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Short communication

Effects of methadone and morphine on c-Fos expression in the rat brain: Similarities and differences

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Abstract:
This is the first study designed to compare the pattern of stimulation of c-Fos in selected brain structures after an acute administration of morphine and methadone. Methadone and morphine induced activation of c-Fos protein in the terminal forebrain projecting areas of the brain dopaminergic system, i.e. the striatum and nucleus accumbens. Taking into account generally accepted differences in the potency of pharmacological effects of the two drugs, it is surprising that this effect was most evident after the dose of 5 mg/kg of either drug.

Key words:
methadone, morphine, acute treatment, c-Fos, rats

Abbreviations: AcbC – nucleus accumbens, core; AcbSh – nucleus accumbens, shell; CPu – caudate putamen, VTA – ventral tegmental area, SNR – substantia nigra parts reticulata

Introduction

Methadone is a well-known opioid receptor agonist, used for many years for the treatment of abstinence symptoms, or as a substitution therapy, in the heroin addicted patients [12]. The worldwide attractiveness of methadone substitution programs is due to methadone's peculiar profile of action. It reduces the craving for heroin without inducing a strong euphoria. Both, morphine and methadone, can render the patients tolerant to opiods, but in the case of methadone this process usually takes much longer. However, the neurobiological background of differences in their clinical and pharmacological profiles is still not well recognized. Both drugs have in some aspects different pharmacological and pharmacokinetic spectra of activity. Morphine has much shorter biological half-life time (1–7 h in humans, and 28 min in mice) [1, 10] than methadone (5–130 h in humans, and 70–90 min in rats) [4, 18]. Morphine and methadone have similar opioid agonistic profile in that they both bind albeit, with different affinities, to the mu, delta, and kappa receptors [13]. It is also not clear whether both com-

pounds affect the same brain structures responsible for processing of motivational input, and if this action is structure-selective.

One of the most valuable methods used to map the sites of action of psychoactive compounds in the preclinical studies, is the measurement of the density of neurons expressing c-Fos protein – a product of expression of the *c-fos* gene, belonging to the family of immediate early genes, a marker of changes in neuronal activity. There is a wealth of studies on the central stimulatory effects of morphine using this immunocytochemical technique [2, 7, 8, 11] but there are no available data on the effect of methadone [Medline, 1966–2005]. It appears, therefore, of interest to compare the distribution of c-Fos protein after morphine and methadone, two mu opioid receptor agonists with different euphoric and addictive potential. This could help to better understand and localize the mechanisms that rule the processes of drug dependence. c-Fos induction after administration of morphine seems to be mediated by dopamine neurons in substantia nigra parts reticulata (SNR) and ventral tegmental area (VTA) that project to caudate putamen (CPu) and nucleus accumbens (Acb), respectively. Morphine is proposed to act on mu opioid receptors located on GABAergic interneurons in SNR and VTA. Inhibition of these GABA interneurons disinhibits SNR and VTA dopamine neurons, producing dopamine release in CPu and Acb [2].

To summarize, the purpose of this study was to compare the pattern of distribution of c-Fos in brain structures engaged in morphine effects, the dopaminergic nuclei (SNR, VTA) and projecting areas (Acb, CPu) of the central dopaminergic system, after an acute administration of morphine and methadone, applied in a wide dose-range. To minimize the interference of confounding factors, the immunocytochemical analysis was performed after a long and thorough habituation of experimental animals to the procedure of drugs injections.

Materials and Methods

Animals

Adult, male Wistar rats (220 ± 30 g) were used in this study. Animals were housed four per cage under standard laboratory condition with 12/12 light/dark cycle (lights on at 7.00, temperature 20°C, humidity 70%), with free access to food and water. All experiments were conducted during the light period of the day-night cycle between 11.00–14.00 h, to minimize the influence of circadian rhythms on c-Fos expression. At this time of the day, the basal c-Fos protein is at the lowest level and subjected to the least variability [8].

The study was carried out in accordance with European Communities Council Directive of 24 November 1986 (86/609/EEC). All experiments were approved by Committee for Animal Care and Use at the Medical University of Warsaw.

Drugs

Morphine sulfate was purchased from Polfa (Warszawa, Poland), methadone hydrochloride was generously provided by Molteni Farmaceutici (Scandiccii, Italy). Rabbit polyclonal anti-c-Fos IgG (sc-52×) was purchased from Santa Cruz, USA, biotinylated anti-rabbit IgGs and avidin-biotin-peroxidase complex from Vector Laboratories, USA .

Administration of morphine and methadone

Each experimental group consisted of 8 animals. To reduce a non-specific c-Fos expression caused by injection stress, after 4 days of acclimatization to the animal house conditions, all animals were subjected to the habituation procedure: daily handling and intraperitoneal injection of saline (2 ml/kg) for 10 days, in the same chamber by the same experimentator. On the 11th day, the rats were divided into experimental groups and received, respectively, saline or drug injection (morphine sulfate, 5, 10 and 50 mg/kg; R,S-methadone hydrochloride, 0.5, 2, 5, and 25 mg/kg). The highest dose of methadone appeared toxic and lethal for most of the animals (n = 6), therefore, the data from this group were omitted.

