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European Journal of Pharmacology 458 (2003) 201–205
www.elsevier.com/locate/ejphar

Short communication

Vas deferens response to selective opioid receptor agonists in adult mice is impaired following postnatal repeated mild stress

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Received 23 July 2002; received in revised form 5 November 2002; accepted 6 November 2002

Abstract

Mild stress repeatedly applied to neonatal rodents induces alterations of central nervous system functions, persisting up to the adult age. Most alterations may be mediated through hormones and neuromediators active on the autonomic nervous system, therefore we tested the efficacy of selective opioid receptor agonists on the vas deferens of adult mice that, as neonates, had undergone daily mild stress until weaning (brief isolation and solvent injection). We found in the adult mouse (90 days old) decreased sensitivity of vas deferens to selective μ-, δ- and κ-opioid receptor agonist drugs. The neonatal administration of an antisense oligodeoxynucleotide adrenocorticotropin-synthesis-inhibitor partly prevented these effects.

Keywords: Opioid; Vas deferens; Ontogenesis; Stress; Antisense oligonucleotide

1. Introduction

Repeated neonatal mild stress, such as manipulation of pups and brief isolation from the dam—with or without sham injection—performed daily for a few weeks, induces brain, behavioural and metabolic alterations which may persist up to adult ages. Central neuromediator/hormonal systems contribute to these phenomena and, among the systems, the hypothalamic–pituitary–adrenal axis and the central opioid system are those most extensively studied (Anand, 2000; Loizzo et al., 2002). Although significant interconnections exist between the central and the autonomic nervous systems for several neurohumors, including opioids (Wittert et al., 1996), there have been only few investigations of the effects induced by chronic mild stress administered during the pre-weaning period on peripheral opioid receptors properties of adult rodents.

We now studied the effects produced by repeated neonatal mild stress on the capacity of vas deferens to respond to μ-, δ- and κ-opioid receptor-selective agonist drugs in the adult period. We had shown (D'Amore et al., 1996; Loizzo et al., 2002) that naloxone prevents alterations induced by repeated stress in the nociceptive threshold, body weight—increase curve, and selective modulation of immune properties: thus, in the present study, a different group of mice received naloxone instead of solvent, to study any possible role played by the endogenous opioid system. Finally, another group of animals received an antisense oligodeoxynucleotide with adrenocorticotropin synthesis blocker properties (AS-ACTH), in order to ascertain the role played by the hypothalamic–pituitary–adrenal axis.

2. Materials and methods

2.1. Drugs and materials

AS-ACTH and its mismatch compound (MS-ACTH) were purchased as phosphorothioates, with collaboration of

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PII: S0014-2999(02)02725-5

EUROBIO Laboratoires (Les Ulis Cedex, France). The synthesis of both compounds was performed following project of one of us (S.S.). The 21-base sequence of AS-ACTH was: TCT GGC TCT TCT CGG AGG TCA; the MS-ACTH served as control and had five bases with exchanged position: TGT GCC TCT TTC CGG TGG ACA. The AS-ACTH was designed on the basis of previous studies showing that an antisense oligodeoxynucleotide complementary to a region of β-endorphin *m*RNA markedly reduced the synthesis of the proopiomelanocortin (POMC)-derived peptides ACTH and β-endorphin (Spampinato et al., 1994).

The selective μ-opioid receptor agonist, dermorphin, the selective δ-opioid receptor agonist, deltorphin-I, and the selective κ-opioid receptor agonist, dynorphin-A, were purchased from Sigma (St. Louis, USA) and used for vas deferens assays. Naloxone, AS-ACTH and MS-ACTH were used for subcutaneous injections; preparations were stored at −30 °C in glass vials and were used only once.

2.2. In vivo experiments

Two series of experiments were performed: (a) preliminary experiments with young adult mice treated for 5 days with AS-ACTH and its mismatch (MS-ACTH, see below) and killed 3 h after the fifth treatment, and (b) experiments with mice stressed and treated when neonates, and finally tested for peripheral (vas deferens) opioid sensitivity as adults. Animal care, environmental conditions and use followed the rules of the Council of European Communities. The experimental procedures were approved by the Bioethical Committee of the Italian National Health Institute.

