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Article

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Vincent Laudenbach,[1] Girolamo Calo,[2] Remo Guerrini,[3] Géraldine Lamboley,[1] Jean-François Benoist,[4] Philippe Evrard,[1] and Pierre Gressens[1]

[1]Institut National de la Santé et de la Recherche Médicale (INSERM) E9935, Hôpital Robert-Debré, Paris, France
[2]Department of Experimental and Clinical Medicine, Section of Pharmacology, and
[3]Department of Pharmaceutical Sciences and Biotechnology Center, University of Ferrara, Ferrara, Italy
[4]Laboratoire de Biochimie Hormonologie, Hôpital Robert-Debré, Paris, France

Address correspondence to: Pierre Gressens, Service de Neuropédiatrie, Hôpital Robert-Debré, 48 Boulevard Sérurier, F-75019 Paris, France. Phone: 33-1-40-03-47-83; Fax: 33-1-40-03-47-74; E-mail: gressens@idf.inserm.fr.

Vincent Laudenbach's present address is: Department of Anesthesiology, Hôpitaux Bichat-Claude Bernard & Robert Debré, Paris, France.

Intracerebral administration of the excitotoxin ibotenate to newborn mice induces white-matter lesions, mimicking brain lesions that occur in human preterm infants. Nociceptin (NC), also called orphanin FQ, is the endogenous ligand of the opioid receptor-like 1 (ORL1) receptor and does not bind classical high-affinity opioid receptors. In the present study, administration of NC exacerbated ibotenate-induced white-matter lesions while coadministration of ibotenate with either of two NC antagonists reduced excitotoxic white-matter lesions by up to 64%. Neither ibotenate plus endomorphin I (a selective μ receptor agonist), nor ibotenate plus naloxone (a classical opioid receptor antagonist) modulated the excitotoxic lesion. Pretreatment with antisense oligonucleotides targeting the NC precursor peptide mRNA significantly reduced ibotenate-induced white-matter damage. Finally, high doses of fentanyl, which stimulates both classical μ opioid receptors and ORL1, exacerbated excitotoxic white-matter lesion. This toxic effect was blocked by inhibiting ORL1 but not classical opioid receptors. Together, these findings show that endogenous or exogenous stimulation of the ORL1 receptor can be neurotoxic and that blocking NC signaling protects the white matter against excitotoxic challenge. These data point to potential new avenues for neuroprotection in human preterm infants at high risk of brain lesions.

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Introduction

Periventricular leukomalacia (PVL) is a hemispheric white-matter lesion that is often cystic and occurs most frequently in infants born before 32 weeks of gestation (1). PVL is the major cause of cerebral palsy among premature infants (2). Based on epidemiological and experimental studies, the pathophysiology of PVL appears to be multifactorial. It potentially involves the combined toxicity of hypoxic-ischemic insults, excess free radicals, maternal-fetal infection with increased cytokine production, and growth factor deficiency (1, 3–8). Excess release of glutamate and the subsequent overactivation of the excitotoxic cascade could represent a common molecular pathway for several of these risk factors (6, 9).

Findings from several experimental studies support this hypothesis. Newborn mice injected intracerebrally with ibotenate, a glutamate analog acting mainly on N-methyl-D-aspartate (NMDA) receptors, develop periventricular cystic white-matter lesions mimicking several aspects of human PVL (10). In this well characterized murine model, several drugs that interfere with the excitotoxic cascade have been shown to be neuroprotective (7, 11–14) while proinflammatory cytokines (15) and iron (16) exacerbated ibotenate-induced white-matter lesions. In order to further understand the pathophysiology of the ibotenate-induced cystic lesions, we targeted the NMDA receptors thought to be present on white-matter glial cells. Newborn mice were injected intracerebrally with sense, antisense, or missense oligonucleotides specific for the NMDA R1 receptor subunit (P. Gressens, unpublished work). The sequences of the oligonucleotides were identical to those previously used by Wahlestedt et al. in an in vitro assay (17). In newborn mice, pretreatment with antisense oligonucleotides was neuroprotective while control sense oligonucleotides had no effect on the ibotenate-induced lesion. Unexpectedly, missense oligonucleotides, which were used as another control, induced a three-fold reduction in the severity of the excitotoxic white-matter lesions. A search of databases with Basic Local Alignment Search Tool (BLAST) revealed that 12 successive nucleotides of the so-called "NMDA R1 missense oligonucleotide" were actually complementary to a triplicated motif of the murine nociceptin (NC) mRNA, suggesting that the blockade of NC production is neuroprotective in the ibotenate model.

NC, also called orphanin FQ, is the endogenous ligand of the opioid receptor-like 1 (ORL1) receptor (18, 19). The ORL1 receptor (also referred to as the OP_4 receptor) has significant sequence homology with the classical μ (OP_3), κ (OP_2) and δ (OP_1) opioid receptors. However, the ORL1 receptor does not bind with high affinity to traditional opioid ligands. Moreover, NC does not bind with high affinity to the classical opioid receptors, and naloxone, the nonselective opioid receptor antagonist, is inactive against the effects elicited by NC (for a review, see ref. 20). In the central nervous system, NC is produced by both neurons and astrocytes (21, 22). In vitro studies suggest that NC production and secretion is induced by cAMP (23, 24). An in situ hybridization study has shown that the ORL1 receptor and NC mRNAs are both expressed in the neopallium of newborn mice (21). At the cellular level, NC inhibits adenylate cyclase (18, 19), activates potassium channels (25), and inhibits calcium channels (26). At a broader level, NC has demonstrated effects on nociception, locomotion, food intake, and memory processes (for a review, see ref. 20). NC has also been shown to interact with the glutamatergic system. For example, (a) NC inhibits glutamate- and kainate-induced currents in spinal cord neurons (27); (b) the NMDA receptor blockade reduces the pronociceptive effects of NC in the land snail (28); (c) incubating neuroblastoma cells with NMDA attenuates NC-induced inhibition of adenylate cyclase (29); and (d) NC can inhibit glutamate release from adult rat cerebral cortex slices (30). Finally, fentanyl, a potent agonist of the classical μ opiate receptors as well as a weak agonist of the NC receptor, is commonly administered to premature neonates to reduce pain and discomfort (31). A recent retrospective study on a small cohort of premature infants (32) suggested that fentanyl treatment could be associated with a higher risk for PVL.

