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RESEARCH ARTICLE

Nicotinamide prevents the effect of perinatal asphyxia on dopamine release evaluated with in vivo microdialysis 3 months after birth

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Abstract The present study shows that nicotinamide prevents the long-term effect of perinatal asphyxia on dopamine release monitored with in vivo microdialysis in the neostriatum of 3-month-old rats. Perinatal asphyxia was induced by immersing foetuses-containing uterine horns removed from ready-to-deliver rats into a water bath for 16 or 20 min. Sibling, spontaneous, and caesarean-delivered pups 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. After weaning, the rats were randomly distributed in laboratory cages for animal care under standard ad libitum laboratory conditions. Approximately 3 months after birth, control and asphyxia-exposed animals were implanted with microdialysis probes into the lateral neostriatum for measuring extracellular monoamine and metabolite levels with HPLC-coupled to an electrochemical detection system under basal, D-amphetamine, and K^+-depolarising conditions. There was an asphyxia-dependent decrease of extracellular dopamine levels, mainly observed during the periods when D-amphetamine (100 μM) or KCl (100 mM) was added into the perfusion medium. Compared to that observed in caesarean-delivered controls, the effect of D-amphetamine on dopamine levels was decreased by approximately 30 and 70% in animals exposed to 16 and 20 min of perinatal asphyxia, respectively. The effect of K^+-depolarisation was decreased by 45 and 83% in animals exposed to the same periods of asphyxia, respectively. Both effects were prevented by nicotinamide, even if the treatment started 24 h after the insult. The present results support the idea of nicotinamide as an interesting molecule, useful for protecting against anoxia/ischemia occurring at neonatal stages. Nicotinamide can help to restore $NADH/NAD^+$ depletion, but also to inhibit PARP-1 overactivation, a mechanism of action that has attracted attention, representing a novel target for neuroprotection following insults involving energy failure.

Keywords Niacinamide · Poly(ADP-ribose) Polymerase (PARP) · Perinatal asphyxia · Dopamine · Striatum · Microdialysis · Rat

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Introduction

Asphyxia implies oxygen deprivation of peripheral and cerebral tissue, resulting in an increased anaerobic metabolism, decreased production of energy-rich phosphate compounds, and accumulation of potentially

toxic metabolites (Carter et al. 1993). Asphyxia also triggers a cascade of biochemical events leading to alterations in cellular function, swelling and necrosis, and/or activation of a metabolic cascade leading to delayed cell death (Lupton et al. 1988; Dell'Anna et al. 1997). The metabolic cascade can also imply an eventual rescue and selective increase of cell viability.

Our previous work has shown that the neurocircuitries of the basal ganglia are particularly vulnerable to perinatal asphyxia, regarding morphological and neurochemical parameters assayed with immunochemistry (Dell'Anna et al. 1995; Chen et al. 1997a,b; Morales et al. 2003; Klawitter et al. 2005) and molecular biology (Andersson et al. 1995; Gross et al. 2000, 2005), in vivo (Dell'Anna et al. 1995, 1997; Chen et al. 1997c), in vitro (Morales et al. 2003; Klawitter et al. 2005), and/or ex vivo biochemistry (Ungethüm et al. 1996; Chen et al. 1997c; Bustamante et al. 2003).

Asphyxia can trigger a cascade of events menacing the stability of the genome, producing the immediate activation of Poly(ADP-ribose) (PAR) polymerases (P) buffering the consequences of the metabolic insult (Amé et al. 2004). The main isoforms are PARP-1 and PARP-2, catalysing the attachment of chains of PAR, by a reaction with NAD^+, to a variety of proteins, including PARP-1 itself. When DNA damage is mild, PARP-1 is involved in the maintenance of chromatin integrity (De Murcia et al. 1994), while excessive activation of PARP-1 leads to NAD^+ exhaustion and energy crisis (Berger 1985), and to a caspase-independent apoptosis (Jiang et al. 1996; Yu et al. 2002; Hong et al. 2004). Hence, PARP-1 inhibition has emerged as a main target for neuroprotection against the consequences of hypoxic/ischemic insults.

Perinatal asphyxia has a great incidence, whenever delivery is prolonged (Berger and Garnier 2000; Volpe 2001; Low 2004; Vannuci and Hagberg 2004). After asphyxia, the infants can develop long-term neurological sequelae, such as cerebral palsy, mental retardation, and epilepsy. In the clinical scenario, after resuscitation, the emphasis is on supportive therapy, although some attempts have been done to prevent, or even to reverse, the neurotoxic cascade elicited by asphyxia. Hypothermia has been shown to be effective by several multicentre trial studies (Gunn and Thoresen 2006), although there is concern for a narrow therapeutic window (Engidawork et al. 2001) and lack of a clear mechanism of action for the effect of hypothermia (see Gluckman et al. 2005).

Nicotinamide has been proposed as protecting against oxidative stress (Wan et al. 1999; Yan et al. 1999), ischemic injury (Sakakibara et al. 2000), and inflammation (Ducrocq et al. 2000) in neonatal brain by replacing $NADH/NAD^+$ (Zhang et al. 1995) or by inhibiting PARP-1 overactivation (Virag and Szabo 2002). Interestingly, we have reported (Bustamante et al. 2003) that nicotinamide prevents several of the changes induced by perinatal asphyxia on monoamine levels in rat brain, even if the treatment is delayed by 24 h, suggesting a clinically relevant therapeutic window.

