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Research report

Prior experience of morphine application alters the c-*fos* response to MDMA (‘ecstasy’) and cocaine in the rat striatum

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Abstract

Repeated morphine application usually leads to the development of tolerance but under certain circumstances sensitization may arise simultaneously. This phenomenon becomes obvious in behavioral tests as increasing locomotor activity and increasing drug self-administration during a course of chronic morphine application. It was suggested recently that sensitization could contribute to addiction. The molecular mechanisms of sensitization may include the long lasting increase in neuronal responsiveness to morphine which was observed in defined brain areas after repeated morphine injections. In this work, we studied whether morphine-sensitized Wistar rats also display an enhanced neuronal activity in response to other drugs of abuse (so called co-sensitization). The substances to be tested were injected as single doses 4 weeks after completion of a 10-day morphine pretreatment. MDMA (3,4-methylenedioxymethamphetamine, 6 mg/kg) as a single test dose yielded a c-*fos* response in a wide range of brain areas. In the caudate putamen, the expression pattern of c-*fos* was clearly altered if the rats had received repeated morphine application previously. In this case, the MDMA-induced c-*fos* expression was markedly confined to the centromedial, mesolimbic aspect of the striatum whereas it had a diffuse appearance in rats not exposed to the opiate earlier. Cocaine application (50 mg/kg) elicited an intense c-*fos* expression in the medial striatum if the animals were morphine-pretreated; it was virtually absent in drug-naive rats after the same cocaine dose. Ten mg/kg cocaine had a similar but weaker effect. No difference in the c-*fos* expression pattern between morphine and saline pretreated animals was observed in the case of a THC (Δ^9-tetrahydrocannabinol, 25 mg/kg) or an LSD (lysergic acid diethylamide, 1 mg/kg) test application. These findings imply that morphine sensitizes the brain towards other addicting drugs. In consequence, morphine sensitization obviously does not solely reflect alterations in μ-opioid receptor signaling. Rather, it seems to reflect further rearrangements within the mesolimbic system.

Themes: Neural basis of behaviour

Topics: Drugs of abuse: opioids and others

Keywords: Co-sensitization; Morphine; MDMA; Cocaine; LSD; THC; c-*fos*; In situ hybridization

1. Introduction

Development of addiction is a main feature of morphine abuse [15]. In studies using experimental animals it was observed that repeated morphine application induces long-lasting alterations of the brain’s responsiveness to another morphine challenge [2,3,5,35,36,38]. Similar mechanisms may be at work in human opiate abusers, too [1,24]. Robinson and Berridge recently have hypothesized that a kind of sensitization could be responsible for the incentive drug taking behavior in human addicts [29]. Morphine sensitization was also detected at the level of gene expression. For example, the dorsal striatum displays at best a low c-*fos* induction after an acute dose of morphine in naive rats but elicits a strong response of c-*fos* and Fos-related antigens in opiate-experienced animals [9,11,25]. It became further obvious that large parts of the limbic system are involved in this response [11]. It is not clear, however, whether this morphine-induced sensitization affects only the brain’s reaction on morphine itself. It is also conceivable that repeated morphine applications induce more general rearrangements within certain brain areas which could lead to an altered response to other

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drugs of abuse, too. This is supported by the finding that morphine and cocaine display co-sensitization in several behavioral experiments [8,20,34]. Furthermore, it was demonstrated that morphine and cocaine induce a very similar set of Fos-related proteins in the brain [18,25]. A human correlate of co-sensitization may be the phenomenon of polytoxicomania which means that addicts often consume a variety of several different drugs of abuse simultaneously [7,19,33,39].

In the present study we aimed to investigate whether co-sensitization is detectable at the level of gene expression, and which brain areas will be involved in this process. Therefore, we studied c-*fos* accumulation throughout the whole brain after an injection of various frequently abused drugs in rats with or without prior morphine experience. Cellular-*fos* constitutes a sensitive indicator for gene induction since it belongs to the family of transcription factors and thereby governs the expression of a wide range of downstream genes. The drugs that we used were MDMA, THC, LSD and cocaine (morphine itself was studied earlier) [14]. These substances cover a wide range of pharmacological effects, namely sedation (morphine), stimulation (cocaine, MDMA) and hallucinogenesis (LSD and, in part, MDMA) [13,16,26,37], and affect different neurotransmitter systems. The pharmacological target of cocaine are dopaminergic and, to a lesser extent, noradrenergic synapses [26]; LSD affects serotoninergic transmission [32]. THC and morphine bind to specific receptors for endogenous cannabinoids [28] and opioids [17], respectively. And MDMA is known to affect the dopamine and serotonin system simultaneously [16,30,31].

The doses of the drugs employed here were chosen according to our previous studies. The substances are largely different in terms of their affinity to the respective receptors and hence also in terms of their behavioral effects. Since gene expression studies usually need higher doses than behavioral observations to gain reliable results, we employ up to five times the dose in our gene expression studies as used in standard behavior test such as locomotion [13]. Animals were left morphine-free for 4 weeks before the test substances were applied to avoid any confusion by interfering signs of morphine withdrawal. The latter are detectable by measuring c-*fos* expression up to 2 weeks after finishing our 10-day pretreatment scheme [11].