Together these data prompted us to investigate the modulating effects of NC on white-matter lesions in the developing mouse brain induced by intrapallial injections of ibotenate. The potential effects of fentanyl were also evaluated in this model.

Methods

Animals and injections. Pregnant Swiss mice were housed in groups and fed with laboratory chow and water ad libitum. Several litters of mouse pups of both sexes were used for the experiments. Experimental protocols were approved by the institutional review board of Hôpital Robert-Depré and complied with guidelines of INSERM. For intraperitoneal (i.p.) injections, a volume of 5 μl was administered to neonatal pups. Intracerebral injections were performed as previously described on postnatal day (P) 3, 4, or 5 (7, 10, 14–16, 33). Pups were anesthetized and kept under a warming lamp. A 26-gauge needle on a 50 μl Hamilton syringe was mounted on a calibrated microdispenser. The needle was inserted 2 mm under the external surface of scalp skin in the frontoparietal area of the right hemisphere 2 mm from the midline in the lateral-medial plane and 3 mm (in the rostro-caudal plane) from the junction between the sagittal and lambdoid sutures. Two 1-μl boluses were injected at a 30 second interval. The needle was left in place for an additional 30 seconds. In cases where several intracerebral injections were performed in the same animal, the initial site of injection was marked with permanent ink. In all these experiments, the tip of the needle reached the periventricular white matter. To confirm the correct positioning of the needle, some animals were injected with toluidine blue. After the injections, pups were allowed to recover from anesthesia and were returned to their dams.

Drugs. Ibotenate (Sigma Chemical Co., Saint Louis, Missouri, USA) was diluted in 0.1 M PBS containing 0.02% acetic acid. H89 (Biomol Research Laboratories, Plymouth Meeting, Pennsylvania, USA) was diluted in PBS containing 5% DMSO. All the other injected drugs and oligonucleotides were diluted in PBS.

Ibotenate, a glutamate analog extracted from the *Muscarina amanita* mushroom, activates both NMDA and metabotropic receptors but not the alpha-3-amino-hydroxy-5-methyl-4-isoxazole (AMPA) and kainate receptors. Previous studies showed that ibotenate-induced neonatal brain lesions are completely blocked by cotreatment with a specific NMDA receptor antagonist (10, 33).

NC was purchased from Bachem (Voisins-le-Bretonneux, France). $NC(1-17)NH_2$ is a selective ORL1-receptor agonist and has demonstrated higher potency than NC in some assays (34, 35). $[desPhe^1]NC(1-17)NH_2$, which lacks the NH_2-terminal Phe residue, has been shown to be inactive at the ORL1 receptor both in vivo and in vitro (36). $[Phe^1\psi(CH_2\text{-}NH)Gly^2]NC(1-13)NH_2$ ($[F/G]NC(1-13)NH_2$) was the first available compound found to be a specific ORL1-receptor antagonist (37). However, more recent studies revealed that this compound can behave as an antagonist, an agonist, or a partial antagonist of the ORL1 receptor, depending on the experimental model used (35, 37, 38). $[Nphe^1]NC(1-13)NH_2$ is a recently described selective NC receptor antagonist that lacks any residual agonist activity (39, 40). $Nphe^1$ indicates that the side chain of Phe^1 has been shifted from the C to the N atom in the $NC(1-13)NH_2$ sequence. Naloxone Benzoylhydrazone (NalBzoH) (or 6-desoxy-6-benzoylhydrazido-*N*-allyl-14-hydroxydihydronormorphinone) was purchased from Sigma Chemical Co. and is an antagonist of the classical opiate receptors and, at high concentrations, of the ORL1 receptor (36). Endomorphin-I is a specific agonist of the μ-opioid receptor (OP_3). $NC(1-17)NH_2$, $[F/G]NC(1-13)NH_2$, $[Nphe^1]NC(1-13)NH_2$, $[desPhe^1]NC(1-17)NH_2$, and Endomorphin-I were prepared at the University of Ferrara, as previously described (37, 41). NocII (Tocris, Bristol, United Kingdom) is an orphan neuropeptide that flanks NC in the precursor pro-NC. It may act through a novel specific receptor (42). Nocistatin (Tocris) is an endogenous peptide derived from the same precursor as NC and is able to block some NC-induced

effects (43). Fentanyl and sufentanil, agonists of the μ opioid receptor used in humans, were purchased from Janssen Pharmaceutica (Boulogne-Billancourt, France). Naloxone, an antagonist of the classical opioid receptors, was purchased from Dupont Pharma (Paris, France).

H89 is a cAMP-dependent protein kinase (PKA) inhibitor. The neurotensin-VIP hybrid (Bachem) is a vasoactive intestinal peptide (VIP) antagonist that has no agonist activity (44–46). Cotreatment with the neurotensin-hybrid and neurotensin does not influence the biological activity associated with the VIP antagonist (47). In the ibotenate murine model, simultaneous injection of ibotenate and VIP at P5 is protective for the white matter, while a single injection of ibotenate and VIP antagonist at P5 augments the lesion (7). However, late prenatal and early postnatal pretreatment of mice for several days with this VIP antagonist leads to a dramatic inhibition of neocortical astrocytogenesis (48, 49) and a lower susceptibility to ibotenate-induced neonatal brain lesions (48). This finding suggests that astrocytes may release factors that augment excitotoxic damage.

The sequences of the oligonucleotides (Genset, Evry, France) targeting the precursor pro-NC mRNA were as follows: 5′- ATG CAG AGC CTG GCG CAG (antisense oligonucleotide 1), 5′- ATG CTG AGC CTG GCG CAG (antisense oligonucleotide 2), 5′- CTG CGC CAG GCT CTG CAT (sense oligonucleotide 3) and 5′- CTG CGC CAG GCT CAG CAT (sense oligonucleotide 4). For in situ detection, antisense oligonucleotide 1 was labeled with fluorescein (Genset).