Thus, we report here on the effect of nicotinamide on asphyxia-induced decrease of dopamine (DA) release in rat neostriatum evaluated 3 months after birth with in vivo microdialysis (Ungerstedt et al. 1982).

Materials and methods

Perinatal asphyxia

Pregnant Wistar rats (UChA, bred at a local colony) within the last day of gestation (G 22), after spontaneous delivery of one or two pups (spontaneously delivered controls), were anaesthetised, euthanized by neck dislocation, and hysterectomised. One or two pups were removed immediately and 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 16 or 20 min. Following asphyxia, pup-containing uterine horns were incised, and the pups delivered and stimulated to breathe. The umbilical cord was ligatured and the animals were left to recover on a heating pad. Forty minutes after delivery, an Apgar evaluation adapted to rats was applied to record several parameters, such as weight, sex, colour of the skin, respiratory frequency, presence of gasping, vocalisations, and spontaneous movements (see Table 1 for details). Finally, surviving pups were given to a surrogate dam for nursing, pending further experiments.

Nicotinamide treatment

Nicotinamide (Sigma, St. Louis, MO, USA) dissolved in saline previously autoclaved was administered at the dose of 0.8 mmol/kg, i.p. (100 mg/kg, i.p.) 24, 48, and 72 h after birth. As a saline control, a sterile saline solution NaCl 0.9% was used to inject control or asphyxia-exposed rats in a volume of 0.1 ml using a 0.5 ml insulin syringe. After 28 days, the rats were separated by sex and housed in plexiglass cages with food and water ad libitum waiting for microdialysis experiments.

In vivo microdialysis

Male, spontaneous-, caesarean-delivered- or asphyxia-exposed (16 or 20 min) saline or nicotinamide treated

Table 1 Apgar scale for evaluating the consequences of perinatal asphyxia 40–80 min after birth

Parameters	Spontaneous delivered pups ($n = 119$; $m = 35–38$)	0-min asphyxia ($n = 550$; $m = 135–137$)	16-min asphyxia ($n = 250$; $m = 75–77$)	20-min asphyxia ($n = 640$; $m = 135–137$)	H-Kruskal–Wallis ANOVA
Weight (g)	5.6 ± 0.1	5.1 ± 0.1	5.1 ± 0.1	5.1 ± 0.1	4.039, n.s.
Sex (% of males)	49.2 ± 6.2%	49.2 ± 5.8%	48.0 ± 6.6%	40.8 ± 3.0%	2.031, n.s.
Rate of survival (%)	100 ± 0%	100 ± 0.2%	99 ± 0.8	**56 ± 3***,**	**45.07**
Respiratory frequency (events/min)	65 ± 2	63 ± 1	**47 ± 2***	**24 ± 1***,**	**92.02**
Presence of gasping (yes) (%)	0 ± 0%	0 ± 0%	**6 ± 2%***	**56 ± 4%***,**	**17.49**
Skin colour					
Pink	100 ± 0%	93 ± 2%	**30 ± 5%***	**1 ± 1%***,**	**82.21**
Pink–blue	0 ± 0%	6 ± 2%	**57 ± 5%***	**39 ± 4%***,**	**47.36**
Blue–pink	0 ± 0%	0 ± 0%	**13 ± 3%***	**29 ± 3%***,**	**15.35**
Blue	0 ± 0%	0 ± 0%	1 ± 1%	**31 ± 3%***,**	**52.87**
Presence of vocalisations (yes) (%)	100 ± 0%	95 ± 2%	**73 ± 4%***	**15 ± 2%***,**	**68.25**
Spontaneous movements					
No movements, akinesia, rigidity (0)	0 ± 0%	0 ± 0%	**29 ± 4%***	**87 ± 2%***,**	**80.5**
Single movement of front legs, or head alone (1)	0 ± 0%	0 ± 0%	**17 ± 3%***	**7 ± 2%***,**	**10.5**
Movement of two body structures (2)	0 ± 0%	3 ± 1%	**29 ± 4%***	**6 ± 1%****	**30.82**
Movement of all body structures (3)	3 ± 2%	3 ± 1%	**20 ± 3%***	**1 ± 1%****	**11.97**
Intensive movements shown by wriggling (4)	97 ± 2%	94 ± 2%	**6 ± 2%***	**0 ± 0%***,**	**98.21**
Lack of reception by surrogate dams (at 24 h)	18.5%	21.1%	29.6%	20.9%	1.49, n.s.

n, number of pups; *m*, number of mothers ($n = 137$)

*$p < 0.05$, versus caesarean delivered pups

**$p < 0.05$, 20 min vs. 16 min of asphyxia (bold, underlined) (Dunn test)

rats weighing 240–300 g were anaesthetised with a mixture of air and isofluorane and placed in a Kopf stereotaxic frame with the skull oriented according to the atlas of Paxinos and Watson (1982). As described before (Herrera-Marschitz et al. 1996), a microdialysis probe (dialysing length, 4 mm; diameter, 0.5 mm; model CMA 12, CMA/Microdialysis AB, Stockholm, Sweden) was implanted into the lateral neostriatum with the following coordinates: A 0.7, L ±3.5, V −7.2 (Paxinos and Watson 1982) with a coronal plane.

