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Modifications of Striatal D_2 Dopaminergic Postsynaptic Sensitivity During Development of Morphine Tolerance–Dependence in Mice

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NAVARRO, M., J. J. FERNÁNDEZ-RUIZ, F. RODRIGUEZ DE FONSECA, M. L. HERNÁNDEZ, M. CEBEIRA AND J. A. RAMOS. *Modifications of striatal D_2 dopaminergic postsynaptic sensitivity during development of morphine tolerance-dependence in mice.* PHARMACOL BIOCHEM BEHAV **43**(2) 603–608, 1992.—Alterations in the activity of striatal dopaminergic neurons have been implicated in the development of morphine tolerance-dependence in rodents. To further explore this possibility, we examined the activity of these neurons in mice exposed to morphine during 4 days (addiction group) and subsequently treated with naloxone (withdrawal group). The efficiency of opiate treatment was assessed behaviorally. Striatal dopaminergic activity was evaluated by measuring: a) the ratio between the amounts of *L*-3,4-dihydroxyphenylacetic acid (DOPAC), the main intraneuronal metabolite of dopamine (DA), and the neurotransmitter itself, as an index of presynaptic activity; and b) the number and affinity of D_1 and D_2 dopaminergic receptors, as well as the amount of their coupled second messenger, cyclic adenosine monophosphate (cAMP), as postsynaptic parameters. Spontaneous motor activity was decreased in chronically morphine-exposed mice. In these animals, the number of striatal D_2 receptors also decreased, with no changes in their affinity, whereas the number and affinity of D_1 receptors remained unchanged. This hyposensitivity of D_2 receptors was paralleled by an increase in the amount of cAMP with a good statistical correlation between both parameters. Treatment with naloxone of morphine-exposed mice resulted in the typical jumping behavior indicative of opiate withdrawal. The differences in D_2 receptors between placebo- and morphine-exposed mice disappeared after naloxone-induced opiate withdrawal, although this effect was due more to the inhibitory effect of naloxone on the density of these receptors in placebo-exposed mice rather than to a stimulatory effect in morphine-addicted mice. The morphine-induced increase in cAMP content also disappeared after naloxone treatment. No significant changes occurred in the presynaptic activity in both states, as reflected by the absence of changes in the DOPAC/DA ratio. However, DA storage decreased after the induction of opiate withdrawal, which could be related to a repeated DA utilization during the naloxone-induced jumping behavior in morphine-addicted mice. In summary, our results provide neurochemical evidence of changes in striatal dopaminergic neurotransmission concomitant to the typical motor alterations produced during morphine tolerance-dependence. These changes were mainly produced at the postsynaptic level, by decreasing the number of D_2 receptors and increasing the cAMP production, during the morphine addiction. These differences disappeared after the naloxone-induced morphine withdrawal, likely by an inhibitory effect of naloxone on D2 receptors in placebo-exposed mice. Moreover, a presynaptic effect could be suspected during the opiate withdrawal in view of the decrease in DA content observed in naloxone-treated morphine-exposed mice.

Morphine Opiate tolerance–dependence Striatum Dopamine D_1 and D_2 dopaminergic receptors Cyclic adenosine monophosphate

PHYSIOLOGICAL variations in the activity of several neurotransmitters in various brain areas have been observed during the development of morphine tolerance-dependence in rodents [for review, see (25)]. Particular attention has been addressed to the striatum because this brain area contains a high density of both dopaminergic nerve terminals originating from the substantia nigra (5) and enkephalinergic neurons (14) with a high density of receptors for both neurotransmitters (31,32). Among other possible interpretations, the existence of alterations in the activity of nigrostriatal dopaminergic neurons during morphine exposure/withdrawal might help explain certain characteristic motor manifestations of opiate tolerance-dependence (11). Thus, there is evidence that opiates can modify several biochemical parameters that reflect presynaptic ac-

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tivity in dopaminergic neurons, such as the synthesis (29), turnover (10), and release (4,30) of dopamine (DA). Other studies show modifications at postsynaptic level by inducing hypersensitivity of D_2 dopaminergic receptors. In rats, chronic treatment with morphine caused an enhanced response to apomorphine as reflected by an increase in the affinity of striatal dopaminergic receptors with no changes in their density (3,26). Similar observations have been made in mice acutely treated with morphine, where this opiate caused an increase in the number of D_2 dopaminergic receptors and potentiated apomorphine-induced climbing behavior (18,19).