Experimental groups. Experiments were run in seven sets of mouse pups of different ages. The first set of P5 pups received an intracerebral injection of 10 μg ibotenate immediately followed by an i.p. administration of one of the following drugs or combination of drugs: 0.25, 2.5, or 25 μg/kg NC; 2.5 μg/kg NC(1-17)NH$_2$; 25 μg/kg [desPhe^1]NC(1-17)NH$_2$; 0.25, 2.5, or 25 μg/kg [F/G]NC(1-13)NH$_2$; 25 μg/kg NC + 25 μg/kg [F/G]NC(1-13)NH$_2$; 25 μg/kg NC + 25 μg/kg naloxone; 2.5, 25, or 250 μg/kg [Nphe^1]NC(1-13)NH$_2$; 25 μg/kg NC + 250 μg/kg [Nphe^1]NC(1-13)NH$_2$; 25 μg/kg NalBzoH; 25 μg/kg endomorphin I; 25 μg/kg naloxone; 25 μg/kg NocII; or 25 μg/kg nocistatin. Controls received PBS. In a second group of pups, 2.5 μg NC and 10 μg ibotenate were injected intracerebrally at P5. In the third group, PBS (controls), 0.1 nmol antisense oligonucleotides 1 and 2, or 0.1 nmol sense oligonucleotides 3 and 4 were injected intracerebrally both at P3 and P4. All pups received an intracerebral injection of 10 μg ibotenate at P5. A fourth experimental group was intracerebrally treated at P3 and P4 with the antisense oligonucleotide 1 labeled with fluorescein or with fluorescein alone. In the fifth group, i.p. injections of PBS, 0.1, 1, or 10 μg/kg fentanyl, 250 μg/kg [Nphe^1]NC(1-13)NH$_2$ + 10 μg/kg fentanyl, 25 μg/kg naloxone + 10 μg/kg fentanyl, or 2 μg/kg sufentanil were performed twice at P4 and once at P5. Immediately following the last

injection, 10 μg ibotenate were intracerebrally administered to all pups. In the sixth group, some pups were treated i.p. with 0.1 μg/kg VIP antagonist or with PBS, twice a day, on days P1 through P4. At P5, these pups were either used for immunolabeling of astrocytes (see below) or received an intracerebral injection of 10 μg ibotenate alone or in combination of one of the following treatment: (a) intracerebral injection of 5 μg H89; (b) i.p. administration of 25 μg/kg [F/G]NC(1-13)NH$_2$; or (c) combination of H89 and [F/G]NC(1-13)NH$_2$ injections. Finally, some pups received at P5 an i.p. injection of 25 μg/kg NC or of PBS alone, without ibotenate injection, in order to test the endogenous excitotoxic properties of NC. These pups were used for cell-death staining (see below).

Although the main purpose of the present study was to establish the modulating effects of NC on the neonatal excitotoxic lesions, a secondary goal was to test the potential neuroprotective properties of ORL1 receptor modulating drugs in an experimental model mimicking the clinical situation of human neonates. Therefore, NC agonists and antagonists were generally given through an i.p. route. Because fentanyl is usually delivered in a prolonged manner in intensive care units, we also choose to inject fentanyl according to a chronic schedule prior to ibotenate injection.

Lesion-size determination. Pups injected with ibotenate at P5 were sacrificed by decapitation 8, 24, 48, 72, or 120 hours after ibotenate injection. Brains were removed and fixed in 4% formalin for 7 days. After embedding in paraffin, 15 μm coronal serial sections were cut, and every third section was stained with cresyl-violet. Brains were completely and serially sectioned from the frontal pole to the occipital lobes. In theory, neocortical and white-matter lesions can be defined by the maximal length of three orthogonal axes: the lateral-medial axis (in a coronal plane), the radial axis (also in a coronal plane, from the pial surface to the lateral ventricle) and the fronto-occipital axis (in a sagittal plane). Due to the difficulty of accurately evaluating the degree of damage to neurons in neocortical layers in the epicenter of the lesion focus, the radial axis did not appear as an objective measure of the lesion size. In preliminary studies (7, 10), we had shown an excellent correlation between the maximal size of the radial and fronto-occipital diameters of the ibotenic-induced lesions. Based on these observations, we serially sectioned the entire brain in the coronal plane. This permitted an accurate and reproducible determination of the maximal sagittal fronto-occipital diameter (which is equal to the number of sections where the lesion was present, multiplied by 15 μm) and was used as an index of the volume of the lesion. Sections were analyzed by two investigators unaware of the identity of the samples. Each experimental group included 8–29 pups from at least two different litters (numbers are given in Figures 2 and 5). Lesion size calculations were obtained from two or more successive experiments. Results were expressed as means ± SEM. Statistical analyses were

performed using analysis of variance (ANOVA) with Dunnet's multiple comparison of means test.

Astrocyte density measurement. Pups that were treated i.p. with either VIP antagonist or PBS (controls) on days P1 to P5 were sacrificed by decapitation at P5. Brains were frozen immediately at –80°C. Following cryostat sectioning and methanol-acetone fixation, sections at the fronto-parietal level (SI) were reacted with rabbit antiserum to glial fibrillary acidic protein (GFAP) (DAKO A/S, Glostrup, Denmark). Sections were incubated for 1 hour at room temperature. Avidin-biotin horseradish peroxidase kits (Vector Laboratories, Burlingame, California, USA) were used according to instructions to detect rabbit or mouse antibodies. Diaminobenzidine (Sigma Chemical Co.) served as the chromogen. GFAP-positive cells were counted in 0.025 mm^2 areas located in (a) the paramedian upper (layers I to IV) neocortex and (b) the paramedian deep (layers V and VI) neocortex and underlying white matter. Five animals were included in each experimental group, and four sections per animal were examined. Sections from control and VIP antagonist–treated animals were processed simultaneously. Results were expressed as means ± SEM and compared with a *t* test.

Phenotype determination of oligonucleotide-internalizing cells. Pups injected at P3 and P4 with the antisense oligonucleotide 1 labeled with fluorescein, or with fluorescein alone, were sacrificed at P5. Paraffin-embedded brains were processed for double immunohistochemistry using anti-fluorescein antibody coupled to alkaline phosphatase (Roche, Meylan, France) plus either anti-GFAP or anti–microtubule-associated protein type 2 (MAP-2, a neuronal marker; Sigma Chemical Co.) antibodies. Anti-GFAP and anti-MAP-2 were detected using avidin-biotin horseradish peroxidase kits as directed. Vector VIP peroxidase substrate (Vector Laboratories) and the combination of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate toluidonium salt (Roche) were used as substrates for the peroxidase and alkaline phosphatase, respectively.

