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Research report

Acute injection of drugs with low addictive potential (Δ^9-tetrahydrocannabinol, 3,4-methylenedioxymethamphetamine, lysergic acid diamide) causes a much higher c-fos expression in limbic brain areas than highly addicting drugs (cocaine and morphine)

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Abstract

It is regarded as a common pharmacological property responsible for the addictive potential of drugs of abuse that they are able to activate brain areas involved in the sensation of pleasure, especially the nucleus accumbens. To investigate the connection between addictive potential and stimulation of critical brain areas in more detail, we studied c-fos accumulation in response to various addicting drugs in direct comparison. The substances were injected into drug-naive rats, and c-fos mRNA levels were measured throughout the brain by in situ hybridization. Cocaine in a high dose of 50 mg/kg yielded only a discrete c-fos expression in the medial and central striatum. Morphine (50 mg/kg) caused a weak c-fos synthesis in the lateral septum. THC (Δ^9-tetrahydrocannabinol), 25 mg/kg, induced c-fos mRNA again in the lateral septum and furthermore in large parts of the striatum including the nucleus accumbens. LSD (lysergic acid diamide), 1 mg/kg, elicited a similar c-fos expression pattern as THC, but there was additionally a very strong hybridization signal in the cerebral cortex, especially in the upper layers, and in the ventral part of the periaqueductal gray. The widest range of brain areas was activated by MDMA (3,4-methylenedioxymethamphetamine, 'ecstasy'), 6 mg/kg. In addition to the regions that responded to LSD, there was a very pronounced c-fos signal in the nucleus accumbens core and shell and in the mammillary nuclei. Taken together, our study revealed that the drugs with the highest addictive potential, cocaine and morphine, yielded a very low c-fos synthesis throughout the brain whereas the brain regions closely linked to pleasure (especially the nucleus accumbens) responded strongly to drugs with an apparently lower addictive potential (THC, LSD, MDMA).

Keywords: MDMA; LSD; THC; Cocaine; Morphine; C-fos; In situ hybridization

1. Introduction

Substances that are abused for their psychoactive properties differ largely in their chemical constitutions and in their pharmacological targets. For example, MDMA (3,4-methylenedioxymethamphetamine), which is also known as ecstasy and acts as a psychostimulant as well as a psychedelic agent, represents an indirect agonist at serotonin and dopamine synapses [27,28]. The hallucinogen LSD (lysergic acid diamide) is a direct agonist on serotonin receptors. Cocaine blocks the reuptake of dopamine and norepinephrine [22]. THC (Δ^9-tetrahydrocannabinol) and morphine are agonists on specific G-protein coupled receptors for endogenous neurotransmitters or neuromodulators [11,24]. Hence, it is not obvious as to what the common pharmacological property of all these mentioned compounds is that makes drugs abused. It is assumed that they all activate the same brain areas, directly or indirectly, which are involved in the representation of pleasurable sensations and might therefore be responsible for addictive behaviour. One brain area, the nucleus accumbens, was