However, the available information is still fragmentary and there is no systematic analysis of the striatal dopaminergic changes, at both pre- and postsynaptic levels, that occur during the different states of morphine exposure: a) opiate tolerance after chronic exposure; and b) opiate dependence expressed as naloxone-induced morphine withdrawal. In this work, we attempt to provide further information on this matter. To this end, we examined the activity of striatal dopaminergic neurons in mice exposed to morphine during 4 days (addiction group) and subsequently treated with naloxone (withdrawal group). Animals exposed to placebo were used as controls for both situations. The ability of morphine to produce the addiction state was assessed by evaluation of motor activity (23), whereas the naloxone-induced opiate withdrawal state was validated by the jumping test (34). Activity of striatal DA was evaluated by analysis of the ratio between the amounts of *L*-3,4-dihydroxyphenylacetic acid (DOPAC), the main intraneuronal metabolite of DA, and the amine itself, as a presynaptic index (28), and the number (B_{max}) and affinity (K_d) of D_1 and D_2 dopaminergic receptors as indices of postsynaptic sensitivity. Because most sources have proposed that D_1 receptors are positively coupled to adenylate cyclase activity and D_2 receptors are negatively coupled [for review, see (2)], we also measured cyclic adenosine monophosphate (cAMP) production as an index of the effectiveness receptor stimulation.

METHOD

Animals and Treatments

Male OF1 mice weighing 30 ± 3 g were used in all studies. Ten animals were housed per cage under a controlled photoperiod (12 L:12 D; light on at 0700 h) and temperature (23 ± 1°C). Standard food and water were available ad lib. Experiments were carried out between 0900 h and 1400 h. Each animal was used only once. Mice were rendered morphine-dependent using the method previously described by Maggiolo and Huidobro (17). This procedure consists of implantation of a pellet containing 75 mg morphine base, prepared as described by Gibson and Tingstadt (8), in the dorsal subcutaneous tissue. Placebo animals were implanted with a pellet of similar weight and size containing the same quantity of sucrose instead of morphine and used as controls. Surgery was performed under light ether anesthesia. Four days after morphine or placebo pellet implantation, half the animals were behaviorally tested and killed and used to examine the chronic effects of morphine exposure (addiction group). The remaining animals for both morphine and placebo groups were IP injected with naloxone (1 mg/kg body weight) and used for the corresponding behavioral test and, 10 min later, killed and used to examine the effects of opiate withdrawal (withdrawal group). In both cases, animals were quickly decapitated and their brains removed and frozen at −70°C until assayed. On the day of analysis, the striatum was dissected out according to Glowinski and Iversen (9) and used for measurements of the above-mentioned indices of striatal dopaminergic activity.

Behavioral Tests

Locomotor activity during a period of 30 min was monitored in mice of the addiction group. Animals were placed in motility cages (26 × 21 × 9.5 cm each) with photocell motility meters (Actimeter Photoelectrique, Apelab., Paris, France). The apparatus was located in a sound-isolated cubicle and the number of crossings was recorded every 15 min. Mice were always placed in the motility cages for a period of 10 min before the onset of the test to become reacclimatized. Opiate withdrawal induction with naloxone was behaviorally validated by the jumping test. Each mouse was placed in a transparent cage (15 × 15 × 42 cm) connected to an automatic jump register-counter on the cage floor (21). The following parameters were registered during a period of 10 min: a) jump to void (jumping off) recorded as absence or presence (±) and expressed as percentages; and b) number of jumps per mouse (jumping up).