2. Materials and methods

2.1. Materials

Chemicals were of the highest purity available and were obtained from Merck (Darmstadt, Germany), unless otherwise stated. Radiolabeled substances were from NEN Life Science Products (Cologne, Germany); the c-*fos* oligonucleotide was synthesized by Eurogentec (Seraing, Belgium). Microscopic slides (Superfrost Plus) were obtained from Menzel (Braunschweig, Germany).

2.2. Animal treatment

For all procedures ethical approval was sought prior to the experiments according to the requirements of the National Act on the Use of Experimental Animals (Germany).

The experiments were conducted with male Wistar rats, 8 weeks old in the beginning of the experiment. The animals were housed in groups of five rats per cage under controlled laboratory conditions in a light- (12-h light/dark cycle), temperature- (20 ± 2°C), and relative air humidity-(55–60%) controlled environment with free access to food and water. Animals were made tolerant to morphine by the procedure outlined below.

2.2.1. Establishment of morphine tolerance

Animals received two daily i.p. applications of increasing doses of morphine according the following treatment schedule: day 1 and 2, 2×10 mg/kg; day 3 and 4, 2×20 mg/kg; day 5 and 6, 2×30 mg/kg; day 7 and 8, 2×40 mg/kg; day 9 and 10, 2×50 mg/kg as recently described [11].

2.2.2. Controls

Another group of animals received saline, i.p., twice daily for 10 days instead.

2.2.3. Application of a test substance

Four weeks after finishing the pretreatment procedure animals received either one injection of saline, 6 mg/kg MDMA, 10 mg/kg or 50 mg/kg cocaine, 1 mg/kg LSD or 25 mg/kg THC. The numbers of animals in each group are indicated in the legend of the respective figure.

Immediately after the test injection the rats were placed in new cages and thereby grouped according to the substance they had received. Care was taken not to bring together animals which were housed in different cages before, that means all animals which received the same substance were familiar with each other.

2.3. Tissue preparation and in situ hybridization

Animals were sacrificed by decapitation 60 min after the application of the test substance. The brains were removed and frozen immediately at −70°C.

In situ hybridization for c-*fos* was performed as described recently [10,12]. All of the sections were processed in the same round of in situ hybridization. In brief, 15-μm thick frontal sections of the brains were fixated in 4% paraformaldehyde for 30 min. An oligonucleotide with the following sequence served as a probe: 5′-GCA GCG GGA GGA TGA CGC CTC GTA GTC CGC GTT GAA ACC CGA GAA-3′ corresponding to base position 141–185

from the c-*fos* mRNA [21]. It was radiolabeled with deoxyadenosine 5′[alpha-[^{35}S]thio]triphosphate using the terminal deoxynucleotidyltransferase reaction. Hybridization was performed over night at 42°C. Thereafter, the sections were washed four times at 55°C in 1× SSC. Finally, the slices were dried and exposed to X-ray films for 26 days.

By using our standard protocol as published earlier [12], we achieved a very even in situ hybridization with a low variation between the animals of the same treatment group.

2.4. Quantification

Quantification of the amount of c-*fos* mRNA in the caudate putamen was performed after in situ hybridization and autoradiography by scanning the exposed X-ray films with a CCD camera. The signal intensities were measured as density profiles of signal intensity across the caudate putamen in mediolateral direction, using the software of NIH Image, version 1.57. From each animal three or four slices were hybridized to ensure uniformity of hybridization. Thereafter, one representative slice was chosen for quantification of each animal. Data were obtained from the right hemisphere for each slice per region per rat. Data are presented as mean±standard error of the mean (S.E.M.) for each treatment group.

3. Results

A single injection of MDMA, cocaine, LSD, THC or, as a control, saline into morphine-pretreated or saline pretreated rats revealed the following differences in the c-*fos* mRNA expression between the morphine-experienced and the drug-naive animals.

3.1. Controls

Control animals which received saline instead of a test substance were included in this study because our previous work had revealed that the expression of the immediate early gene c-*fos* is inducible for the reason of handling and injection stress in a variety of brain areas [11,14].

In animals pretreated with saline and tested with a saline injection 4 weeks later (Fig. 1A and C, two different animals from this treatment group), c-*fos* expression was induced strongly in the piriform cortex and, weaker, in the cingulate cortex. Furthermore, a c-*fos* signal in the septal area and in the basal ganglia was obvious, extending in a diffuse manner from the lateral septum into the medial, ventromedial and dorsal portion of the striatum. A similar pattern was seen in animals which were pretreated with morphine and received a test dose of saline 60 min before decapitation (Fig. 1B and D, two different animals from this treatment group).