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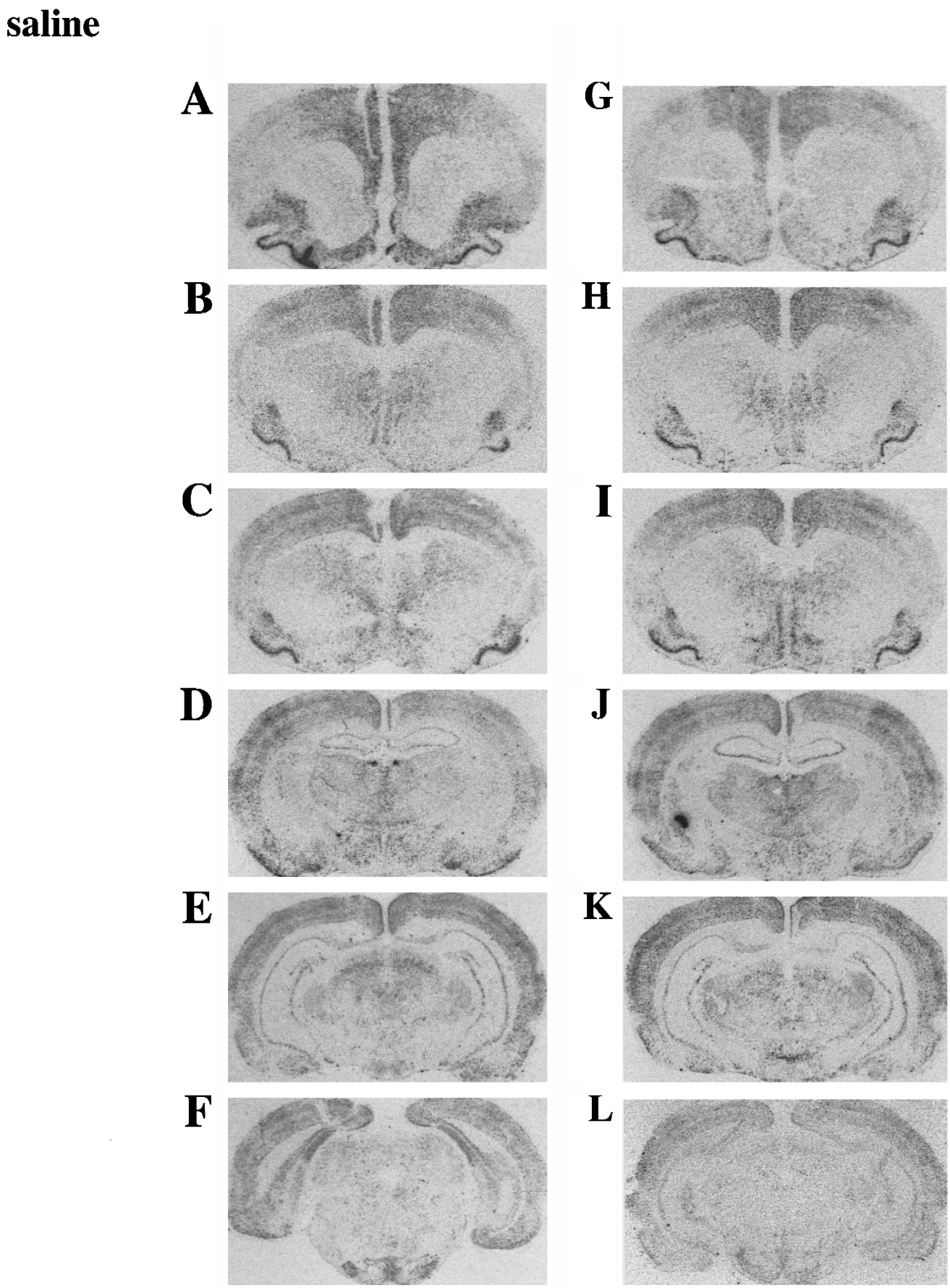
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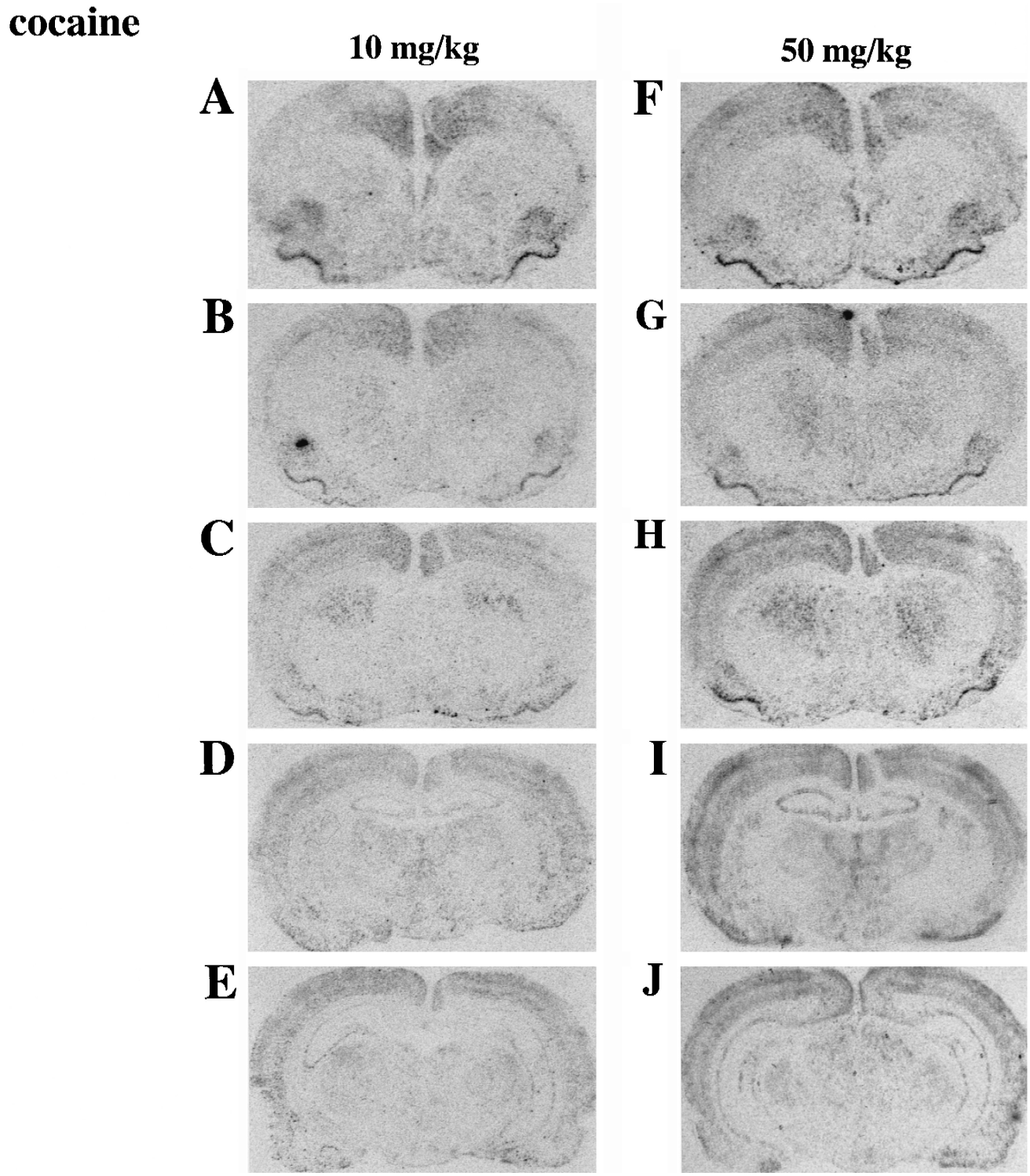


Fig. 2. Induction of c-fos expression by the application of 10 mg/kg or 50 mg/kg cocaine. The figure shows photographs from five different coronal sections of the brain (from top to bottom) after in situ hybridization for c-fos. Left column, 10 mg/kg cocaine, right column, 50 mg/kg cocaine. Schematic drawings to identify important brain areas on the depicted slices are provided in Fig. 5.