DA and DOPAC Measurements

DA and DOPAC contents in the striatal tissue were analyzed using high-performance liquid chromatography (HPLC) with electrochemical detection. Tissues from two animals were pooled and homogenized in 10 vol ice-cold 0.2 N perchloric acid with 0.5 mM sodium bisulfite and 0.45 mM EDTA. Dihydroxybenzylamine was added as an internal standard to correct procedural losses. The homogenates were then centrifuged and the supernatants injected into the HPLC system. Details of the HPLC system have been previously published (6). Values are expressed as ng/mg tissue weight.

D_1 and D_2 Dopaminergic Binding Site Analysis

Measurements of D_1 and D_2 binding sites were performed according to the procedures described by Reader et al. (24) and Leysen et al. (15), respectively, with slight modifications. Radioactive ligands were [^{3}H]SCH 23390 (60 Ci/mmol) for D_1 and [^{3}H]spiroperidol (27.5 Ci/mmol) for D_2, both purchased from New England Nuclear (Boston, MA). The concentration range was 0.125–3.0 nM and 0.05–0.80 nM, respectively. Protein concentration, measured by the Lowry method (16) in the incubated membrane fractions, was 0.2–0.3 mg/ml incubation and 0.15–0.20 mg/ml, respectively. For measurement of nonspecific binding, 30 μM (±)−SK&F38393 and 1 μM (+)−butaclamol, both purchased from Research Biochemicals, Inc. (Natick, MA), were used, respectively. To block the binding of spiroperidol to 5-hydroxytryptamine$_2$ (5-HT_2) serotoninergic receptors, 10 nM ketanserin, kindly supplied by Janssen Farmaceutica (Madrid, Spain), was added to all the tubes in the D_2 assay. The final volume of incubation medium was 0.5 ml. Details of both methods have been previously reported (7,27). A Scatchard analysis of the data, using linear regression, was performed to evaluate the dissociation constant (K_d), expressed as nM units, and the number of binding sites (B_{max}), expressed as fmol/mg protein.

cAMP Analysis

The amount of cAMP was analyzed with a kit based on the protein binding assay described by Tovey et al. (33). Tissues were homogenized in 20 vol 0.2 N perchloric acid containing 0.5 mM sodium bisulfite and 4 mM EDTA and adjusted

TABLE 1

(A) MOTOR ACTIVITY IN MICE EXPOSED TO CHRONIC MORPHINE/PLACEBO (ADDICTION GROUP) AND (B) JUMPING BEHAVIOR IN NALOXONE-TREATED CHRONICALLY MORPHINE/PLACEBO-EXPOSED MICE (WITHDRAWAL GROUP)

(A)	Motor Activity	+Placebo	+Morphine
	15 min	139.95 ± 15.49 [22]	41.39 ± 11.17 [18]*
	30 min	248.82 ± 22.86 [22]	86.28 ± 21.33 [18]*
(B)	Jumping Behavior	+Naloxone	+Morphine + Naloxone
	Jumping off (+)	2 (3.6%) [30]	22 (44.6%) [30]*
	Jumping off (−)	24 (42.9%) [26]	5 (8.9%) [30]*
	Jumping up	0.2 ± 0.2 [19]	91.8 ± 12.5 [17]*

Details in the text. Values are expressed as a) cumulative scores at 15 and 30 min for motor activity; b) percentages of jump to void (jumping off), recorded as absence or presence (+/−); and c) number of jumps per mouse (jumping up). They are means ± SEM with the number of determinations per group in brackets. Statistical analyses used were as follows: one-way ANOVA for motor activity and jumping up and Fisher's exact test for the remaining parameters.

*$p < 0.001$.

to pH 7 with the same volume of 0.2 N KOH containing 4 mM EDTA. This sample was used for the cAMP assay. Values are expressed as pmol/mg tissue weight.

