Neuroprotection of the Developing Brain by Systemic Administration of Vasoactive Intestinal Peptide Derivatives

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ABSTRACT

Periventricular leukomalacia (PVL), a necrotic and often cystic lesion of the cerebral white matter occurring in very premature babies, is the leading cause of cerebral palsy in this population. Increased glutamate release and the excitotoxic cascade thus triggered may be critical factors in the development of PVL. The glutamatergic analog ibotenate injected intracerebrally into newborn mice produces white matter cysts that mimic human PVL. Concomitant injection of vasoactive intestinal peptide (VIP), a trophic factor, protects the white matter against excitotoxic lesions. The goal of the present study was to assess the protective properties of systemically injected VIP analogs against ibotenate-induced excitotoxic white matter lesions in newborn mice. VIP analogs were selected on the basis of their low susceptibility to endopeptidases and their potential ability to cross biological membranes. RO-25-1553, a long-lasting cyclic VIP analog, and stearyl-norleucine-VIP, a fatty derivative of VIP, reduced ibotenate-induced white matter cysts by up to 87% and 84%, respectively, when injected i.p. immediately after ibotenate. By comparison, i.p. coadministration of VIP and ibotenate was not protective against the excitotoxic insult. Furthermore, RO-25-1553 and stearyl-norleucine-VIP still induced significant neuroprotection of the developing white matter when injected systemically 8 and 12 h, respectively, after ibotenate, establishing these peptides as therapeutic agents in this murine model. VIP analogs may have therapeutic potential in human premature babies at high risk for PVL.

The development of new strategies in the prevention and treatment of perinatal brain damage remains a health care priority. Cerebral palsy is still prevalent and its incidence is increasing in some countries (Hagberg et al., 1996), in part because of the increased survival of extremely low-birth-weight infants (Pharoah et al., 1990). Although new treatments have improved pulmonary outcomes in infants of 26 to 32 weeks' gestational age (Crowley, 1995), the risk of brain lesions remains high in this population (Zupan et al., 1996).

Periventricular leukomalacia (PVL), a necrotic and often cystic lesion of the neocortical white matter, is a major cause of neurological handicap in premature infants. Its pathophysiology may be multifactorial, involving both prenatal and perinatal factors that may include genetic determinants, perfusion failure, growth factor deficiency, and maternal infection (Nelson and Ellenberg, 1986; Evrard et al., 1992, 1995; Murphy et al., 1995; Volpe, 1995; Zupan et al., 1996).

Several risk factors for PVL may share excitatory amino acids as a common final pathway leading to white matter damage. We recently used ibotenate to produce an animal model of excitotoxic brain lesions (Marret et al., 1995b). Ibotenate, a glutamate analog, activates both N-methyl-D-aspartate and metabotropic receptors but not the α3-amino-hydroxy-5-methyl-4-isoxazole and kainate receptors. Ibotenate administered after completion of neuronal migration produces transcortical necrosis that mimics the cortical damage observed most commonly in human babies born after 32 weeks of gestation (Volpe, 1995) and, more importantly, results in cystic white matter lesions strikingly similar to some types of PVL. Although this excitotoxic mouse model is not a perfect phenocopy of PVL in human preterm infants, it is one of a very small number of animal models specifically designed to study this human disease.

In this model of excitotoxic white matter lesion, various molecules with a potential for interfering with N-methyl-D-aspartate receptors, including magnesium sulfate, prevented ibotenate-induced white matter lesions when they were injected before or very shortly after the excitotoxin (Marret et
al., 1995a, 1997). However, these drugs were effective only when present at the site of the insult during the very early stages of the excitotoxic cascade, limiting their use in medicine. Growth factors may have greater potential as therapeutic agents for PVL because they have been found to prevent the delayed cell death often observed in neonatal brain lesions (Edwards and Mehmet, 1996) or to promote repair of damaged periventricular white matter.

