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RESEARCH ARTICLE

Nicotinamide prevents the long-term effects of perinatal asphyxia on apoptosis, non-spatial working memory and anxiety in rats

Paola Morales · Nicola Simola · Diego Bustamante · Francisco Lisboa · Jenny Fiedler · Peter J. Gebicke-Haerter · Micaela Morelli · R. Andrew Tasker · Mario Herrera-Marschitz

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Abstract There is no established treatment for the long-term effects produced by perinatal asphyxia. Thus, we investigated the neuroprotection provided by nicotinamide against the effects elicited by perinatal asphyxia on hippocampus and behaviour observed at 30–90 days of age. Asphyxia was induced by immersing foetuses-containing uterine horns, removed from ready-to-deliver rats into a water bath at 37°C for 20 min. Caesarean-delivered siblings were used as controls. Saline or nicotinamide (0.8 mmol/kg, i.p.) was administered to control and asphyxia-exposed animals 24, 48, and 72 h after birth. The animals were examined for morphological changes in hippocampus, focusing on delayed cell death and mossy fibre sprouting, and behaviour, focusing on cognitive behaviour and anxiety. At the age of 30–45 days, asphyxia-exposed rats displayed (1) increased apoptosis, assessed in whole hippocampus by nuclear Hoechst staining, and (2) increased mossy fibre sprouting, restricted to the *stratum oriens* of dorsal hippocampus, assessed by Timm's staining. Rats from the same cohorts displayed (3) deficits in non-spatial working memory, assessed by a novel object recognition task, and (4) increased anxiety, assessed by an elevated plus-maze test when examined at the age of 90 days. Nicotinamide prevented the effects elicited by perinatal asphyxia on apoptosis, working memory, and anxiety.

P. Morales and N. Simola contributed equally to this work.

P. Morales · D. Bustamante · F. Lisboa · M. Herrera-Marschitz (✉)
Programme of Molecular and Clinical Pharmacology, ICBM, Medical Faculty, University of Chile, PO Box 70,000, Santiago 7, Chile
e-mail: mh-marschitz@med.uchile.cl

P. Morales
e-mail: pmorales@med.uchile.cl

D. Bustamante
e-mail: dbustama@med.uchile.cl

F. Lisboa
e-mail: flisboag@gmail.com

N. Simola · M. Morelli
Department of Toxicology, Center of Excellence on Neurobiology of Dependence, University of Cagliari, Via Ospedale 72, 09124 Cagliari, Italy
e-mail: nicola.simola@virgilio.it

M. Morelli
e-mail: morelli@unica.it

R. A. Tasker
Department of Biomedical Sciences, University of Prince Edward Island, 550 University Avenue, Charlottetown, PEI C1A4P3, Canada
e-mail: tasker@upei.ca

J. Fiedler
Department of Biochemistry and Molecular Biology, Chemical and Pharmaceutical Sciences Faculty, University of Chile, Santiago, Chile
e-mail: jfiedler@ciq.uchile.cl

P. J. Gebicke-Haerter
Department of Psychopharmacology J 5, Central Institute for Mental Health, 68159 Mannheim, Germany
e-mail: peter.gebicke@zi-mannheim.de

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Abbreviations

AS	Asphyxia saline
AN	Asphyxia nicotinamide
CA	Cornus amonnis
CN	Caesarean-delivered nicotinamide
CNS	Central nervous system
CS	Caesarean-delivered saline
DG	Dentate gyrus
IML	Inner molecular layer
MFS	Mossy fibre sprouting
n.s.	Non-significant
OML	Outer molecular layer
P	Postnatal day
PARP-1	Poly(ADP-ribose) polymerase-1
PBS	Phosphate buffered saline
S1, S2	Session 1, 2
SEM	Standard error of the mean
SL	Stratum lucidum
SO	Stratum oriens

Introduction

Perinatal asphyxia is a major cause of death and neurological injury in newborn babies, frequently associated with difficult or prolonged delivery. Its international incidence is 2–6/1,000 term births (de Haan et al. 2006). Clinical evidence shows that infants surviving perinatal asphyxia may suffer severe neurological sequelae, including cerebral palsy, encephalopathy, motor alterations, seizures, or cognitive alterations of variable severity, such as attention deficit, hyperactivity, mental retardation and/or neuropsychiatric syndromes with delayed clinical onset (du Plessis and Volpe 2002; Van Erp et al. 2002; Kaufman et al. 2003; Vannucci and Hagberg 2004). In rats, neonatal anoxia induces hyperactivity, working memory deficits, and anxiety (Dell'Anna et al. 1991; Iuvone et al. 1996; Hoeger et al. 2000, 2006; Venerosi et al. 2004). Indeed, in a recent report, we observed long-term deficits in non-spatial memory and motor coordination, but not in gross motor behaviour or spatial memory (Simola et al. 2008), suggesting specific effects of perinatal asphyxia on neuro-behavioural functions.

There is clinical and experimental evidence indicating that the neurocircuitries of the hippocampus are vulnerable to hypoxia/ischaemia (Pulsinelli et al. 1982; Van Erp et al. 2002; Harry and Lefebvre d'Hellencourt 2003), perhaps explaining the cognitive and mental disorders described as sequelae of perinatal asphyxia (Almli et al. 2000; Balduini et al. 2000; Mañeru et al. 2001; Weitzdoerfer et al. 2004; Calvert and Zhang 2005; Simola et al. 2008). The effect of neonatal hypoxia on hippocampal cell death has been observed 1 week (Dell'Anna et al. 1997; Morales et al. 2005), 1 month (Bjelke et al. 1991; Johansen et al. 1992; Morales et al. 2008) and 3 months (Kohlhauser et al. 1999; Hoeger et al. 2006) after the insult. In parallel, an increase of pro- and anti-apoptotic protein levels has been observed, suggesting an apoptosis mechanism (Nakajima et al. 2000; Northington et al. 2001; Morales et al. 2008). Several compensatory mechanisms have been suggested to be triggered for protecting from cell death, including neurogenesis and neuritogenesis (Pokorny et al. 1982; Biernaskie and Corbett 2001; Kee et al. 2001; Jin et al. 2001; Yagita et al. 2001; Nakatomi et al. 2002; Daval et al. 2004; Daval and Vert 2004; Morales et al. 2005, 2007, 2008).