2.2.1. Effect of oligodeoxynucleotide treatments on pituitary ACTH and plasma corticosterone

The activity of AS-ACTH, versus MS-ACTH or vehicle was evaluated by administering them subcutaneously (s.c.) (0.1 nmol g^{-1}) for 5 days to groups consisting of six 30-day-old mice. Mice were killed by rapid decapitation 3 h after the last treatment, the anterior pituitary was homogenized in 0.1 M acetic acid (90° C) and processed as described (Spampinato and Goldstein, 1983). Immunoreactive (*ir*)-ACTH levels were measured by radio-immunoassay (RIA) of the pituitary acetic acid extracts by double-antibody precipitation using human ACTH antiserum and human ACTH standard obtained from Dr. AF Parlow (Harbor-UCLA Medical Center) and [^{125}I]iodotyrosyl^{2}–ACTH-(1-39) (Amersham Biosciences, Milano). The antibody recognizes mouse ACTH-(1-39), and there is no cross-reactivity with other peptides derived from the proopiomelanocortin precursor. At the time of the decapitation, trunk blood was collected from each mouse into edetate calcium disodium tubes placed on ice, spun in a refrigerated centrifuge, and plasma was collected and stored at −80 °C. Corticosterone levels were assayed in duplicate in a single assay using a RIA kit (ICN, Costa Mesa, CA, USA). Intra-assay coefficients of variations were <2.0%.

2.2.2. Neonatal treatment and post-weaning protocol

Experiments were performed during the winter, because opioid receptor sensitivity is maximal in this period (De Ceballos and De Felipe, 1985). Multiparous CD1 mice (Charles River Italia, Calco, Como) were transferred to our laboratory on the 14th day of gestation. After birth, litters of similar size (12 ± 1 pups) were combined, randomly culled to five male pups, and cross-fostered.

Litters were randomly assigned to one of the following groups: (1) Control: the pups were left undisturbed with their mother in the home cage, except for cage cleaning twice a week. (2) Water-treated: pups of each litter were removed daily from the home cage and grouped in a container with fresh bedding material; each pup was weighed and s.c. injected with distilled water, 1 ml kg^{-1} using a microsyringe with a 27-gauge needle; after 10 min the pups were returned to the home cage with the mother. (3) Naloxone-treated: pups of each litter were manipulated as those in group water-treated except for injection of (−)-naloxone hydrochloride, instead of water, 1 mg kg^{-1} dose, as weight of the base. (4) AS-ACTH-treated group: pups were manipulated as those in the group naloxone-treated except for injection of oligodeoxynucleotide, 0.1 nmol g^{-1} dose.

From weaning (21 days of age) animals of each group were re-housed in post-weaning cages with five male mice per cage. From 91 to 95 days of age, on consecutive days, one animal from each group was weighed, and was killed by rapid decapitation. The vas deferens was rapidly removed and mounted for drug testing.

2.3. In vitro experiments

Preparations from vas deferens were set up as previously described (Negri et al., 1997). Vasa were mounted in an organ bath of 10 ml capacity, at 37 °C, bathed in modified Krebs solution (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 0.93, $NaHCO_3$ 25, glucose 11, gassed with 95% O_2 and 5% CO_2. Longitudinal contractions were recorded isometrically at a tension of 0.5 g with a strain gauge transducer (DY Basile 1, Milano, Italy) and displayed on a recording microdynamometer (Unirecord, Basile, Milano, Italy). The intramural nerves were stimulated with trains of rectilinear pulses. After an equilibration period of 30 min, stimulation trains were given at intervals of 20 s and consisted of six stimuli of 1 ms duration with intervals of 10 ms. Drugs were added to the 10 ml bath in a volume of 50 μl in distilled water, and washed away after 5-min contact. Concentration–response curves were made in all the experiments in order to allow calculation of IC_{50} values and confidence limits.

2.4. Statistical analysis

Concentration–responses curves were analyzed with a linear regression method (Tallarida and Murray, 1986) and IC_{50} and confidence limits were calculated only from the

linear portion of the dose–response curves. Analysis of variance (ANOVA) and Dunnett's test were used to estimate the significance level of the differences between groups. The accepted level of significance was 0.05.