Vasoactive intestinal peptide (VIP) is a 28-amino acid peptide that has trophic properties on cultured astrocytes and neurons (Brenneman et al., 1985; Brenneman and Eiden, 1986) and promotes early embryonic growth (Gressens et al., 1993, 1994). We have previously shown that VIP protected the developing white matter against ibotenate-induced lesions if it was administered within 8 h after the ibotenate injection (Gressens et al., 1997a). Further studies revealed that VIP prevented early ibotenate-induced astrocyte death and promoted subsequent axonal repair (Gressens et al., 1998). VIP-induced neuroprotection against excitotoxic lesions of the developing white matter was independent from cyclic AMP (cAMP) production but required protein kinase C activations (Gressens et al., 1997a, 1998). Two receptors with similar affinities for VIP and pituitary adenylate cyclase-activating peptide (PACAP) have been cloned and called VPAC1 and VPAC2 (Ishiara et al., 1992; Lutz et al., 1993). Two receptors with similar affinities for VIP and pituitary adenylate cyclase-activating peptide (PACAP) have been cloned and called VPAC1 and VPAC2 (Ishiara et al., 1992; Lutz et al., 1993). VPAC1 is limited by its susceptibility to endopeptidases and its poor passage across biological membranes. Several recently described VIP analogs (O'Donnell et al., 1994; Gozes et al., 1997) exhibit more promising properties in terms of resistance to endopeptidases and its poor passage across biological membranes. Several recently described VIP analogs (O'Donnell et al., 1994; Gozes et al., 1997) exhibit more promising properties in terms of resistance to endopeptidases and its poor passage across biological membranes.

The goal of the present study was to test the ability of these VIP analogs to protect the developing murine white matter against ibotenate-induced lesions. Local intracerebral injection followed by a delayed intracerebral or i.p. injection of RO-15-1553, [Arg^{16}]RO-25-1553, or stearyl-norleucine-VIP and myristyl-norleucine-VIP were performed as described previously (Gozes et al., 1994; Gozes et al., 1994). [Arg^{16}]RO-25-1553 was synthesized in the same way as RO-25-1553, except that an arginine residue was incorporated instead of lysine.

**Materials and Methods**

**Drug Administration and Histological Procedures.** Several litters of Swiss mouse pups of both sexes were used for the experiments. As described previously (Marret et al., 1995a,b; Gressens et al., 1997a, 1998), on postnatal day 5 the pups were anesthetized by ether inhalation and kept under a warming lamp. Intracerebral injections were performed with a 26-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 in the frontoparietal area of the right hemisphere, 2 mm from the midline in the lateral-medial plane and 3 mm from the junction between the sagittal and lambdoid sutures in the rostro-caudal plane. Two 1-µl boluses were injected 30 s apart. The needle was left in place for 30 s after the second bolus. In the animals that received a second delayed intracerebral injection (see below), the initial injection site was easily recognized based on the presence of a punctate blood clot under the skin. In all of these experiments, the tip of the needle penetrated the periventricular white matter. In some experimental groups (see below), 3 µl of PBS, pH 7.35, containing VIP or VIP analogs were injected i.p. After the injections, the pups were allowed to recover from the anesthesia and were then returned to their mothers. Five days later (on postnatal day 10), surviving pups were sacrificed by decapitation and their brains were fixed in Formalin for 72 h.

**Experimental Groups.** Ibotenate (lot 94H37971; Sigma, St. Louis, MO) and VIP (Peninsula Laboratories, St. Helens, U.K.) were diluted in 0.02% acetic acid/0.1 M PBS. Stearyl-norleucine-VIP was diluted in 0.002% acetic acid/0.008% dimethyl sulfoxide/8% ethanol. Myristyl-norleucine-VIP, RO-25-1553, and [Arg^{16}]RO-25-1553 were diluted in 0.1 M PBS. Synthesis and purification of stearyl-norleucine-VIP, myristyl-norleucine-VIP, and RO-25-1553 were performed as described previously (Gozes et al., 1994; Gozes et al., 1997). [Arg^{16}]RO-25-1553 was synthesized in the same way as RO-25-1553 except that an arginine residue was incorporated instead of a glutamine in position 16. The amino acid sequences of VIP and the VIP analogs are given in Fig. 1.