Cell-death staining. Twenty-four hours following i.p. injection of NC or PBS, pups were sacrificed and brains were fixed in formalin and embedded in paraffin. Cell death was detected using an in situ cell death detection kit (Roche). Deparaffinized sections were treated for 20 minutes at 37°C with 20 mg/ml proteinase K and incubated for 2 minutes on ice with 0.1% Triton X-100. DNA strand breaks were identified by labeling, for 60 minutes at 37°C, of free 3′-OH termini with terminal deoxynucleotidyl transferase (Tdt) and fluorescein-labeled nucleotides. Incorporated nucleotides were detected using an anti-fluorescein antibody conjugated with alkaline phosphatase, using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate toluidonium salt as the substrates. Terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) positive cells were counted in a 0.01 mm^2 area in the neocortical layers and in the underlying white matter of the paramedian somatosensory area. Ten nonadjacent fields from five brains were studied in each group. Results were expressed as means ± SEM and compared with a *t* test.

Studies on cultured astrocytes. Cortices from P0–P2 mouse pups were dissected and meninges were eliminated. Brain tissues underwent a dissociation procedure, both enzymatical (0.25% trypsin followed by DNAse) and mechanical (20 passages through a glass pipette). Following centrifugation, cells were suspended in Minimum Eagle Medium (Life Technologies, Cergy Pontoise, France) enriched with 10% horse serum, and plated in 60-mm dishes (for protein extraction) coated with poly-DL-Ornithine (Sigma Chemical Co.) or on 12-mm coverslips (for immunocytochemistry). Cultures reached confluence within 18–21 days. Astroglial cultures were then incubated for 72 hours in 1 μM NC antisense or sense oligonucleotides (sequences were identical to those used for in vivo experiments), in the presence or absence of 10 μM forskolin. Experiment cultures were repeated twice. For protein extraction, cells were scrapped in 50 mM TrisH-Cl (pH 8), 100 mM NaCl, 5 mM DTT, 1× proteases inhibitor mix (Roche), and 0.5% Igepal CA 630 (Sigma Chemical Co.). Following centrifugation (10 minutes at 450 *g*), cells were suspended in 25 mM Hepes pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, and 1% Triton, 1× proteases inhibitor mix. An additional centrifugation (16,000 *g* for 20 minutes) was performed, supernatants were collected, and protein content was measured with the Bradford method. For each experimental condition, 1 μl containing 0.8 μg total protein was spotted on a polyvinylidene difluoride (PVDF) membrane (Novex, San Diego, California, USA). Membranes were incubated with polyclonal anti-NC antibody (Bio-Trend, Köln, Germany) diluted 1:20,000. Anti-NC antibodies were detected using avidin-biotin horseradish peroxidase kits (Vector Laboratories) as directed and diaminobenzidine as a chromogen. For immunocytochemistry, astroglial cells were fixed with acetic acid and ethanol and then incubated in 1:1,500 anti-NC antibody. Detection of anti-NC antibody was performed as above. A total of eight coverslips were analyzed for each experimental condition. For each coverslip, the proportion of labeled cells was determined in two separate 0.0625 mm^2 fields by an observer unaware of the experimental conditions. A total of 531–586 cells were counted in each experimental condition. Results were expressed as means ± SEM. Statistical analyses were performed using ANOVA with Dunnet's multiple comparison of means test.

Results

Clinical manifestations. In this study, the overall mortality was low (less than 5%). Clinical comparisons of different treatment groups with animals injected with ibotenate alone revealed no statistically significant differences in tests of contingency (exact Fisher test) (data not shown). Epileptic manifestations, including clonic

or tonic seizures and apneas, were observed during the first 24 hours after ibotenate injection in almost all treated animals. There was no variation detected in the intensity, clinical phenotype, or incidence of epileptic manifestations across treatments.

Pharmacological manipulation of the ORL1 receptor. Control pups treated at P5 with ibotenate and sacrificed 5 days later displayed both a cortical lesion and a periventricular white-matter cyst (Figure 1a and Figure 2, a and b). The cortical plate lesion was characterized by a neuronal depopulation in all neocortical layers.

Cotreatment with ibotenate and i.p. NC of P5 animals induced a dose-dependant increase of the white-matter lesion size without affecting the cortical plate lesion (Figure 1b and Figure 2, a and b). Intracerebral injection of 2.5 µg NC mimicked i.p. injections of NC, with a significant increase of the white-matter lesion size (mean length of the lesion ± SEM: $498 \pm 48\,\mu m$ in controls vs. $734 \pm 6\,\mu m$ in NC-treated pups; $P < 0.0001$) and no significant effect on the cortical plate lesion ($866 \pm 50\,\mu m$ in controls vs. $905 \pm 102\,\mu m$ in NC-treated pups). Similarly, i.p. $NC(1-17)NH_2$ exacerbated the excitotoxic white-matter lesion while $[desPhe^1]NC(1-17)NH_2$ had no detectable effect on ibotenate-induced white-matter lesions (Figure 2, a and b). In contrast, cotreatment with ibotenate and i.p. $[F/G]NC(1-13)NH_2$ or $[Nphe^1]NC(1-13)NH_2$ protected the white matter against the excitotoxic challenge in a dose-dependant manner but had no detectable effect on the cortical plate lesion (Figure 1c and Figure 2, a and b). High dose i.p. NalBzoH was also neuroprotective for the developing white matter in this model (Figure 2, a and b). In P5 pups, cotreatment with ibotenate, NC and $[F/G]NC(1-13)NH_2$, or $[Nphe^1]NC(1-13)NH_2$ abolished the deleterious effects of NC on the white-matter lesion (Figure 2a). In control animals injected with ibotenate, the white-matter lesion increased in size during the first 24 hours and remained stable thereafter (Figure 2c). Treatment with ibotenate plus i.p. $[Nphe^1]NC(1-13)NH_2$ induced a reduction of the white-matter lesion size at all ages studied with no significant changes over time (Figure 2c).

