Molecular chaperone heat shock protein 70 participates in the labile phase of the development of behavioural sensitization induced by a single morphine exposure in mice

Wang-Jun Qin1*, Yan-Ting Wang1*, Min Zhang1, Rui-Ting Wen1, Qing Liu1, Yu-Ling Li1, Feng Chen2, Andrew J. Lawrence2 and Jian-Hui Liang1

1 National Institute on Drug Dependence, Peking University, Beijing, People’s Republic of China
2 Florey Neuroscience Institutes and Centre for Neuroscience, University of Melbourne, Parkville, Victoria, Australia

Abstract

De-novo protein synthesis is required in the development of behavioural sensitization. A prior screening test from our laboratory has implicated heat shock protein 70 (Hsp70) as one of the proteins required in this behavioural plasticity. Thus, this study was designed to extend our understanding of the role of Hsp70 in the development of behavioural sensitization induced by a single morphine exposure in mice. First, by employing transcription inhibitor actinomycin D (AD) and protein synthesis inhibitor cycloheximide (CHX), we identified a protein synthesis-dependent labile phase (within 4 h after the first morphine injection) in the development of behavioural sensitization. Second, Hsp70 protein expression in the nucleus accumbens correlated positively with locomotor responses of sensitized mice and, more importantly, the expression of Hsp70 increased within 1 h after the first morphine injection. Third, AD and CHX both prevented expression of Hsp70 and disrupted the development of the single morphine induced behavioural sensitization, which further implied Hsp70 was highly associated with behavioural sensitization. Finally, the selective Hsp70 inhibitor pifithrin-β (PES) i.c.v. injected in mice prevented the development of behavioural sensitization induced by a single morphine exposure, probably functioning as a molecular chaperone.

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Introduction

Drug addiction is widely considered as a chronic brain disease associated with long-lasting neuroplasticity and behavioural plasticity (Pierce & Vanderschuren, 2010; Russo et al. 2009; Schmidt & Pierce, 2010). Exposure to drugs of abuse results in a long-lasting enhancement of the behavioural response to a subsequent injection, a phenomenon termed behavioural sensitization (Robinson & Berridge, 1993). Behavioural sensitization induced by a single exposure to drugs of abuse is a form of long-lasting changes at neurochemical, electrophysiological and behavioural levels (Bloise et al. 2007; Pereira et al. 2004; Robinson et al. 1982; Ungless et al. 2001; Vanderschuren et al. 2001). Therefore, behavioural sensitization induced by a single drug exposure in rodents, due to its simplicity in experimental paradigm and effectiveness of drug response, has been considered as a representative model for exploration of mechanisms that may be pertinent to drug addiction (Jing et al. 2011; Luo et al. 2011; Valjent et al. 2010).

The temporal pattern of behavioural sensitization is generally identified to encompass two major phases,
namely, development and expression, in which the former refers to immediate molecular and/or cellular effects that induce behavioural sensitization, while the latter is considered as long-term consequences of molecular and/or cellular effects defining the eventual behavioural alterations (Kalivas & Stewart, 1991). Interestingly, a wealth of data have demonstrated that the development of behavioural sensitization can be easily disrupted by some substances, such as L-type Ca\(^{2+}\) channel blockers (nimodipine, nifedipine, verapamil; Zhang et al. 2003), \(\gamma\)-aminobutyric acid transaminase inhibitor valproate (Li et al. 2004), D\(_3\) receptor antagonist nafadotride (Li et al. 2010), \(\mu\)-opioid receptor antagonist CTOP (Johnson & Napier, 2000) and histone deacetylase inhibitor sodium butyrate (Jing et al. 2011). Additionally, we have recently validated that de-novo protein synthesis is critically involved in the mechanism underlying the development of behavioural sensitization following a single morphine exposure in mice (Luo et al. 2011). Thus, we hypothesize that there is a labile phase, probably a de-novo protein synthesis-dependent one, in the development of behavioural sensitization induced by a single morphine exposure.