![Fig. 1.](https://example.com/fig1.png)
Five to 23 pups from at least two different litters were used in each experimental group. Values were obtained from two or more successive experiments.

Results

Clinical Manifestations. Mortality was low (9.5%), and no significant differences were observed in a test of contingency (Fisher’s exact test) when the various treatment groups were compared to the animals injected with ibotenate alone (data not shown). Tonic and/or clonic fits and apneas were observed in almost all treated animals. Prolonged apnea during the first 24 h following ibotenate administration was responsible for the vast majority of deaths. No significant differences in intensity, clinical presentation, or incidence of epileptic manifestations were observed among the different experimental groups. No other side effects were recorded in the treated pups.

Excitotoxic Lesions Induced by Ibotenate. All postnatal day 5 animals injected with ibotenate and sacrificed 5 days later displayed large periventricular white matter cysts (mean length of the lesion in the sagittal fronto-occipital axis, 602 ± 44 μm) (Figs. 2A and 3). Cortical lesions characterized by neuronal loss affecting all cortical layers (mean length of the sagittal fronto-parietal axis of the lesion, 1127 ± 70 μm) (Figs. 2A and 4) were also observed in these animals.

Neuroprotective Effects of VIP and VIP Analogs. As previously shown (Gressens et al., 1997a, 1998), intracerebral coadministration of ibotenate and VIP to postnatal day 5 animals provided excellent protection against excitotoxic white matter cysts (87% decrease in the mean length of the sagittal fronto-parietal axis of the lesion) (Figs. 2B and 3A) but not against neuronal loss (Figs. 2B and 4A). When VIP was given i.p., no protection of the developing brain was observed (Figs. 3B and 4B).

RO-25-1553 and stearyl-norleucine-VIP administered intracerebrally or i.p. exhibited a potent dose-dependent protective effect against ibotenate-induced lesions of the developing white matter (Figs. 2 C and D, and 3); 81% and 93% decreases in the mean length of the lesion in the sagittal fronto-parietal axis with intracerebral injection of 1 μg of RO-25-1553 or 0.1 μg stearyl-norleucine-VIP, respectively; 87% and 84% decreases with i.p. administration of 10 μg of RO-25-1553 or 1 μg of stearyl-norleucine-VIP, respectively). Furthermore, significant protection against excitotoxic white matter damage was observed when RO-25-1553 or stearyl-norleucine-VIP was injected up to 12 h or 8 h, respectively, after ibotenate administration (Fig. 3). When injected systemically within the first 8 h following ibotenate administration, 10 μg of RO-25-1553 provided moderate but significant protection of the cortical plate against ibotenate-induced neuronal death (Fig. 4B). Intracerebral or i.p. administration of stearyl-norleucine-VIP or intracerebral injection of RO-25-1553 did not affect excitotoxic cortical lesions (Figs. 2, C and D and 4 A, C, and D).

Intraperitoneal injection of 10 μg of [Arg16]RO-25-1553 significantly protected the developing white matter (72% decrease in the mean length of the sagittal fronto-parietal axis of the lesion when performed simultaneously with the ibotenate injection), but not the cortical plate, against excitotoxic lesions (Figs. 2E and 5). Similar to RO-25-1553 and stearyl-norleucine-VIP, [Arg16]RO-25-1553 was protective even when given several hours after the ibotenate injection (Fig. 5).

Intracerebral cotreatment with ibotenate and myristyl-norleucine-VIP had no detectable effect on cortical plate and white matter excitotoxic lesions (Figs. 2F and 6).

Systemically injected VIP derivatives did not induce any detectable histological changes in the untreated contralateral cerebral hemisphere.

Discussion

Our data show that systemically injected VIP analogs effectively protect the developing white matter against excitotoxic lesions in a mouse model mimicking periventricular leukomalacia in human premature newborns. This protective effect occurred even when the VIP analogs were given several hours after the excitotoxic insult.