The coinjection of i.p. endomorphin I, naloxone, NocII, or nocistatin plus ibotenate had no detectable effect on the excitotoxic white-matter (Figure 2d) or cortical plate (data not shown) lesions. Coadministration of ibotenate, NC, and naloxone did not prevent the NC-induced exacerbation of the excitotoxic white-matter lesion (Figure 2d). Intraperitoneal pretreatment of pups with three 10 µg/kg fentanyl injections led to a significant exacerbation of the ibotenate-induced white-matter lesion (Figure 2e). The effect of fentanyl was blocked by cotreatment with $[Nphe^1]NC(1-13)NH_2$ but not with naloxone (Figure 2e). Interestingly, pups treated with ibotenate + fentanyl + $[Nphe^1]NC(1-13)NH_2$ had a white-matter lesion size very similar to animals cotreated with ibotenate + $[Nphe^1]NC(1-13)NH_2$, suggesting that $[Nphe^1]NC(1-13)NH_2$ was

able to antagonize the toxic effects of both the endogenous NC and the exogenous fentanyl (Figure 2e). Intraperitoneal pretreatment of pups with three 2-µg/kg sufentanil injections (a dose considered as equipotent to 10–20 µg/kg fentanyl in terms of pain alleviation) did not exacerbate the ibotenate-induced white-matter lesion (Figure 2e).

In vivo blockade of the NC production. When compared with controls, newborn mice pretreated with antisense oligonucleotides targeting the precursor pro-NC mRNA were significantly protected against the white-matter change related to excitotoxic challenge (Figure 2f). Sense oligonucleotides had no detectable effect on the excitotoxic lesions (Figure 2f).

Cell distribution of injected oligonucleotides. Following intracerebral administration of antisense oligonucleotide labeled with fluorescein, double immuno-histochemistry revealed that, in the injected hemisphere, a large proportion of astrocytes in both the white matter and the cortical plate had taken up the oligonucleotide (Figure 3, a and b) while very few neurons were double-labeled (Figure 3c) (in serial sections from five brains, only two neurons had incorporated the labeled oligonucleotide). In contrast, the injection of fluorescein alone induced a very limited
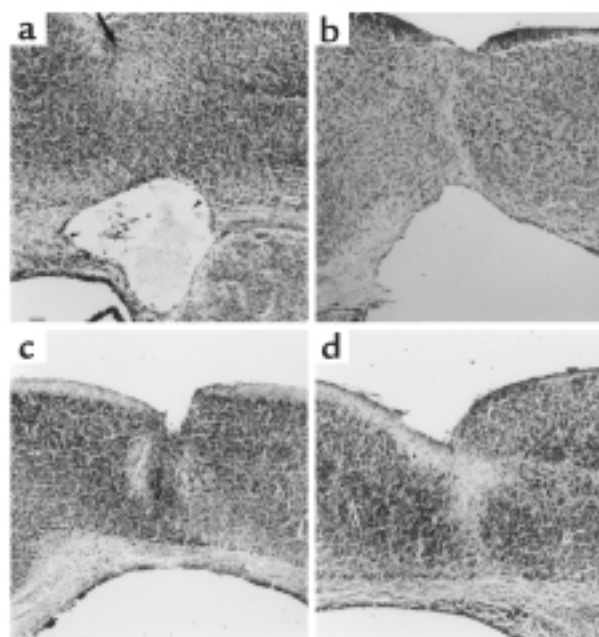

Figure 1
NC exacerbates ibotenate-induced white-matter lesions and the blockade of endogenous NC neurotransmission is protective. Cresyl violet–stained sections showing brain lesions induced by ibotenate injected at P5 and studied at the age of P10. (**a**) Brain injected with ibotenate alone, showing typical neuronal loss in layers II–VI (arrow) and white-matter cystic lesion (arrowheads). (**b** and **c**) Brains cotreated with ibotenate and 25 µg/kg NC (**b**) or 250 µg/kg $[Nphe^1]NC(1-13)NH_2$ (**c**). (**d**) Brain pretreated with precursor pro-NC mRNA antisense oligonucleotide at P3 and P4, and injected with ibotenate at P5. Bar, 40 µm.

labeling of a few cells at the site of injection (data not shown), suggesting that the above described widespread astrocytic distribution of the fluorescent tag reflected the integrity of the link between the oligonucleotide and fluorescein.

In vitro blockade of the astrocytic NC production. Preincubation of cultured astrocytes with antisense oligonucleotides targeting the precursor pro-NC mRNA significantly reduced the proportion of astrocytes labeled with anti-NC antibody when compared with control cultures or cultures exposed to sense oligonucleotides (Figure 4, a–c). Dot-blot analysis showed that antisense oligonucleotides, but not sense

oligonucleotides, reduced the NC content of cultured astrocytes, both in basal conditions and after exposure to forskolin (Figure 4d).

Effects of astrocyte depopulation. Compared with control pups injected with PBS, pups pretreated with the VIP antagonist had significant astrocytic depopulation in the superficial cortical layers (layers I to IV) (Figure 5). This finding confirms data reported previously (48). Astrocytic density in the deep cortical layers (layers V and VI) and in the periventricular white matter was not affected by VIP blockade (Figure 5).