A previous reverse transcription polymerase chain reaction array analysis from our laboratory has found that Hsp70 out of 84 candidate genes altered in association with behavioural sensitization induced by a single morphine exposure in mice (Luo et al. 2011). Also, the increased protein expression of Hsp70, traditionally considered as an inducible protein mediating cell stress (Kiang & Tsokos, 1998), in the nucleus accumbens (NAc) parallels behavioural sensitization induced by a single morphine exposure (Luo et al. 2011). Additionally, several lines of evidence have demonstrated that most psychoactive substances, such as morphine (Ammon et al. 2003), psychostimulants (Miller et al. 1991) and ethanol (Calabrese et al. 2000; Holownia et al. 1995), induce expression of Hsp70. Furthermore, Ammon-Treiber and colleagues have found that a single morphine treatment induces a time-related augmentation of Hsp70 mRNA expression, which is attenuated by naloxone (Ammon-Treiber et al. 2004). Herein, we propose that Hsp70 may be causally linked to opiate-induced plasticity resulting in behavioural sensitization.

Here, we sought to investigate the time-course of protein synthesis in the development of behavioural sensitization induced by a single morphine exposure, so as to characterize the temporal involvement of Hsp70 in several stages of the development phase. As such, the potential participation of Hsp70 in behavioural sensitization induced by a single morphine exposure is supported, which may provide a new insight to understand the molecular mechanisms underlying drug-induced plasticity and the biological functions of molecular chaperones.

**Materials and method**

**Animals**

Male C57BL/6j mice, initially weighing between 18 and 20 g, were obtained from Beijing HFK Bioscience Co., Ltd. (China). The animals were housed (four to six per cage) in a light (12 h on–12 h off; lights on 08:00 hours), temperature (22 ± 1 °C) and relative air humidity (50 ± 10%) controlled environment with free access to food and water. All mice were habituated to the housing conditions for 3–4 d before experiments and each mouse was used for only one experiment. All experiments were conducted according to the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1996). The experimental procedures were approved by the local Committee of Beijing on Animal Care and Use. Every effort was made to relieve animal suffering and to reduce the number of animals used.

**Drugs**

Actinomycin D (AD), cycloheximide (CHX) and pifithrin-\(\mu\) (PES) were purchased from Sigma Chemical Co. (USA). Morphine hydrochloride was obtained from Qinghai Pharmaceutical Plant (China). AD, CHX and morphine were dissolved in saline (0.9% NaCl) just before experiments and injected in a volume of 0.1 ml/10 g (i.p.). PES was dissolved in 10% dimethyl sulphoxide and i.c.v. injected in a volume of 10 \(\mu\)l/mouse.

**The i.c.v. injection**

The i.c.v. administration was performed as described in Haley & McCormick (1957). The unilateral injection site was 1 mm beside the midpoint of a line drawn through the anterior base of the ears and verified by injecting 1% Methylene Blue. The experiment was performed when the success rate for injection was consistently >95%.

**Locomotor activity measurement**

Locomotor activity was measured in four identical chambers (25 × 25 × 45 cm, without ceiling) sound-proofed in closed cabinets using Digbehv spontaneous activity monitors (DigBehv-LG; Shanghai Jiliang Software Technology Co. Ltd., China). Horizontal
locomotor activity was recorded with a video camera placed above the chamber and analysed with the DIGbehv software (version 2.0; Shanghai Jiliang Software Technology Co. Ltd., China).

**Experimental protocol**

To establish the model of behavioural sensitization to a single morphine exposure, mice were administered morphine (20 mg/kg i.p.) or saline for the first injection on day 1 and challenged with a lower dose of morphine (5 mg/kg i.p.) on day 8. Mice were put into test chambers immediately after injections to measure the locomotor activity for 60 min (5-min intervals) on both day 1 and day 8. Behavioural sensitization was determined by differences in the locomotor response to the challenge morphine between morphine and saline pretreated groups.

To investigate the role of protein synthesis in the development of behavioural sensitisation induced by a single morphine exposure, AD (12 and 24 mg/kg i.p.) or CHX (2 and 3 mg/kg i.p.) was administered 0.5 h before the first morphine injection on day 1. To determine the time-course of dependence on protein synthesis, the minimal effective dose of AD (24 mg/kg i.p.) or CHX (3 mg/kg i.p.) was given at different time-points after the first morphine injection on day 1.