Fig. 1. Induction of c-fos expression in control rats that received saline instead of a drug. Shown are the results of the in situ hybridization for c-fos on sections from six different coronal levels of the brain (from top to bottom) from two different animals of this treatment group (left and right column, respectively). Schematic drawings to identify important brain areas on the depicted slices are provided in Fig. 5.

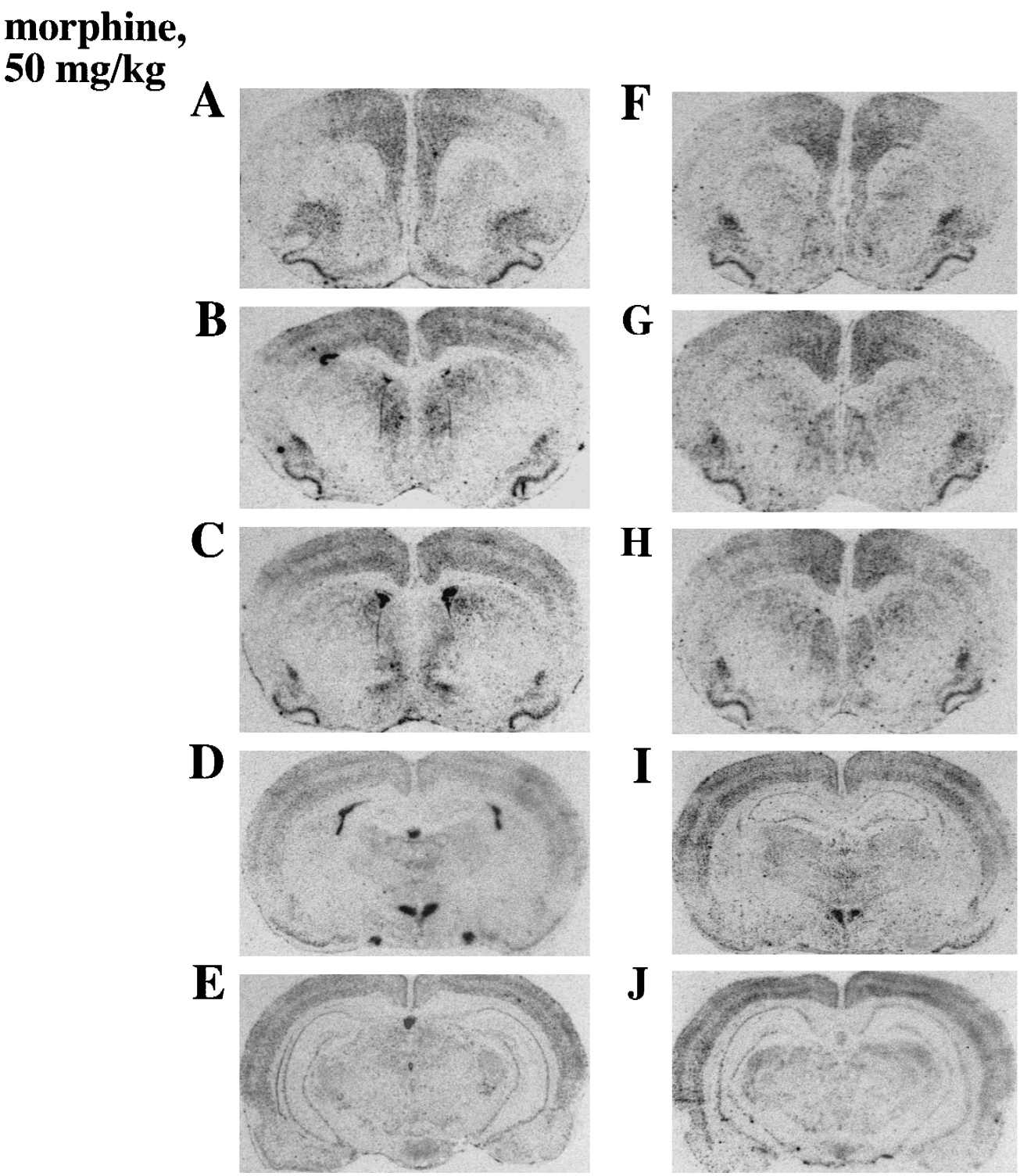
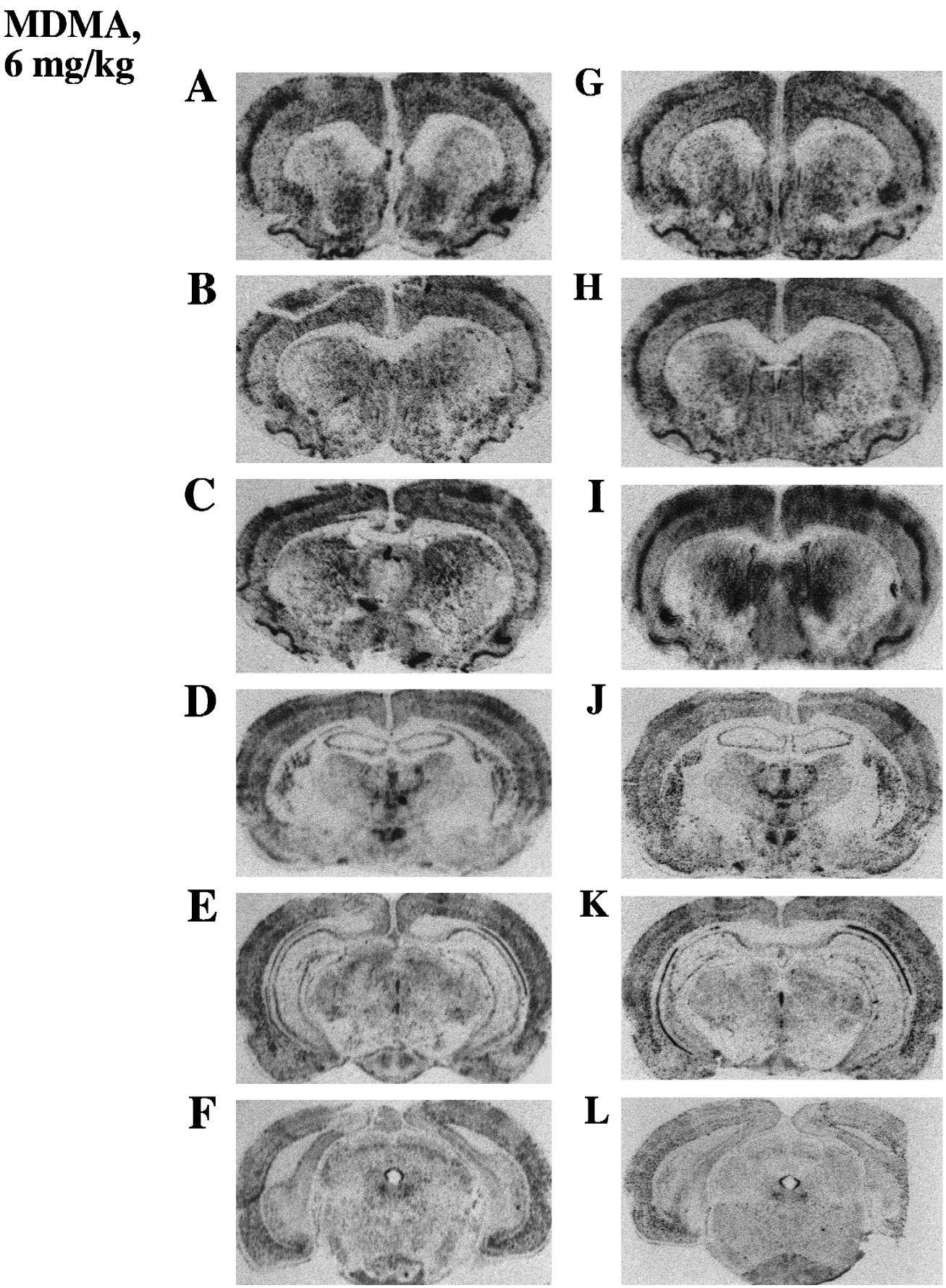