As previously shown (48), pretreatment of neonatal mice with the VIP antagonist significantly protected
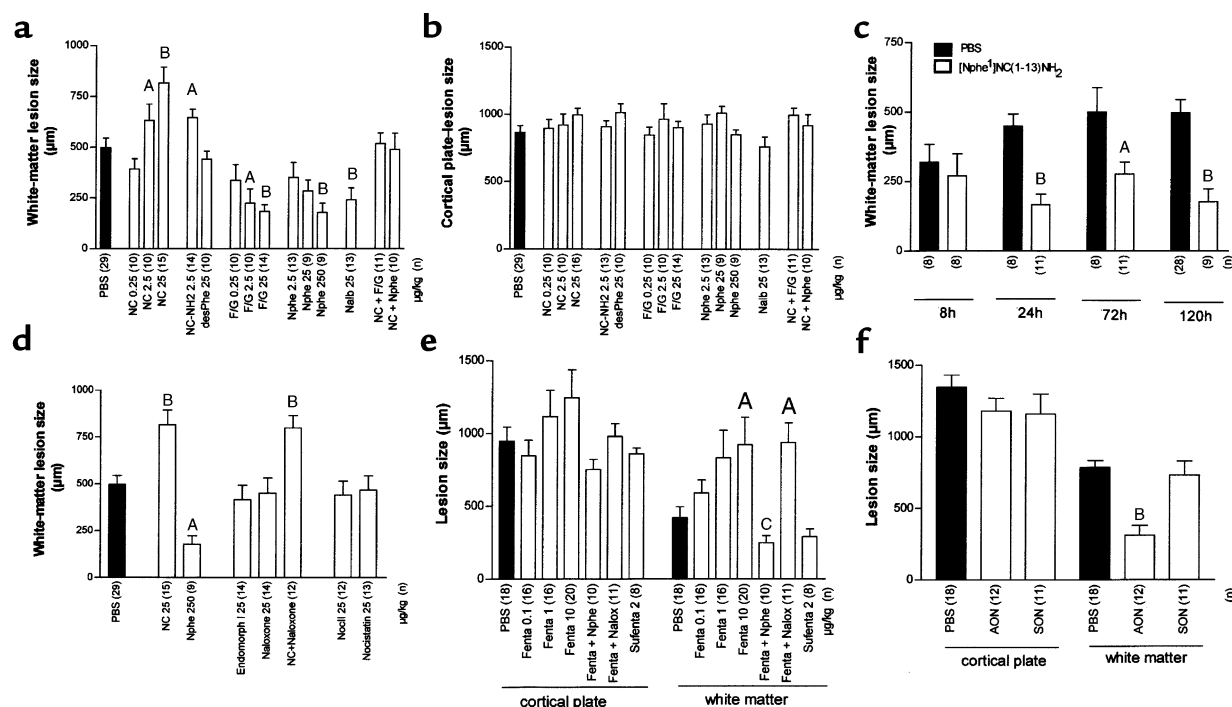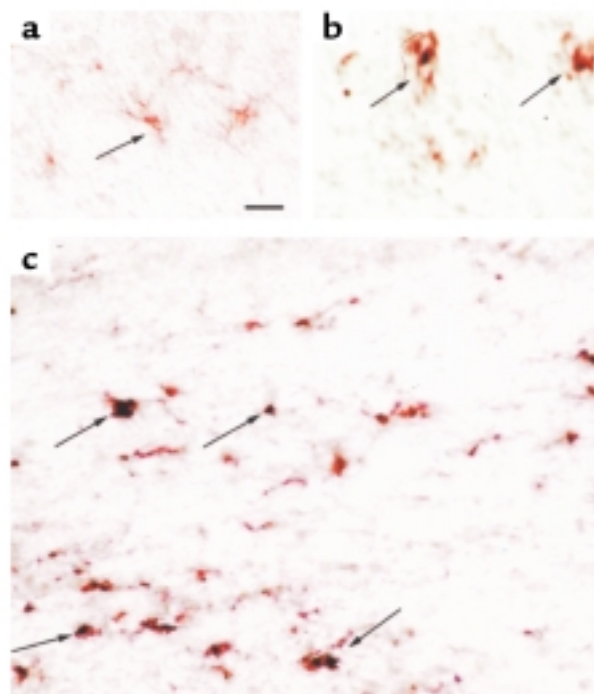

Figure 2

Quantitative analysis of the modulatory effects of NC agonists and antagonists on ibotenate-induced lesions. Bars represent mean length of the lesion along the sagittal fronto-occipital axis ± SEM. Numbers in parentheses are the numbers of animals used in each experimental group. (**a** and **b**) Effects of NC analogs and NC antagonists on excitotoxic white-matter (**a**) and cortical plate (**b**) lesions. Pups cotreated with NC and an NC antagonist received the highest dose of each peptide. Statistically significant differences between controls and experimental animals are shown ([A]$P < 0.05$, [B]$P < 0.01$ in ANOVA with Dunnett's multiple comparison test). NC-NH2, NC(1-17)NH$_2$; desPhe, [desPhe[1]]NC(1-17)NH$_2$; F/G, [F/G]NC(1-13)NH$_2$; Nphe, [Nphe[1]]NC(1-13)NH$_2$; and Nalb, NalBzoH. (**c**) Mean length of the white-matter lesion at different times (indicated on the x axis) after ibotenate injection in pups cotreated with PBS (filled bars) or [Nphe[1]]NC(1-13)NH$_2$ (open bars) injections. The two-way ANOVA showed a significant Treatment by Time interaction ($P < 0.01$). Contrast analyses revealed significant differences between controls and [Nphe[1]]NC(1-13)NH$_2$-treated pups at 24 hours ($P < 0.01$), at 72 hours ($P < 0.05$), and at 120 hours ($P < 0.01$). (**d**) Effects of classical opioid agonists and antagonists on white-matter excitotoxic lesions. Pups cotreated with NC and naloxone received 25 mg/kg of each drug. Statistically significant differences between controls and experimental animals are shown ([A]$P < 0.05$, [B]$P < 0.01$ in ANOVA with Dunnett's multiple comparison test). Endomorph I, endomorphin I. (**e**) Effects of fentanyl and sufentanil on cortical plate and white-matter excitotoxic lesion. Statistically significant differences from controls or pups treated with 10 μg/kg fentanyl are shown ([A]$P < 0.05$ or [C]$P < 0.01$ in ANOVA with Dunnett's multiple comparison test, respectively). Fenta, fentanyl; Nalox, naloxone; Sufenta, sufentanil. When not indicated on the graph, the dose of fentanyl was 10 μg/kg, the dose of [Nphe[1]]NC(1-13)NH$_2$ was 250 μg/kg and the dose of naloxone was 25 μg/kg. (**f**) Effects of pretreatment with precursor pro-NC mRNA antisense oligonucleotides (AON) or sense oligonucleotides (SON) on ibotenate-induced lesions. Statistically significant differences between controls and experimental animals are shown ([B]$P < 0.01$ in ANOVA with Dunnett's multiple comparison test).