An Hsp70 selective inhibitor PES (12.5, 25 and 50 μg/mouse i.c.v.) was used for assessing whether Hsp70 protein was involved in the development of behavioural sensitization. The minimal effective dose of PES (25 μg/mouse i.c.v.) was chosen to examine the time-course of requirement for Hsp70 during the development of the single morphine-induced behavioural sensitization. To test the effects of PES itself on locomotor activity and on locomotor response to challenge morphine, mice received PES or vehicle injection on day 1 and morphine or saline on day 8 and locomotor activity was measured immediately after injections.

**Tissue preparation**

The time-effect and dose-effect of Hsp70 following a single morphine exposure were conducted to evaluate the association between Hsp70 expression and behavioural sensitization. For measurement of the time-effect of Hsp70 expression upon morphine exposure, mice received a single morphine injection (20 mg/kg i.p.) and brains were removed after +0.5, +1, +4 or +24 h. Control animals were killed +0.5 h after saline injections. For measurement of the dose response of Hsp70 upon a single morphine exposure, mice received a single injection of 5, 10, 20 or 40 mg/kg morphine or saline and brains were removed 0.5 h later.

To evaluate the effect of AD/CHX on the expression of Hsp70 induced by a single morphine exposure, mice received a morphine or saline injection 0.5 h following a single injection with AD/CHX or saline. Brains were removed 0.5 h after the morphine or corresponding saline injection.

For all the experiments associated with determining Hsp70 protein expression, coronal slices containing the NAc were prepared according to the mouse brain atlas of Paxinos & Franklin (2001) and then the NAc was quickly removed, frozen immediately with liquid nitrogen and stored at −80 °C until processed.

**Western blot**

Total protein was extracted by homogenizing the tissue sample in ice-cold RIPA lysis buffer and protein content was measured using BCA protein assay kit (Applygen Technologies Inc., China). Protein samples were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (10%) at 120 V and transferred onto nitrocellulose membranes. The membranes were washed with TBS/T (Tris-buffered saline mixed with 0.05% Tween-20) containing 5% non-fat dry milk for 1 h to block non-specific antibody binding sites, incubated with antibodies against Hsp70 protein (1:1000; R&D, USA) or β-actin (1:5000; Santa Cruz, USA) at 4 °C overnight and then treated with HRP-conjugated secondary antibody (1:2000; Santa Cruz, USA) for 1 h at room temperature. The blots were probed by chemiluminescent detection method (Applygen Technologies Inc., China) and then exposed to X-ray films. The content of the Hsp70 protein in blots was quantified by a Gel Doc 2000 densitometer (Bio-Rad, USA) and normalized to signals of β-actin protein.

**Statistical analysis**

The data of locomotor activity were analysed by two-factor repeated-measures analysis of variance (ANOVA) with time as a repeated measure (drug treatment x time) (Figs. 1, 6, 7c). All column graphs except Figs. 5 and 7 were tested by one-way ANOVA and post-hoc Bonferroni’s test. Fig. 7b was analysed by independent-samples t test. Figs. 5 and 7c were analysed by two-way ANOVA followed by an independent-samples t test. Bivariate correlation (Pearson) was used in Fig. 4c to test the relationship between expression of Hsp70 and behavioural sensitization induced by different doses of morphine. In Fig. 2, the inhibitory rate of the AD/CHX group was calculated.
based on the value of the saline group (taken as 0%) and the independent-samples t test was applied to determine differences between the two groups at each time-point. The significant level was taken as $p < 0.05$.

Results

Protein synthesis-dependent labile phase in the development of behavioural sensitization induced by a single morphine exposure in mice

We first examined whether protein synthesis inhibitors have an inhibitory effect on the development of behavioural sensitization induced by a single morphine exposure in mice. Mice were pretreated with AD (12, 24 $\mu$g/kg i.p.) or CHX (2, 3 mg/kg i.p.) 0.5 h before the morphine injection on day 1 (Fig. 1a). Neither AD nor CHX affected the locomotor response of mice to the acute morphine treatment (Fig. 1b, c), but both attenuated the increased locomotor response of mice to the morphine challenge on day 8. As shown in Fig. 1d, e, a two-factor repeated-measures ANOVA indicated significant differences among the study groups (time: $F_{11,44} = 11.419$, $p = 0.000$; treatment: $F_{5,44} = 18.931$, $p = 0.000$; time × treatment interaction: $F_{33,444} = 1.786$, $p = 0.005$ for Fig. 1d and