Fig. 3. Induction of c-fos expression by the application of 50 mg/kg morphine. The figure shows photographs from five different coronal sections of the brain (from top to bottom) after in situ hybridization for c-fos from two different animals of this treatment group (left and right column, respectively). Schematic drawings to identify important brain areas on the depicted slices are provided in Fig. 5.

Fig. 4. Induction of c-fos expression by the injection of 6 mg/kg MDMA ('ecstasy'). The figure shows photographs from six different coronal sections of the brain (from top to bottom) after in situ hybridization for c-fos from two different animals of this treatment group (left and right column, respectively). Schematic drawings to identify important brain areas on the depicted slices are provided in Fig. 5.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already identified as a common target for stimuli that are linked to pleasure [1,4,12,34,35] and, as a possible consequence, to addiction [10,21]. To search for brain areas that are activated in correlation with the application of a drug of abuse, we investigated c-fos mRNA expression by in situ hybridization in rats that have received a single injection of an addictive drug. The substances tested ranged from morphine and cocaine, which are regarded as highly addicting to drugs with lower addictive potential (MDMA, LSD and THC) which do usually not cause the loss of a productive life [22]. The doses of the drugs that we used in this study were derived from our previous experiments with morphine which revealed that detectable changes in gene expression usually require much higher doses of the drug than behavioural tests do to yield unequivocal results. Therefore, the dose of each drug was increased four to five times as compared with the doses often used in drug discrimination experiments (see for example, Refs. [17,29,30] for MDMA, LSD and cocaine, respectively).

2. Materials and methods

2.1. Animals

The experiments were conducted with male Wistar rats (Shoe:Wist(Shoe)). The animals were housed in groups of five rats per cage under controlled laboratory conditions in a light- (12 h on/12 h off), temperature- (20 ± 2°C) and relative air humidity- (55–60%) controlled environment with free access to food and water.

Animals received either one saline ($n = 4$), MDMA (6 mg/kg; $n = 3$), LSD (1 mg/kg; $n = 2$), THC (25 mg/kg; $n = 2$), cocaine (10 mg/kg; $n = 3$; resp. 50 mg/kg; $n = 3$) or morphine (50 mg/kg; $n = 4$) injection, i.p., 60 min before decapitation. Immediately after the injection, the rats were placed in new cages and thereby grouped according to the substance they have received. Care was taken not to bring together animals which were housed in different cages before, which means that all animals which received the same drug were familiar with each other.