3.2. MDMA-induced c-fos expression pattern

A test dose of 6 mg/kg MDMA elicited a marked c-*fos* expression in a wide array of brain regions as already described [13]. In brief, a hybridization signal was seen in all cortical areas, most intense in the upper layers of the frontal and parietal cortices, in the core and, weaker, in the shell of the nucleus accumbens, diffusely in the whole striatum, in the lateral septum, in the paraventricular nucleus of the hypothalamus, in the mammillary nuclei and in the ventral part of the central gray. In these areas except the striatum, the c-*fos* mRNA expression was induced with the same intensity and pattern in animals with as well as without morphine experience.

In the caudate putamen the spatial distribution of the c-*fos* hybridization signal clearly differed between the morphine and the saline pretreated animals. As shown in Fig. 2, the c-*fos* signal induced by MDMA was concentrated to the centromedial aspect of the caudate putamen if the animals had received a pretreatment with morphine (pictures A–D). In contrast, a diffuse hybridization signal encompassing a large portion of the striatum occurred after an MDMA test injection in saline pretreated animals (pictures E–H). The c-*fos* expression was in each case weak in the lateral striatum and increased gradually towards the centromedial striatum. Morphine pretreatment caused this gradient to become much steeper.

To quantify the increasing gradient of c-*fos* expression from the lateral to the centromedial aspect of the caudate putamen, we determined the density profile of the hybridization signal across the corpus striatum in mediolateral direction as shown schematically in Fig. 3(picture B). The resulting profile plots, which are exemplified in Fig. 2(pictures C, D, G and H), were evaluated by determining their maximal slope.

Fig. 3A shows the means (±S.E.M.) from all animals of the treatment groups. It can be derived from this figure that the gradient was approximately twice as steep in saline-pretreated animals after MDMA application as compared with drug-naive controls. The maximal slope of the profile, however, increased markedly in rats with morphine history and MDMA test application. In this case, the slope was approximately fivefold the basal value and more than twice the value of saline pretreated, MDMA injected rats. In contrast, morphine experienced rats that had received a saline injection remained at the same level as drug-naive controls.

3.3. Cocaine-induced c-fos expression pattern

A test application of 10 mg/kg cocaine in saline-pretreated animals caused only a minor c-*fos* expression which was hardly distinguishable from saline background (Fig. 4A). The same test dose applied after morphine pretreatment induced a stronger c-*fos* synthesis in a large part of the caudate putamen. This area encompassed the