The microdialysis probes were perfused with a Ringer solution (pH = 6.7–7.0) at a flow rate of 2 μl/min using a microinjection pump (model CMA 100, CMA/Microdialysis AB). Sixty micro litre perfusates (each 30 min) were collected using a microfraction collector (model CMA 140, CMA/Microdialysis AB). One hundred and eighty minutes after the implantation of a microdialysis probe, 100 μM of D-amphetamine diluted in the Ringer solution was perfused into the neostriatum via the probes for a 30-min period. Two further Ringer-alone samples were taken and then 100 mM KCl was added to the perfusion medium to induce K^+-depolarisation (270–300 min after the beginning of the microdialysis experiment). Changes of the perfusion medium were performed with a syringe selector (model CMA 111, CMA/Microdialysis AB). The rats were maintained under isoflurane anaesthesia throughout the experiments by free breathing into a mask fitted over the nose of the animal (1.5–2.0% isofluorane in an air flow of 1.5 l/min). Body temperature was maintained at 37°C by using a temperature control system (CMA 150, CMA/Microdialysis AB) and breathing, heart frequency, and motility were permanently observed. Samples were collected every 30 min (60 μl) and assayed for monoamines and metabolites.

Biochemical analysis

Dopamine and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxytryptamine metabolite, 5-hydroxyindoleacetic

acid (5-HIAA) were measured with high-performance liquid chromatography (HPLC) coupled to electrochemical detection as previously described (Bustamante et al. 2002) using a liquid chromatography pump adjusted to 0.45 ml/min (model CMA 250 CMA/Microdialysis AB), an autoinjector (model CMA 200, CMA/Microdialysis AB), a Synergy 4-Hydro-RP column (Phenomenex, Torrance, CA, USA), and a carbon electrode adjusted to 700 mV (BAS, Tokyo, Japan). The identification and quantification of the substances was achieved by comparison with standard solutions. Peak integration was performed in a PC with an ad-hoc analogous-digital card and a CSW® software (Pronexus, Stockholm, Sweden); the detection limit was 0.2 nM for DA, DOPAC and 5-HIAA and 1 nM for HVA.

Drugs

Standard substances for analytical determination (DA, DOPAC, HVA, and 5-HIAA), nicotinamide, and D-amphetamine sulphate were purchased from Sigma. Doses were corrected for the free bases of each drug.

Statistics

The levels of the assayed substances are expressed as the concentrations found in the perfusates (mean ± SEM). Basal values refer to the values obtained before drug administration, and are set as 100%. The results were analysed using one-way analysis of variance (F-ANOVA) and a post hoc test (Bonferroni's test for multiple pair-wise comparisons) when required. The results obtained by the Apgar scale were analysed with Kruskal–Wallis one-way ANOVA and Dunn's post hoc test when required. A level of $p < 0.05$ was considered critical for statistical significance.

The protocols were approved by a Local Ethics Committee for Experiment with Laboratory Animals at the Medical Faculty (Protocol CBA#0136, FMUCH) and by the ad-hoc commission of the Chilean Council for Science and Technology Research (CONICYT), endorsing the principles of laboratory animal care (NIH; no. 86-23; revised 1985).

Results

Evaluation of the immediate consequences of asphyxia

Table 1 shows an Apgar scale adapted for evaluating the outcome of birth in rats. Among several monitored parameters, the rate of survival, which is the variable with the largest physiological relevance, was significantly decreased following 20 min of asphyxia (~50%).

After perinatal asphyxia, the establishment of pulmonary respiration was delayed, demanding intensive resuscitation work for starting and maintaining breathing. Breathing was characterised at the beginning by gasping, supported by diaphragm and abdominal muscles. This abnormal type of breathing was still observed 40–80 min after birth in ~50% of the pups recovering from 20 min of asphyxia. Respiratory frequency was decreased, mainly following 20 min of asphyxia, but also significantly decreased following 16 min of asphyxia. At birth, the colour of the skin reflects peripheral blood perfusion, and was used as an indicator of cardiovascular function. A pink skin colour indicates a successful perfusion, while a blue skin colour indicates a cyanotic condition. Thus, a pink-blue, a blue-pink, or a blue skin indicate a graded impairment of peripheral blood perfusion, reflecting a decrease of oxygenated blood perfusion, mainly affecting pups recovering from 20 min of asphyxia. These observations indicate that 40–80 min after birth, respiratory and cardiovascular functions are still compromised, prolonging or perpetuating a condition of hypoxia and ischemia, probably affecting the CNS after resuscitation is established. Indeed, following perinatal asphyxia, there was a decrease of vocalisation and spontaneous movements, and an increase of muscle rigidity, mainly affecting hind legs. Following 20 min of asphyxia, the majority of the pups were still akinetic and showed signs of rigidity 40–80 min after birth. The rejection rate by the surrogate dams, recorded 24 h after birth, was, however, ~20% for all experimental groups, probably indicating a skilful nursery rather than a feature related to the physiological condition of the pups. No rejection was observed if the pups were well received during the first 24 h. After that, control and asphyxia exposed pups were randomly distributed for saline (1 ml/kg, i.p.) or nicotinamide (0.8 mmol/kg, i.p.) treatment, one dose daily, 24, 48, and 72 h after birth.

