EXPERIMENTAL STUDY

Morphine-induced stimulation of pituitary–adrenocortical activity is mediated by activation of nitric oxide in the early stages of postnatal life in the rat

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Abstract

Objective: The first aim of the present study was to determine if morphine, a prototypic μ -opioid agonist drug, affects pituitary–adrenocortical activity in developing rat pups (first and second weeks of postnatal life). The second aim of this study was to explore, *in vivo*, if nitric oxide (NO) could be involved in the neurohormonal response to morphine in the early stages of postnatal life.

Methods: Plasma ACTH and corticosterone concentrations were determined by RIA in rat pups ($n = 5$ –14 rats/experimental group) after they had been killed by decapitation. In a first experiment, 1-day and 1- and 2-week-old rats were treated s.c. with morphine (20 mg/kg) or with vehicle (0.9% NaCl) and killed 5–90 min later. In a second experiment, 2-week-old pups were pretreated s.c. with naltrexone (NAL; 0.4 mg/kg or 10 mg/kg), and injected 1 h later with either morphine (20 mg/kg) or vehicle, and killed 30 min later. Some pups injected with only NAL were killed 60 or 90 min later. On the other hand, pups injected with NAL (10 mg/kg) or NAL and morphine were killed 30 min later. In a third experiment, 2-week-old pups were pretreated s.c. with N- ω -nitro-L-arginine methyl-ester (L-NAME; 30 mg/kg or 100 mg/kg), and injected 1 h later with either morphine (20 mg/kg) or vehicle, and killed 30 min later. Moreover, some pups injected with L-NAME (100 mg/kg) or L-NAME with morphine were killed 30 min later. In a final experiment, pups were injected s.c. with either S-nitroso-N-acetylpenicillamine (SNAP; 5 mg/kg) or vehicle, and killed 60 or 90 min later.

Results: Morphine administered to rat pups elicited marked rises in both ACTH and corticosterone secretion. Moreover, these responses increased with advancing postnatal age. In 2-week-old rat pups, NAL, a competitive antagonist at μ -opioid receptors, administered alone increased both plasma ACTH and corticosterone concentrations 30 min later. L-NAME, a specific NO synthase inhibitor, did not affect plasma ACTH and corticosterone concentrations 30 min later when administered alone. NAL, when concomitantly administered with morphine, was unable to block morphine responses. In contrast, morphine responses were blocked by pretreatment (60 min before) with NAL or with L-NAME. Acute injection of SNAP increased both ACTH and corticosterone release.

Conclusion: Our results suggest that opioids have controversial effects on pituitary–adrenocortical activity in the early postnatal period in the rat, and that endogenous NO is one of the major factors in the response of the pituitary–adrenocortical axis to morphine.

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Introduction

In adult rats, acute administration of morphine has been reported to stimulate the hypothalamo–pituitary–adrenal (HPA) axis (1) but to inhibit the release of gonadotrophins (2). In rats, the HPA axis is already functional in fetuses in late gestation (3). We have previously reported that the exposure of pregnant rats to morphine induces both atrophy and hypoactivity of the adrenals for corticosterone release in newborns at birth and during the early postnatal period (4). As such effects of morphine on the HPA axis were not observed in newborns from adrenalectomized females (5), a

direct action of this drug on the fetal brain when administered to the mother is unlikely. However, according to several studies, opiate drugs such as heroin or morphine can be transferred through the placenta from the pregnant female to the fetus (6).

Sexual differentiation of the brain, which occurs perinatally in several species, including the rat, can be affected by exposure of the developing nervous system to opiates (2). Three major opioid receptor subtypes, μ (μ), κ (κ) and δ (δ) have been pharmacologically characterized (7). The μ -opioid receptor shows high affinity for morphine-like drugs as well as for dynorphin A and for several endogenous opioid

Figure 1 Plasma ACTH (A) and corticosterone (B) concentrations in 1-day-old newborn rats (J1) before (○) and after morphine injection (20 mg/kg; morphine group, ■) or 0.9% NaCl injection (vehicle group, ●). Values are means $\pm$ S.E.M. ($n = 10-14$). (**) $P < 0.01$ vs time 0 min. * $P < 0.05$, ** $P < 0.01$ morphine vs vehicle group at the same time.

peptides, including mainly pro-opiomelanocortin-derived β -endorphin.