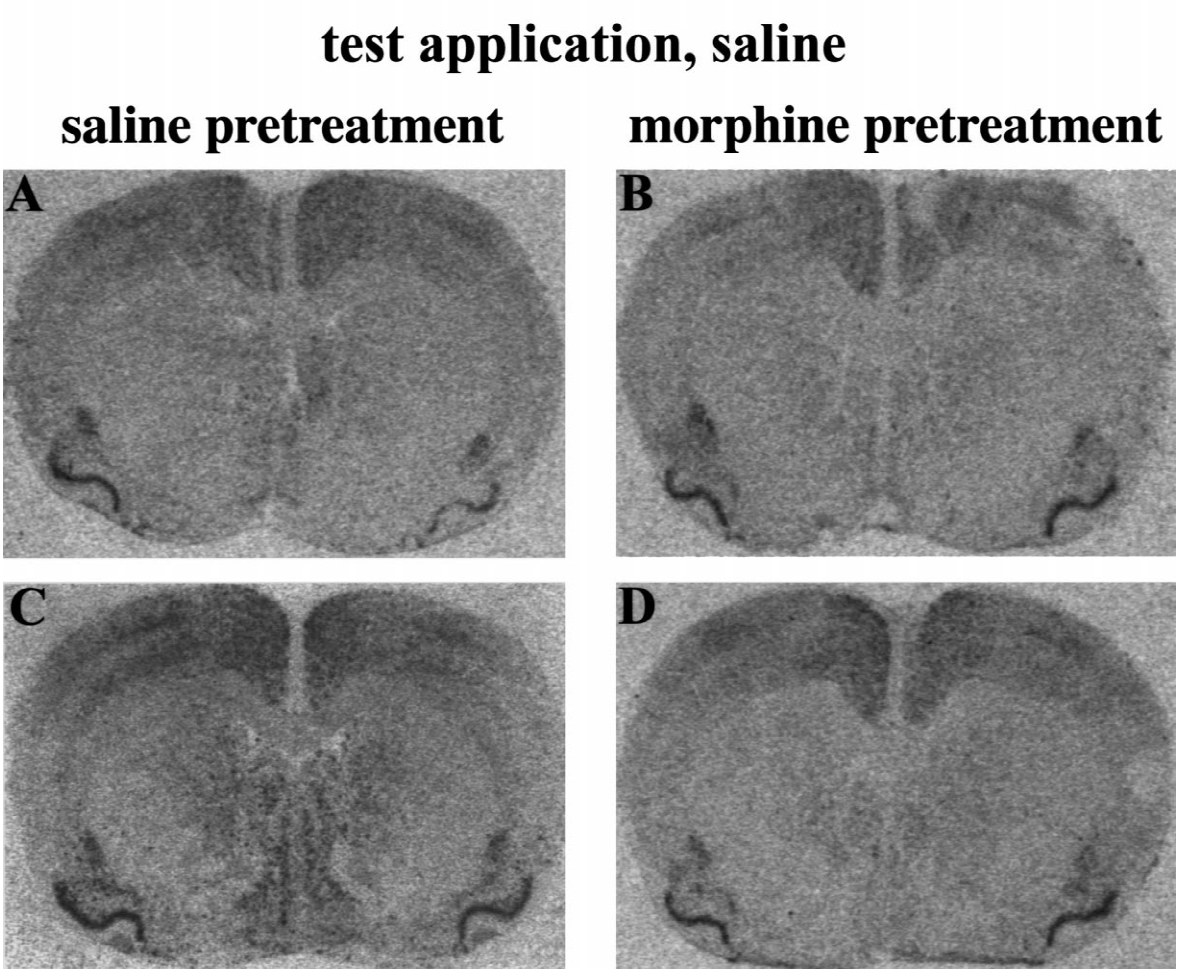

Fig. 1. Test application of saline as a control in repeatedly morphine pretreated and in drug-naive rats. In situ hybridizations for c-*fos* on coronal sections at the striatal level is shown. Each treatment groups (saline pretreated and morphine pretreated) consisted of three animals. Sections in the left column (A, C) are from two animals treated for 10 days with saline 4 weeks prior to a test application of saline. Sections of the right column (B, D) are from two rats treated with ascending morphine doses (from 10 to 50 mg/kg) for 10 days 4 weeks before a saline test dose. Animals were decapitated 60 min after the saline test application.

medial and central aspects of the striatum. The c-*fos* signal expanded from dorsal to ventral but spared the lateral and dorsolateral part of the caudate putamen as well as the nucleus accumbens (Fig. 4B).

Because 10 mg/kg of cocaine produced only a rather weak c-*fos* response, we tested 50 mg/kg to obtain a more pronounced signal. However, even this extraordinary high dose again caused only a very weak c-*fos* induction in saline pretreated animals (Fig. 4C) [13]. This cocaine dose injected into morphine-pretreated animals (Fig. 4D) led to a strong increase in c-*fos* synthesis in the same brain area as described for 10 mg/kg cocaine. Additionally, a weaker signal in the core of the nucleus accumbens became visible.

The high cocaine dose of 50 mg/kg caused tonic-clonic seizures in two out of six animals. In this case, there was a strong c-*fos* expression in the whole caudate putamen including the nucleus accumbens displaying strikingly uniform intensity. Furthermore, all cortical regions, most intensely the upper layers, contained c-*fos* mRNA as well as the lateral septal nuclei. Therefore, the c-*fos* signal induced by the convulsions was clearly distinguishable from the cocaine effect. Animals which showed signs of seizures were excluded from this study.

3.4. LSD- and THC-induced c-fos expression pattern

After a test application of either LSD (1 mg/kg) or THC (25 mg/kg) no changes in expression pattern and intensity were seen irrespective whether the rats were pretreated with saline or morphine (Fig. 5). Thus, the increase of c-*fos* mRNA in many brain areas was only due to the single LSD or THC application and was not influenced by the morphine pretreatment.

4. Discussion

The present study revealed that morphine is able to alter the responsiveness of the brain to MDMA and cocaine