Immunohistochemistry

After decapitation (2 h after saline or drug injection), the brains were removed, immediately immersed in isopentane over dry ice and stored at –70°C. The immunocytochemical reaction was performed on slide-mounted brain sections as described earlier [14]. Coronal 15 µm cryostat sections from each animal were cut and mounted on silan-coated slides, and fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH = 7.4) for 15 min. They were then

washed (2 × 15 min) in 0.01 M PBS (pH = 7.4), incubated in 3% H_2O_2 solution for 30 min to block the activity of endogenous peroxidase, washed again in 0.01 M PBS (2 × 15 min), and incubated in a 3% normal goat serum (NGS) blocking solution. Subsequently, slide-mounted brain sections were incubated in rabbit polyclonal anti-c-Fos IgG diluted at 1:20,000 at temperature 4–8°C for 72 h, washed in 0.01 M PBS (3 × 15 min), then incubated with biotynylated anti-rabbit IgGs for 2 h, rinsed in 0.01 M PBS solution (2 × 15 min), and incubated with avidin-biotin-peroxidase complex for 1 h. Finally, after being washed in 0.05 M Tris solution pH 7.4 (2 × 15 min) slide-mounted brain sections were immunoreacted with a 0.03% diaminobenzidine hydrochloride (DAB) and 0.003% H_2O_2 in Tris solution. The slides were then dehydrated through an alcohol series, dewaxed in xylene, and coverslipped in the histofluid mountant. c-Fos-like immunoreactivity was assessed by light microscopy (Olympus BX-51 light microscope, Camedia Master C-3040 digital camera) at a magnification of 40 ×. To ensure the objectivity of c-Fos quantification method, the measurement was performed by two independent researchers blind to the treatment group. The number of c-Fos-positive nuclei was counted bilaterally with the use of computerized image analysis system (Olympus DP-Soft version 3.2 software) from two sections per rat in the examined brain regions. Two adjacent coronal 15 µm cryostat slices were cut for each section based on the atlas of Paxinos and Watson from each animal. Using DP-soft software the area of the outlined region was calculated [the whole area for: nucleus accumbens, shell (AcbSh), nucleus accumbens, core (AcbC), ventral tegmental area (VTA), substantia nigra, parts reticulata (SNR)], as shown in Figure 1A. In case of the caudate putamen (CPu), the examined area was sampled using 0.2 × 0.2 mm frame (Fig. 1A). Then c-Fos-positive nuclei were counted for each region and each rat separately, and expressed as the number of positive neurons per $1mm^2$. Examples of c-Fos immunoreactivity in CPu are presented in Figure 1 B–D.

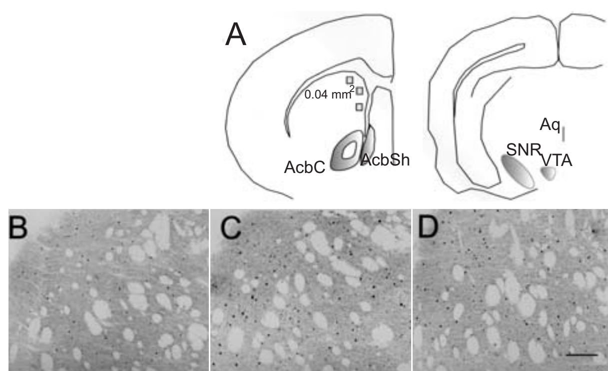

Fig. 1. (**A**) A drawing illustrating brain regions analyzed for c-Fos expression; (**B**) representative photomicrographs of c-Fos immunoreactivity in CPU from control group; (**C**) 5 mg/kg methadone; (**D**) 5 mg/kg morphine; Bar indicates 200 µm

Statistical analysis

The data for each analyzed group were shown as the means ± SEM The data were analyzed by one-way ANOVA followed by *post-hoc* Sheffe test (Statistica for Windows, Release 6, Stat-Soft Inc., USA). The probability value of $p \leq 0.05$ was considered significant.

Results

One-way ANOVA showed a significant morphine-induced increase in the number of c-Fos-positive nuclei in CPu [$F(3,27) = 4.69$, $p < 0.01$], AcbC [$F(3,23) = 4.76$, $p < 0.01$], and AcbSh [$F(3,23) = 2.97$, $p = 0.05$], only. *Post-hoc* test revealed that the doses of 5 mg/kg (CPu, $p < 0.01$; AcbC, $p < 0.01$, AcbSh, $p < 0.02$ *vs*. saline control), and 50 mg/kg of morphine (CPu, $p < 0.01$) were effective. Likewise, methadone induced c-Fos in the CPu and AcbC [CPu, $F(3,26) = 14.22$, $p < 0.001$; AcbC, $F(3,25) = 3.67$, $p < 0.05$], with the dose of 5 mg/kg exerting statistically significant effects (CPu, $p < 0.01$; AcbC, $p < 0.05$). The effects of methadone and morphine are shown in Figure 2.