The injection of the drugs had the expected pharmacological effects in terms of behaviour. MDMA elicited a somewhat stereotypical behaviour like crawling and there was salivation. LSD injection caused interrupted crawling, strong salivation and piloerection. After the application of THC, the rats were sedated. Cocaine induced increased locomotion including frequent rearings in both doses. Morphine had a highly sedating effect.

2.2. In situ hybridization

In situ hybridization for c-fos was performed as described recently [6,7]. 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′ [14]. It was radiolabelled 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 $1 \times$ SSC. Finally, the slices were dried and exposed to X-ray films for 26 days.

By using our standard protocol as published earlier [7], we achieved a very even in situ hybridization with a low variation between the animals of the same treatment group. To allow an estimation of this variation, we will depict brain slices from two animals for each substance applied in Section 3. An independent series of experiments yielded the same findings.

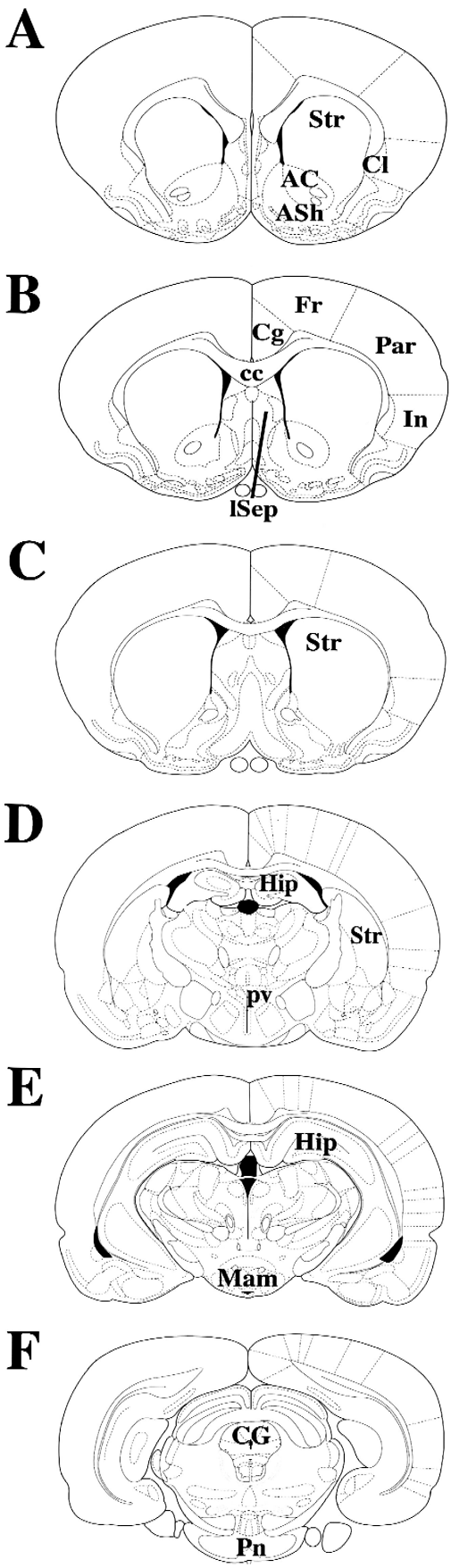
3. Results

The following c-fos expression patterns in the brain were observed 60 min after an acute dose of the drugs listed below. As a control, saline instead of a drug was injected.

Saline controls were included to determine c-fos expression due to stress of handling and injection or due to novelty. They showed c-fos mRNA synthesis in the following brain areas (in the order of decreasing intensity): in the piriform cortex, in the pontine nuclei, in the entorhinal cortex, in the cerebellum, in the claustrum, in the cingulate and frontal cortices, in the dorsal thalamus, in the dorsomedial part of the striatum, in the geniculate nuclei and in the posteromedial cortical nuclei of the amygdala (Fig. 1). For the description of the drug effects, the brain regions that already appeared after saline application will be omitted.

Cocaine application in a high dose of 50 mg/kg led to weak c-fos expression as compared with the other drugs and was hardly to distinguish from saline background

Fig. 5. Schematic drawings corresponding to the coronal sections shown in Figs. 1–4, 6 and 7. To identify important brain regions, sketches were adapted from Ref. [23] and representative structures were indicated as follows: Cg, cingulate cortex; Fr, frontal cortex; Par, parietal cortex; In, insular cortex; Str, striatum; AC, core of the nucleus accumbens; ASh, shell of the nucleus accumbens; Cl, claustrum; lSep, lateral septal nuclei; cc, corpus callosum; pv, paraventricular hypothalamic nucleus; Hip, hippocampal formation; Mam, mammillary nuclei; CG, central gray; Pn, pontine nuclei.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(Fig. 2F–J). Only medial and central aspects of the striatum appeared slightly stronger in cocaine-treated vs. saline-treated animals (compare Fig. 1B,C,H,I [saline] with Fig. 2G,H [50 mg/kg cocaine]). A dose of 10 mg/kg had no clear effect (Fig. 2A–E).