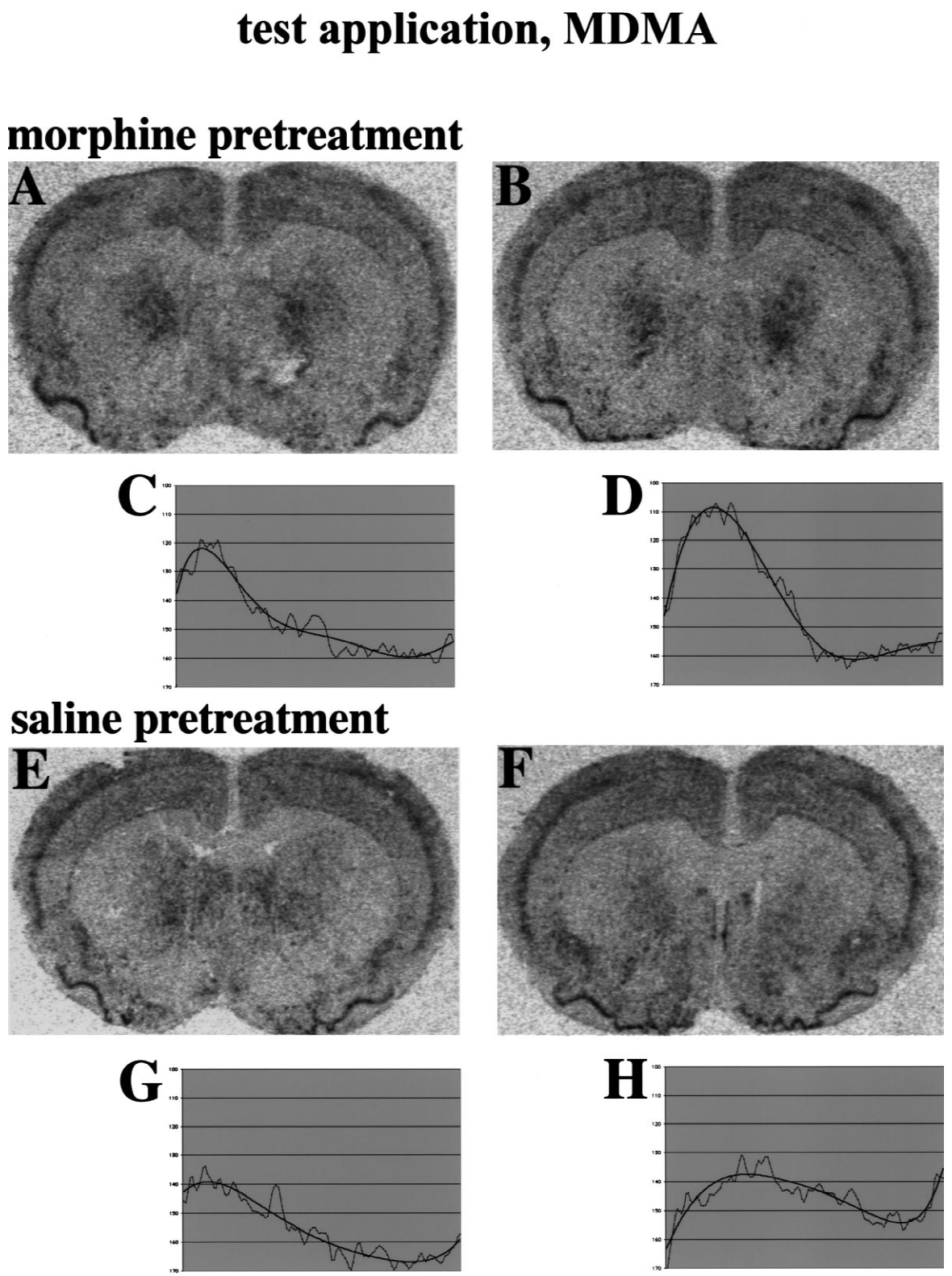

Fig. 2. Induction of c-*fos* mRNA by a test application of 6 mg/kg MDMA (3,4-methylenedioxymetamphetamine) in morphine-experienced and in drug-naive rats. The figure shows in situ hybridizations on coronal sections of two representative animals of each group (morphine pretreated, pictures A and B, or saline pretreated, pictures E and F) after the MDMA test dose at the level of the striatum and, immediately below, the corresponding density profiles across the right caudate putamen (pictures C and D for morphine pretreated animals and pictures G and H for saline pretreated rats). The profile curves were obtained by measuring the signal intensities along the horizontal bar that is indicated in Fig. 3B (adapted from Ref. [27], with permission). All animals were decapitated 60 min after the last injection.