Mossy fibre sprouting (MFS) is a plastic change observed in hippocampus following epilepsy and other neurotoxic insults. It consists of abnormal growth of axons of dentate gyrus (DG) granule neurons, forming synaptic connections with dendrites of neurons of the inner molecular layer of the DG and of the *stratum oriens* (*SO*) of CA3. It has been suggested that MFS is a reaction to hippocampal cell loss (Cavazos and Sutula 1990; Nadler 2003; Chen et al. 2004; Zappone and Sloviter 2004), resulting in an increased, probably protective, inhibitory tone (Sloviter 1992), although MFS has also been reported in the absence of cell loss (Bernard et al. 2007). The relationship between MFS, cell death and neurobehavioural effects observed after perinatal asphyxia is not yet known. Therefore, this study aimed to evaluate whether sprouting of mossy fibres in the hippocampus is also affected by perinatal asphyxia.

Although the understanding of the pathophysiology of perinatal asphyxia is gradually increasing, therapeutic options for preventing or mitigating the effects produced by the insult are limited. Nicotinamide has been proposed to protect against oxidative stress, ischaemic injury and inflammation, by replacing the depletion of the NADH/NAD^+ pair produced by poly(ADP-ribose) polymerase-1 (PARP-1), which is activated to repair hypoxic injury-induced DNA damage (Zhang et al. 1995). Indeed, nicotinamide has been shown to prevent several of the changes induced by perinatal asphyxia on monoamines, even if the treatment is delayed for 24 h, suggesting a clinically relevant therapeutic window (Bustamante et al. 2003, 2007; Klawitter et al. 2006, 2007).

Thus, the main aim of this study was to evaluate the neuroprotection provided by nicotinamide against the long-term effect of perinatal asphyxia, focusing on delayed cell death and MFS in hippocampus, as well as on cognitive performance and anxiety.

Methods

Perinatal asphyxia

Pregnant Wistar rats (bred as a local colony) within the last day of gestation (G 22) were euthanised by neck dislocation and hysterectomised. One or two pups were removed immediately and were used as non-asphyxiated caesarean-delivered controls, and the uterine horns containing the remaining foetuses were immersed in a water bath at 37°C for 20 min. Following asphyxia, the uterine horns were incised; the pups were removed, and stimulated to breathe.

An Apgar scale for rodents

Approximately 1 h (60–80 min) after delivery, an Apgar scale adapted to rats (Herrera-Marschitz et al. 1993; Loidl et al. 1994; Dell'Anna et al. 1997; Engidawork et al. 2001) was applied to monitor several parameters, such as weight, sex, colour of the skin, respiratory frequency and presence of gasping, vocalisation, muscular rigidity and spontaneous movements. Thereafter, surviving pups were given to a surrogate dam for nursing, pending further experiments.

The Apgar scale is a critical parameter for this experimental model, because it evaluates whether the pups were subjected to mild or to severe asphyxia, determined by the percentage of survival and recovery (Herrera-Marschitz et al. 1993). Recovery may also imply a prolonged hypoxic/ischaemic condition, as long as surviving pups may show a decreased breathing rate, decreased cardiovascular function and low peripheral and/or central blood perfusion. Furthermore, the Apgar implies an evaluation of the condition shown by the caesarean-delivered control pups, which has to be similar to that shown by vaginally delivered—but different to that by asphyxia-exposed pups. Thus, the Apgar evaluation is a requirement when using the present model of perinatal asphyxia, making it possible to compare results obtained by different laboratories and/or different treatments. The Apgar scale was applied to all male and female surviving pups 60–80 min after birth, but the evaluations of delayed neurochemical and behavioural effects of perinatal asphyxia only involved male pups, avoiding confusing factors related to hormonal and/or cycling status when performing experiments with rats at puberty or adult stages.

Nicotinamide treatment

Nicotinamide [0.8 mmol/kg, i.p. (100 mg/kg); niacinamide, Sigma, St. Louis, MO, USA] or the corresponding vehicle (20 ml/kg of 0.9% NaCl solution) was administered to asphyxia-exposed or caesarean-delivered pups, with the following administration schedule: 24, 48 and 72 h after birth.

Histochemistry

Intracardial formalin fixation

At approximately 1 month after delivery, the rats were deeply anaesthetised with chloral hydrate (400 mg/kg i.p.) and intracardially perfused with 100 ml of 0.1 M of PBS (pH 7.4), followed by 200 ml of 4% paraformaldehyde (Sigma, in 0.1 M of PBS, pH 7.4). The brain was removed from the skull, post-fixed in a formalin solution overnight, and immersed in 30% sucrose in 0.1 M of PBS at 4°C for 2–3 days, to be then embedded in cryomatrix (Thermo Electron Corp, Pittsburgh, PA, USA) and stored at −80°C. Coronal sections (20 μm thick) were taken from the frozen fixed brains, between Bregma −4.52 and −2.56 (Paxinos and Watson 1986), pending further analysis.

Apoptotic cells

Hoechst #33342 (Bisbenzimide, Sigma) was used for investigating nuclear morphology, revealing chromatin condensation during apoptosis, which is detected as an intensively bright blue staining. Coronal sections were stained with Hoechst #33342 (2.5 μg/μl) and cover-slipped with a fluorescent mounting medium (DAKO, DAKO Corp). Apoptotic nuclei were identified using the criteria proposed by Greiner et al. (2001). Briefly, one of the following criteria had to be fulfilled to count for apoptotic cells: (1) tightly condensed nuclear material; (2) darkly stained spherical or clumped nuclear material, and/or (3) fragmented nuclei. Stereological quantification was conducted as previously described (Morales et al. 2005). As shown before (Morales et al. 2008), there is a good agreement between Hoechst #33342 and the TUNEL-based detection kits for characterising delayed, apoptotic-like, cell death. An average of the number of apoptotic cells was calculated from 20 slices/animal (5 sections of 20 μm, every 100 μm), inspected at 100×, and expressed as mean ± standard error of the means (SEM).

Tissue processing for Timm's staining

A series of rats was euthanized by decapitation (following deep anaesthesia) at postnatal day 45 (P45). The brain was rapidly removed for dissecting and straightening the hippocampi in a tissue cassette, which was then immersed in 0.4% sodium sulphide solution for 20 min (Sperber et al. 1991), before being transferred to a 10 ml solution containing 10% buffered neutral formalin saturated with 30% sucrose for 24 h. The tissue, coated with cryomatrix (Thermo Electron Corp.), was frozen using liquid nitrogen, and stored at −80°C until sectioning in a cryostat. Hippocampal sections (5 sections of 40 μm, every 400 μm) were cut on a coronal plane through the entire extent of the

hippocampi. The sections were mounted on gelatine coated slides (0.5% gelatine) and allowed to air dry overnight, to then be treated for Timm's staining in the dark for 80 min, constantly stirred on a magnetic stir plate set at 200 rpm (see Bernard et al. 2007). The staining solution (modified from Holmes et al. 1998) contained: (1) 120 ml of 50% gum arabic solution (Sigma-Aldrich, Oakville, ON, Canada); (2) 10 ml of citric acid (51 g/100 ml H_2O); (3) 10 ml of sodium citrate (47 g/100 ml H_2O); (4) hydroquinone (7 g/100 ml) (Sigma-Aldrich); and (5) 212.25 mg silver nitrate (Sigma-Aldrich) dissolved in 10 ml H_2O. After staining, the sections were washed for 45 min in gently running tap and then deionised water, dehydrated in serial ethanol solutions, cleared twice in xylene, and cover-slipped using Entellan (Merck, Darmstadt, Germany). Timm's staining selectively labels the high concentration of zinc located in the dentate granule cell axon terminals (mossy fibres).

