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Abstract

Excitotoxicity is a key molecular mechanism of perinatal brain damage and is associated with cerebral palsy and long term cognitive deficits. VIP induces a potent neuroprotection against perinatal excitotoxic white matter damage. VIP does not prevent the initial appearance of white matter lesion but promotes a secondary repair with axonal regrowth. This plasticity mechanism involves an atypical VPAC2 receptor and BDNF production. Stable VIP agonists mimic VIP effects when given systemically and exhibit a large therapeutic window. Unraveling cellular and molecular targets of VIP effects against perinatal white matter lesions could provide a more general rationale to understand the neuroprotection of the developing white matter against excitotoxic insults.

Incidence and pathophysiology of perinatal brain damage

The incidence of motor and/or cognitive deficits linked to perinatal brain injury increased during the nineties and currently seems to remain stable [1-3]. This data can be explained by progress in the field of neonatal intensive care, leading to an increase of very preterm neonate survival. Despite a significant improvement of their neurological outcome, ten percents of preterm neonates less than 1,500 grams later exhibit cerebral palsy, and about 50% develop cognitive and behavioral deficits [4]. Periventricular white matter is preferentially affected in preterm infants whereas cortico-subcortical gray matter lesions are most often observed in term babies.

The pathophysiology of perinatal brain damage is multifactorial, involving both prenatal and perinatal factors that may include genetic determinants, perfusion failure, growth factor deficiency, and maternal-fetal infection/inflammation [5-10]. Several risk factors for perinatal brain damage seem to share excitatory amino acids as a common final pathway leading to brain matter damage.

Animal model of excitotoxic lesions during brain development

Glutamate is the main excitotoxin during development. Among the different types of ionotropic glutamate receptors, the N-methyl-D-aspartate (NMDA) receptor mediates a large part of excitotoxic neuronal injury during development. Glutamate metabotropic receptors have been reported to stimulate phosphoinositide hydrolysis and to potentiate the NMDA effects.

Different glutamate agonists have been used to produce excitotoxic brain damage, both during development and in the adult rodent. In particular, ibotenate, an agonist of the N-methyl-D-aspartate (NMDA) complex receptor and of the group I metabotropic receptor, has been used to study the spectrum of excitotoxic disturbances at different ages of cerebral development.

During maturation of neuronal layer V and during migration of neurons destined to granular and supragranular layers, newborn hamsters intracerebrally injected at P0 with ibotenate display arrests of migrating neurons at different distances from the germinative zone [11]. High doses of ibotenate induce periventricular and subcortical neuronal heterotopias while low doses of ibotenate produce intracortical heterotopias and molecular layer ectopias. The resulting cytoarchitectonic patterns mimic some migration disorders encountered in humans [12, 13].

After completion of neuronal layer V and during the full settlement of supragranular layers, P0 mice and rats injected with ibotenate disclosed a laminar neuronal depopulation of layer V-VIa sharply mimicking human microgyria [14-16].

Injected after completion of migration (P5-P10 in mouse or rat), ibotenate produced a neuronal loss in all neocortical layers [14], mimicking neocortical lesions occurring in the term human newborns. Furthermore, at this developmental stage, ibotenate induced the formation of white matter cysts, mimicking some aspects of periventricular white matter observed in human preterm infants [17].

Neuroprotective effects of vasoactive intestinal peptide against excitotoxic brain damage

Vasoactive intestinal peptide (VIP) is a central nervous system neurotransmitter and neuromodulator with neurotrophic properties, including stimulation of astrocytic mitoses [18], increase of neuronal survival [19-21], promotion of early embryonic growth [22, 23], and neuronal differentiation of murine embryonic stem cells [24]. VIP was also shown to attenuate excitotoxic pulmonary edema [25], suggesting some interactions between transduction pathways of VIP and glutamate. Based on these data, the potential protective effects of VIP against excitotoxic damage have been evaluated in the developing rodent brain.