Fig. 1. Effect of actinomycin D (AD) or cycloheximide (CHX) on the development of behavioural sensitization induced by a single morphine exposure in mice. (a) Experimental paradigm: $n = 12$. (b) or (c) AD (12 and 24 $\mu$g/kg) or CHX (2 and 3 mg/kg) had no effect on the hyperlocomotion induced by a single morphine (Mor) treatment. (d) or (e) AD (12 and 24 $\mu$g/kg) or CHX (2 and 3 mg/kg) attenuated behavioural sensitization induced by a single Mor exposure, with 24 $\mu$g/kg AD or 3 mg/kg CHX reaching a significant level. The doses of AD and Mor marked in the inset of Fig. 1d, together with the doses of CHX and Mor marked in the inset of Fig. 1e are for discrimination of different treatments on day 1; thus, the 5 mg/kg challenge Mor treated in all groups on day 8 is not mentioned here. Data are expressed as means ± S.E.M. *** $p < 0.001$ vs. saline–saline group; # $p < 0.05$ vs. saline–Mor 20 mg/kg group.
A post-hoc analysis revealed that mice pretreated with saline and morphine exhibited increased locomotor activity compared to the saline–saline group when challenged with morphine on day 8 ($p<0.001$), indicating behavioural sensitization was established. The lower dose of AD (12 µg/kg), given 0.5 h before the first morphine injection, had a trend to reduce the increased locomotor response to challenge morphine, while a higher dose of AD (24 µg/kg) made a significant difference ($p<0.05$). Similarly, CHX given 0.5 h before the first injection of morphine impaired morphine-induced behavioural sensitization (the inset of Fig. 1c) ($F_{3,44}=17.740$, $p=0.000$), in which the higher dose of CHX (3 mg/kg) produced a significant difference ($p<0.05$).

To identify the protein synthesis-dependent labile phase in the development of behavioural sensitization induced by a single morphine exposure, the minimal effective dose of AD (24 µg/kg i.p.) or CHX (3 mg/kg i.p.) was administered to mice $-0.5$, $+1$, $+2$, $+4$, $+6$, $+8$ or $+24$ h after the first morphine treatment on day 1 and all mice were challenged with morphine on day 8 (Fig. 2c). To compare effects of AD or CHX at various time-points on the development of behavioural sensitization, locomotor responses to challenge morphine of the AD group (LRAD group) or CHX group (LRCHX group) were transformed to inhibitory rates based on the value of the saline (Sal) group (taken as 0%):

$$\text{Inhibitory rate} = \frac{\text{LRAD group or LRCHX group} - \text{LRSal group}}{\text{LRSal group}} \times 100\%$$

As shown in Fig. 2b, AD given $-0.5(t=2.964$, $p=0.007)$, $+1(t=3.752$, $p=0.001)$ or $+2$ h ($t=2.782$, $p=0.011$) after the first morphine injection on day 1 significantly reduced the locomotor response of mice to challenge morphine by 22.5, 27.7 or 22.8% compared to the corresponding saline group, indicating that behavioural sensitization induced by a single morphine exposure was impaired. When given $+4$, $+6$, $+8$ or $+24$ h after the first morphine injection on day 1, AD failed to produce any significant difference. CHX exhibited a longer effective time window on the development of behavioural sensitization induced by a single morphine exposure than AD (Fig. 2c).

Independent-samples $t$ test revealed that there was a significant difference between the CHX group and the corresponding saline group in the locomotor response to challenge morphine when CHX was injected $-0.5(t=2.964$, $p=0.007)$, $+1(t=4.551$, $p=0.000)$, $+2(t=2.546$, $p=0.018)$ or $+4$ h ($t=2.646$, $p=0.015$) after the first morphine injection.

