism), from the competitive inhibition curves of ¹²⁵I-VIP, ¹²⁵I-PHI, or ¹²⁵I-PACAP 27 binding by unlabelled VIP, PHI, or PACAP 27 in E17, P5, or adult mouse brain membranes.

^b Asterisks indicate difference from corresponding E17 group; **p* < 0.05, ***p* < 0.01, ****p* < 0.001 in ANOVA with Newman-Keuls multiple comparison test.

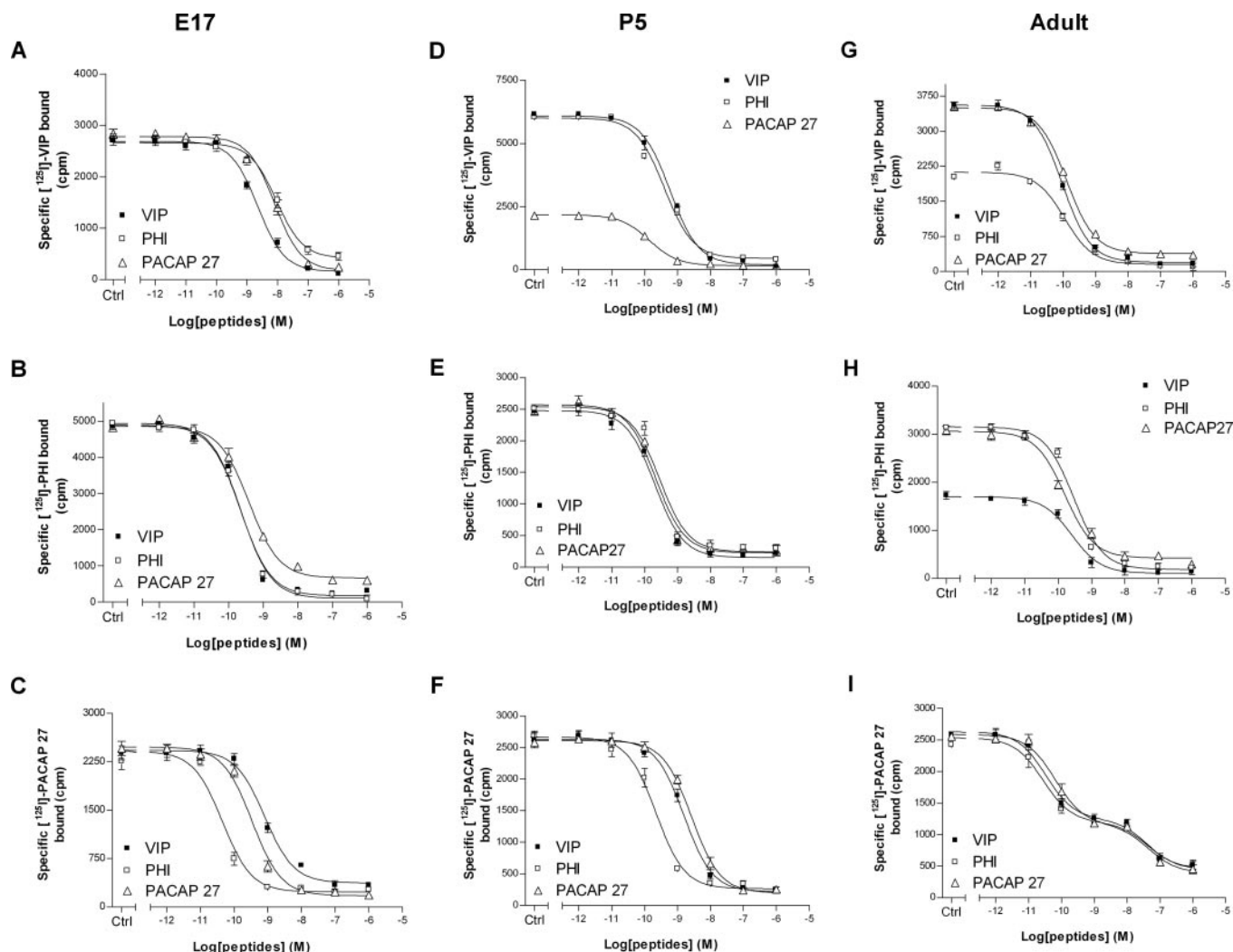


Fig. 6. Inhibition of ¹²⁵I-VIP (A, D, and G), ¹²⁵I-PHI (B, E, and H) or ¹²⁵I-PACAP 27 (C, F, and I) by unlabeled VIP, PHI or PACAP 27, respectively, in E17, P5, and adult Swiss mouse brain membranes. Data represent the mean $\pm$ S.E.M. of three independent experiments each performed in triplicate.

agonists, the loss of VIP neuroprotective effects in C57BL/6 VPAC₂^{-/-} mice and the presence of VPAC₂ receptor mRNA in the P5 white matter.