Statistics

Data were assessed by Student's *t*-test, one-way analysis of variance (ANOVA) or Fisher's exact test, as required. The test used for each parameter is mentioned in the corresponding legend. Analysis of statistical correlation between number of D_2 receptors and cAMP amount was done in all groups.

TABLE 2

DA AND DOPAC CONTENTS, RATIO BETWEEN BOTH (DOPAC/DA), NUMBER (B_{max}) AND AFFINITY (K_d) OF D_1 AND D_2 DOPAMINERGIC RECEPTORS, AND cAMP CONTENT IN THE STRIATUM OF CHRONICALLY MORPHINE/PLACEBO-EXPOSED MICE (ADDICTION GROUP)

Parameters	+Placebo	+Morphine
DA	6.14 ± 0.50	6.30 ± 0.60
DOPAC	0.55 ± 0.07	0.72 ± 0.14
DOPAC/DA	0.09 ± 0.01	0.11 ± 0.01
D_1 B_{max}	2079 ± 221	2015 ± 248
D_1 K_d	1.19 ± 0.09	1.03 ± 0.17
D_2 B_{max}	685 ± 88	435 ± 77*
D_2 K_d	0.44 ± 0.07	0.33 ± 0.06
cAMP	2.14 ± 0.31	3.16 ± 0.48†
r-value	−0.81 ($p < 0.05$)	−0.78 ($p < 0.05$)

Details in the text. Values are expressed as ng/mg tissue weight for DA and DOPAC contents, fmol/mg protein for B_{max} and nM units for K_d, and pmol/mg tissue weight for cAMP. They are means ± SEM of more than eight determinations per group. Statistical differences were assessed by Student's *t*-test. The *r*-value and statistical significance obtained for correlation analysis between number of D_2 receptors and cAMP content are noted at the end of the table.

*$p < 0.01$.

†$p < 0.05$.

RESULTS

Behavioral Validations of Morphine Tolerance/Dependence

Chronic exposure to morphine significantly decreased spontaneous motor activity compared with placebo-exposed mice, as reflected by the cumulative count scores obtained at both 15 and 30 min after the onset of test (Table 1). Induction of opiate withdrawal with naloxone was accompanied by the characteristic jumping behavior, as reflected by the increase in jumping off (+) and up and the decrease in jumping off (−) observed in morphine-exposed mice compared with placebo-treated animals (Table 1). Both observations agree with previous data from several studies (21,23,34) and allow the validation of both situations of physical tolerance-dependence to opiates (addiction and withdrawal states).

Neurochemical Analysis of Striatal DA in Morphine Tolerance-Dependence

The number of striatal D_2 receptors decreased, but with no changes in their affinity, in morphine-addicted animals (Table 2), whereas the number and affinity of D_1 receptors remained unchanged (Table 2). This decrease in D_2 receptors was paralleled by an increase in the amount of cAMP (Table 2), with a good statistical correlation for both parameters (Table 2). The differences in D_2 receptors between placebo- and morphine-exposed mice disappeared after the blockade of opioid receptors with naloxone (Table 3), which was able to induce an opiate withdrawal state. However, this effect was due more to a naloxone-induced decrease in the density of D_2 receptors of placebo-exposed mice than to a naloxone-induced reversion of morphine effects in opiate-addicted mice. In parallel, the morphine-induced increase in cAMP amount also disappeared after naloxone treatment (Table 3). No significant changes occurred in the presynaptic activity in both states, as reflected by the absence of changes in the DOPAC/DA ratio (Tables 2 and 3), although DA storage decreased after induction of opiate withdrawal (Table 3).