In the P0 hamster, co-treatment with VIP and a high dose of ibotenate produced a pattern of neuronal heterotopias similar to the one observed in animals treated with low doses of ibotenate alone. Pups co-injected with a low dose of ibotenate and a neurotensin-VIP hybrid VIP antagonist displayed cortical dysgeneses similar to those observed in animals treated with high doses of ibotenate alone. These data show that VIP can modulate migration disorders induced by ibotenate administration [26].

In the P0 mouse, co-treatment with ibotenate and VIP induced a dose-dependent reduction of the cortical lesion size (77% decrease of the lesion sizes with 1µg VIP) [27]. With the highest dose of VIP, 47% of co-treated animals displayed completely normal cortex.

In the P5 mouse, co-treatment with ibotenate and VIP had only a moderate effect on the ibotenate-induced neuronal death [27]. In contrast, VIP provided a very significant and dose-dependent protection against the excitotoxic white matter cyst (85% decrease of the white matter cyst size with 1µg VIP). With the highest dose of VIP, 38% of co-treated animals displayed completely normal white matter. Co-treatment with a neurotensin-VIP hybrid VIP antagonist [27] and ibotenate aggravated the excitotoxic lesion (64% increase of white matter cyst size), suggesting that endogenous VIP partially protect the developing white matter against excitotoxic insults.

VIP neuroprotective effects are mediated by VPAC2 receptors coupled to different transduction pathways

Prepro-vasoactive intestinal peptide (VIP) mRNA codes for two neuropeptides: VIP and peptide histidine isoleucine (PHI) in rodents or VIP and peptide histidine methionine (PHM) in humans. Two VIP receptors, shared with a similar affinity by pituitary adenylate cyclase-activating polypeptide (PACAP), have been cloned: $VPAC_1$ and $VPAC_2$ [28]. PHI binds to these receptors with a lower affinity. Furthermore, PACAP-27 and PACAP-38, but not VIP, bind with high affinity to a specific PACAP receptor called the PAC_1 receptor. VPAC receptors are preferentially coupled to Gαs protein that stimulates adenylate cyclase activity and induces cAMP increase [28]. VPAC receptors can also be coupled to Gαq and Gαi proteins that stimulate the inositol phosphate / calcium / protein kinase C (PKC) pathways.

In the P0 hamster, the modulating effects of VIP on excitotoxin-induced heterotopias were mimicked by forskolin, PACAP-38 and by a specific $VPAC_2$ receptor agonist but not by a $VPAC_1$ agonist, and were blocked by a protein kinase A (PKA) inhibitor. Taken together, these data suggest that VIP and PACAP can attenuate ibotenate-induced heterotopias in newborn hamster and that this effect is mediated by the $VPAC_2$ receptor utilizing the cAMP-PKA pathway.

In contrast, in the P5 mouse, forskolin had no detectable effect on ibotenate-induced white matter lesions, suggesting that cAMP production was not involved in VIP-induced neuroprotection [26]. Further supporting this hypothesis, stearyl norleucine VIP, a specific VIP agonist that does not activate adenylate cyclase, mimicked VIP neuroprotective effects [26]. A large range of concentrations of PKA inhibitor, calmodulin-dependent PK inhibitor and phosphatidylinositol 3-OH kinase inhibitor had no significant effect on VIP neuroprotection against white matter excitotoxic cystic lesion [29]. In contrast, a PKC inhibitor and a MAPK kinase (or Mek-1) inhibitor abolished VIP protective effects in a dose-dependent manner [29]. *In vitro* and in *vivo* studies revealed that VIP elicited in white matter astrocytes PKC activation of PKC but not of MAPK. In addition to a PKC-like activation in white matter cells at the site of injection, VIP also elicited a PKC-like and MAPK-like activation in cortical plate neurons at distance from the site of injection. In neuronal cultures, while VIP and conditioned medium from control astrocytes had no detectable effect on the activation of PKC and MAPK, medium conditioned by astrocytes cultured with VIP induced a significant PKC and MAPK activation [29].