Opioid receptors are first detectable within the rat central nervous system on embryonic day 14 (8). The distribution of μ -opioid receptor mRNA in the rat brain increases between embryonic days 16 and 22 and continues to increase until postnatal day 7 to a level equivalent to the adult one (9). The coexistence of μ - and κ - but not δ -opioid sites has been reported in the human fetal brain (10), and during early neonatal life

in the brain of both rat (11) and mouse (12). The development of opiate receptors in the rat brain may be sex hormone dependent, as 6-day-old females display more opiate-binding sites than age-matched males (13).

As the effects of opioids on neuroendocrine functions during the postnatal period are poorly documented (14), the first aim of the present work was to investigate, *in vivo*, the consequence of acute morphine administration on hypophysial corticotrophin (ACTH)

Figure 2 Plasma ACTH (A) and corticosterone (B) concentrations in 1-week-old newborn rats (W1) before (○) and after morphine injection (20 mg/kg; morphine group, ■) or 0.9% NaCl injection (vehicle group, ●). Values are means $\pm$ S.E.M. ($n = 8-13$). (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ vs time 0 min. * $P < 0.05$, ** $P < 0.01$ morphine vs vehicle group at the same time.

release and corticosterone secretion in newborn rats at several stages of postnatal life (first and second week of life).

Experiments performed *in vitro* have suggested that morphine coupling to nitric oxide (NO) can stimulate both corticotrophin-releasing factor (CRF) and gonadotrophin-releasing hormone release from the median eminence of adult rats (15). The presence of NO synthase has been demonstrated in several areas of the adult rat brain, including the hypothalamic

paraventricular nucleus, and in a subpopulation of CRF-expressing neurones (16). Nevertheless, with respect to the role of endogenous NO, conflicting results have been reported, suggesting either stimulatory or inhibitory effects of NO on CRF release (17).

Thus, the second aim of this work was to test whether NO contributes to the release of ACTH and corticosterone in early postnatal life, and whether NO could mediate the endocrine effect of morphine in the newborn rat.

Figure 3 Plasma ACTH (A) and corticosterone (B) concentrations in 2-week-old newborn rats (W2) before (○) and after morphine injection (20 mg/kg; morphine group, ■) or 0.9% NaCl injection (vehicle group, ●). Values are means $\pm$ S.E.M. ($n = 7-12$). (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ vs time 0 min. ** $P < 0.01$, *** $P < 0.001$ morphine vs vehicle group at the same time.

Materials and methods

Animals

Experiments were performed on Wistar rats bred in our laboratory. Animal accreditation by the French Ministry of the Agriculture (no. 04860) has been granted to our laboratory for experimentation with rats. Newborns spontaneously delivered vaginally were used 1 day (J1), 1 week (W1) or 2 weeks (W2) after birth.

Treatment of the newborns

In a first experiment, morphine sulphate (Sanofi-Francopria, Paris, France) was dissolved in physiological saline (0.9% NaCl) used as vehicle. Morphine is known as prototypic μ -opioid receptor agonist, which is also an agonist at the κ -opioid receptor (18). On the basis of preliminary results showing that a single s.c. injection of 20 mg/kg morphine sulfate is more effective than a lower dose of 10 mg/kg at stimulating secretion of

Figure 4 Plasma ACTH (A) and corticosterone (B) concentrations in 2-week-old newborn rats (W2) before (0 min) and 60 min following 0.9% NaCl injection (vehicle) (60 min) at a time when the pups were subjected to a second injection of vehicle or morphine (20 mg/kg) before being killed 30 min later (90 min). Values are means $\pm$ S.E.M. ($n = 6$). (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ vs time 0 min. [+++] $P < 0.001$ vs time 60 min. **** $P < 0.001$ vehicle + morphine vs vehicle + vehicle group at the same time.