DISCUSSION

Alterations in the activity of the nigrostriatal dopaminergic system have been observed during morphine exposure/with-

TABLE 3

DA AND DOPAC CONTENTS, RATIO BETWEEN BOTH (DOPAC/DA), NUMBER (B_{max}) AND AFFINITY (K_d) OF D_1 AND D_2 DOPAMINERGIC RECEPTORS, AND cAMP CONTENT IN THE STRIATUM OF NALOXONE-TREATED CHRONICALLY MORPHINE/PLACEBO-EXPOSED MICE (WITHDRAWAL GROUP)

Parameters	+Naloxone	+Morphine +Naloxone
DA	7.29 ± 0.42	5.79 ± 0.48*
DOPAC	0.67 ± 0.07	0.59 ± 0.07
DOPAC/DA	0.09 ± 0.01	0.10 ± 0.07
D_1 B_{max}	2196 ± 276	1700 ± 287
D_1 K_d	1.15 ± 0.08	1.12 ± 0.14
D_2 B_{max}	405 ± 56	493 ± 82
D_2 K_d	0.37 ± 0.05	0.44 ± 0.06
cAMP	2.62 ± 0.40	2.44 ± 0.81
r-value	−0.48	+0.043

Details in the text. Values are expressed as ng/mg tissue weight for DA and DOPAC contents, fmol/mg protein for B_{max} and nM units for K_d, and pmol/mg tissue weight for cAMP. They are means ± SEM of more than eight determinations per group. Statistical differences were assessed by Student's *t*-test. The *r*-value and statistical significance obtained for correlation analysis between number of D_2 receptors and cAMP content are noted at the end of the table.

*$p < 0.01$.

drawal in rodents, which might explain certain characteristic motor manifestations of opiate tolerance-dependence (see the introductory section). In the present study, we tried to combine both pre- and postsynaptic determinations to examine the biochemical site of opiate-induced modifications in the nigrostriatal system. The results obtained support the occurrence of dopaminergic variations during the development of morphine tolerance-dependence, although the neuronal location of these effects seems to be different during the morphine addiction—postsynaptic effect—compared with the morphine withdrawal—presynaptic effect.

Previous studies from our group and others have provided evidence concerning that acute morphine exposure in mice increases the density of striatal D_2 receptors and the spontaneous motor activity [(19), Navarro and coworkers, unpublished observations] and potentiates apomorphine-induced climbing behavior (18). However, we found in the present study that chronic exposure to this opiate produced opposite changes to those observed after acute exposure at both behavioral and neurochemical levels. This points out the existence of differences between acute and chronic morphine treatments and indirectly suggests that tolerance or a compensatory response to this opiate have probably been developed. Thus, the number of striatal D_2 receptors decreased—with no changes in their affinity—in parallel with a marked decrease in spontaneous motor activity after chronic morphine exposure. This is a specific effect on D_2 receptors because the number and affinity of D_1 receptors remained unchanged. This decreased number of D_2 receptors was accompanied by a corresponding increase in cAMP concentrations, concordant with the well-known negative effect of D_2 receptors on adenylate cyclase activity (2). Analysis of the statistical correlation between both parameters revealed that they are negatively correlated, which supports the notion that the changes in cAMP production could reflect, in this case, a D_2 dopaminergic coupling rather than an opiate effect.

This inhibitory effect of chronically administered morphine on D_2 receptors seems to be exclusively exerted at the postsynaptic level because no changes in the presynaptic activity, as reflected by the absence of variation in the DOPAC/DA ratio, were observed in these animals. Neuronal cells containing D_2 receptors in the striatum are basically cholinergic neurons, although nigrostriatal neurons also contain them, acting as presynaptic receptors (22). However, it is unlikely that the autoreceptor would have been modified by morphine exposure because of the absence of changes at the presynaptic level whose processes (synthesis and release) are modulated by this receptor.

The fact that the modifications observed after chronic morphine exposure mainly affected the D_2 receptors would suggest a possible role of these receptors not only in the behavioral manifestations associated with the opiate addiction but also in their genesis. Several studies support this hypothesis and in this sense the existence of analgesic effects after stimulation of D_2 receptors with several agonists has recently been described (20). Moreover, Martin and Takemori (19) found that treatment with apomorphine decreased the amount of naloxone required to induce withdrawal-induced jumping in mice acutely pretreated with opiates. They concluded that the increase in D_2 receptors after acute opiate exposure could modify per se the degree of tolerance and dependence that develops in response to opiate administration.