3. Results

In preliminary experiments, daily s.c. administration of AS-ACTH to adult mice for 5 days significantly reduced the anterior pituitary content of *ir*-ACTH versus that in stressed (vehicle treated) mice, while MS-ACTH did not induce consistent effects (Fig. 1, Panel A). Plasma corticosterone levels, taken as an index of activity of the pituitary–adrenal axis (Ladd et al., 2000), were significantly reduced by AS-ACTH treatment, whereas MS-ACTH did not affect plasma corticosterone levels (Fig. 1, Panel B). In another study (manuscript submitted), we found increased pituitary ir-ACTH and plasma corticosterone levels in adult mice following repeated neonatal mild stress, and AS-ACTH treatment was able to prevent these effects.

In our experiments, the vas deferens of adult control mice was more sensitive to deltorphin-I than deferens taken from neonatally stressed (sham-injected) animals (IC_{50} = 0.23 nM versus 0.53; $P < 0.05$, after ANOVA) (Table 1). AS-ACTH was effective to prevent the stress-induced hyposensitivity (IC_{50} = 0.31 nM; $P < 0.05$ versus stressed mice; not significant versus controls). Neonatal exposure to naloxone induced partial, non-significant antagonism (IC_{50} = 0.45 nM). Neonatal exposure to stressful procedures induced hyposensitivity of vas deferens receptors to selective μ- and δ-opioid receptor agonists, dermorphin and dynorphin, as well. However, in this case, neither naloxone nor AS-ACTH neonatal treatment was able to prevent this effect (Table 1).

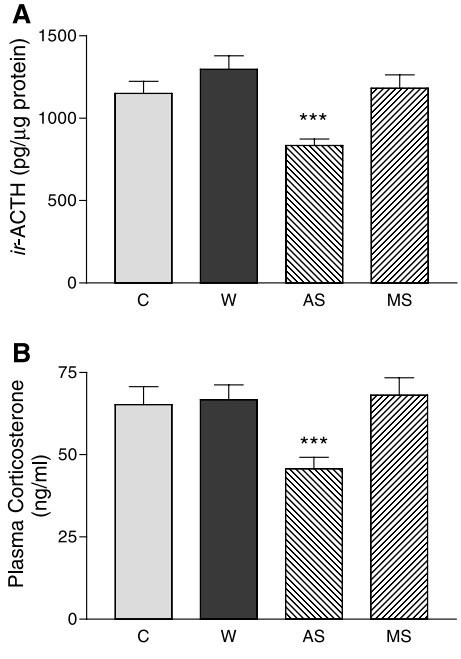

Fig. 1. Effect of oligodeoxynucleotide treatment on pituitary ir-ACTH content and corticosterone plasma levels in adult mice. The compounds or water were administered s.c. for 5 days. Mice were killed 3 h after the last treatment. C: Control; W: water-treated mice; AS: mice treated with ACTH-antisense oligodeoxynucleotide (0.1 nmol g^{-1}); MS: mice treated with ACTH-mismatch oligodeoxynucleotide (0.1 nmol g^{-1}). Data are expressed as means ± S.E.M. (n = 6). ***$P < 0.001$ vs. C; Dunnet's test after ANOVA.

Table 1
Estimated IC_{50} values (nM) and confidence limits (in brackets) for μ-, δ- and κ-opioid receptor agonist activity on vas deferens

	C	W	NA	AS
μ-Opioid receptor agonist				
Dermorphin*	8.1 (5.0–13.0)	35.2 (11.8–104)	25.4 (16.6–38.6)	43.1 (29.7–62.4)
δ-Opioid receptor agonist				
Deltorphin-I*	0.23 (0.14–0.37)	0.53 (0.43–0.67)	0.45 (0.32–0.64)	0.31 (0.26–0.38)
κ-Opioid receptor agonist				
Dynorphin*	45.1 (33.3–61.0)	96.8 (35.0–270)	99.4 (83.5–118)	111 (24.5–507)

After ANOVA

For dermorphin: C versus W, NA, AS, $P < 0.05$
For deltorphin I: C versus W, NA; W versus AS: $P < 0.05$
For dynorphin: C versus NA: $P < 0.05$ (IC_{50} = 0.45 nM)

Dose–effect responses were obtained with drugs tested on vas deferens of 90-day-old mice from four groups: control mice (C); mice receiving daily isolation and sham injection up to the 21st day of life (W); mice receiving isolation and naloxone (NA); or isolation and AS-ACTH (AS). Data are for five animals per group.