In vivo microdialysis

After weaning, the rats were randomly distributed in laboratory cages for animal care under the standard ad libitum laboratory conditions. Two to three months after birth, control and asphyxia-exposed animals were implanted with microdialysis probes into the lateral neostriatum for measuring extracellular monoamine levels and metabolites under basal, D-amphetamine, and K^+-depolarising conditions.

Table 2 shows extracellular DA and metabolite levels observed under basal and D-amphetamine

(100 μM) conditions in neostriatum of saline (a)- or nicotinamide (b)-treated control (spontaneous- and caesarean-delivered) and asphyxia-exposed (16 and 20 min) rats. Figure 1 shows the time-course of DA levels monitored through the experimental conditions.

Under basal conditions, DA was detected in the nM, while the metabolites were detected in the sub μM range. DA, but not metabolite levels, were increased (>tenfold) by local administration of D-amphetamine (100 μM). As tested for multiple comparisons (F-ANOVA), no significant differences were observed between saline-treated, spontaneous- and caesarean-delivered rats. Thus, in the present study, all further comparisons were against the caesarean-delivered control condition if not specifically stated.

As shown in Table 2a, F-ANOVA revealed significant differences between saline-treated, caesarean-delivered, and asphyxia-exposed groups, evaluated under basal and D-amphetamine-stimulated conditions, allowing further post hoc comparisons. Under the basal condition, DA levels were decreased by ~40% in saline-treated, asphyxia-exposed (42% for 16 min; 44% for 20 min of asphyxia), compared to the corresponding caesarean-delivered control animals. The effect of perinatal asphyxia was even more prominent when DA levels were compared under the D-amphetamine condition. Under that condition, DA levels were decreased by 33 and 68% following 16 and 20 min of perinatal asphyxia, respectively (Fig. 1a). DOPAC levels were also significantly decreased following 20 min of asphyxia (Table 2a).

As shown in Table 2b, no significant differences (F-ANOVA) were observed between nicotinamide-treated, caesarean-delivered, and asphyxia-exposed groups, although the net effect of D-amphetamine was slightly decreased in asphyxia-exposed, compared to that observed in the corresponding caesarean-delivered nicotinamide-treated control animals (by ~38 and 37%, for 16 and 20 min of asphyxia treated with nicotinamide, respectively) (cf. Fig. 1b versus a). Multiple comparison analysis (F-ANOVA) revealed, however, significant (between-group) differences between nicotinamide-treated, spontaneous- versus caesarean-delivered groups, affecting HVA levels, which were decreased in nicotinamide-treated, caesarean-delivered animals.

Table 3a shows the effect of K^+-depolarisation following saline treatment. Multiple comparison analysis (F-ANOVA) revealed significant differences between caesarean-delivered, 16 and 20 min asphyxia groups, allowing further post hoc analysis. Under the selected basal condition (240–270 min), DA levels were decreased by ~45% in both asphyxia-exposed, compared to caesarean-delivered control animals treated with saline. The effect of perinatal asphyxia was even more prominent when analysed under the K^+-depolarising condition, revealing that DA levels were decreased by 83% after 20 min of asphyxia, compared to the corresponding caesarean-delivered control. DOPAC levels were significantly decreased in animals exposed to 20 min of asphyxia, whether compared to