ACTH and corticosterone in newborn rats, we selected the highest dose for this study. Newborns ($n = 7$ –14 rats/group) were treated with morphine given s.c. (20 mg/kg in 0.025 ml (J1 and W1) or 0.050 ml saline (W2)) or the same volume of vehicle (0.9% NaCl) for controls. Pups were killed 5, 15, 30, 60 or 90 min after the injection of the drug or the vehicle. Some littermate newborns ($n = 7$ rats), which were killed without previous treatment, were used as absolute controls. Morphine use accreditation by the French Ministry of Health (no. 93 00 196 S) has been granted to our laboratory for experimentation with rats. In a

second experiment, 2-week-old newborns were pre-treated with naltrexone hydrochloride (NAL; Sigma Chemical Co., St Louis, MO, USA), a competitive antagonist at μ -, κ - and δ -opioid receptors (0.4 mg/kg or 10 mg/kg s.c. in 0.050 ml 0.9% NaCl used as vehicle). One hour later, newborns were injected with either morphine (20 mg/kg s.c. in 0.050 ml vehicle) or the same volume of vehicle ($n = 9$ –12 rats/group), and killed 30 min later. Some pups ($n = 12$ rats) injected with only NAL were killed 60 or 90 min later. On the other hand, pups ($n = 8$ rats/group) injected with NAL (10 mg/kg) or NAL and morphine were killed 30 min

Figure 5 Plasma ACTH (A) and corticosterone (B) concentrations in 2-week-old newborn rats (W2) before (0 min) and 30 min after injection of 0.9% NaCl, morphine (20 mg/kg), NAL (10 mg/kg), L-NAME (100 mg/kg), morphine and NAL, or morphine and L-NAME. Values are means $\pm$ S.E.M. ($n = 5-12$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs time 0 min. [+] $P < 0.05$, [++] $P < 0.01$ vs respective controls (vehicle, NAL, L-NAME).

later. In a third experiment, 2-week-old newborns were pretreated with N- ω -nitro L arginine methyl-ester (L-NAME) hydrochloride (Sigma), an NO synthase inhibitor (30 mg/kg or 100 mg/kg s.c. in 0.050 ml 0.9% NaCl used as vehicle). One hour later, newborns were injected with either morphine (20 mg/kg s.c. in 0.05 ml vehicle) or the same volume of vehicle ($n = 9-11$ rats/group). The treated newborns were killed 30 min later. On the other hand, pups injected with L-NAME (100 mg/kg) or L-NAME with morphine were killed 30 min later ($n = 7$ rats/group). In a final experiment, pups were injected with either S-nitroso-N-acetylpenicillamine (SNAP; Sigma), an NO donor (5 mg/kg s.c. in 0.05 ml vehicle) or the same volume of vehicle ($n = 10-13$ rats/group). Injected pups were killed 60 or 90 min later.

Plasma collection

Trunk blood samples were collected between 0900 and 1200 h, after carotid section, in polyethylene tubes

pre-rinsed with EDTA 5% (w/v). The blood samples were centrifuged at 3500 g for 10 min at 4 $^{\circ}\text{C}$ and kept at -30°C until hormone assays. The sex of the pups was determined by examination of the genitals.

Radioimmunoassays

ACTH was measured in unextracted plasma by radioimmunoassay (RIA) using an ACTH kit (ACTHK-PR; Cis Bio International, Gif sur Yvette, France; sensitivity 10 pg/ml). The characteristics of the antiserum have been reported previously (19). The intra- and inter-assay variations were, respectively, 4.3% ($n = 6$) and 11.7% ($n = 9$). Corticosterone assay in plasma was preceded by extraction in ethylacetate after delipidation in iso-octane. The percentage recovery of a known amount of this steroid was over 95%. Corticosterone levels were determined by RIA using a highly specific corticosterone antiserum according to a previously detailed procedure (20) with a detection threshold of

Figure 6 Plasma ACTH (A) and corticosterone (B) concentrations in 2-week-old newborn rats (W2) before (0 min) and 60 min following NAL (10 mg/kg) injection (60 min) at a time when the pups were subjected to a second injection of 0.9% NaCl or morphine (20 mg/kg) before being killed 30 min later (90 min). Values are means $\pm$ S.E.M. ($n = 9-12$). (*) $P < 0.05$, (***) $P < 0.001$ vs time 0 min. [+++] $P < 0.001$ vs time 60 min. ** $P < 0.01$ morphine vs vehicle group at the same time.

1 ng/ml. The intra- and interassay variations were, respectively 2.4% ($n = 12$) and 4.4% ($n = 4$).