4. Discussion

A mildly stressful procedure applied to mice during neonatal period is able to induce consistent hyposensitivity of vas deferens to selective opioid receptor agonists, 70 days after the end of procedures. Pharmacological analysis of activity in vas deferens is complicated by the presence of, in addition to δ-opioid receptors, μ- and κ-opioid receptors (for a review see Smith and Leslie, 1993). The absolute potencies of μ-opioid and κ-opioid receptor agonists are lower in vas deferens than in guinea-pig ileum; this is postulated to result from differences in tissue receptor reserve (Smith and Leslie, 1993). In agreement with literature data, we found higher IC_{50} values for the μ-opioid receptor agonist, dermorphin, and the κ-opioid receptor agonist, dynorphin, in comparison to the δ-opioid receptor agonist, deltorphin-I (Table 1). The hypothesis that dermorphin and dynorphin may act through δ-opioid receptors may be ruled out. In fact, it has been shown that dermorphin has no relevant activity on vas deferens from mice deficient in μ-opioid receptors (Maldonado et al., 2001), and that dynorphin has a large potency shift (~ 6 times less potent) on vas deferens exposed to an alkylating

agent that inactivates μ- and κ-opioid receptors. (Goldstein and James, 1984).

AS-ACTH may partly prevent δ-receptor down-regulation, presumably by blocking ACTH and glucocorticoid production, but has no consistent activity on vas deferens sensitivity to μ-opioid and κ-opioid receptor selective agonists. This is in agreement with our previous observation that glucocorticoids may interact differently with different opioid receptors: in acute experiments dexamethasone enhanced the analgesic effect of κ-opioid agonists, while it prevented the analgesia induced by selective μ- and δ-opioid receptor agonists in adult mice (Pieretti et al., 1994). Moreover, during weaning, rodents activate a δ-opioid receptor that mediates specific functions (e.g., swim-stress-induced analgesia; Goody and Kitchen, 2001), and this in turn may interact with HPA hormones.

The present results suggest that HPA is involved in the regulation of peripheral opioid receptors as well. Early postnatal environmental events may affect the development of the HPA axis activity in later life, and may induce long-term behavioural and endocrine effects (Ladd et al., 2000). Glucocorticoids represent the end-product of the HPA axis and, along with the sympathetic system, participate in the adaptive response to stress. These systems interact at several levels: glucocorticoids may regulate the enkephalinergic phenotype of sympathetic neurons (Henion and Landis, 1992). Recent studies revealed that repeated neonatal stress involves different types of peripheral receptors. Weizman et al. (1999) found that rats exposed to handling in the neonatal period, in adulthood showed an increase in adrenal and renal peripheral benzodiazepine receptor density and a reduction in the expression of this receptor in the gonads. The role of long-term changes in peripheral benzodiazepine receptors found by Weizman et al. (1999), and the role of long-term changes in peripheral opioid receptor sensitivity described in the present experiment, are both poorly understood. Moreover, we can hypothesize that both neuromediator system changes represent phenotype expression for adaptations to stressful situations in adulthood.

We cannot affirm whether the effect we observed was a direct effect on opioid receptors (to be better defined in the future also using selective opioid receptor antagonists), or an indirect effect, perhaps mediated through malfunction of an aminergic mechanism triggered by repeated stress (D'Arbe et al., 1999), which in turn induces opioid receptor down-regulation. Another condition to be investigated is the possibility that the travel of pregnant females from the producer to our animal house might have involved some prenatal stress, and therefore could have influenced some effects induced by the experimental procedure. This feature, however, was identical for all experimental groups.

In conclusion, our experiments suggest that repeated perinatal mild stress applied to rodents is able to produce long-lasting functional alteration of peripheral-vas deferens-sensitivity to selective opioid receptors agonists, and that these alterations are in part prevented by a peripheral treatment with AS-ACTH, which was indeed effective on POMC synthesis. Future investigation should ascertain whether these alterations can lead to impaired function of vas deferens, such as, for example, to impaired fertility of the adult animal.

Acknowledgements

This paper was realized in part with a contribution from the Italian National Health Institute Research Programme ''Developmental Pharmacology'', and in part with a contribution from the Italian Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST, anno 1999, Prot. 9905111147-066). Editorial assistance by Ms. Carla Campanella is acknowledged. Thanks are due to Mr. Stefano Fidanza and Mr. Adriano Urcioli for care of animals.

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