Quantification of mossy fibre sprouting

The quantification of MFS was performed on samples from dorsal, mid and ventral hippocampus, each region comprising 5 sections, up to a total of 15 sections per animal, measuring the distribution and density of Timm's stained granules. Digital images were taken using a Nikon 7.0 P digital camera (Nikon Canada Inc., Montreal, QC, Canada) coupled to a light microscope (Zeiss Photomicroscope 3, Carl Zeiss Inc., Thornwood, New York, USA). Images were transferred to a TIFF file format using Adobe Photoshop®. MFS into the *SO*, *stratum lucidum* (*SL*) of CA3, and inner molecular layer (IML) of the supra and infrapyramidal blades of DG was analysed by taking eight evenly spaced optical density readings (0.1 mm in diameter) for each of the areas using the Scion Image software (Scion Corporation, Frederick, Maryland, USA). A grey scale was used for measuring the intensity of the signals (pixels) observed in the *SO*, *SL* and IML, i.e. the darker staining the higher optical density; the lighter staining the lower optical density. Since the outer molecular layer (OML) of the hippocampus is not labelled by Timm's staining, the signal in that area was considered as a background, to be subtracted from the optical density evaluated for IML, *SO*, *SL* regions. The sections were also scored using a modified version of Holmes' semi-quantitative scoring method (Holmes et al. 1998, 1999), but the results reported here are based on the density analysis, because similar results were obtained regardless of the scoring system used.

Behavioural analysis

Cohorts from the same experimental groups were kept alive for behavioural analysis performed at approximately 3-months of age.

Novel object recognition task

Measurement of novel object recognition is widely used for evaluating non-spatial working memory in rodents (Ennaceur and Delacour 1988). Novel object recognition experiments were performed in a black wooden box (length 60 cm, width 40 cm, height 30 cm) with the floor covered with sawdust. Objects to be discriminated were made of plastic or glass, differing in shape and colour. Objects had no physiological relevance and were not associated with any rewarding or aversive stimuli. Two days before the test, the rats were allowed to explore the experimental box, twice for 5 min, in order to habituate them to the new environment. On the testing day, each rat was placed in the box for two 4 min sessions, letting it to explore various types of objects freely. During the first session (S1) two copies of the same object were presented, whereas during the second session (S2) a novel object was introduced, together with a copy of the already presented object. S1 and S2 sessions were separated by a 60 min interval, based on previous experiments performed by our group with the same experimental model (Simola et al. 2008). Sniffing, gnawing, and/or touching any of the objects was considered as a sign of exploration, but not when the rat only turned around the objects. To avoid the presence of olfactory cues, objects were thoroughly cleaned after each session. The position (right or left) of old or novel objects was counterbalanced to prevent biased preferences. Rat performance was videotaped, quantifying the time spent exploring the objects during S1 and S2 sessions. A ratio of the time spent with the novel by the time spent with the already presented object (during the S1 session) was calculated for the S2 session.

Elevated plus maze performance

The elevated plus-maze represents a reliable experimental tool for evaluating anxiety in rodents (Dawson and Tricklebank 1995; Rodgers and Dalvi 1997). Briefly, the apparatus consists of two opposed open (length 50 cm, width 10 cm) and two opposed enclosed (length 50 cm, width 10 cm, enclosed by 40 cm high walls on their length) arms, made of white PVC. The four arms converge onto a central square (10 × 10 cm), thus reproducing the shape of a plus sign. The apparatus was elevated 50 cm above the floor. Rats having no prior experience with an elevated plus-maze were placed in the central square and let free to explore the whole apparatus for a 5 min test session. The performance of the rats was videotaped. The percentage of arm entries, as well of the time spent in open or close arms was calculated with respect to the total number of entries and the total time spent in the arms, respectively. A reduced exploration of open arms is considered as an index of anxiety (Pellow et al. 1985).

Spontaneous behavioural alternation

Evaluation of spontaneous behavioural alternation in a Y maze is commonly employed in experiments evaluating the short-term spatial memory in rodents (Maurice et al. 1994; Yamada et al. 1996). The apparatus consists of three equally black PVC made arms (length 50 cm, width 20 cm, height 35 cm), named A, B and C, converging onto a central triangular area. The floor of the maze was covered with sawdust, which was changed between the tests. Rats were placed in the central area and let free to explore the whole apparatus for an 8 min trial. The performance of the rats was videotaped. The percentage of spontaneous behavioural alternation was calculated based on the sequence of arm entries as reported by Yamada et al. (1996).

Statistical analysis

All data are expressed as mean ± SEM for the following experimental groups: (1) saline-treated, caesarean-delivered (CS); (2) nicotinamide-treated, caesarean-delivered (CN); (3) saline-treated, asphyxia-exposed (AS), and (4) nicotinamide-treated, asphyxia-exposed (AN) animals. For Apgar studies, comparisons were analysed with a non-parametric Mann–Whitney test, applied to discontinuous scales. Multiple comparisons were first analysed for treatment effect (F-ANOVA), followed by Newman–Keuls (N–K) or Tukey's HSD post hoc test when appropriated (GraphPad Prism; version 4.0, 2003; S. Diego, CA, USA). Significance was set at $P < 0.05$.

All experiments were conducted in accordance with the guidelines for care and use of experimental animals of the European Communities Directive (86/609/EEC; D.L., 27.01.1992, number 116). The experimental protocol was approved by a Local National Committee for Ethic Experiments with Laboratory Animals (Protocol CBA# 0136, FMUCH), endorsing the principles of laboratory animal care (NIH; No. 86-23; revised 1985).