The biochemical designs of stearyl-norleucine-VIP and RO-25-1553, although aimed at achieving similar properties (i.e., resistance to endopeptidases and/or better diffusion through biological membranes), are basically different. RO-25-1553 is a long-acting cyclic VIP analog (O’Donnell et al., 1994). Stearyl-norleucine-VIP is derived from VIP by means of two chemical modifications, namely, addition of an N-terminal long-chain fatty acid (stearyl group) and substitution of norleucine for the methionine in position 17. These two compounds have been characterized, albeit to different extents, in terms of binding affinities, receptor coupling, and biological properties. RO-25-1553 is a selective agonist for the VPAC1 receptor subtypes with low affinity for VPAC2 receptors (Gourlet et al., 1997). It stimulates the production of cAMP in transfected cells expressing VPAC2 receptors (Gourlet et al., 1997). Its effects on cAMP-independent path-
ways have not been directly studied. RO-25-1553 has been shown to have biological effects, including an ability to induce muscle relaxation in isolated trachea (O'Donnell et al., 1994) and to stimulate in vivo neocortical astrocytogenesis (Zupan et al., 1998). Stearyl-norleucine-VIP binds with high affinity to both VPAC₁ and VPAC₂ receptors but is a partial agonist for recombinant VIP receptors (Gourlet et al., 1998). Stearyl-norleucine-VIP promotes survival of cultured neurons through cAMP-independent mechanisms (Gozes et al., 1995b) and prevents in vivo neuronal degeneration associated with β-amyloid toxicity, (Gozes et al., 1996). As previously mentioned, VIP neuroprotection of the white matter against excitotoxic lesions is cAMP-independent and is probably not mediated by any of the two cloned VPAC receptors because PACAP does not mimic VIP effects in this model (Gressens et al., 1997a and 1998). Further isolation and characterization of this putative specific VIP receptor not shared by PACAP will be necessary to compare the pharmacological effects of stearyl-norleucine-VIP and RO-25-1553 on this receptor; these studies will perhaps explain why the clearly different biochemical properties described thus far for stearyl-norleucine-VIP and RO-25-1553 result in closely similar profiles of neuroprotection against excitotoxic white matter lesions in our mouse model.

Interestingly, RO-25-1553 induced moderate but significant protection of the cortical plate when injected i.p. We suggest that this neuroprotective effect was mediated by systemic effects of RO-25-1553 because 1) intracerebral injections of RO-25-1553, stearyl-norleucine-VIP, or VIP failed to protect the cortical plate against ibotenate-induced lesions and 2) systemic injection of stearyl-norleucine-VIP failed to modify the excitotoxic neuronal lesion. This last observation probably reflects the differences in biological and biochemical properties between RO-25-1553 and stearyl-norleucine-VIP.
In [Arg^{16}]RO-25-1553, the introduction of an arginine residue in position 16 results in increased affinity for VIP receptors (Gourlet et al., 1996). [Arg^{16}]RO-25-1553 has been found to exhibit greater affinity for recombinant VPAC1 and VPAC2 receptors than RO-25-1553 (IC_{50} values of 0.3 and 1.0 nM, respectively, for VPAC2 receptors and 30 and 300 nM, respectively, for VPAC1 receptors) (Gourlet et al., 1996). In our mouse model of excitotoxic lesions, [Arg^{16}]RO-25-1553 was slightly less potent than RO-25-1553 in protecting the developing white matter, when given by the systemic route. This difference in neuroprotective effects may be ascribable to the above-described differences in biochemical effects on recombinant receptors or to small variations in vivo properties such as resistance to peptidases or ability to cross the blood-brain barrier.