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Fig. 2. Time-course of actinomycin D (AD) or cycloheximide (CHX) on the development of behavioural sensitization induced by a single morphine (Mor) exposure in mice. (a) Experimental paradigm: ($-$/+$+$): before/after the injection with Mor or saline; $n=12$. (b) When given at $-0.5$, $+1$ or $+2$ h after the first Mor injection, AD (24 µg/kg) impaired the development of behavioural sensitization. (c) Behavioural sensitization was attenuated by CHX (3 mg/kg) when it was given within 4 h after the first Mor exposure. Data relative to corresponding saline group (taken as 0%) are expressed as means ± S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. corresponding saline group.

time: $F_{11,44}=10.084$, $p=0.000$; treatment: $F_{3,44}=17.741$, $p=0.000$; time × treatment interaction: $F_{33,44}=0.760$, $p=0.832$ for Fig. 1c). Difference of the total locomotor activity between groups was further analysed by one-way ANOVA and post-hoc Bonferroni’s test. In the inset of Fig. 1d, a significant difference was demonstrated by one-way ANOVA ($F_{3,44}=18.940$, $p=0.000$).
Hsp70 protein expression in mice at different time-points after the first morphine injection (20 mg/kg i.p.) by Western blot. As shown in Fig. 3, Hsp70 protein expression was significantly elevated at +0.5 or +1 h after the morphine injection and returned to normal level at the time-points of 4 and 24 h (n = 9). Data normalized to the control group (taken as 100%) are expressed as means ± S.E.M. * p < 0.05 vs. control group.

Time- and dose-dependent Hsp70 expression in the NAc was involved in the development of behavioural sensitization induced by single morphine exposure in mice

To assess the change of Hsp70 protein sensitization in the development of behavioural sensitization, we analysed the expression of Hsp70 in the NAc of mice at different time-points after the first morphine injection (20 mg/kg i.p.) by Western blot. As shown in Fig. 3, Hsp70 protein expression was significantly elevated at +0.5 or +1 h after the single morphine treatment (one-way ANOVA: F_{1,44} = 4.900, p = 0.003; Bonferroni’s test: p = 0.046 or 0.016, respectively). However, there was no significant difference in Hsp70 expression at +4 or +24 h after the single morphine treatment compared to the control group. These results indicate that Hsp70 was highly expressed within the first 1 h of the development of behavioural sensitization to a single morphine exposure.

We further detected the expression of Hsp70 protein in the NAc induced by a series of doses of morphine. Fig. 4a shows that morphine treatments enhanced the expression of Hsp70 in a dose-related manner. Expression of Hsp70 significantly increased when mice were treated with 10, 20 and 40 mg/kg morphine. The peak point of Hsp70 expression was at the dose of 20 mg/kg. Similarly, as shown in the locomotor response upon challenge morphine, behavioural sensitization to a single morphine exposure gradually increased at the doses of 5, 10 and 20 mg/kg and began to decrease at the high dose of 40 mg/kg, in which 10, 20 and 40 mg/kg produced a significant difference (Fig. 4b). This pattern, to a large extent, was consistent with that of Hsp70 expression. Interestingly, the correlation between expression of Hsp70 and behavioural sensitization induced by different doses of morphine was significantly positive in Pearson’s analysis (r = 0.917, p = 0.028, n = 5; Fig. 4c), which suggests that Hsp70 was dynamically involved in the development of behavioural sensitization induced by a single morphine exposure.

Protein synthesis inhibitors prevented Hsp70 expression and behavioural sensitization induced by a single exposure to morphine in mice

As expression of Hsp70 is suggested to be potentially involved in the development of behavioural sensitization induced by a single morphine exposure, and previous experiments demonstrated that AD or CHX inhibited the development of behavioural sensitization (Fig. 1d or e), we therefore investigated whether AD or CHX inhibited the expression of Hsp70 in the development of behavioural sensitization. Mice received an injection of AD/CHX or saline, followed by administration of morphine or saline and expression of Hsp70 was examined 0.5 h after the morphine or corresponding saline injection. As shown in Fig. 5, morphine treatment significantly increased the expression of Hsp70 in the NAc and the increase was attenuated by AD (Fig. 5a) or CHX (Fig. 5b) given at 0.5 h before morphine administration (AD: F_{1,14} = 4.716, p = 0.040; morphine: F_{1,24} = 4.386, p = 0.047; AD × morphine: F_{1,24} = 8.809, p = 0.007 or CHX: F_{1,14} = 14.053, p = 0.001; morphine: F_{1,20} = 24.799, p = 0.000; CHX × morphine: F_{1,20} = 1.042, p = 0.320). These results, together with previous ones showing that AD or CHX inhibited behavioural sensitization, further implied that AD or CHX targeted, at least partially, on Hsp70 to function the inhibitory effect on behavioural sensitization following a single morphine exposure.