Results

Short-term effect produced by perinatal asphyxia

A rodent Apgar scale

An Apgar scale was applied 60–80 min after birth, a period long enough for observing a full recovery after a caesarean-induced delivery (Table 1). When performed at 37°C, the rate of survival after 20 min of perinatal asphyxia decreased to approximately 70% for AS ($n = 77$) versus CS ($n = 93$) pups (the nomination AS and CS is kept here, although no saline or any other treatment was started before 24 h after delivery). The surviving AS pups slowly recovered with decreased respiratory frequency, sustained by remarkable gasping, still observed at the 60–80 min period after birth. No gasping was observed for CS pups. The Apgar scale further revealed an impairment of systemic and CNS function, since the AS pups were cyanotic (blue/pale skin), noiseless, akinetic, with rigidity mainly affecting the hind limbs, clearly contrasting to that observed for CS pups, which, like vaginally delivered pups, promptly showed coordinated movements, vocalisation, a pink coloured skin, and a rhythmic and sustained breathing minutes after delivery, in agreement with previous reports (Dell'Anna et al. 1997; Engidawork et al. 2001). Both AS and CS animals were accepted and nursed by surrogate mothers. Drug administration, which started 24 h after delivery, did not affect nursing or survival. As an average, each dam provided two male or female pups for each experimental group (CS, CN, AS, AN). The Apgar scale was applied to all male and female surviving pups 60–80 min after birth, but for the evaluation of delayed neurochemical and behavioural parameters only male pups were sampled for the experimental cohorts. Thus, each dam provided approximately one pup for each cohort. Body weights for each experimental condition along the observation periods are shown in Table 2.

Table 1 Apgar scale applied 60–80 min after perinatal asphyxia

Parameters	0-min asphyxia (CS, $n = 93$; $m = 12$)	20-min asphyxia (AS, $n = 77$; $m = 12$)
Weight (g)	5.73 ± 0.08	5.76 ± 0.06
Sex (% males)	55 ± 4.8	45 ± 4.8
Rate of survival (%)	100 ± 0	[a]*75 ± 9.0*
Respiratory frequency (events/min)	62.4 ± 2.7	[a]*14.3 ± 2.0*
Presence of gasping (yes) (%)	0 ± 0	[a]*95 ± 3*
Skin colour (%)		
Pink	100 ± 0	[a]*0 ± 0*
Pink-blue	0 ± 0	[a]*77 ± 10*
Blue-pink	0 ± 0	[a]*22 ± 10*
Blue	0 ± 0	0 ± 0
Vocalisations (yes) (%)	94.4 ± 5.6	[a]*2.7 ± 1.9*
Akinesia, rigidity (yes) (%)	0 ± 0	[a]*100 ± 0*
Spontaneous movements (intensive movements shown by wriggling) (yes) (%)	100 ± 0	[a]*0 ± 0*

n Number of pups, *m* number of mothers

[a] $P < 0.05$, compared to CS (italicised) (Mann–Whitney test)

Table 2 Body weight (g) (mean ± SEM) measured along different observation periods

Animals	60–80 min	24 h	30 days	45 days	60 days
CS	5.73 ± 0.08; *n* = 77	5.95 ± 0.14; *n* = 34	78.98 ± 0.37; *n* = 28	119.32 ± 0.46; *n* = 9	200.1 ± 0.05; *n* = 11
CN		6.04 ± 0.19; *n* = 29	76.8 ± 0.53; *n* = 19	114.42 ± 0.29; *n* = 6	198.5 ± 0.08; *n* = 11
AS	5.76 ± 0.06; *n* = 93	5.89 ± 0.13; *n* = 38	74.8 ± 0.29; *n* = 36	106.61 ± 0.46; *n* = 9	199.2 ± 0.08; *n* = 11
AN		5.76 ± 0.12; *n* = 46	66.32 ± 0.27; *n* = 18	101.79 ± 0.56; *n* = 8	199.2 ± 0.09; *n* = 11
F-ANOVA	n.s.	n.s.	n.s.	n.s.	n.s.

Male and female pups were evaluated at the 60–80 min period after birth, named caesarean-delivered (CS) and asphyxia-exposed (AS) pups, although the treatment, saline (CS, AS) or nicotinamide (CN, AN) started 24 h after birth. From that period, only male rats were used along the experiments (comparisons amongst animal groups, F-ANOVA; n.s., non significant)

Histochemistry

Apoptosis

The hippocampi of CS, AS, CN, AN ($n = 4$, each condition) animals were examined for apoptotic morphology after nuclear Hoechst staining 1 month after birth (Fig. 1). The stereological analysis demonstrated that there was a significant increase (by ~36%) in the number of apoptotic nuclei of whole hippocampus of AS compared to CS animals. Nicotinamide prevented the increase in the number of apoptotic nuclei induced by asphyxia, since no difference was observed between AN, CS, and CN rats (Fig. 1).

Mossy fibre sprouting

MFS was evaluated in dorsal, mid and ventral hippocampus, in *SO*, *SL* and IML of DG (supra- and infrapyramidal band) (Table 3; Figs. 2, 3). Five sections were analysed in dorsal, mid and ventral hippocampus, completing 15 samples per animal. Representative images of Timm's staining from dorsal hippocampus are shown in Fig. 2, for CS (A, C) and AS (B, D) animals. There was an increase of sprouting in *SO* of AS, compared to that of CS animals, evaluated 45 days after birth, which was confirmed by the quantitative analysis throughout the hippocampus (Table 3), showing an increase (by ~40%) of sprouting restricted to *SO* in dorsal hippocampus of AS ($n = 7$), compared to CS ($n = 8$) rats. As shown in Fig. 3, however, the effect of perinatal asphyxia on MFS was not altered by the nicotinamide treatment. Indeed, the increase was similar in AS and AN animals (by ~50%), compared to CS rats. No effect was observed in any other region.

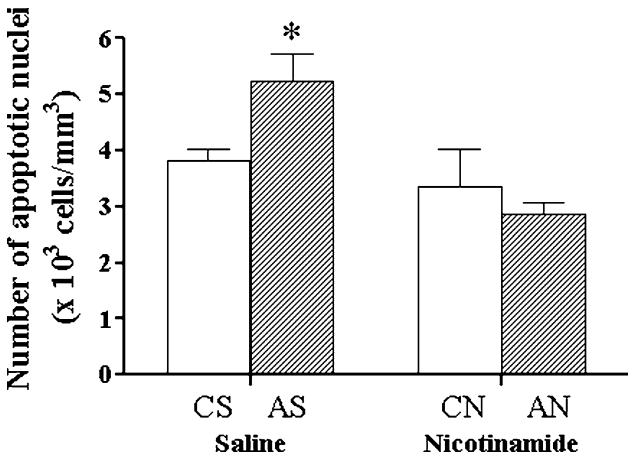

Fig. 1 Number of cells with apoptotic nuclei ($\times 10^3$ cells/mm^3) in hippocampus of control (treated with saline, CS, 3.83 ± 0.37, $n = 4$; or nicotinamide, CN, 3.37 ± 1.34, $n = 4$) (*open bars*), and asphyxia-exposed (treated with saline, AS, 5.23 ± 0.97, $n = 4$; or nicotinamide, AN, 2.87 ± 0.41, $n = 4$) (*dashed bars*) rats at P30. Multiple comparison analysis (F-ANOVA = 5.39, $m,n = 4,16$, $P < 0.01$) revealed a treatment effect. Post hoc analysis (Newman–Keuls) revealed significant differences between AS and CS (N–K = 3.23, $P < 0.05$), AS and AN (N–K = 5.4, $P < 0.05$), as well as CN (N–K = 4.3, $P < 0.05$) groups. *$P < 0.05$ for the comparison between AS and CS