Morphine even in a high dose of 50 mg/kg (Fig. 3) revealed only a weak c-fos signal in the lateral septum (Fig. 3B,C,G,H) in accordance with our previous findings [6]. The paraventricular nucleus of the hypothalamus also showed a hybridization signal which apparently encom-

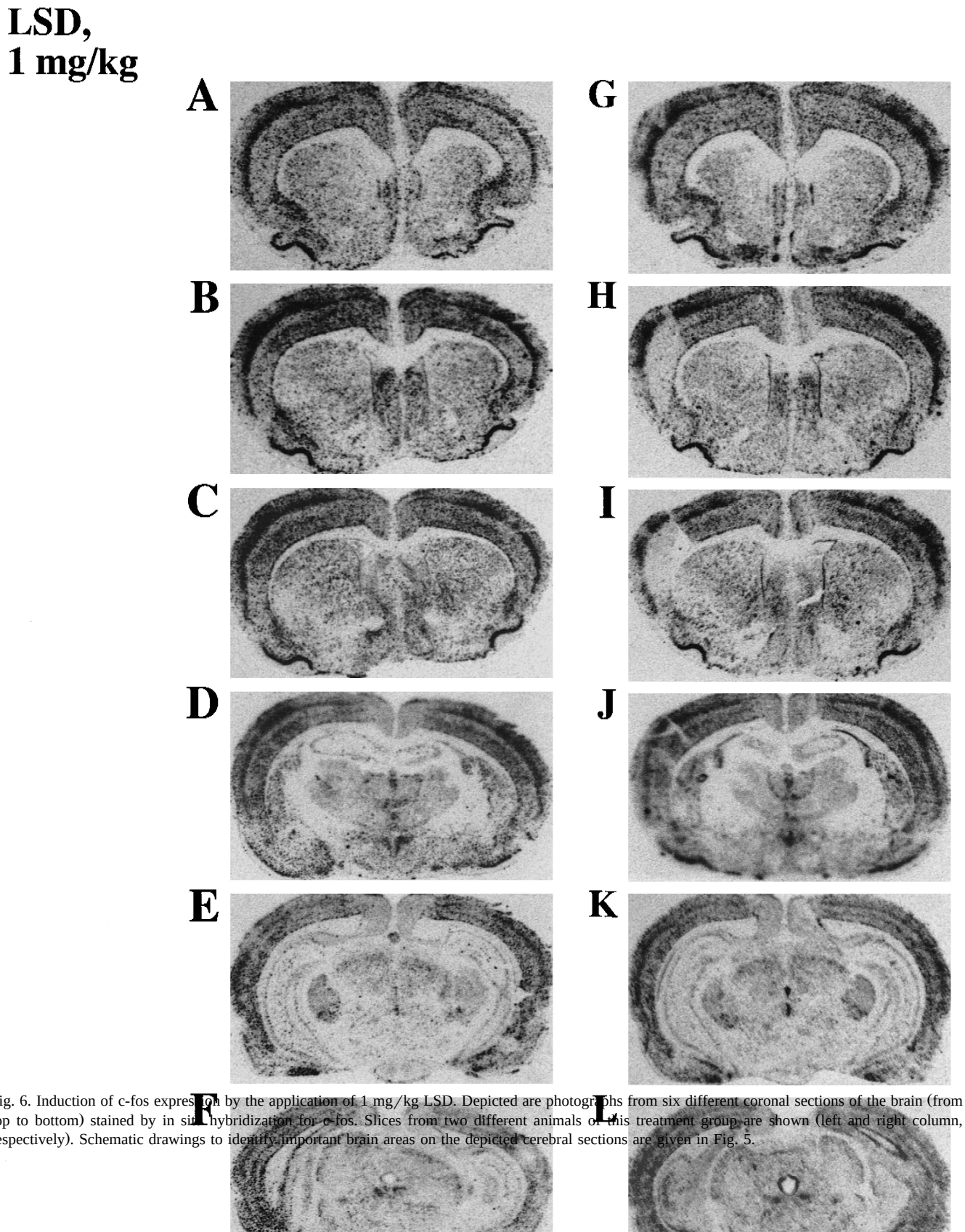

Fig. 6. Induction of c-fos expression by the application of 1 mg/kg LSD. Depicted are photographs from six different coronal sections of the brain (from top to bottom) stained by in situ hybridization for c-fos. Slices from two different animals of this treatment group are shown (left and right column, respectively). Schematic drawings to identify important brain areas on the depicted cerebral sections are given in Fig. 5.

passed both magnocellular and parvocellular sections of this nucleus (Fig. 3D,I).

MDMA (6 mg/kg, Fig. 4) caused a strong c-fos expression in all cortical areas which was most intense in the upper layers (layers two to four as revealed by comparison with Nissl-stained sections; not shown) of the frontal and parietal cortices. There was also pronounced c-fos expression in the dorsal and medial part of the striatum, and, stronger than in the striatum, in the core and shell of the nucleus accumbens. Furthermore, the lateral septum, the paraventricular nucleus of the hypothalamus, the mammillary nuclei and the ventral part of the central gray did also display a marked c-fos response (for identification of the brain areas see schematic drawings in Fig. 5). Notably, all mentioned brain areas except dorsolateral and lateral parts of the striatum and the parietal cortex belong to the limbic system or are closely associated with it.