Selective blockade of Hsp70 function in the labile phase disrupted the development of behavioural sensitization induced by a single morphine exposure in mice

First, the dose-effect of PES on the development of behavioural sensitization induced by a single dose-effect of PES on the development of behavioural sensitization induced by a single
morphine exposure was investigated. As shown in Fig. 6b, one-way ANOVA demonstrated that the single morphine exposure (20 mg/kg i.p.) significantly increased locomotor activity of mice (F_{4,52} = 10.400, p = 0.000). Post-hoc Bonferroni’s test revealed a significant difference between the vehicle–saline group and the vehicle–morphine 20 mg/kg group. PES at the doses of 12.5 and 25 mg/mouse slightly reduced the single morphine-induced hyperlocomotion and a high dose of PES (50 mg/mouse) had a significant effect (p < 0.01). After a 7-d drug-free period, all mice were challenged with morphine (5 mg/kg i.p.). A two-factor repeated-measures ANOVA demonstrated a significant effect for treatment (F_{4,52} = 8.852, p = 0.000), a significant effect for time (F_{11,572} = 2.325, p = 0.008) and a significant time × treatment interaction (F_{44,572} = 2.991, p = 0.000; Fig. 6c). In the inset of Fig. 6c, the total locomotor activity of the study groups was further analysed by one-way ANOVA (F_{4,56} = 8.855, p = 0.000) and post-hoc Bonferroni’s test. The vehicle–morphine 20 mg/kg group exhibited significantly increased locomotor activity in response to challenge morphine, indicating that behavioural sensitization induced by the single morphine exposure was established. The PES 12.5 mg/mouse–morphine 20 mg/kg group tended to suppress locomotor activity upon challenge morphine compared to the vehicle–morphine 20 mg/kg group, while the PES 25 mg/mouse–morphine 20 mg/kg group and PES 50 mg/mouse–morphine 20 mg/kg morphine group had significant effects. These results suggest that PES dose-dependently impaired the development of behavioural sensitization induced by a single morphine exposure.

Based on results of the dose–effect experiment, we chose 25 mg/mouse PES to investigate the time-course of PES on the development of behavioural sensitization following a single morphine exposure. First, effects of 25 mg/mouse PES itself on spontaneous locomotor activity and challenge morphine-induced hyperactivity were examined (Fig. 7a). As shown in Fig. 7b, mice treated with PES (25 mg/mouse, i.c.v.) exhibited similar locomotor activity with that of the

Fig. 4. Expression of Hsp70 in the nucleus accumbens (NAc) correlates with behavioural sensitization induced by a series of doses of morphine (Mor). (a) Dose–effect of a single Mor exposure on the expression of Hsp70 in the NAc in the development of behavioural sensitization (n = 7). (b) Dose–effect of a single Mor exposure on behavioural sensitization (n = 12). (c) Correlation plot between expression of Hsp70 in the NAc and behavioural sensitization. Both expression of Hsp70 and the development of behavioural sensitization exhibited a dose-related profile. The peak points in expression of Hsp70 and behavioural sensitization presented at the dose of 20 mg/kg. Data in Fig. 4a were normalized to the saline group (taken as 100%). All data are expressed as means±S.E.M. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. control group.
vehicle-treated group, indicating PES itself had no effect on spontaneous locomotor activity of mice. On day 8, both groups treated with morphine (5 mg/kg i.p.) showed significantly increased locomotor activity than the groups treated with saline (PES: $F_{1,36} = 0.774, p = 0.385$; morphine: $F_{1,36} = 24.123, p = 0.000$; PES × morphine interaction: $F_{1,36} = 0.749, p = 0.393$; Fig. 7c). Moreover, there was no difference in locomotor activity between vehicle–morphine 5 mg/kg and PES–morphine 5 mg/kg groups, demonstrating that PES pretreatment did not affect the hyperactivity induced by challenge morphine.