Behavioural analysis

Novel object recognition

When tested for novel object recognition, AS rats spent significantly less time exploring a novel object compared to CS rats (F-ANOVA = 4.31, $P < 0.05$, $m,n = 4,24$), indicating that AS rats did not recognise the differences between the objects (Table 4A). Nicotinamide did not affect object recognition when administered to CN rats, but prevented the effect of perinatal asphyxia, since AN animals performed like CS and CN animals (Table 4A).

Elevated plus maze performance

Perinatal asphyxia affected two of the parameters evaluated by the elevated plus maze: (1) entries (F-ANOVA = 10.23, $P < 0.05$, $m,n = 4,28$), and (2) time spent (F-ANOVA = 5.85, $P < 0.05$, $m,n = 4,28$) in the open arms (Table 4B). As compared to CS, AS, but not AN, rats displayed a significant reduction in the percentage of entries to the open arms ($P < 0.05$, Tukey-HSD). AS, AN, but also CN rats showed a significant reduction in the time spent in the open arms as compared to CS animals ($P < 0.05$, Tukey-HSD) (see Table 4B).

Table 3 Mossy fibre sprouting

Experiment	Hippocampus region		
	Dorsal	Middle	Ventral
CS			
SO	53.05 ± 9.09 (*n* = 8)	55.38 ± 5.25 (*n* = 8)	33.19 ± 3.77 (*n* = 8)
SL	108.3 ± 5.23 (*n* = 9)	105.3 ± 6.8 (*n* = 6)	72.9 ± 8.62 (*n* = 7)
$IML_{(supra)}$	29.24 ± 2.97 (*n* = 5)	20.78 ± 1.77 (*n* = 6)	22.83 ± 2.11 (*n* = 8)
$IML_{(infra)}$	23.08 ± 3.98 (*n* = 7)	14.28 ± 3.26 (*n* = 7)	12.1 ± 1.59 (*n* = 8)
CN			
SO	51.27 ± 1.70 (*n* = 9)	50.34 ± 2.83 (*n* = 9)	30.7 ± 3.42 (*n* = 9)
SL	100.1 ± 7.98 (*n* = 6)	95.21 ± 6.2 (*n* = 6)	74.63 ± 7.67 (*n* = 6)
$IML_{(supra)}$	27.66 ± 5.01 (*n* = 6)	24.78 ± 2.9 (*n* = 6)	20.08 ± 3.0 (*n* = 6)
$IML_{(infra)}$	24.91 ± 5.79 (*n* = 6)	13.48 ± 2.88 (*n* = 6)	14.32 ± 2.44 (*n* = 6)
AS			
SO	[a]*76.35 ± 5.43* (*n* = 7)	49.34 ± 2.83 (*n* = 7)	31.57 ± 3.42 (*n* = 7)
SL	107.1 ± 7.98 (*n* = 9)	92.22 ± 6.6 (*n* = 7)	74.63 ± 7.67 (*n* = 7)
$IML_{(supra)}$	29.36 ± 4.01 (*n* = 6)	24.78 ± 2.9 (*n* = 7)	24.08 ± 2.63 (*n* = 7)
$IML_{(infra)}$	26.91 ± 6.79 (*n* = 6)	16.48 ± 2.95 (*n* = 6)	15.36 ± 2.24 (*n* = 7)
AN			
SO	[a]*78.25 ± 3.13* (*n* = 9)	47.14 ± 2.13 (*n* = 9)	38.3 ± 3.76 (*n* = 9)
SL	102.1 ± 5.08 (*n* = 8)	88.2 ± 5.21 (*n* = 7)	69.94 ± 7.07 (*n* = 7)
$IML_{(supra)}$	28.16 ± 5.01 (*n* = 7)	23.87 ± 3.9 (*n* = 7)	22.08 ± 2.61 (*n* = 7)
$IML_{(infra)}$	25.71 ± 5.43 (*n* = 6)	15.48 ± 2.95 (*n* = 6)	14.31 ± 2.34 (*n* = 6)

Densitometric analysis of sprouting density (pixels) in dorsal, middle and ventral regions of *Stratum Oriens*, *SO*; *Stratum Lucidum*, *SL*; inner molecular layer (supra), [$IML_{(supra)}$]; inner molecular layer (infra), [$IML_{(infra)}$] of caesarean-saline, CS; caesarean-nicotinamide, CN; asphyxia-saline, AS; asphyxia-nicotinamide, AN treated rats at P45

[a] $P < 0.05$, compared to CS (italicised) (F-ANOVA, followed by Newman–Keuls; see legend for Fig. 3)