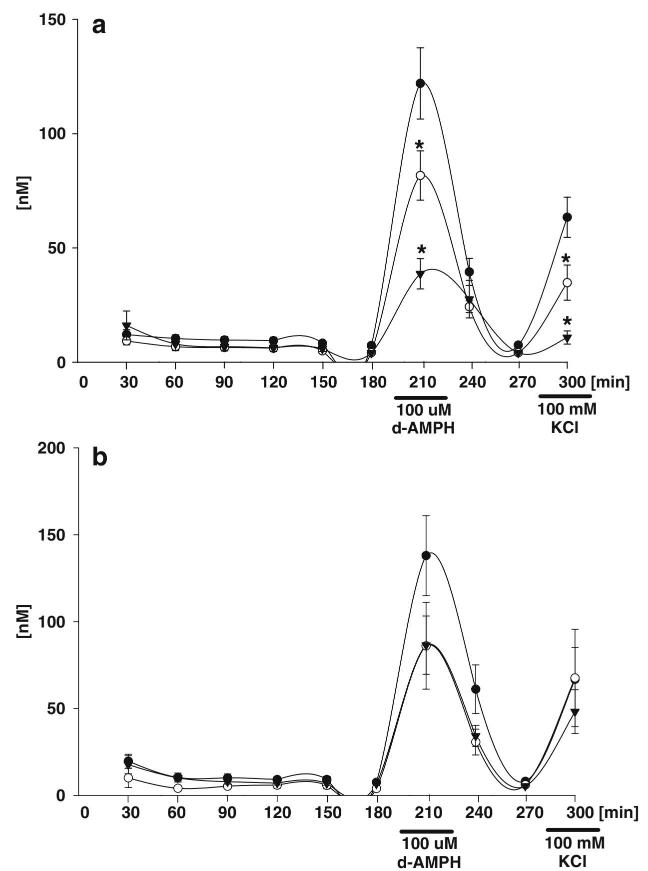

Fig. 1 Time-course of dopamine release monitored with microdialysis under basal, D-amphetamine (D-Amph) (100 μM), and K^+-depolarising (100 mM KCl) conditions in neostriatum of 3-month-old caesarean-delivered control and asphyxia-exposed rats treated neonatally with saline (1 ml/kg, i.p. sterile NaCl; 24, 48 and 72 h after birth) (**a**) or nicotinamide (0.8 mmol/kg, i.p.) (**b**). *Filled circle*, caesarean-delivered control rats (saline, $n = 16$; nicotinamide, $n = 8$); *open circle*, 16-min asphyxia-exposed rats (saline, $n = 10$; nicotinamide, $n = 6$); *filled triangle*, 20-min asphyxia-exposed rats (saline, $n = 10$; nicotinamide, $n = 14$). A spline equation was applied for best fitting the plotted curves. *Abscissa*: time (min) after microdialysis probe implantation. *Ordinate*: dopamine (nM); *Vertical lines* show SEM. $*p < 0.05$, F-ANOVA, followed by Benferroni post hoc test to compare with the corresponding caesarean-delivered control

Table 2 Effect of saline (a) or nicotinamide (b) treatment on striatal extracellular monoamine and metabolite levels (nM) monitored with in vivo microdialysis under basal and D-amphetamine (D-Amph) conditions 2–3 months after perinatal asphyxia

	Basal levels (150–180 min)	D-Amph (180–210 min)	
	(nM)	(nM)	%
(a) Saline-treated			
Saline-treated spontaneous delivered rats (Sp S) (body weight: 259 ± 17; $n = 6$)			
Dopamine	6.8 ± 0.8	**101.2 ± 23.6***	**1,460 ± 265***
DOPAC	394 ± 24	373 ± 28	95 ± 4
HVA	378 ± 43	366 ± 29	97 ± 6
5-HIAA	265 ± 35	271 ± 31	100 ± 5
Saline-treated caesarean delivered rats (CS 0 min) (body weight: 305 ± 8; $n = 18$)			
Dopamine	7.3 ± 0.8	**122 ± 15.6***	**1,635 ± 152***
DOPAC	472 ± 55	452 ± 67	95 ± 7
HVA	378 ± 38	305 ± 12	78 ± 5
5-HIAA	230 ± 21	198 ± 15	83 ± 6
F-ANOVA $_{\text{(SP S versus CS 0 min)}}$ ($m, n = 2, 24$)			
Dopamine	0.11, n.s.	0.47, n.s.	
DOPAC	1.72, n.s.	1.19, n.s.	
HVA	0.0, n.s.	3.78, n.s.	
5-HIAA	0.68, n.s.	4.53, n.s.	
Saline-treated 16-min asphyxia (AS 16 min) (body weight: 258 ± 10; $n = 10$)			
Dopamine	**4.2 ± 0.5****	**81.7 ± 10.8***	**2,068 ± 205***
DOPAC	416 ± 46	400 ± 35	97 ± 2
HVA	**573 ± 35****	**532 ± 24****	94 ± 5
5-HIAA	336 ± 27	304 ± 31	92 ± 8
Saline-treated 20-min asphyxia (AS 20 min) (body weight: 270 ± 18; $n = 10$)			
Dopamine	**4.1 ± 0.4****	**38.7 ± 6.6***,**	**852 ± 134***
DOPAC	**252 ± 42****	**243 ± 44****	97 ± 8
HVA	355 ± 65	349 ± 67	96 ± 3
5-HIAA	206 ± 50	225 ± 52	116 ± 24
F-ANOVA $_{\text{(CS 0, AS 16, AS 20 min)}}$ ($m, n = 3, 38$)			
Dopamine	**6.3, $p \le 0.05$**	**9.28, $p \le 0.05$**	
DOPAC	**6.01, $p \le 0.05$**	**5.32, $p \le 0.05$**	
HVA	**7.23, $p \le 0.05$**	**8.68, $p \le 0.05$**	
5-HIAA	3.12, n.s.	1.87, n.s.	
(b) Nicotinamide-treated			
Nicotinamide-treated spontaneous delivered rats (body weight: 236 ± 16; $n = 6$)			
Dopamine	6.5 ± 0.5	**156 ± 20***	**2,269 ± 227***
DOPAC	388 ± 9	397 ± 4	103 ± 3
HVA	596 ± 46	632 ± 36	109 ± 10
5-HIAA	303 ± 37	346 ± 47	115 ± 13
Nicotinamide-treated caesarean delivered rats (CN 0 min) (body weight: 267 ± 21; $n = 8$)			
Dopamine	7.5 ± 1.2	**138 ± 23***	**1,631 ± 130***
DOPAC	411 ± 27	408 ± 25	99 ± 1
HVA	**398 ± 33*****	**362 ± 37*****	92 ± 6
5-HIAA	270 ± 20	257 ± 27	97 ± 7
F-ANOVA $_{\text{(SP N versus CN 0 min)}}$ ($m, n = 2, 14$)			
Dopamine	0.92, n.s.	0.26, n.s.	
DOPAC	0.56, n.s.	0.15, n.s.	
HVA	**12.97, $p \le 0.05$**	**26.66, $p \le 0.05$**	
5-HIAA	0.61, n.s.	2.66, n.s.	
Nicotinamide-treated 16-min asphyxia (AN 16 min) (body weight: 245 ± 19; $n = 6$)			
Dopamine	4.0 ± 1.1	**86.1 ± 25***	**2,116 ± 225***
DOPAC	353 ± 28	326 ± 43	91 ± 8
HVA	518 ± 38	514 ± 35	103 ± 3
5-HIAA	272 ± 40	273 ± 46	100 ± 9
Nicotinamide-treated 20-min asphyxia (AN 20 min) (body weight: 298 ± 11; $n = 14$)			
Dopamine	6.6 ± 0.8	**86.5 ± 16.8***	**1,567 ± 175***
DOPAC	370 ± 25	349 ± 25	95 ± 4
HVA	442 ± 41	468 ± 36	93 ± 5
5-HIAA	257 ± 29	257 ± 28	100 ± 7

