Neuroscience 284 (2015) 107–124

TEMPORAL AND ANATOMIC PATTERNS OF IMMEDIATE-EARLY GENE EXPRESSION IN THE FOREBRAIN OF C57BL/6 AND DBA/2 MICE AFTER MORPHINE ADMINISTRATION

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Abstract—Although morphine was previously reported to produce an instant induction of *c-fos* in the striatum, our recent studies have demonstrated that the expression of numerous immediate early genes (IEGs) is significantly elevated at delayed time-points (several hours) after morphine administration. To better dissect the time-course of opioid-produced IEG induction, we used *in situ* hybridization to examine the expression of the IEGs *c-fos*, *zif268* and *arc* in the mouse forebrain at several time-points after acute morphine injection. To link drug-produced behavioral changes with the activity of specific neuronal complexes, this study was performed comparatively in the C57BL/6 and DBA/2 mouse strains, which differ markedly in their locomotor responses to opioids and opioid reward. Our study demonstrates that morphine produces two episodes of IEG induction, which are separate in time (30 min vs. 4–6 h) and which have different neuroanatomic distribution. At 30 min, one or more IEGs were induced in circumscribed subregions of the dorsal striatum (dStr) and of the nucleus accumbens (NAc) shell, as well as in the lateral septum. The observed inter-strain differences in IEG expression at 30 min support earlier proposals that activation of the dorsomedial striatum may mediate morphine-elicited locomotor stimulation (both effects were present only in the C57BL/6 strain). In contrast, NAc shell activation does not appear to be linked to morphine-elicited changes in locomotor behavior. The second IEG induction (of *arc* and of *zif268*) was more widespread, involving most of the dStr and the cortex. The second IEG induction peaked earlier in the DBA/2 mice than in the C57BL/6 mice (4 h compared with 6 h) and displayed no apparent relation to locomotor behavior. This delayed episode of IEG activation, which has largely been overlooked thus far, may contribute to the development of long-term effects of opioids such as tolerance, dependence and/or addiction.

Key words: immediate early gene, inbred strain, morphine, opioid, striatum.

INTRODUCTION

The C57BL/6 (B6) and DBA/2 (D2) inbred strains of mice differ markedly in their behavioral responses to opiates and to other addictive substances (Crawley et al., 1997). Thus, these strains represent a useful model to study genetically determined differences in drug abuse vulnerability. The administration of morphine elicits strong locomotor activation in B6, but not D2, mice (Oliverio and Castanello, 1974; Brase et al., 1977; Murphy et al., 2001). The B6 mice are also more sensitive to the rewarding effect of morphine and show more pronounced effects of chronic morphine treatment: locomotor sensitization and physical dependence (Oliverio and Castanello, 1974; Brase et al., 1977; Phillips et al., 1994; Murphy et al., 2001; Orsini et al., 2005; Solecki et al., 2009). These large behavioral differences suggest that morphine may affect brain activity states differently in the two mouse strains when acting acutely and that the drug may produce divergent neuroadaptations upon chronic treatment.

In an attempt to link opiate-produced behavioral changes with the activity of specific neuronal complexes, we used the B6 and D2 strains to study the acute morphine-stimulated expression of immediate early genes (IEGs) in forebrain regions important for locomotion and for reward. IEGs are induced rapidly and transiently at the transcriptional level as a result of the synaptic activation of a neuron and therefore are considered markers of neuronal activation (Sagar et al., 1988; Chaudhuri, 1997; cf Ziółkowska and Przewłocki, 2002).

Earlier studies of morphine-produced IEG expression changes in rodents demonstrated the induction of the *c-fos* gene (and, to a lesser extent, of *junB* and *c-jun*) in the dorsomedial striatum and, less consistently, in several other brain regions, including the nucleus accumbens (NAc), cortex, septum and thalamus (Chang

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Abbreviations: ANOVA, analysis of variance; B6, the C57BL/6 mouse strain; cAMP, cyclic adenosine monophosphate; CPP, conditioned place preference; D2, the DBA/2 mouse strain; dm dStr, dorsomedial dorsal striatum; dStr, dorsal striatum; IEGs, immediate early genes; LS, lateral septum; MAP kinase, mitogen-activated protein kinase; MOR, μ opioid receptor; NAc, nucleus accumbens; PKA, protein kinase A; PSL, photostimulated luminescence.

http://dx.doi.org/10.1016/j.neuroscience.2014.09.069
0306-4522/

et al., 1988; Liu et al., 1994; Garcia et al., 1995; Bontempi and Sharp, 1997; Spielewoy et al., 2000; Leite-Morris et al., 2002; Moulédous et al., 2010). This *c-fos* induction was reported to have a time-course typical of IEGs, whose mRNA levels peak at 30–60 min from stimulation and usually return to baseline by 1.5–3 h (Chang et al., 1988; Garcia et al., 1995; Erdtmann-Vourliotis et al., 1998). This time profile is consistent with the concept that morphine-produced IEG up-regulation in some brain regions reflects neuronal circuit activation responsible for motor activity changes and for opioid reward, both of which are virtually immediate morphine effects. We expected that IEG induction should be less pronounced or absent in the D2 mouse strain in the functionally relevant brain regions compared with the B6 strain, commensurate with the differential behavioral response of the two strains to morphine.