Regarding the possible molecular mechanisms underlying opiate effects on striatal dopaminergic activity, the fact that the opiate-induced changes in this activity were mostly produced at the postsynaptic level suggests a possible heterosynaptic regulation. Two neurotransmitters may interact without a synaptic contact through crossing effects between the intracellular mechanisms coupled to their receptors in the same postsynaptic cell. Examples of heterosynaptic regulation have been described for various neurotransmitters, such as DA and acetylcholine in the striatum and autonomic ganglia [for review, see (12)]. One possibility would be that stimulation of opioid receptors with morphine may alter, via activation or inhibition of the corresponding G proteins, the efficiency of the coupling between D_2 receptors and adenylate cyclase activity. This hypothesis requires further experimental study.

The blockade of opioid receptors with naloxone induced opiate withdrawal, as reflected by the characteristic jumping behavior (13,34). As expected, the effects observed in the addicted mice—the decrease in D_2 receptors and the increase in cAMP concentrations—disappeared after induction of this state. However, in the case of D_2 receptors the loss of statistical differences between placebo- and morphine-exposed mice after naloxone treatment was due more to a naloxone-induced decrease in placebo-exposed mice than to an increase in morphine-exposed mice. In these last animals, naloxone was apparently unable to reverse the morphine-induced decrease in D_2 receptors despite the fact that it was able to precipitate the opiate withdrawal. The reason for this absence of naloxone effect in morphine-exposed mice remains to be determined, although it is possible that it might be related to modifications in the coupling of opioid receptors to their transduction mechanisms as a consequence of the prolonged morphine exposure.

Anyway, the effect of naloxone in placebo-exposed mice supports the idea of a possible modulation of D_2 dopaminergic receptors by opioid receptors as suggested above. Moreover, the observation that naloxone treatment decreased the number of D_2 receptors in placebo-exposed mice agrees with the observations of Martin and Takemori (18,19), who found that the

acute treatment with an opiate agonist such as morphine increased the number of striatal D_2 dopaminergic receptors and potentiated apomorphine-induced climbing behavior after 3 h of treatment in mice. Another important observation in the withdrawal group is the fact that the correlation between the D_2 receptors and cAMP contents in the addicted mice also disappeared, reflecting a possible opioid receptor-mediated naloxone-induced modification in cAMP production. This loss of correlation was observed not only in morphine-exposed mice but also in placebo-treated mice.

Presynaptic activity was not modified after the induction of opiate withdrawal, as reflected by the absence of changes in the DOPAC/DA ratio, although the DA content decreased. This latter effect could presumably be related to an activation of D_2 presynaptic receptors, which negatively modulate neurotransmitter synthesis (22). This is concordant with the findings of Ahtee and coworkers (1), who found decreased synthesis of DA in both striatal and limbic neurons in rats withdrawn from chronic morphine exposure. However, the fact that D_2 receptors were not modified after naloxone treatment to morphine-addicted mice precludes this possibility. An alternative explanation could be that the decrease in DA content might reflect a loss of DA stores in nigrostriatal terminals as a consequence of a repeated DA utilization in these neurons during the jumping behavior characteristic of naloxone-induced opiate withdrawal. However, the possibility that it could be related to a decreased DA reuptake, leading to decreased DA storage, with no changes in release, should be also considered.

In summary, our results evidence the existence of alterations in striatal dopaminergic neurotransmission during morphine tolerance/dependence; this might explain their associated characteristic changes in motor behavior. These changes would be produced at the postsynaptic level, by decreasing the number of D_2 receptors and increasing cAMP production, during the morphine addiction. These differences disappeared after naloxone-induced morphine withdrawal, likely by an inhibitory effect of naloxone on D_2 receptors in placebo-exposed mice, whereas a decrease in DA storage was associated with the induction of opiate withdrawal.

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