Discussion

It has been well recognized that accompanying stressors can enhance the effects of psychoactive agents on c-Fos in Acb and CPu [16]. These points to an important role of proper control of the experimental procedure to assure the adequate specificity and intensity of the examined c-Fos expression. Both, stress of injection and morphine, were shown to stimulate

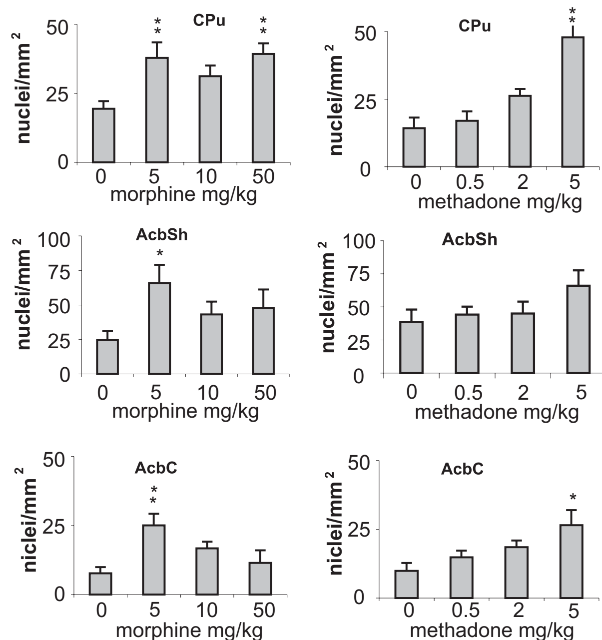

Fig. 2. Levels of c-Fos/ mm^2 (mean ± SEM) in the examined brain regions after different doses of morphine (mg/kg) and methadone (mg/kg). Values different significantly from saline. * $p < 0.05$, ** $p < 0.01$

c-Fos [9]. Another intervening factor may be the novelty of experimental design [6]. It is conceivable, therefore, that the effect of morphine in not well habituated animals may be significantly enhanced. This may explain the differences between the results of the present and some previously published papers. For example, contrary to some authors [5] and us, some other authors observed a significant effect of 10 mg/kg of morphine on c-Fos in the rat striatum [8, 11]. However, given the lack of habituation of animals to the experimental conditions in that experiment, it is possible that the effect of morphine was enhanced by stress-induced increase in dopamine and endogenous opioids [19]. In this context, it is noteworthy that our animals were very thoroughly habituated to the experimental procedure for 14 days. Such procedure helped to minimize the influence of other stressful stimuli, as shown by the low basal activation of c-Fos in control animals. However, such interpretation does not explain the inverted bell-like shape of a dose-response curve for the effect of morphine.

We used a racemic mixture of (R)- and (S)-methadone in this study, because in such a form the drug is being administered in most substitution programs, even though it is the (R)-isomer that accounts for most, if not all, opioid effects of this substance. Morphine and methadone produced induction of c-Fos in the same terminal forebrain projecting areas of the brain dopaminergic system, i.e. the striatum and nucleus accumbens. Neither of these substances produced any activation in VTA and SNR. The c-Fos induction in the CPu was observed at two doses of morphine (low and high). These data agree with the results of other authors, in that the dose-response relationship of morphine-induced c-Fos expression is not linear [15, 17]. In the case of methadone, only the highest tolerated dose was effective. Activation of the c-Fos expression in the AcbC was induced by the same doses of morphine and methadone (5 mg/kg), but in the AcbSh statistically significant effect was observed only with morphine. The latter observation seems particularly interesting, since the data indicate that c-Fos activation by a variety of drugs of abuse is selective for the shell *vs*. core of the nucleus accumbens. The observed effects of methadone and morphine were the most pronounced after the same dose of 5 mg/kg of both drugs, whereas it is well known from the preclinical and clinical studies that methadone is about two times as potent as morphine as an analgesic compound [3]. To make the matters more complicated, the dose of 25 mg/kg of methadone appeared lethal for most of the animals, while the dose of 50 mg/kg of morphine was much better tolerated. It appears therefore, that the potency of action of both opioid receptor ligands depends, for reasons yet unknown, on the neurobiological variable under study (pain, euphoria, lethality etc.). Identification of the kind of neurons being activated needs future research, including co-localization studies.

It is obvious that the development of dependence is a multifactorial process of response to a prolonged exposure to psychoactive substances. This study is only the first step on the road to a full-fledged comparison of the effects of morphine and methadone at a molecular level. It was limited to a comparison of the effects of an acute administration of both substances, under conditions designed to minimize a potential influence of external factors. The usefulness of methadone in drug substitution programs is based on the similarities (prevention of craving) and differences (smaller euphoric effects and slower development of tolerance) of its action to morphine. As the results of this study show, the induction of the c-Fos expression by both substances also exhibits both similarities and differences. Further studies, particularly on the effects of chronic administration of methadone and mor-

phine, are needed to explain the lesser euphoric and addictive potential of methadone.

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