Statistical analysis

All data are presented as means $\pm$ S.E.M. Multiple analysis of variance was performed followed by a Dunnett's test using a computer program. The Student's *t*-test was also used when appropriate. $P < 0.05$ were considered statistically significant.

Results

As no significant differences between sexes were observed for plasma concentrations of ACTH and corticosterone before and after treatment with either morphine or vehicle, values for male and female pups were pooled. Morphine given to 1-day-old newborns induced a slow and slight increase in the circulating concentration of ACTH which was significant only 90 min after drug injection (Fig. 1A). Morphine-treated pups showed significant increase in the plasma

Figure 7 Plasma ACTH (A) and corticosterone (B) concentrations in 2-week-old newborn rats (W2) before (0 min) and 60 min following L-NAME (100 mg/kg) injection (60 min) at a time when the pups were subjected to a second injection of 0.9% NaCl or morphine (20 mg/kg) before being killed 30 min later (90 min). Values are means $\pm$ S.E.M. ($n = 9-11$). (**) $P < 0.01$, (***) $P < 0.001$ vs time 0 min. [+] $P < 0.05$, [++] $P < 0.01$, [+++] $P < 0.001$ vs time 60 min.

concentration of corticosterone at 30 min after injection (Fig. 1B). Morphine also activated the pituitary–adrenal axis in both 1-week-old (Fig. 2) and 2-week-old newborns (Fig. 3). However, morphine-induced ACTH and mainly corticosterone release were highest and more lasting in the latter pups (Fig. 3). Moreover, vehicle injection induced a slight but significant increase of both ACTH and corticosterone release at 30, 60 and 90 min in 2-week-old newborns (Fig. 3) but not in younger ones (Figs 1 and 2). Morphine-induced ACTH increase was more drastic in pups pretreated

with vehicle (Fig. 4A) than in naive pups (Fig. 3A). NAL given alone to 2-week-old newborns at 0.4 mg/kg had no significant effect on the plasma concentration of ACTH and corticosterone 90 min after the injection (data not shown). In contrast, at 10 mg/kg, NAL induced 30 min later (but not 60 or 90 min later) a significant rise in both plasma ACTH (Fig. 5A) and corticosterone (Fig. 5B) concentrations, but was unable to prevent morphine responses when this drug was administered concomitantly (Fig. 5). Vehicle injection given 60 min after an injection of NAL induced an

Figure 8 Plasma ACTH (A) and corticosterone (B) concentrations in 2-week-old newborn rats (W2) before (○) and after SNAP injection (5 mg/kg; SNAP group, ■) or 0.9% NaCl injection (vehicle group, ●). Values are means $\pm$ S.E.M. ($n = 10$ – 13). (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ vs time 0 min. ** $P < 0.01$, *** $P < 0.001$ SNAP vs vehicle group at the same time.

increase in the circulating concentration of ACTH and corticosterone 30 min later (Fig. 6); these were significantly larger than the increases observed 30 min after single injection of vehicle (Fig. 5). Nevertheless, morphine-induced activation of the pituitary–adrenal axis was prevented by pretreatment with NAL at a dose of 10 mg/kg (Fig. 6) but not at the dose of 0.4 mg/kg (data not shown). At a 90-min period, L-NAME at 30 mg/kg had no significant effect on morphine-induced ACTH release and corticosterone release (data not shown). By contrast, a higher dose of

L-NAME (100 mg/kg) induced, by itself, a slight but significant increase in both plasma ACTH concentrations at 30 (Fig. 5A), 60 and 90 min after administration (Fig. 7A) and plasma corticosterone concentration at 60 min after injection (Fig. 7B). In newborns pretreated with the higher dose of L-NAME, and thereafter with morphine, circulating concentrations of ACTH (Fig. 7A) and corticosterone (Fig. 7B) were not significantly different from those observed in corresponding controls (pretreatment with L-NAME and thereafter with vehicle). SNAP injection at 5 mg/kg

significantly increased ACTH release (Fig. 8A) as well as corticosterone release (Fig. 8B) at a 90-min period in 2-week-old pups.

Discussion

According to the present data, injections of vehicle solution produced a slight but significant release of ACTH and corticosterone in 2-week-old newborns but not in younger pups, indicating that, at this stage, injection *per se* could induce a stress response.