VIP effects on white matter were mimicked with a similar potency by $VPAC_2$ agonists and PHI but not by $VPAC_1$ agonists [30, 31]. Surprisingly, VIP-induced neuroprotection was not mimicked by a large range of doses of PACAP 27 or PACAP 38 [27, 31]. This atypical pharmacology of VIP-induced neuroprotection in P5 mice raised several hypotheses: i) activation of PAC_1 receptors could have a toxic effect on the excitotoxic lesions while activation of VPAC receptors could be neuroprotective, leading to a lack of detectable effect for PACAP. In this context, it has been shown that VIP can provide cellular protection through a specific splice variant of the PAC_1 receptor [32]. ii) During some stages of brain development, the binding of VIP or PACAP to VPAC receptors leads to activation of separate transduction pathways. iii) VIP acts through a yet to be identified specific VIP receptor which is not recognized by PACAP. Indeed, Ekblad et al. [33] characterized a PACAP 27 preferring receptor and a VIP specific receptor, distinct from those that have been cloned ($VPAC_1$, $VPAC_2$, and PAC_1 receptors), in intestine of rat and $PAC_1^{-/-}$ mice.

The first stated hypothesis that activation of PAC_1 receptors could have a toxic effect on the excitotoxic lesions while 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 $PAC_1^{-/-}$ mice [31]. In contrast, VIP neuroprotective effects are completely abolished in mice lacking $VPAC_2$ receptor [31]. In situ hybridization confirms the presence of $VPAC_2$ mRNA in the postnatal day 5 white matter [31]. When analyzed between embryonic life and adulthood, VIP specific binding site density peaks at postnatal day 5 [31]. These data suggest that, in this model, VIP-induced neuroprotection is mediated by $VPAC_2$ receptors. The pharmacology of this $VPAC_2$ receptor seems unconventional as i) PACAP does not mimic VIP effects, ii) PHI acts with a comparable potency and iii) PACAP 27 modestly inhibits the VIP specific binding while for PHI or VIP, inhibition is complete.

Potential mechanisms underlying the atypical pharmacology of VIP effects in P5 mice

In order to explain the observed characteristics of $VPAC_2$ receptors involved in VIP-induced neuroprotection in the P5 mouse, some hypotheses can be formulated: i) During some stages of brain development, the binding of VIP or PACAP to $VPAC_2$ receptors leads to activation of separate transduction pathways. This differential coupling could

be secondary to $VPAC_2$ receptors dimerization (homo- or heterodimers) or to their interaction with larger oligomeric complexes, as demonstrated for other types of GPCRs [34]. 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. ii) VPAC receptors can dimerize with receptor activity modulating proteins (RAMPs), which leads to the modulation of cell signalling through a commutation of the coupling of a GPCR to different G proteins [35]. iii) An alternative hypothesis has been suggested by recent studies. A first study identified a deletion variant of the mouse $VPAC_2$ receptor in immune cells [36]. This natural deletion abrogates VIP-induced cAMP production without apparent alterations of expression or ligand binding. Secondly, Langer and Robberecht [37] showed that mutations in the proximal domain of the third intracellular loop of the $VPAC_1$ 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 Gαi 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 $VPAC_2$ receptor transcript, through RNA editing for instance, might be able to induce VIP specificity and modulate the coupling with different G proteins.

VIP-induced neuroprotection involves plasticity mechanisms

The study of the chronological evolution of the white matter lesion size showed that VIP did not prevent the initial formation of the lesion but induced a secondary axonal regrowth with repair of the white matter cyst [29]. VIP co-treatment also prevented the ibotenate-induced astrocytic cell death in the white matter and the secondary reactive gliosis. The survival-promoting effect of VIP observed on the astrocytes at the border of the white matter cyst during the first 24 h after ibotenate injection could be critical for the secondary repair : i) these surviving astrocytes could secrete growth factors promoting axonal regrowth; ii) they will serve as template for axonal regrowth; iii) they could limit the reactive gliosis which would impair axonal growth. VIP seemed to play a role of coordinating repair by acting on astrocytes to promote their survival and to induce the release of growth factors. These glia-derived released factors will activate neuronal pathways leading to axonal regrowth.