Table 2 continued

	Basal levels (150–180 min)	D-Amph (180–210 min)	
	(nM)	(nM)	%
5-HIAA	257 ± 29	257 ± 28	100 ± 7
F-ANOVA $_{(CN\ 0,\ AN\ 16,\ AN\ 20\ min)}$ ($m, n = 3, 28$)			
Dopamine	3.04, n.s.	1.87, n.s.	
DOPAC	1.14, n.s.	1.55, n.s.	
HVA	1.41, n.s.	0.79, n.s.	
5-HIAA	0.07, n.s.	0.06, n.s.	

Spontaneous and caesarean-delivered siblings were used as controls. The effect of D-Amph is expressed as the percentage of the respective basal value immediately before adding the drug into the perfusion medium

*$p < 0.05$, compared to basal values

**$p < 0.05$, compared to the effect observed after a caesarean-delivered condition (0-min asphyxia)

***$p < 0.05$, compared to the effect observed after spontaneous delivery (Sp S or Sp N) (F-ANOVA; followed by Bonferroni post hoc test) (underlined and bold)

caesarean- or 16 min asphyxia-exposed animals (Table 3a).

Table 3b shows the effect K^+-depolarisation following nicotinamide treatment. Multiple comparison analysis (F-ANOVA) revealed that there were no differences between nicotinamide-treated, caesarean-delivered, and asphyxia-exposed groups, although the effect of K^+-depolarisation appeared to be decreased by 28% after 20 min of asphyxia, compared to the corresponding control (cf. Fig. 1b versus a). Multiple comparison analysis revealed significant differences between nicotinamide-treated, spontaneous- versus caesarean-delivered groups, affecting HVA levels, which were decreased in nicotinamide-treated, caesarean-delivered animals.

Discussion

The present results show that nicotinamide prevents the long-term effect of perinatal asphyxia on DA release evaluated with in vivo microdialysis 3 months after birth. DA release was monitored in the neostriatum, the main target of the nigro-striatal DA system (Ungerstedt 1971), shown to be particularly vulnerable to global anoxia/ischemia occurring at neonatal (Gunn et al. 1991; Pasternak et al. 1991; Pastuzko 1994; Cowan et al. 2003; Miller et al. 2005; Barkovich 2006) and adult (Pusinelli et al. 1982; see Haddad and Jiang 1993; Calabresi et al. 2000; Venkatesan and Frucht 2006) stages.

It was found here that there was an asphyxia-dependent decrease of extracellular DA levels, mainly observed during the periods when D-amphetamine (100 μM) or KCl (100 mM) was added into the perfusion medium. Compared to that observed in caesarean-delivered control rats, the effect of D-amphetamine on DA levels was decreased by approximately 30 and 70% in animals exposed to 16 and 20 min of perinatal asphyxia, respectively. The effect of K^+-depolarisation was decreased by 45 and 83% in animals exposed to 16 and 20 min of perinatal asphyxia, respectively. Both effects were prevented by nicotinamide, even if the treatment started 24 h after the insult.

The present results confirm and expand previous observations indicating that perinatal asphyxia produces long-term effects on neurotransmission systems of the basal ganglia of the rat, whether evaluated with in vivo microdialysis (Loidl et al. 1994; Chen et al. 1997b), ex vivo biochemistry (Ungethüm et al. 1996), or histochemistry (Chen et al. 1995, 1997a; Kohlhauser et al. 1999a,b). D-amphetamine was used to stimulate Ca^{2+}-independent DA release from a newly synthesised cytosolic pool (Zetterström et al. 1983; Hurd and Ungerstedt 1989), while K^+-depolarisation was used to mainly stimulate DA release from the Ca^{2+}-dependent vesicular pool (Butcher et al. 1988; Herrera-Marschitz et al. 1992). As shown here, it was found before that the effect of perinatal asphyxia on monoamine levels was magnified when monitored under D-amphetamine (Herrera-Marschitz et al. 1994; Loidl et al. 1994) or K^+-depolarising (Chen et al. 1997b) conditions, revealing a prominent deficit in synthesis and releasable pools of DA. These observations are not trivial because they agree with a common clinical observation (Roland et al. 1998) that challenging is a requirement for unmasking deficits, which can be compensated or hidden under homeostasis, but not under demanding physiological and/or environmental conditions.