Our results are in accordance with an *in vitro* study suggesting VPAC₂ involvement in neuroprotection (Zusev and Gozes, 2004). They studied activity-dependent neuroprotective protein (ADNF) expression in astrocytes cell cultures. Indeed, 1) ADNF was previously shown to be a VIP-responsive gene in astrocytes derived from the cerebral cortex of newborn rats (Bassan et al., 1999); and 2) VIP neuroprotective properties are mediated through interaction with glial cells (Brenneman et al., 1987; Gozes et al., 1991; Gressens et al., 1998). They showed that VIP induced changes in ADNF expression in astrocyte cell cultures via the VPAC₂ receptor (Zusev and Gozes, 2004).

However, *in vivo*, the pharmacology of the involved VPAC₂ receptors is atypical compared with data obtained in transfected cultured cells (Ishihara et al., 1992; Lutz et al., 1993; Vaudry et al., 2000; Laburthe et al., 2002) because 1) using the same mouse model of neonatal excitotoxic brain lesions, we previously showed that VIP neuroprotective effects against excitotoxic white matter damage are not mediated by cAMP-protein kinase A pathway but by a phospholipase C-

PKC transduction pathway (Gressens et al., 1997, 1998); 2) neither PACAP 27 nor PACAP 38 mimics VIP effects; 3) PHI mimics VIP effects with a similar potency; and 4) in P5 pups, PACAP 27 modestly inhibited the specific high ¹²⁵I-VIP binding, whereas PHI or VIP totally inhibited it.

The previously stated hypothesis that activation of PAC₁ receptors could have a toxic effect on the excitotoxic lesions, whereas activation of VPAC receptors could be neuroprotective, leading to a lack of detectable effect for PACAP38, can be ruled out by the lack of protective effects of PACAP 38 in PAC₁^{-/-} mice.

As mentioned above, the NPR-C, which binds to ANP, has been proposed to be involved in VIP-induced calcium entry response in gastric smooth muscle (Murthy et al., 1998). However, in the present study, ANP and des-ANP that classically bind NPR-C did not reproduce VIP neuroprotective effects, and ANP was even toxic at high dose.

Therefore, to explain the observed characteristics of VPAC₂ receptors in the present study, some hypotheses can be formulated. 1) During some stages of brain development, the binding of VIP or PACAP to VPAC₂ receptors leads to activation of separate transduction pathways. This differential coupling could be secondary to VPAC₂ receptors dimer-

ization (homo- or heterodimers) or to their interaction with larger oligomeric complexes, as demonstrated for other types of G protein-coupled receptors (for review, see Milligan, 2004). A variant of this hypothesis would be a developmental change in the G proteins available for the receptor to couple to in the relevant cells. 2) An alternative hypothesis has been suggested by recent studies. A first study identified a deletion variant (receptor lacking amino acids 367–380 at the carboxyl-terminal end of the seventh transmembrane domain) of the mouse VPAC₂ receptor in immune cells (Grininger et al., 2004). This natural deletion abrogates VIP-induced cAMP production without apparent alterations of expression or ligand binding. Second, Langer and Robberecht (2005) showed that mutations in the proximal domain of the third intracellular loop of the VPAC₁ receptor reduced the capability of VIP to increase adenylate cyclase activity without any change in the calcium response, whereas mutations in the distal part of the loop markedly reduced the calcium increase and G_{ai} coupling but only weakly reduced the adenylate cyclase activity. Based on these studies, we can hypothesize that a yet-to-be-identified substitution or deletion in the newborn mouse VPAC₂ receptor transcript, through RNA editing for instance, might be able to induce VIP specificity and modulate the coupling with different G proteins.

Together, these data strongly support the hypothesis that, in newborn mice, VIP neuroprotective effects against an excitotoxic insult are mediated by VPAC₂ receptors showing atypical pharmacological properties. Further studies will be required to further characterize these VPAC₂ receptors to unravel the molecular basis of their intriguing pharmacology.

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