BDNF mediates VIP-induced neuroprotection

Several lines of evidence supported the role of BDNF as a mediator of VIP effects in the P5 mouse [38]: i) BDNF mimicked VIP neuroprotective effects on ibotenate-induced white matter damage; ii) BDNF did not prevent the initial lesion formation but induced a secondary repair; iii) BDNF effects were blocked by MAPK inhibitors; iv) VIP effects were blocked by neutralizing anti-BDNF antibodies; v) VIP induced the release of BDNF in cultured astrocytes; vi) VIP and ibotenate co-treatment increased the *in vivo* expression of BDNF mRNA. Interestingly, PACAP has also been shown to increase BDNF production [39].

Neuroprotective properties of VIP derivatives and clinical relevance

Despite the neuroprotective properties of VIP, its use as a drug is limited by its susceptibility to endopeptidases and its poor passage across biological membranes. Several recently described VIP analogues exhibit more promising properties in terms of resistance to endopeptidases and lipophilic status. They include cyclic molecules, such as RO-25-1553, and fatty molecules, such as stearyl-norleucine-VIP. 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 analogue [40]. 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 $VPAC_2$ receptor subtypes with low affinity for $VPAC_1$ receptor subtypes [41]. It stimulates the production of cAMP in transfected cells expressing $VPAC_2$ receptors [41]. Its effects on cAMP-independent pathways 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 [40] and to stimulate *in vivo* neocortical astrocytogenesis [42]. Stearyl-norleucine-VIP binds with high affinity to both $VPAC_1$ and $VPAC_2$ receptors but is a partial agonist for recombinant VIP receptors [43]. Stearyl-norleucine-VIP promotes survival of cultured neurons through cAMP-independent mechanisms [44] and prevents *in vivo* neuronal degeneration associated with beta-amyloid toxicity [45].

RO-25-1553 and stearyl-norleucine-VIP administered intracerebrally or intraperitoneally exhibited a potent dose-dependent protective effect against ibotenate-induced lesions of the developing white matter [27, 30]. Furthermore, significant protection against excitotoxic white matter damage was observed when RO-25-1553 or stearyl-norleucine-VIP were injected up to 12 or 8 hours, respectively, after ibotenate administration. These data showed that systemically-injected VIP analogues effectively protect the developing white matter against excitotoxic lesions in a mouse model mimicking brain damage frequently observed in human preterm infants. This protective effect occurred even when the VIP analogues were given several hours after the excitotoxic insult.

Activity-dependent neuroprotective protein (ADNP) has been shown to be induced by VIP and to be essential for embryogenesis and brain development while NAP, an active motif of ADNP, is neuroprotective in a broad range of neurodegenerative disorders [46-49]. Interestingly, NAP was shown to have potent neuroprotective effects against ibotenate-induced excitotoxic damage in the cortical plate and the white matter of P5 mice [50] as well as in a model of neonatal hypoxic-ischemic insult [51]. NAP (generic name davunetide) has been used in clinical trials in patients suffering from amnestic mild cognitive impairment, a precursor to Alzheimer's disease, without significant side effect [52].

Concluding remarks

As a working hypothesis, we propose that VIP neuroprotection against excitotoxic white matter lesions in the P5 mouse involves several steps : 1) VIP binds to an atypical $VPAC_2$ receptor on astrocytes and activates a PKC pathway; 2) PKC activation promotes astrocytic survival and astrocytic release of soluble factors, including BDNF;

3) released factors such as BDNF activate MAPK and PKC cascades in neurons; 4) MAPK cascade and, possibly PKC, activation leads to axonal repair.

By unraveling cellular and molecular targets of VIP effects against white matter lesions mimicking those observed in human preterm infants, the above-summarized studies could provide a more general rationale to understand the neuroprotection of the developing white matter against excitotoxic insults. Furthermore, these data suggest that some VIP analogues may prove useful in the prevention and/or treatment of white matter damage in human premature infants.

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