Figure 8 shows the effects of PES (25 µg/mouse, i.c.v.) administered at different time-points after the first morphine injection on the development of behavioural sensitization. Morphine pretreatment on day 1 induced a significant behavioural sensitization upon challenge morphine in all experiments, indicating behavioural sensitization was successfully established. In Fig. 8b, c, the PES 25 µg/mouse–morphine 20 mg/kg group showed a significantly lower locomotor activity than the vehicle–morphine 20 mg/kg group, which indicated that PES administered immediately or +1 h after the first morphine injection prevented the behavioural sensitization induced by a single morphine exposure. In contrast, when PES was given +4 or +24 h after the first morphine injection, no significant difference was found between the vehicle–morphine 20 mg/kg group and PES 25 µg/mouse–morphine 20 mg/kg group (Fig. 8d, e), indicating the time window of inhibitory effect of PES on behavioural sensitization was within the labile phase of the development of the single morphine induced behavioural sensitization.

**Discussion**

Here we show that AD and CHX suppressed behavioural sensitization induced by a single morphine exposure within a specific time frame. The effective time windows in which AD and CHX had an inhibitory effect on behavioural sensitization were respectively within 2 and 4 h after the first morphine exposure. Herein, we ascertain that the development of the single morphine-induced behavioural sensitization can be divided into two important phases: a protein synthesis-dependent labile phase and a protein synthesis-independent stable phase. The two phases are demarcated at the time-point of 4 h after the first morphine injection, at least under the current paradigm. AD interacts with DNA and inhibits gene transcription, further resulting in protein synthesis inhibition (Sobell & Jain, 1972), while CHX inhibits mRNA translation on 80S ribosomes, thereby having a direct effect on protein synthesis inhibition (Suzuki et al. 1992). Therefore, the time window for CHX having effect on behavioural sensitization is longer than that of AD. The doses of AD (12 and
24 μg/kg i.p.) and CHX (2 and 3 mg/kg i.p.) employed in the present study are considered as not having effect on spontaneous locomotor activity, since much higher doses of AD (300 μg/kg i.p.) and CHX (15 mg/kg i.p.) tested in other studies presented no lethality and no obvious influence on spontaneous locomotor activity in mice (Capasso et al. 1996; Kuo et al. 2007). Additionally, a previous study from our laboratory has demonstrated that AD (12 μg/kg i.p. once daily for 3 d) and CHX (2 mg/kg i.p. once daily for 3 d) also did not produce obvious signs of toxicity or alter locomotor activity in mice (Luo et al. 2011). Taken together, by utilizing AD or CHX to intervene in the development of the single morphine-induced behavioural sensitization, we demonstrated the existence of a protein synthesis-dependent labile phase and inferred there might be at least one key protein mediating behavioural sensitization induced by a single morphine exposure.

As the development of the single morphine-induced behavioural sensitization consists of a labile phase and a stable phase, it is natural to associate this phenomenon with the course of learning and memory. Generally, the acquisition of memory gradually shapes from a transient, reversible state to a long-lasting, non-reversible state and the prerequisite of this transformation is de-novo protein synthesis...
It has been well documented that mechanisms of learning and memory are critically involved in drug-induced behavioural disorders (Nestler, 2002). Yu-Min Kuo and colleagues have previously found that cocaine induced conditioned place preference (CPP), a widely accepted behavioural paradigm to study drug-associated memories, was impaired by anisomycin within 2 h after each drug-place pairing. Our results on behavioural sensitization, together with the indication on CPP (Kuo et al. 2007), substantiate the phenomenon that a protein synthesis-dependent labile phase is included in mechanisms underlying drug-induced behavioural plasticity.

Hsp70, also termed Hsp72, Hsp70-1 or HspA1A, weighing approximately 70 kDa, is an ATP-dependent molecular chaperone being at low or even undetectable levels in most normal cells and tissues. However, Hsp70 is highly expressed as a protective protein following a variety of metabolic or exogenous insults to cells, such as elevated temperature, nutrient deprivation, toxins, heavy metals, oxidative stress, seizure, ischaemia, infection or other cellular stresses (Arya et al. 2007; Morimoto & Santoro, 1998). The functions of Hsp70 are generally thought to assist cells in nascent polypeptides folding and/or damaged proteins refolding, to prevent protein from aggregation and/or self-association, to facilitate protein transportation into intracellular locations for degradation and to assist forming protein complexes (Brodsky & Chiosis, 2006; Garrido et al. 2006; Mayer Bukau, 2005; Powers Workman, 2007; Schmitt et al. 2006). Previous studies from our laboratory have demonstrated that changes of Hsp70 in the NAc are parallel to behavioural sensitization induced by a