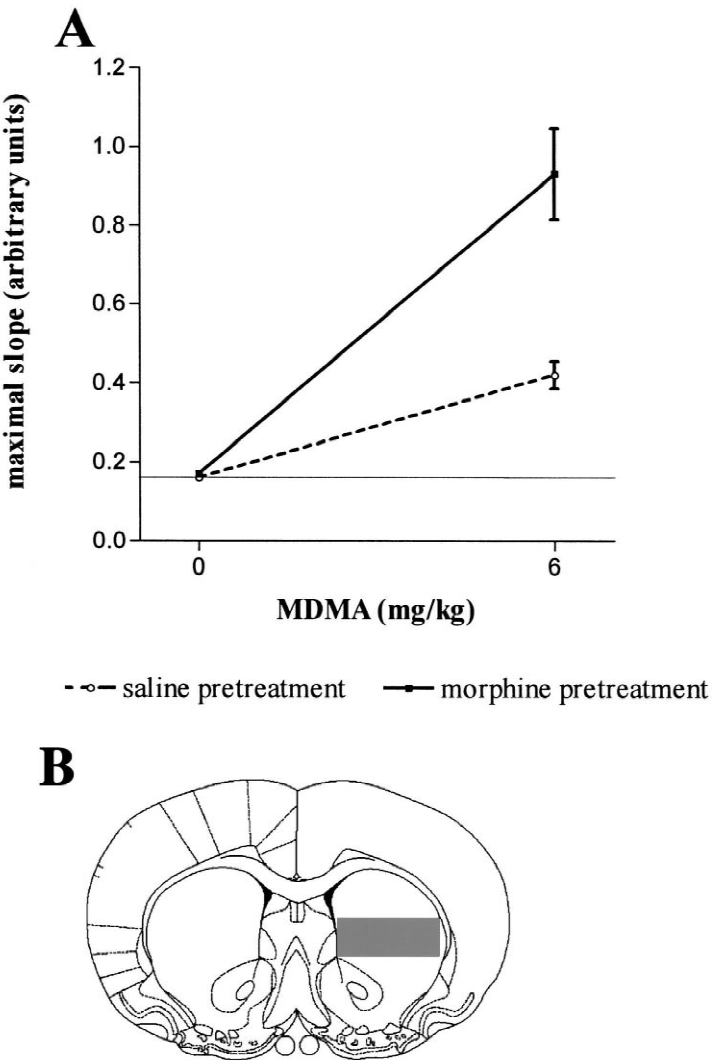

Fig. 3. Quantification of the c-*fos* expression density profiles across the caudate putamen. (A) Cellular-*fos* mRNA synthesis was induced by a test dose of either 0 (saline) or 6 mg/kg MDMA in animals having received morphine (solid line) or saline (dashed line) pretreatment, respectively, 4 weeks earlier. Each data point was derived by averaging the maximal slopes of the density gradients of three or four animals. Data are given as mean±S.E.M. (B) The lateromedial gradient of c-*fos* expression across the striatum was obtained as a density profile from each slice along the gray horizontal bar drawn in the picture.

along with the well-established sensitization towards itself (see also Refs. [9,11,13,25]). Notably, two other drugs of abuse, LSD and THC, did not yield any co-sensitization with morphine; this means that there was no difference in the LSD- or THC-induced c-*fos* expression pattern irrespective whether the rats were chronically pretreated with morphine or not.

MDMA and cocaine differ to some extent from each other in their sites of action although they are quite similar in their pharmacological effects. It will be outlined in the following which common properties of these two substances might be responsible for their co-sensitization with morphine. Cocaine is known to act as an indirect agonist at noradrenergic, dopaminergic and serotoninergic synapses by inhibiting the reuptake and therefore the removal of the respective transmitter from the synaptic cleft [26]. This implies that cocaine will act only on synapses in which transmitter is released by another stimulation. In contrast, MDMA is able to release transmitter (dopamine and serotonin) by itself; like cocaine, it also blocks the reuptake of these transmitters. Furthermore, MDMA inhibits a carrier for serotonin on the neurotransmitter storage vesicles [16,30,31,37]. Therefore, indirect action in dopamine and serotonin synapses are a common mechanism of MDMA and cocaine. Serotonin, however, might be less important because LSD, acting on serotonin receptors only [6], did not show co-sensitization with morphine. In consequence, dopamine could be involved, and this is further supported by our finding that the co-sensitization between morphine and MDMA as well as between mor-