Figure 3

Cell distribution in brains injected with fluorescein-labeled NC mRNA antisense oligonucleotides. Pups were intracerebrally injected with labeled antisense oligonucleotides prior to processing the brains for immunohistochemistry. Arrows point to examples of labeled cells. (**a**) GFAP immunostaining in the P5 neopallium, showing astrocytes labeled in red. (**b**) Typical example of double labeling for GFAP (red deposit as in **a**) and fluorescein (blue deposit) in the P5 neopallium, showing numerous double-labeled cells. (**c**) Double labeling for MAP-2 (red deposit) and fluorescein (blue deposit) in the P5 neopallium, showing the only two double-labeled neurons that were detected in five injected and serially sectioned brains. Bar, 40 μm.

the white matter against excitotoxic lesions (Figure 6). Similarly, inhibition of PKA by H89 was also neuroprotective (Figure 6). The neuroprotection afforded by the VIP blockade and H89 was in the same range (50% and 60%, respectively, reduction of the ibotenate-induced lesion size) as the neuroprotection obtained with NC antagonist (63% reduction of the lesion size) (Figure 6). Furthermore, any combination of two of these three neuroprotective strategies did not result in any more protection than any single drug used alone (Figure 6).

Effects of NC on brain cell death. In order to test the potential neurotoxic effects of NC in the absence of ibotenate challenge, TUNEL staining was performed 24 hours after an i.p. injection of NC or PBS. Figure 7 shows the absence of a detectable effect of NC treatment on developmental neopallial cell death.

Discussion

The most salient finding of the present study is the demonstration that exogenous NC augments neonatal excitotoxic white-matter lesions and that the blockade of NC endogenous signaling with either ORL1-receptor antagonists or with antisense oligonucleotides targeting the NC precursor peptide protects the developing periventricular white matter against ibotenate insult. In contrast, endomorphin I, a classical opioid receptor agonist, and naloxone, an antagonist, had no detectable effect on the excitotoxic brain lesions. Furthermore, naloxone did not prevent the deleterious effect of NC on ibotenate-induced white-matter lesions.

Previous studies have suggested some interactions between nociceptinergic and glutamatergic pathways in different neuronal populations (27–30). However,

Figure 4

Antisense oligonucleotides targeting the precursor pro-NC mRNA decrease NC production in cultured astrocytes. (**a** and **b**) Typical examples of astrocytes labeled with anti-NC antibody following exposure to control conditions (**a**) or to antisense oligonucleotides (**b**). Bar, 5 μm. (**c**) Quantitative analysis of the proportion of astrocytes labeled with anti-NC antibody following exposure to control conditions (ctrl), antisense oligonucleotides (AON) or sense oligonucleotides (SON). Bars represent mean ± SEM. Statistically significant differences between control and experimental conditions are shown ([A]$P < 0.001$ in ANOVA with Dunnett's multiple comparison test). (**d**) Dot-blot analysis of NC content of cultured astrocytes exposed to antisense oligonucleotides, sense oligonucleotides, or control conditions. Cells were cultured in the presence (+) or absence (–) of forskolin. One μl containing 0.8 μg total protein was spotted on the membrane for each experimental condition.
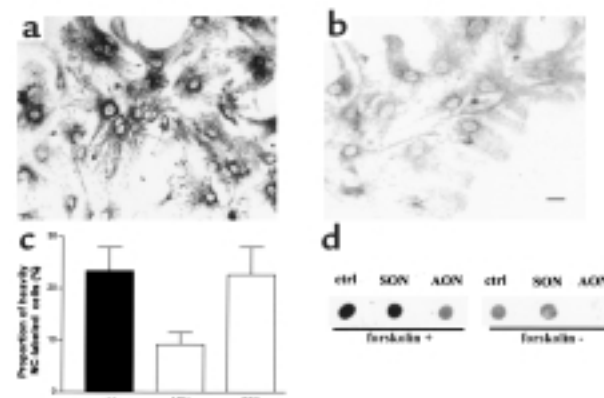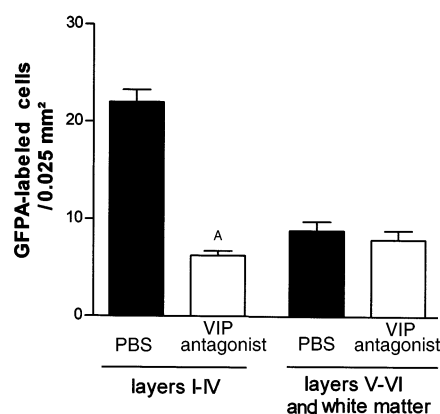
Figure 5
VIP blockade inhibits neocortical astrocytogenesis. Quantitative analysis of GFAP-labeled cell density in the superficial (layers I–IV) and deep (layers V–VI and white matter) neopallium at P5. Values are shown as mean ± SEM. Statistically significant differences between controls and experimental animals are shown ([A]$P < 0.001$ in a t test).

to our knowledge, the present study is the first demonstration of an exacerbating effect of NC on NMDA-mediated excitotoxicity at the level of developing white matter. Furthermore, the lack of detectable effects of NC agonists and antagonists on ibotenate-induced cortical plate lesions suggest that the role of NC in the periventricular white matter uses specific mechanisms. In the present ibotenate model, cortical plate lesions are secondary to massive calcium influx into neurons bearing NMDA receptors. In contrast, in the white matter, ibotenate does not directly induce axonal lesions but triggers microglial activation that is responsible for secondary astrocytic cell death and axonal breakdown (50). ORL1 receptors have only been described on neurons, but the target of NC in the present study is likely to be neuronal bodies and/or axons. Altogether, the data from this murine model suggest that NC is able to modulate white-matter axonal damage induced by microglia-derived factors but not the NMDA-mediated excitotoxic cascade in cortical plate neurons.

As previously mentioned, the ORL1 receptor does not bind with high affinity to traditional opioid ligands (for a review, see ref. 20), and NC does not bind with high affinity to the classical opioid receptors (18, 19). Naloxone and endomorphin I had no detectable effect on ibotenate-induced lesions, sug-

gesting the specific involvement of ORL1 receptors in the present study.