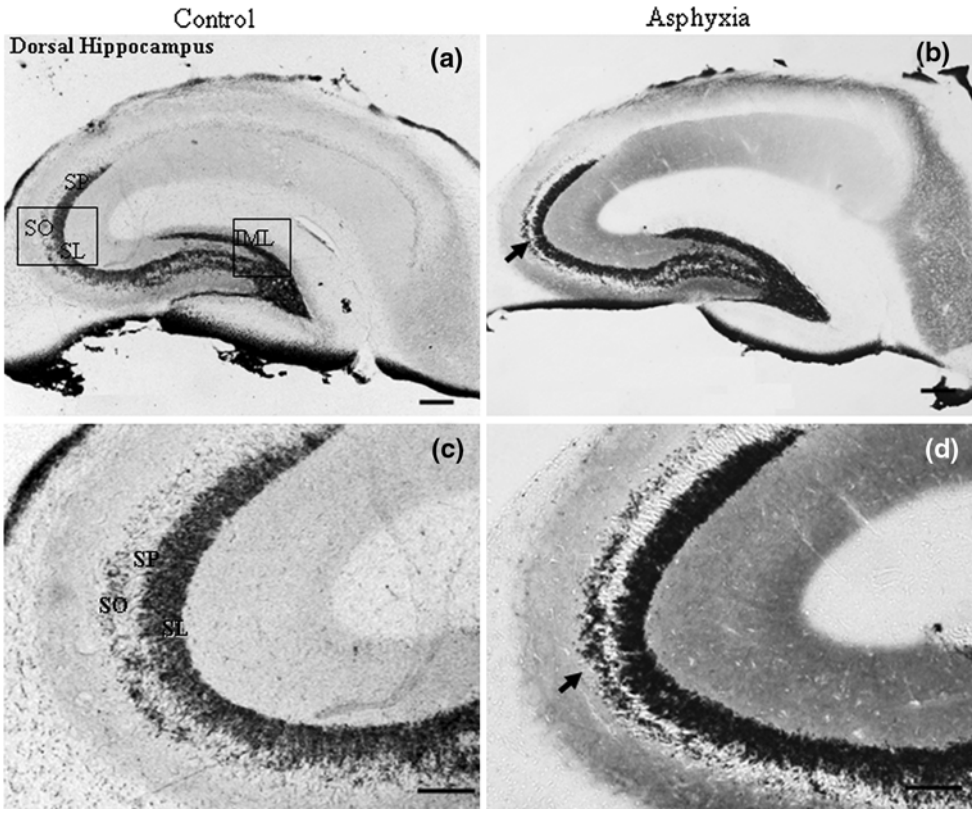

Fig. 2 Photomicrographs illustrating Timm's stain labelled fibre's sprouting in *stratum oriens* (*SO*), *stratum lucidum* (*SL*) and inner molecular layer (IML) of dorsal hippocampus of saline-treated-control (**a**) and asphyxia-exposed (**b**) rats. (**c**) and (**d**) are magnifications of *SO* from **a** and **b**, respectively. *SP*, *stratum pyramidal*. *Scale bar* 200 μm

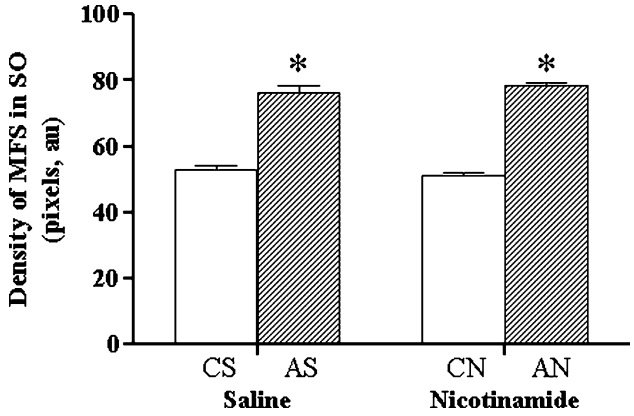

Fig. 3 Densitometric analysis of mossy fibre sprouting (MFS) density (pixels, arbitrary units, au) in *stratum oriens* (*SO*) of dorsal hippocampus of control (treated with saline, CS, 53.0 ± 3.3, $n = 8$, or nicotinamide, CN, 51.3 ± 1.7, $n = 9$) (*open bars*), and asphyxia-exposed (treated with saline, AS, 76.35 ± 5.4, $n = 7$, or nicotinamide, AN, 78.3 ± 3.1, $n = 9$) (*dashed bars*) rats at P45. Multiple comparison analysis (F-ANOVA = 144.8, $m,n = 4, 28$, $P < 0.001$) revealed a treatment effect. Post hoc analysis (Newman–Keuls) revealed significant differences between AS versus CS (N–K = 18.2, $P < 0.001$), and AN versus CN (N–K = 23.1, $P < 0.001$) groups. *$P < 0.05$ for the corresponding control

Spontaneous behavioural alternation

No effect was observed on spontaneous behavioural alternation evaluated in a Y maze. CS, CN, AS and AN animals displayed a comparable rate of spontaneous alternation (F-ANOVA = 0.31, n.s., $m,n = 4,28$) (Table 4C).

Discussion

In the present study, we investigated the protection provided by nicotinamide on long-term effects elicited by perinatal asphyxia, focusing on hippocampus delayed, apoptotic-like, cell death, MFS and cognitive performance and anxiety. Nicotinamide prevented the effect of perinatal asphyxia on (1) delayed cell death; (2) non-spatial working memory and (3) anxiety; but (4) not on MFS, that was increased in *SO* of the dorsal hippocampus by perinatal asphyxia.

Interruption of oxygen availability for 20 min at birth produced a significant decrease in the rate of survival, despite intensive clinical manoeuvres for stimulating breathing and recovery. When breathing was started, asphyxia-exposed animals showed a low breathing rate and gasping, sustained by diaphragm and abdominal muscles.

Compared to CS siblings, the rate of breathing was decreased by ~70%, probably prolonging a hypoxic condition. Cardiovascular function was also compromised by perinatal asphyxia, since a cyanotic coloured skin was observed long after recovering from asphyxia, indicating a decreased perfusion blood and decreased oxygenation of peripheral tissue. There was a decrease of vocalisation and spontaneous movements, as well as increased muscle rigidity when the pups were examined 60–80 min after birth, as previously reported (Loidl et al. 1994; Dell'Anna et al. 1997; El-Khodor and Boksa 1997), indicating an impairment of CNS functions. In a recent paper, it was shown that the onset of several reflexes, including negative geotaxis,

Table 4 Behavioural analysis of caesarean-saline (CS), caesarean-nicotinamide (CN), asphyxia-saline (AS) and asphyxia-nicotinamide (AN) treated 3 month old rats

Behavioural Test	CS	CN	AS	AN
(A) Novel object recognition				
S2% (old object)	31.8 ± 5.5 ($n = 9$)	29.1 ± 3.8 ($n = 9$)	44.6 ± 2.3 ($n = 11$)	35.6 ± 6.4 ($n = 6$)
S2% (new object)	69.4 ± 3.1 ($n = 9$)	70.9 ± 3.8 ($n = 9$)	[a]*55.4 ± 2.3* ($n = 11$)	64.4 ± 6.4 ($n = 6$)
(B) Elevated plus-maze				
Open arm entries (in %, compared to enclosed arm entries)	43.0 ± 3 ($n = 7$)	38 ± 5 ($n = 9$)	[a]*28 ± 2* ($n = 11$)	31 ± 3 ($n = 9$)
Open arm time (in %, compared to enclosed arm time)	29.0 ± 5 ($n = 7$)	[a]*14.0 ± 3* ($n = 9$)	[a]*14.3 ± 3* ($n = 11$)	[a]*14.3 ± 3* ($n = 9$)
(C) Spontaneous behavioural alternation				
Open arm entries (in %, compared to the total number of arm entries)	80.5 ± 5.5 ($n = 7$)	79.3 ± 5.8 ($n = 9$)	68.9 ± 5.0 ($n = 11$)	71.2 ± 5.0 ($n = 9$)