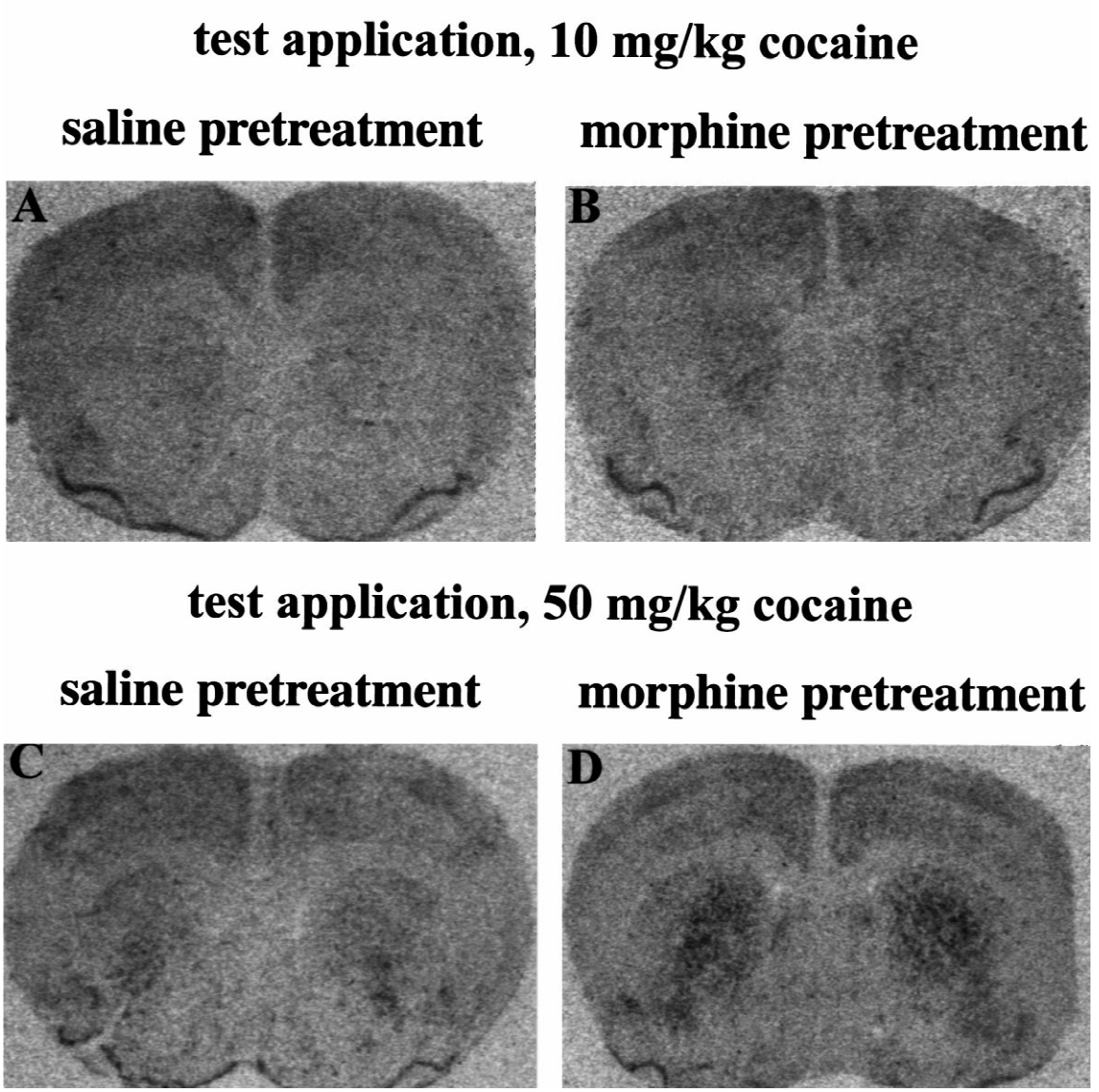

Fig. 4. Induction of c-*fos* mRNA by a test application of 10 or 50 mg/kg cocaine in morphine-experienced and in drug-naive rats. The figure displays in situ hybridizations for c-*fos* on coronal sections taken at the level of the striatum. The left column (pictures A and C) shows slices from animals which had received saline pretreatment 4 weeks before the cocaine injection; the right column (pictures B and D) depicts slices from animals which had received morphine pretreatment 4 weeks before the cocaine injection. The animals shown in pictures A and B received 10 mg/kg cocaine, and the animals depicted in pictures E and F received 50 mg/kg cocaine as a test dose. Each group consisted of three animals. All animals were decapitated 60 min after the last injection.

phine and cocaine was only obvious in the caudate putamen, a brain area exceptionally rich in dopamine receptors.

Our findings further imply that the subchronic morphine pretreatment scheme did apparently not only alter neuronal circuits that are responsible for morphine's action itself (e.g. by altering opioid receptor signaling) but did also influence circuits used by MDMA and cocaine. The latter may include dopaminergic paths as outlined above. The dopaminergic transmitter system is assumed to be closely involved in the phenomenon of drug addiction. In consequence, if repeated morphine application modifies neuronal circuits that are involved in the process of addiction, the observed c-*fos* expression in the centromedial striatum may identify a brain region which is important for the development of drug dependence.

The question remains by which means morphine affects MDMA or cocaine action since neither has morphine similar pharmacological targets as both other drugs nor is morphine directly acting on dopaminergic or serotoninergic synapses, the targets of cocaine and MDMA. Rather, a more complex interaction has to be postulated which needs time to develop. The latter results from the observation that repeated morphine applications are required. If one consid-