On the other hand, our recent studies demonstrated elevated levels of several IEG mRNAs (including *c-fos*) in the mouse striatum at delayed time-points after the administration of morphine or heroin (Ziółkowska et al., 2005, 2012; Piechota et al., 2010). In fact, measurements using striatal extracts have shown that the transcript levels of many IEGs reached their maximum at 4 h after injection of both opiate drugs, whereas these levels were not markedly elevated earlier (Piechota et al., 2010; Ziółkowska et al., 2012). When combined with previous reports of IEG induction occurring at 30–60 min after morphine administration, these observations may suggest that morphine produces more than one episode of IEG induction, possibly of different magnitudes, dynamics and/or tissue localization. Thus, tracing the time-course of morphine-produced IEG expression changes and delineating the distribution of these changes in the forebrain became the second goal of our study.

For this purpose, levels of selected IEG mRNAs were assessed using *in situ* hybridization at different times (between 30 min and 8 h) after acute morphine administration. Three IEGs were chosen for the analysis: *c-fos*, *zif268* (also called *egr1*) and *arc* (coding for the activity-regulated cytoskeleton-associated protein Arc). We expected that induction patterns of the different IEGs would not be identical due to differences in the molecular mechanisms of their transcriptional regulation and, thus, that the use of several markers would reveal a more complete picture of brain activity. The *c-fos* gene was included as the only IEG for which abundant information regarding its regulation by morphine (at least at early time-points) was available for comparison. Moreover, the selected genes are representative of two functional classes of IEGs, two of the genes coding for transcription factors (*c-fos* and *zif268*) and the third gene belonging to the effector IEGs, specifically coding for the dendritic protein Arc, which has been implicated in postsynaptic density function (Herdegen and Leah, 1998; Lanahan and Worley, 1998).

The entire time-course study was performed in the B6 and D2 strains of mice, although the inter-strain comparison seemed crucial primarily at the early time-points, when IEG induction occurs concomitantly with morphine-produced behavioral changes. Although the quantitative analysis of IEG expression was performed in all forebrain regions, where pronounced induction was noted, special attention has been given to subregions of the dorsal striatum (dStr) and of the NAc, which had previously been shown to respond to morphine with IEG induction and which are likely to participate in mediating opioid-produced locomotion and reward, i.e., behaviors that differ between the B6 and D2 mouse strains.

EXPERIMENTAL PROCEDURES

Animals

Male C57BL/6J (B6) and DBA/2J (D2) mice (bred at the Medical Research Center, Warsaw, Poland), which were 10–12 weeks old and weighed from 20 to 25 g, were used throughout the experiments. Animals were kept under standard conditions on a 12-h light/dark cycle, with *ad libitum* access to food and water. One to two weeks were allowed for acclimatization before the experiments. All procedures used in this study were in full accordance with the ethical standards established in the respective European (directive No. 2010/63/UE) and Polish regulations. The experimental protocol was approved by the Ethics Committee on Animal Studies at the Institute of Pharmacology, Polish Academy of Sciences (Kraków, Poland).

Treatment

Mice were pretreated with saline injections (10 μl/g) three times daily for 4 days. On day 5, the mice received a single injection of saline or of morphine (morphine hydrochloride, Polfa, Kutno, Poland; 20 mg/kg, s.c.). For *in situ* hybridization, groups of mice (n = 5–6) were sacrificed by decapitation at 30 min, 1.5 h, 4 h, 6 h or at 8 h after injection. A separate saline control group was prepared for each time-point and an additional naive (untreated) group from each strain served as another control. Locomotor activity measurements were performed in separate groups of the B6 and D2 mice (n = 8–10) injected with saline or with morphine (20 mg/kg, s.c.).

Locomotor activity measurement

Saline- or morphine-injected B6 and D2 mice were tested individually in Med Associates (St. Albans, VT, USA) place preference chambers that measured 17 cm × 12.5 cm × 12 cm and that were equipped with six pairs of light sources and photoelectric cells distributed opposite to each other 1 cm above the cage floor. Locomotor activity counts were recorded after the subsequent interruption of two adjacent photobeams by the animal. The measurements were performed at 15-min intervals for 5 h after injection.

In situ hybridization

After the mice were sacrificed, the brains were removed and frozen on dry ice. Then, the brains were cut into 12-μm-thick coronal sections using a cryostat microtome