LSD (1 mg/kg, Fig. 6) induced c-fos mRNA in the following parts of the brain: in all cortical regions, most pronounced frontal and parietal and in the upper layers (layers two to four); in the lateral septal area; throughout the striatum including the nucleus accumbens; in the paraventricular nucleus of the hypothalamus; and in a circumscribed neuron population in the ventral central gray (Fig. 6F,L). The appearance of the latter structure may, as for MDMA, be due to the high density of serotonin receptors ventral to the aqueduct [9].

THC (25 mg/kg, Fig. 7) elicited c-fos expression in the lateral septal area, in the paraventricular hypothalamic nucleus, in the striatum, and, modestly, in the nucleus accumbens (Fig. 7A,F). Furthermore, in the mediodorsal thalamic nuclei the c-fos expression appeared stronger than in saline controls (not shown).

The weak c-fos expression after cocaine application was unexpected since many reports describe an intense signal in response to this drug. However, to achieve this, a binge injection (this means repeated applications within a short interval) or similar repetitive regimens were often used to investigate cocaine effects [25,32]. But the response to repeated application apparently represents a kind of rapid sensitization apart from the direct effect of the drug and was for this reason not used in this work.

Taken together, most of the addictive drugs tested induced a modest to strong c-fos expression in several distinguished brain areas after the application of a single dose. Strikingly, the only compounds that elicited a very weak c-fos response were the two highly addicting drugs morphine and cocaine.

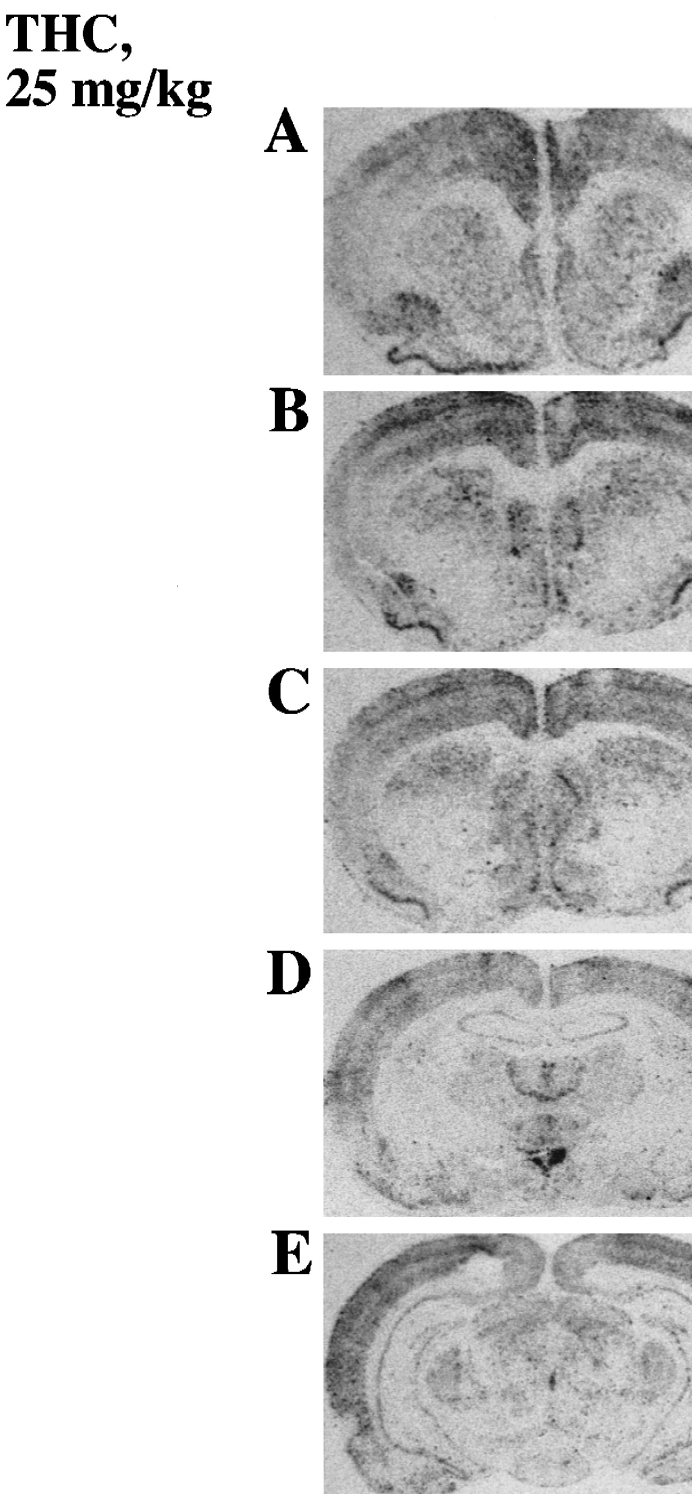

Fig. 7. Induction of c-fos expression by the injection of 25 mg/kg THC. The figure shows photographs from five different coronal sections of the brain (from top to bottom) stained by in situ hybridization for c-fos. Slices from two different animals of this treatment group are depicted (left and right column, respectively). Schematic drawings to identify important brain areas on the slices shown are given in Fig. 5.

4. Discussion

In the present work, a direct comparison of c-fos induction in response to different drugs of abuse was performed. The findings may be summarized below.

First, MDMA, LSD and THC, i.e., the drugs with low addictive potential, were able to stimulate c-fos synthesis in many brain areas. In contrast, cocaine and morphine induced only a very faint c-fos expression as compared with the low addicting drugs and involved much less brain areas.