In the present experimental protocol, the caesarean-delivered condition provides the closer control to the asphyxia-induced condition, although spontaneous-

Table 3 Effect of saline (a) or nicotinamide (b) treatment on striatal extracellular monoamine and metabolite levels (nM) monitored with in vivo microdialysis under basal and K^+-depolarising (KCl) conditions 2–3 months after perinatal asphyxia

	Basal level (240–270 min)	K^+-depolarisation (270–300 min)	
	(nM)	(nM)	%
(a) Saline-treated			
Saline-treated spontaneous delivered rats (body weight: 259 ± 17; n = 6)			
Dopamine	6.4 ± 1.1	**87.6 ± 29.1***	**1,359 ± 398***
DOPAC	312 ± 32	312 ± 34	100 ± 5
HVA	318 ± 17	**235 ± 15***	**79 ± 6***
5-HIAA	262 ± 27	228 ± 27	80 ± 4
Saline-treated caesarean delivered rats (CS 0 min) (body weight: 305 ± 8; n = 16)			
Dopamine	7.5 ± 0.9	**63.4 ± 8.8***	**1,056 ± 96***
DOPAC	433 ± 56	397 ± 45	83 ± 4
HVA	367 ± 50	247 ± 35	69 ± 3
5-HIAA	241 ± 19	172 ± 12	66 ± 9
F-ANOVA $_{\text{(SP S versus CS 0 min)}}$ (m, n = 2, 22)			
Dopamine	0.5, n.s.	0.97, n.s.	
DOPAC	3.99, n.s.	2.39, n.s.	
HVA	1.03, n.s.	0.11, n.s.	
5-HIIA	0.36, n.s.	2.80, n.s.	
Saline-treated 16-min asphyxia (AS 16 min) (body weight: 258 ± 10; n = 10)			
Dopamine	**4.2 ± 0.4****	**34.8 ± 7.7***	**809 ± 15***
DOPAC	393 ± 31	361 ± 27	92 ± 4
HVA	500 ± 32	401 ± 29	77 ± 4
5-HIAA	312 ± 31	268 ± 30	84 ± 6
Saline-treated 20-min asphyxia (AS 20 min) (body weight: 270 ± 18; n = 10)			
Dopamine	**4.1 ± 0.4****	**10.8 ± 2.9***,**	**249 ± 52***,**
DOPAC	**276 ± 55****	**202 ± 56****	74 ± 15
HVA	316 ± 58	222 ± 64	73 ± 16
5-HIAA	199 ± 56	135 ± 50	70 ± 17
F-ANOVA $_{\text{(CS 0, AS 16, AS 20 min)}}$ (m, n = 3, 36)			
Dopamine	**7.07, $p \le 0.05$**	**11.16, $p \le 0.05$**	
DOPAC	**3.43, $p \le 0.05$**	**6.15, $p \le 0.05$**	
HVA	**3.82, $p \le 0.05$**	**4.18, $p \le 0.05$**	
5-HIAA	1.85, n.s.	**3.89, $p \le 0.05$**	
(b) Nicotinamide-treated			
Nicotinamide-treated spontaneous delivered rats (body weight: 236 ± 16; n = 6)			
Dopamine	6.4 ± 1.1	**91.4 ± 27.9***	**1,569 ± 583***
DOPAC	363 ± 31	347 ± 47	96 ± 3
HVA	598 ± 43	462 ± 51	77 ± 7
5-HIAA	374 ± 32	290 ± 28	81 ± 9
Nicotinamide-treated caesarean delivered rats (CN 0 min) (body weight: 267 ± 21; n = 8)			
Dopamine	8.1 ± 1.2	**66.8 ± 17.3***	**842 ± 116***
DOPAC	406 ± 28	379 ± 36	92 ± 4
HVA	**370 ± 29*****	**255 ± 24*****	69 ± 4
5-HIAA	304 ± 17	221 ± 16	72 ± 2
F-ANOVA $_{\text{(SP N versus CN 0 min)}}$ (m, n = 2, 14)			
Dopamine	1.04, n.s.	0.58, n.s.	
DOPAC	1.04, n.s.	0.3, n.s.	
HVA	**20.84, $p \le 0.05$**	**15.89, $p \le 0.05$**	
5-HIIA	3.72, n.s.	4.55, n.s.	
Nicotinamide-treated 16-min asphyxia (AN 16 min) (body weight: 245 ± 19; n = 6)			
Dopamine	6.1 ± 1.2	**67.6 ± 28***	**1,135 ± 418***
DOPAC	394 ± 29	360 ± 35	86 ± 6
HVA	500 ± 51	456 ± 55	84 ± 7
5-HIAA	239 ± 51	203 ± 52	85 ± 10
Nicotinamide-treated 20-min asphyxia (AN 20 min) (body weight: 298 ± 11; n = 14)			
Dopamine	5.4 ± 0.6	**48.2 ± 12.6***	**867 ± 178***
DOPAC	343 ± 21	306 ± 23	86 ± 3
HVA	434 ± 38	353 ± 41	81 ± 3
5-HIAA	264 ± 28	231 ± 26	88 ± 3

Table 3 continued

	Basal level (240–270 min)	K$^+$-depolarisation (270–300 min)	
	(nM)	(nM)	%
F-ANOVA $_{(CN\ 0,\ AN\ 16,\ AN\ 20\ min)}$ (m, n = 3, 28)			
Dopamine	2.63, n.s.	0.44, n.s.	
DOPAC	1.86, n.s.	1.85, n.s.	
HVA	0.86, n.s.	1.83, n.s.	
5-HIAA	0.72, n.s.	0.17, n.s.	