(A) Novel object recognition: A reduced time spent exploring a new object is indicative of non-spatial memory deficits. Novel object recognition was assessed following a 60 min interval between S1 and S2 sessions. AS rats spent significantly less time exploring the novel object as compared to CS rats. That difference was not observed when comparing CN versus AN animals. (B) Elevated plus maze: Entries and time spent in the open arms were recorded. A reduced entry and reduced time spent in open arms is indicative of anxiety. AS rats showed a decrease in entries to the open arms, as compared to CS rats. No differences were observed between CS, CN and AN rats. AS, but also CN and AN rats showed a decrease in the time spent in the open arms, as compared to CS animals. (C) Spontaneous behavioural alternation in a Y maze: A high value of alternation is indicative of a good functionality of spatial short-term memory and, more general, of a good cognitive function. No differences were observed amongst the experimental groups, indicating that perinatal asphyxia did not influence the spatial component of cognition

[a] $P < 0.05$, compared to CS (italicised) (F-ANOVA, followed by Tukey's HSD when required)

righting and sensory reflexes, fore- and hindlimb grasping and gaiting are delayed by 1–4 days in animals exposed to severe perinatal asphyxia, when compared to control rats (Kiss et al. 2009), suggesting that the early impairment of CNS functions is long-lasting, although a fast catch-up-growth may occur along development.

Apoptosis

The stereological analysis demonstrated that there was a significant increase of the number of apoptotic nuclei in whole hippocampus of AS compared to CS animals when evaluated 1 month after birth, in agreement with previous reports evaluating the effect of neonatal hypoxia on hippocampal cell death 1 week (Dell'Anna et al. 1997; Morales et al. 2005), 1 month (Bjelke et al. 1991; Johansen et al. 1992; Morales et al. 2008) and 3 months (Kohlhauser et al. 1999; Hoeger et al. 2006) after birth. We recently reported on a parallel increase of pro- and anti-apoptotic protein levels in hippocampus of asphyxia-exposed rats (Morales et al. 2008), in particular of the pro-apoptotic protein BAD, whose total, but not phosphorylated, levels were increased. Indeed, in that report it was found that there was a concomitant decrease of phosphorylated-BAD (at Ser^{112}). Phosphorylation of BAD is an endogenous anti-apoptotic mechanism, creating binding sites for the chaperone 14-3-3, by which BAD is retained in the cytoplasm, preventing its heterodimerisation with BCL-2 and/or BCL-X_L in the mitochondrial membrane (Zhu et al. 2002; Hirai et al. 2004). That heterodimerisation is a critical factor increasing the permeability of the mitochondrial membrane to cytochrome *c* and other pro-apoptotic factors (see Daval et al. 2004; Chen et al. 2005; Morales et al. 2008). We found here that the increase in apoptosis induced by perinatal asphyxia was prevented by nicotinamide.

Mossy fibre sprouting

Mossy fibres originate from granule cells of DG, terminating primarily in the *SL* on the apical dendrites of pyramidal neurons of CA3. Mossy fibres also project distally to the *SO*, impinging on basal dendrites of pyramidal neurons of CA3 (West et al. 1981; West 1983). It has been shown in rats that the intensity of mossy fibre innervation of CA3 neurons is positively correlated with spatial navigation in a Morris water maze (Prior et al. 1997). An increased mossy fibre innervation has been observed in various inbred strains of mice (DBA and C57) after acquisition of a radial arm task (Crusio et al. 1987) and water maze reversal learning (Schopke et al. 1991), both hippocampal-dependent tasks. MFS has also been observed in the dorsal hippocampus after overtraining in a water maze, suggesting that mossy fibre synaptogenesis can be related to spatial long-term memory formation (Ramirez-Amaya et al. 2001; Holahan et al. 2006; Rekart et al. 2007). It has been speculated that MFS leads to the formation of novel recurrent excitatory connections between granule cells, contributing to hippocampal hyperexcitability (Tauck and Nadler 1985; Cavazos et al. 1991; Sutula et al. 1992; Scharfman et al. 2003), although aberrant dentate granule cells target inhibitory interneurons of the IML of the DG, suggesting an increased inhibitory tone following epileptic seizures (Sloviter 1992; Kotti et al. 1997; Sloviter et al. 2006).

In the present study, perinatal asphyxia induced an increase of sprouting in *SO* of dorsal hippocampus, 45 days after birth. No changes in Timm's staining were detected in *SO* of the middle or ventral hippocampus, or in IML, suggesting that perinatal asphyxia promotes the formation of new recurrent excitatory branches from mossy fibre projection to the CA3 region, perhaps improving the connectivity between the affected regions. In agreement, Epsztein et al. (2006) showed that CA3 undergoes reorganization after global ischaemia, suggesting that formation of new recurrent excitatory circuits may be a widespread phenomenon produced by brain injury. An increased Timm's staining has been reported in IML of hippocampus of animals exposed to unilateral hypoxia/ischaemia at P7 (Kadam and Dudek 2007) and at P30 (Williams et al. 2004). An increased Timm's staining has also been detected in IML and *SO* regions of hippocampus following domoic acid induced epilepsy (Bernard et al. 2007). The increased sprouting in *SO* of dorsal hippocampus observed in the present study reflects, perhaps, an improved connectivity with the CA3 region, explaining, as shown here, why short-term spatial memory was not affected by perinatal asphyxia. Nicotinamide did not have any effect on MFS of the hippocampus.

Novel object recognition

In the present study, rats exposed to perinatal asphyxia displayed an impaired performance in a novel object recognition task, suggesting a long-term deleterious effect on non-spatial working memory. The novel object recognition paradigm is based on the rat spontaneous exploratory behaviour, without any involvement of spatial memory, primary reinforcing or stressful cues, such as food deprivation and/or electric shocks (Ennaceur and Delacour 1988; Morrow et al. 2002), hence selective for non-spatial memory. We previously reported that asphyxia-exposed rats displayed deficits in novel object recognition, but not in general motor activity (Simola et al. 2008), it is, therefore, suggested that the impairment in novel object recognition observed here is not due to a decrease in spontaneous exploratory activity. Simola et al. (2008) also showed that a deficit in novel object recognition could only be observed

when 60 min, but not when 15 min, elapsed between the S1 and S2 sessions, indicating that the deficit is not due to a reduced interest for novel objects, in agreement with previous observations that the raw exploration time of objects is not affected by perinatal asphyxia (Simola et al. 2008). Thus, the impairment in novel object recognition likely reflects a specific deficit in non-spatial working memory.