Second, there was a marked overlap between the c-fos expression patterns induced by the different compounds; e.g., the nucleus accumbens, large parts of the striatum, the lateral septum and the paraventricular nucleus of the hypothalamus responded to MDMA, LSD and THC; furthermore, the central aspect of the caudate putamen responded to cocaine, too; the lateral septum and the paraventricular hypothalamic nucleus were also activated by morphine.

The observed expression patterns in response to THC and MDMA [5], respectively, were in accordance with previous reports about FOS-like immunoreactivity distribution after application of these drugs. Cellular-fos mRNA induction by THC was investigated by Mailleux et al. [15] and FOS protein was studied with immunohistochemistry by McGregor et al. [16], Miyamoto et al. [19] and Porcella et al. [26]. At a dose of 5 mg/kg, the c-fos expression observed was relatively weak in the striatum. In the cingulate cortex, there was a marked c-fos expression already under conditions of saline injection; this expression was obviously further slightly enhanced by the injection of THC [15]. This is in good accordance with our findings. We applied the fivefold dose of 25 mg/kg not to miss any effect on c-fos expression and accordingly we observed a more pronounced c-fos expression in the striatum and in the cingulate cortex. A total of 6 mg/kg MDMA induced a c-fos mRNA signal at the level of the striatum in our experiments similar to the results of Dragunow et al. [5] using immunohistochemistry. However, Dragunow et al. [5] applied a very high dose of 25 mg/kg. Moreover, we identified several other brain regions which also responded to MDMA application. No data are available about the effect of LSD on c-fos expression.

The observed c-fos expression patterns after injection of several drugs of abuse may be due to different causes.

First, brain areas could respond because they are rich in the receptor for the respective drug. This is probably the reason for the c-fos expression seen in the ventral periaqueductal gray after an LSD or MDMA injection since this part of the brain was found to contain large amounts of serotonin [9]. Such a direct activation of the c-fos pathway may also take place in the striatum after cocaine application because of the high density of dopaminergic synapses there.

Second, part of the c-fos synthesis may reflect the response of the animal to the injection procedure and may therefore represent either handling and injection stress or novelty. These reasons for c-fos synthesis are independent from the drug injected, and the corresponding hybridization signals were therefore readily detected in our saline-treated control rats.

Third, c-fos accumulation could be due to drug-induced stress because we used relatively high doses of each substance. The paraventricular hypothalamic nucleus contains CRH (corticotropin releasing hormone) neurons and will therefore be activated in stressful situations. This nucleus was indeed stimulated by all drugs except cocaine. However, it is not clear whether stress is aversive for the animal in any instance. For example, it is conceivable that the stimulating actions of ecstasy represent a sort of stress, but these psychoactivating properties are obviously a major reason for human abusers to take this drug.

Fourth, c-fos synthesis in the brain may reflect locomotion. The strongest locomotor response was seen with cocaine, but cocaine application yielded only a weak c-fos accumulation, indicating that mere locomotion is not sufficient to induce marked c-fos expression in the brain. On the other hand, stereotypical behaviour could be a reason for altered gene expression in the caudate putamen. A similar stereotypic behaviour (crawling) was observed after LSD and MDMA but the pattern of c-fos expression in the caudate putamen was different for each drug. LSD yielded a relatively even signal in all parts of the striatum whereas after MDMA application the nucleus accumbens and centromedial aspects of the striatum appeared. Therefore, it is unlikely that c-fos synthesis in the striatum secondary to drug-induced stereotypical behaviour is a major reason for the observed c-fos accumulation in response to different substances.

To summarize the above considerations, the observed c-fos expression patterns after the drug applications did not match the distribution of the receptors for the respective compound very well. On the other hand, secondary effects such as c-fos synthesis induced by locomotion also seem to play no major role. In consequence, the different drugs probably activate neuronal pathways which converge onto certain brain areas including parts of the caudate putamen and the lateral septum to induce c-fos accumulation there. An important role for the lateral septum in mediating sensations of pleasure is implicated by the fact that it was the first structure to be discovered which allowed electrical self-stimulation in rats [18].

The low c-fos expression in the nucleus accumbens and in other parts of the brain in response to morphine and cocaine implicates that stimulation of such reward areas thought to correlate with the induction of pleasant feelings by a drug is not necessary to render it highly addictive. Although MDMA, LSD and THC are taken by abusers to experience fun or pleasure and although these drugs elicit molecular signs in rats which presumably correspond to such emotions, the substances are not highly addictive in the way that they do not cause the loss of a productive life

[22]. In contrast, morphine and cocaine, the classical highly addictive drugs, had no major effect on the described brain areas associated with pleasure if applied as acute doses. On the other hand, both compounds are able to induce massive and increasing drug craving in human beings, and, as an experimental correlate, they are able to induce behavioural sensitization [3,13,30,31,33]. A molecular form of sensitization towards morphine was described recently by Curran et al. [2] and by our group [6,8]. Notably, the first report of c-fos induction in response to morphine [14] was based on a triplicate injection and not on a single dose of the substance. Molecular sensitization at the level of c-fos expression was also described for cocaine [2,20]. Therefore, it appears that it is not so important that brain areas associated with reward are acutely activated by a 'hard' drug to explain its addictive potential but instead, it seems to be the ability of the substance to elicit sensitization in certain brain areas after repeated application.

Acknowledgements

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