In previous studies (34, 35), $NC(1-17)NH_2$ displayed a higher potency than NC, probably due to a greater metabolic stability. Such a benefit was not observed in the ibotenate model, suggesting that NC degradation following systemic injection in neonatal mice is not a fully mature phenomenon. In different experimental paradigms, $[F/G]NC(1-13)NH_2$ has been shown to behave as an antagonist, an agonist, or a partial antagonist of the ORL1 receptor (35, 37, 38). In the present study, $[F/G]NC(1-13)NH_2$ clearly behaved as a NC receptor antagonist. The data further confirmed the pure antagonist properties of $[Nphe^1]NC(1-13)NH_2$ (39, 40) and the lower potency of this compound when compared with $[Phe^1\psi(CH_2-NH)Gly^2]NC(1-13)NH_2)$ (37, 39).

Astroglial origin of endogenously released NC. In this murine model, the neuroprotection induced by NC antagonists and by antisense oligonucleotides targeting NC mRNA strongly suggests that endogenous NC is playing a deleterious role on the excitotoxic lesion. NC is present in neurons and astrocytes (21, 22), and in vitro studies suggest that NC production and secretion is induced by cAMP in both cell types (23, 24). The findings from this study support the hypothesis that, following ibotenate injec-

Figure 6
Effects of astrocyte depopulation and PKA inhibition on the excitotoxic lesions. Bars represent mean length of the lesion along the sagittal fronto-occipital axis ± SEM. Numbers in parentheses are the numbers of animals used in each experimental group. Statistically significant differences between controls and experimental animals are shown ([A]$P < 0.05$, [B]$P < 0.01$ in ANOVA with Dunnett's multiple comparison test). Ant, antagonist.
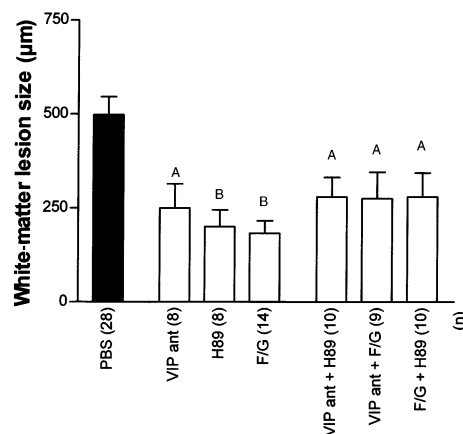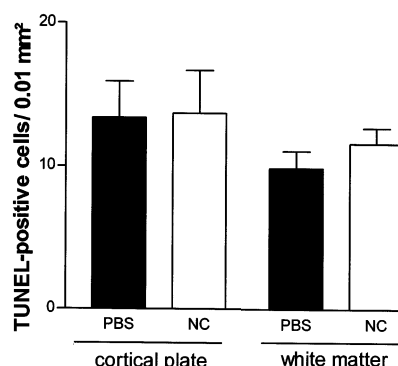
Figure 7

NC treatment does not affect developmental cell death in P5 mouse pups. Quantitative analysis of TUNEL-positive cell density in the somatosensory cortical layers and underlying white matter of pups treated with PBS (filled bars) and NC (open bars). Values shown are means ± SD.

tion, NC is released predominantly by astrocytes through a cAMP-dependant mechanism. This conclusion is based on several pieces of evidence derived from our ibotenate model: (a) antisense oligonucleotides targeting NC mRNA are taken up readily by astrocytes but not by neurons; (b) the inhibition of neopallial astrocyte production and/or differentiation leads to neuroprotection of the white matter against excitotoxic lesions; (c) the blockade of cAMP-dependent PKA also decreases ibotenate-induced lesions of the white matter; and (d) the reduction in size of white-matter lesions produced by astrocytogenesis inhibition or PKA blockade is similar to that provided by the ORL1-receptor blockade, but combined neuroprotective strategies did not produce further reduction in lesion size. The precise molecular mechanism linking NMDA receptor activation and NC release by astrocytes, which do not have NMDA receptors (51), is still unknown. Similarly, the physiological role of astroglia-derived NC in neocorticogenesis is also unknown.

Potential implications for human preterm neonates. A large body of evidence supports the hypothesis that our murine model of ibotenate-induced white-matter lesion mimics several aspects of human PVL (7, 10–13, 50), and there are some reasons to be hopeful about treating this condition. For example, the present data suggest that compounds such as [Nphe^1]NC(1-13)NH$_2$ and [F/G]NC(1-13)NH$_2$ can cross the neonatal blood-brain barrier. Assuming that we can extrapolate from mechanisms operating in the neonatal mouse brain to human preterm infants, it is possible that NC release by astrocytes could be related to the formation of human PVL. If this is true, selective NC receptor antagonists may represent a new class of neuroprotective agents to treat and prevent PLV in human neonates.

In order to reduce pain and discomfort, numerous human preterm infants have received fentanyl for several days in intensive care units. There is no doubt that fentanyl is highly beneficial to alleviate pain. However, the findings from this study and others (32) suggest that the deleterious effects of high doses fentanyl on ibotenate-induced white-matter lesions are mediated through ORL1-receptor activation and

suggest an association between fentanyl and a higher risk of PVL in human preterm infants. Nevertheless, important questions remain. Can we extrapolate from the data obtained in our animal model to human preterm infants? Should a prospective study be conducted in human preterm infants to study potential deleterious effects of fentanyl on PVL occurrence? Should we treat human preterm infants with an opioid drug devoid of any ORL1-receptor agonist activity instead of fentanyl? In our model, sufentanil does not have any detectable effect on excitotoxic brain lesions, although a recent study suggested that sufentanil could modulate ORL1 receptor–mediated effects in SK-N-SH cells (52). Buprenorphine, which was widely used as an analgesic molecule in newborns and infants, has also been shown to have ORL1 agonist activity (31). On the other hand, [Nphe^1]NC(1-13)NH$_2$ produces an antinociceptive action and potentiates morphine-induced analgesia (39) and a recent study described a new class of drugs with opioid agonist/ORL1 antagonist activities (53), thus opening new possibilities in the field of neuroprotection and analgesia in neonates.

Conclusion. The present study demonstrates that, in a murine model of human PVL, NC production blockade and ORL1-receptor antagonists protect the developing white matter against excitotoxic challenge, while NC and fentanyl exacerbate lesions through ORL1-receptor activation. The NC/ORL1 system could represent a new target for the neuroprotection of human premature infants at high risk for PVL.

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