Such data are consistent with the existence of a 'stress hypo-responsive period' in early postnatal life (21, 22). In this study, we have shown that an acute administration of morphine was able to induce a drastic and long-lasting pituitary–adrenal stimulation in 2-week-old newborns; by contrast, HPA axis activation was more transient and/or less robust in younger pups. ACTH and corticosterone release induced by morphine has been reported previously in 10-day-old rat pups (23). In agreement with our results, these authors reported that morphine-induced corticosterone release was lower on day 5 than on day 15 of postnatal life (23).

As NAL stimulated the HPA axis in pups, one can speculate that endogenous opioids may exert a tonic inhibition of this axis in newborns, as was reported in pregnant rats (24). In contrast, a stimulatory effect of endogenous opioids on the HPA axis response to physical stress has been reported in virgin rats (25).

On the other hand, NAL, when administered previously, also prevented morphine-induced stimulation of ACTH and corticosterone release in 2-week-old newborns. One can speculate that μ -opiate receptors may be involved in the control of pituitary–adrenal function in early postnatal development; however, they showed controversial effects. This hypothesis is consistent with the development of μ -receptors in the rat brain during the perinatal period (9) as well as of other opiate receptors including κ -receptors, which are detected in the brain of neonatal rats (11), and may be implicated in the control of the pituitary–adrenal axis within the first week of postnatal life (23). In adults, according to experiments performed *in vivo* and *in vitro*, the facilitation of ACTH secretion by morphine was correlated with the release of CRF (15, 26, 27). However, a sparse opioid binding has been shown in the anterior pituitary gland (28) and opioids were not found to affect the release of ACTH from the pituitary *in vivo* (29). Moreover, we have previously reported that the hypothalamic content of CRF increases drastically in the developing rat from day 17 of gestation to week 4 post-partum (30), consistent with the hypothalamic control of the corticostimulating function of the pituitary gland as early as day 19 of gestation (3, 31). Thus, morphine-induced activation of hypothalamic–adrenocortical activity in the neonate probably occurs via the release of CRF.

Controversial data with morphine (μ - and κ -opioid receptor agonist) and NAL (μ -, κ - and δ -opioid receptor antagonist) suggest that, under our experimental conditions, the exogenous opiate (morphine) and the opioid peptides interacting with several types of opioid receptors affect the activity of the HPA axis differently in the developing brain.

Present data also suggest that, in neonates, morphine may act via the release of NO. This hypothesis is consistent with both the presence of neuronal NO synthase expression in the brain during embryonic development (32) and the prevention of morphine activation of the HPA axis by L-NAME, an NO synthase inhibitor (present data). The rise of circulating ACTH and corticosterone 30 min after vehicle injection in control pups previously treated with NAL or L-NAME illustrates the effects of two successive stressors. SNAP, an NO donor, also increased the release of ACTH and corticosterone in newborns and this mimics morphine's actions.

Our results are consistent with *in vivo* studies (33) showing that nitroprusside, an NO generator, induces CRF release. These results are also consistent with the work of Prevot *et al.* (15) indicating that, *in vitro*, morphine coupling to NO stimulates CRF release from adult rat median eminence fragments, as does the NO donor SNAP, and that morphine-induced CRF release was blocked by naloxone and L-NAME. In contrast, Costa *et al.* (34) reported that NO donors and precursors did not alter, *in vitro*, basal CRF release from the rat hypothalamus.

Because of our experimental conditions we cannot specify at what level of the neonatal brain the putative morphine-induced NO-mediated CRF release was occurring, and we cannot be precise about which is the origin of NO production (neuronal or endothelial source).

In conclusion, the present data suggest that both the endogenous opioid system and the NO one may be involved together in the modulation of the hypothalamo–pituitary adrenal axis in early postnatal development in rats.

In contrast, in 2-week-old newborns, plasma luteinizing hormone concentrations, which were higher in female pups than in male ones, were not significantly affected by several treatments which included morphine, NAL, L-NAME and SNAP at the previously reported doses and time-periods (authors' unpublished data). These data suggest that the gonadotropic axis matures differently from the corticotropic one during the first 2 weeks of postnatal life in rats.

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