It is well known that hippocampal function is required for spatial and non-spatial working memory (Olton and Feustle 1981). It has been shown that the CA1 subregion is required for processing information provided by the CA3, containing processed information, and by the perforant path, containing unprocessed sensory information (Vinogradova 2001). The processing of the converging inputs by the CA1 subregion may lead to (1) novelty detection (Vinogradova 2001); (2) match–mismatch comparison between current sensory information and learned expectations (Hasselmo and Schnell 1994), and/or (3) facilitation of retrieval mechanisms (Lee and Kesner 2002). Delayed cell death has been observed following perinatal asphyxia in CA1, CA3 and DG regions (Morales et al. 2008), suggesting a neuroanatomical substrate for the impairment of non-spatial working memory observed here. It was also found here that nicotinamide could prevent the deficit of novel object recognition induced by perinatal asphyxia.

Anxiety-related behaviour

The evaluation of elevated plus-maze performance of asphyctic rats revealed a behavioural profile characterised by a reduction of both entries and time spent in the open arms with respect to CS siblings. That behavioural profile suggests an increased level of anxiety (Pellow et al. 1985; Cole et al. 1995). In agreement, Weitzdoerfer et al. (2004) reported an increase of social and anxiety-related behaviours in 2-year-old rats exposed to perinatal asphyxia. Venerosi et al. (2004, 2006) reported that adult rats subjected to perinatal asphyxia displayed an increased emission of ultrasonic vocalisations in response to a stressful condition, suggesting also a high level of anxiety.

The increase of anxiety-related behaviour in asphyctic rats reported here might be due to changes in neurocircuitries participating in emotional control, involving neurons of the ventral hippocampus, the prefrontal cortex and amygdala, regulated by a widespread dopaminergic innervation (Sanders et al. 2003; Kalisch et al. 2006). Since the dopamine pathways have shown to be particularly vulnerable to perinatal asphyxia (Chen et al. 1997; Kohlhauser et al. 1999; Gross et al. 2005; Klawitter et al. 2005, 2007), it is tempting to hypothesise that the anxiety-like behaviour observed here is linked to an impairment of dopamine transmission. Interestingly, nicotinamide prevented the effect of perinatal asphyxia on open arms entries, but not on the time spent in the open arms.

Spontaneous behavioural alternation

Asphyxia-exposed rats did not display any impairment in Y maze performance, suggesting that spatial short-term memory is not affected by perinatal asphyxia, in agreement with previous reports (Boksa et al. 1995; Loidl et al. 2000; Hoeger et al. 2006; Simola et al. 2008).

Nicotinamide treatment

Nicotinamide prevented the effects of perinatal asphyxia (1) on delayed, apoptosis-like, cell death, stereologically quantified in whole hippocampus 1 month after birth; (2) on non-spatial working memory; and (3) on anxiety-related behaviour, evaluated 3 months after birth, even when the treatment was started 24 h after the insult, suggesting a clinically relevant therapeutic window.

Nicotinamide has been shown to protect against oxidative stress (Yan et al. 1999; Wan et al. 1999), ischaemic injury (Sakakibara et al. 2000) and inflammation (Ducrocq et al. 2000). We reported on a prominent effect of nicotinamide on decreased dopamine release induced by perinatal asphyxia (Bustamante et al. 2007) in vivo, and on the length of dopamine neurites evaluated with organotypic cultures, in vitro (Klawitter et al. 2007). The effect of nicotinamide can been explained by replacing the depletion of the $NADH/NAD^+$ pair produced by PARP-1, which is activated whenever there is a metabolic menace to the integrity of the genome (De Murcia and Menissier de Murcia 1994; Kihara et al. 1994; Akhter et al. 2001). Nicotinamide would antagonise the $NADH/NAD^+$ exhaustion and the consequent energy crisis produced by the excessive activation of PARP-1 (Berger 1985; Zhang et al. 1995).

In the present study, nicotinamide antagonised the increase in the number of apoptotic-like nuclei/mm^3 measured in whole hippocampus 1 month after perinatal asphyxia, in agreement with previous reports showing that nicotinamide protects against necrosis and apoptosis in a stroke model performed with adult rats (Yang et al. 2002). Feng et al. (2006) also showed an antagonising effect of nicotinamide on increased caspase-3 activity induced by a carotid artery ligation/hypoxia procedure performed in rats at P7, although the effect was evaluated 24 h after the insult only. In contrast to the effect on apoptosis induced by perinatal asphyxia, nicotinamide did not have any significant effect on MFS, for which we do not have any obvious explanation, although nicotinamide was administered during a short period (a single daily dose, 24, 48 and 72 h after birth), while mossy fibre sprouting implies a slow growth, probably beyond the time-window when the pups were protected by nicotinamide. Furthermore, we previously reported that the same nicotinamide treatment reversed asphyxia-induced effects observed in neostriatum, but not

in substantia nigra (Klawitter et al. 2007), suggesting that the protection provided by nicotinamide is region specific, perhaps associated to the developmental stage of the corresponding region. Nicotinamide exerted a partial counteraction of asphyxia-induced anxiety. Nicotinamide did not affect the time spent in the arms, but only arm entries, suggesting that mechanisms other than PARP-1 overactivation might be involved in the increase of anxiety observed here.

The mechanisms explaining the protection provided by nicotinamide have to be further investigated, including histone deacetylation, a mechanism proposed for introducing nicotinamide as a treatment of Alzheimer's disease and other taupathies (Green et al. 2008). The present results support, however, the idea that nicotinamide has an interesting pharmacological profile; in agreement with the idea that PARP-1 inhibition constitutes a therapeutic strategy against long-term deleterious consequences of perinatal asphyxia, as already proposed for several other pathophysiological conditions (Virag and Szabo 2002).

Compared to more selective compounds (Takahashi et al. 1999; Ducrocq et al. 2000; Abdelkarim et al. 2001; Kamanaka et al. 2004; Iwashita et al. 2004; Nakajima et al. 2005), nicotinamide has a low potency to inhibit PARP-1; therefore, more potent compounds should be investigated on the long-term deleterious effects produced by perinatal asphyxia. The low potency of nicotinamide to inhibit PARP-1 can, however, be a useful feature when administered during development, because the drug will only antagonise the effects produced by PARP-1 overactivation, without impairing DNA repair and cell proliferation. Furthermore, nicotinamide can constitute a lead for exploring compounds with similar pharmacological profile.

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