Spontaneous and caesarean-delivered siblings were used as controls. The effect of KCl is expressed as the percentage of the respective basal value immediately before adding KCl (100 mM) into the perfusion medium

*$p < 0.05$, compared to basal values

**$p < 0.05$, compared to the effect observed after a caesarean-delivered condition (0-min asphyxia)

***$p < 0.05$, compared to the effect observed at the corresponding spontaneous delivery control (Sp S; Sp N) (F-ANOVA; followed by Bonferroni post hoc test) (underlined and bold)

delivered controls were also obtained from the same experimental dam. The time-course of the procedure was, however, delayed for the caesarean-excised animals, which reflected in the body-weight, approximately 0.5 g higher in spontaneous- versus caesarean-delivered animals. Indeed, spontaneous delivery can proceed for some hours before the rat is subjected to the caesarean delivery.

While no differences were observed between saline-treated, spontaneous- versus caesarean-delivered animals, multiple comparison analysis revealed significant differences between nicotinamide-treated, spontaneous- versus caesarean-delivered groups, affecting HVA levels, which were decreased in nicotinamide-treated, caesarean-delivered animals. In a previous work, we reported slightly higher tyrosine hydroxylase mRNA levels in neostriatum of caesarean-delivered, compared to spontaneously born animals, although no short-term changes were observed in tyrosine hydroxylase gene expression (Gross et al. 2005). Several authors have reported long-term changes in dopaminergic function and biochemistry due to the caesarean procedure (see Boksa and El-Khodor 2003), and therefore, spontaneously born animals have always been included along the experimental protocol. In the present study, the only difference between both control conditions was on HVA levels following nicotinamide treatment, an observation that is still unexplained.

In a previous paper (Bustamante et al. 2003), we investigated the effect of nicotinamide on the long-term effects of perinatal asphyxia on basal ganglia monoamine contents, demonstrating a protective effect. Here, we have complemented that finding, demonstrating protection against the deleterious effect of perinatal asphyxia on DA release.

Nicotinamide has been proposed as protecting against oxidative stress (Wan et al. 1999; Yan et al. 1999), ischemic injury, and inflammation (Ducrocq et al. 2000; Sakakibara et al. 2000) in neonatal rat brain. Nicotinamide can help to restore NADH/NAD$^+$ depletion, but also to inhibit PARP-1 overactivation (see Virag and Szabo 2002). That PARP-1 inhibitory mechanism has attracted attention, because it can represent a novel target for neuroprotection following insults involving energy failure.

Upon re-oxygenation, there is a cascade of biochemical events worsening the biological outcome elicited by a deficient metabolism. One of the biochemical events involves the PAR polymerase family, which is activated whenever there is a risk for DNA damage. Indeed, the polymerase isoforms, PARP-1 and PARP-2, are immediately activated when the integrity of the genome is menaced and/or damaged (Kihara et al. 1994; Akhter et al. 2001; Amé et al. 2004). PARP-1 catalyses the attachment of chains of PAR, by reaction with NAD$^+$, to a variety of nuclear proteins, including PARP-1 itself. When DNA damage is mild, PARP-1 is involved in the maintenance of chromatin integrity (De Murcia et al. 1994; Ying et al. 2005). Excessive activation of PARP-1 leads, however, to NAD$^+$ exhaustion and energy crisis (Berger 1985), and to a caspase-independent apoptosis, via translocation of the mitochondrial pro-apoptotic protein, apoptosis-inducing factor (AIF), to the nucleus, initiating nuclear condensation (Jiang et al. 1996; Yu et al. 2002; Hong et al. 2004). Interestingly, we have reported that perinatal asphyxia leads to delayed neuronal death, mainly affecting neostriatum and neocortex (Dell'Anna et al. 1997).

The strongest evidence for the hypothesis that PARP-1 inhibition can constitute a target for neuroprotection following hypoxic/ischemic insults is from studies showing that the outcome of ischemic injury is decreased in PARP(-/-) mice (Eliasson et al. 1997), supporting previous evidence that PARP inhibitors,

with increasing degrees of potency, decrease brain damage and improve the neurological outcome of perinatal brain injury (Zhang et al. 1995; Ducrocq et al. 2000; Sakakibara et al. 2000). Apart from inhibiting PARP-1 overactivation (see Virag and Szabo 2002), nicotinamide has been proposed as an agent against oxidative stress (Wan et al. 1999; Yan et al. 1999), ischemic injury (Sakakibara et al. 2000), and inflammation (Ducrocq et al. 2000) in neonatal rat brain. However, the use of nicotinamide has been challenged because of its low potency, limited cell uptake, and short cell viability, stimulating the investigation for more specific compounds inhibiting PARP-1 overactivation (Takahashi et al. 1999; Ducrocq et al. 2000; Abdelkarim et al. 2001; Iwashita et al. 2004; Kamanaka et al. 2004; Nakajima et al. 2005).

However, the present results still support the idea of nicotinamide as an interesting molecule, because the effects reported here show a wide therapeutic window (24 h). Its pharmacodynamic properties can provide advantages over more selective compounds. Nicotinamide low potency in inhibiting PARP-1 can be useful when administered to developing animals, because the drug will only antagonise the effect of PARP-1 overactivation, without impairing DNA repair and cell proliferation. Furthermore, nicotinamide can constitute a lead for exploring compounds with similar pharmacological profile.

In conclusion, nicotinamide prevents (with a wide therapeutic window) long-term DA neurotransmission deficits induced by perinatal asphyxia. The effect of nicotinamide enlightens the enzyme PARP-1 as a novel target for neuroprotection following insults involving energy failure.

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