Although both fatty derivatives displayed fairly similar binding affinities and effects on cAMP production in cells expressing recombinant VIP receptors (Gourlet et al., 1998), myristyl-norleucine-VIP did not protect the developing white matter even when injected directly into the brain. This finding supports a previous suggestion that it is difficult to predict the biological effects of these fatty derivatives because of their partial agonist properties (Gourlet et al., 1998). Alternatively, cerebral VIP receptors may behave differently in vivo from recombinant receptors expressed on cultured cells.

Taken in concert, these data suggest that some VIP analogs may prove useful in the prevention and/or treatment of PVL in human premature babies. As previously mentioned, the search for treatments for PVL is a health care priority given the tremendous morbidity and neurological handicap associated with PVL. VIP or VIP analogs have been reported to exhibit therapeutic properties in animal models of asthma.

Fig. 4. Quantitative analysis of cortical plate lesions in animals treated with ibotenate and RO-25-1553 (A and B) or stearyl-norleucine-VIP (C and D). Columns represent the mean length of the lesion along the sagittal fronto-occipital axis ± S.E.M. Numbers in columns are the numbers of animals used in each experimental group. Asterisks indicate differences from controls (***p < .01 in ANOVA with Dunnett’s multiple comparison test). In all experimental groups, ibotenate was injected into the brain. VIP and VIP derivatives were administered intracerebrally (A and C) or i.p. (B and D); these peptides were injected at the same time as ibotenate (T0 h) or at 2, 4, 8, or 12 h after ibotenate administration (T2 h, T4 h, T8 h, or T12 h, respectively).
(O’Donnell et al., 1994), sexual impotence (Gozes et al., 1994), developmental microcephaly (Gressens et al., 1994), and neuronal degeneration associated with β-amyloid toxicity (Gozes et al., 1996) or gp120 toxicity (Brenneman et al., 1988; Dibbern et al., 1997), indicating that these compounds, given systemically, may be useful in a broad range of conditions. However, before using VIP derivatives in phase I clinical trials, several critical points will have to be investigated in animal models. Although no side effects occurred in our mouse model or in other studies (Gozes et al., 1995a), studies in larger animals are needed to confirm the lack of toxicity of VIP and VIP analogs in experimental models. Similarly, we have limited knowledge about the pharmacology and kinetics of these compounds given as systemic injections.

In conclusion, our study demonstrates that systemically injected VIP analogs prevent excitotoxic brain lesions in a developing mouse model that closely mimics human PVL. Furthermore, these VIP analogs are neuroprotective if they are administered within the first 8 to 12 h following the excitotoxic insult, suggesting that they may have therapeutic potential. RO-25-1553, [Arg\textsuperscript{16}]RO-25-1553, and stearyl-norleucine-VIP may prove to be pharmacological tools that deserve evaluation in human premature infants at high risk for PVL.

Acknowledgments

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References


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**Fig. 5.** [Arg\textsuperscript{16}]RO-25-1553 effects on ibotenate-induced lesions in the white matter (A) or cortical plate (B). Columns represent the mean length of the lesion along the sagittal fronto-occipital axis ± S.E.M. Numbers in columns are the numbers of animals used in each experimental group. Asterisks indicate differences from controls (**p < .01 in ANOVA with Dunnett’s multiple comparison test). Ibotenate was injected intracerebrally and [Arg\textsuperscript{16}]RO-25-1553 i.p. T0 h: injection of ibotenate and [Arg\textsuperscript{16}]RO-25-1553 at the same time; T4 h, T8 h, and T12 h: injection of [Arg\textsuperscript{16}]RO-25-1553 4, 8, or 12 h, respectively, after ibotenate administration.

**Fig. 6.** Quantitative analysis of excitotoxic brain lesions with (closed columns) or without (open columns) cotreatment with myristyl-norleucine-VIP. Both drugs were given intracerebrally. Columns represent the mean length of the lesion along the sagittal fronto-occipital axis ± S.E.M. Numbers in columns are the numbers of animals used in experimental groups.
development, with a special emphasis on the excitotoxic cascade at the consecutive developmental steps, in Aktuelle Neuropädiatrie (Rating D ed) pp 22–33, Ciba-Geigy Verlag, Wehr.