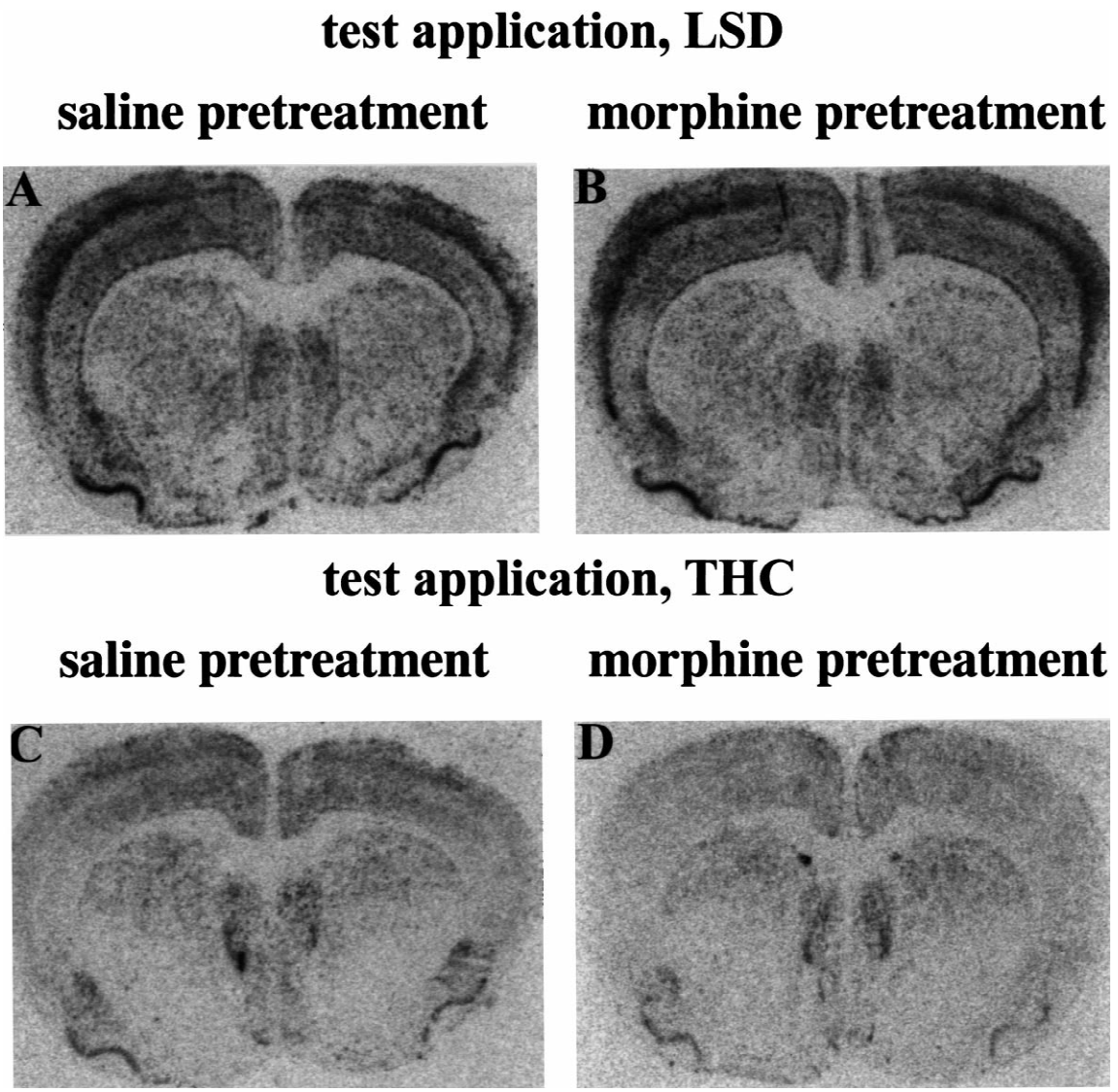

Fig. 5. Induction of c-*fos* mRNA by a test application of LSD (1 mg/kg) or THC (25 mg/kg) in morphine-experienced and in drug-naive rats. The coronal striatal sections shown in pictures A and C are from animals which were saline-pretreated, and the sections depicted in B and D are from morphine-experienced rats. In the upper row (A and B) the test dose was 1 mg/kg LSD, and in the lower row (C and D) it was 25 mg/kg THC. Each treatment group consisted of two animals. All animals were decapitated 60 min after the last injection. Note that no differences are visible between morphine-experienced and drug-naive animals.

ers the c-*fos* expression pattern that results from a single MDMA injection in a drug naive rat (described recently by our group [13]), then it becomes obvious that it appears largely similar to the pattern induced by LSD. This presumably reflects the serotoninergic aspect of MDMA action. But in addition to the brain areas that are stimulated by both, LSD and MDMA, the latter caused a relatively strong c-*fos* expression in the caudate putamen, which probably reflects the dopaminergic part of the MDMA effect. The observed gradient of c-*fos* expression within the striatum from medial to lateral may at least in part result from the fact that the lateral striatum was found to expresses slightly more (inhibitory) D2-receptors than the medial striatum (our data not shown) in accordance with the literature [4,22,23]; the distribution of (excitatory) D1-receptors was virtually equal throughout the whole caudate putamen. To summarize these findings, it appears that the c-*fos* expression pattern in response to MDMA in naive rats fairly well represents the distribution of the target receptors (dopamine and serotonin receptors) of MDMA throughout the brain.

The picture became different if the rats were repeatedly treated with morphine before they received the MDMA test dose. A very strong c-*fos* accumulation in the centromedial caudate putamen appears, and the underlying stimulation of the region can no longer be explained by a

correspondingly high receptor density. Rather, a more integrative signal processing pattern seems to come into play.

To produce such an integrative response within mesolimbic parts of the caudate putamen to drug application, repeated morphine application is required, and this chronic injection scheme seems to alter the circuitry within the limbic or mesolimbic system to a certain extent (note that these alterations last for at least 4 weeks). If however the circuitry within mesolimbic areas is altered in some way as reflected by the altered response to MDMA and cocaine, then one can assume that the response of these parts of the brain will also be modified towards endogenous stimulation. It is therefore conceivable that the processing of emotions could be affected in a similar manner and this in turn could represent a neuronal correlate for persistent drug craving in addicts. Finally, phenomena similar to the co-sensitization described here could be responsible for polytoxicomania which is often observed in addicts and means that these individuals consume a large variety of different drugs simultaneously.

To summarize our findings, our results demonstrate that rats sensitized to morphine respond to certain other drugs of abuse with a c-*fos* mRNA expression pattern which is clearly different from that which results from the application of this drug in morphine-naive animals. The fact that not the intensity of the response changed by morphine pretreatment but the expression pattern means that other brain areas begin to respond to a given drug after a history of morphine pretreatment.

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