Fig. 8. Time-course of pifithrin-α (PES) on the development of behavioural sensitization induced by a single morphine (Mor) exposure in mice. (a) Experimental paradigm: n = 11–12. PES injected immediately (b) or at +1 h (c), but not at +4 h (d) or +24 h (e) after the first Mor injection impaired the development of behavioural sensitization. Data are expressed as means ± S.E.M. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. vehicle-saline group; ## p < 0.01 vs. vehicle–Mor 20 mg/kg group.
single morphine exposure (Luo et al. 2011). The NAc mainly receives dopaminergic projections from the ventral tegmental area and glutamatergic projections from the prefrontal cortex, amygdala and hippocampus. A number of studies have implicated the role of the NAc, a critical component of the mesocorticolimbic system, in mediating the reinforcing and behavioural sensitizing effects of drugs of abuse (Hyman & Malenka, 2001; Valjent et al. 2006). In our study, to confirm the correlation of Hsp70 in the NAc and behavioural sensitization, we found both the extent of behavioural sensitization and the expression of Hsp70 elevated in a dose-related manner following the first morphine injection at doses of 5, 10, 20, 40 mg/kg. Moreover, the analysis of bivariate correlation (Pearson’s) showed that Hsp70 expression was significantly correlated with behavioural sensitization. Furthermore, AD and CHX both impaired behavioural sensitization and the expression of Hsp70 following the first morphine injection. Taken together, these data support the notion that Hsp70 is involved in behavioural sensitization following a single morphine exposure.

To test this supposition further, we used PES, a selective Hsp70 inhibitor, to block the function of Hsp70. PES can interact with Hsp70 directly and/or disrupt the association between Hsp70 and several of its co-chaperones and substrate proteins (Leu et al. 2009). We found that PES significantly attenuated behavioural sensitization induced by a single morphine exposure when given immediately or 1 h, but not 4 or 24 h, following the first morphine exposure. Herein, we infer that Hsp70 performs its function in the development of behavioural sensitization within a relatively short time frame following the first morphine injection. Amazingly, when compared to the time-course of Hsp70 expression in the development of behavioural sensitization, we found that the time window of PES having an inhibitory effect on behavioural sensitization was precisely the time frame when expression of Hsp70 was highly elevated. Thus, we confirm that Hsp70 is functionally involved in behavioural sensitization following a single morphine exposure. Interestingly, as mentioned above, the labile phase of the single morphine-induced behavioural sensitization is within 4 h after the first morphine exposure, and Hsp70 plays a key role in the development of behavioural sensitization at the early stage, implying there might be other key proteins mediating the labile phase at the later period. The exact key proteins are worth further investigation.

Importantly, we demonstrated that PES had no effect on spontaneous locomotor activity on day 1 compared to groups given vehicle. Moreover, both groups exhibited substantially higher locomotor activity upon the challenge dose of morphine on day 8, thus ruling out the possibility that the disruption of PES on behavioural sensitization was non-specific or off target.

Some investigations have indicated that the increased expression of Hsp70 following morphine exposures is attributed to cytotoxic effects of drugs of abuse (Ammon-Treiber & Hollt, 2005; Ammon-Treiber et al. 2004). Conversely, according to other studies, morphine protects cells from apoptosis (Chen et al. 2008; Zhang et al. 2008). Ammon-Treiber and colleagues even rule out the possibility that Hsp70 expression induced by a single morphine exposure is caused by the effect of elevated body temperature, which is considered as a form of stress (Ammon-Treiber et al. 2004). With regard to our study, the alterations of Hsp70 expression in the NAc induced by a single morphine injection are precisely consistent with the time-course of Hsp70 performing its function on the development of the single morphine-induced behavioural sensitization, indicating that the increased expression of Hsp70 is likely responsible for morphine-induced behavioural sensitization. Additionally, Hsp70 expression in the NAc in both morphine pretreated (the present study) and morphine sensitized (our previous study) mice was suppressed by AD or CHX. Even more, increased expression of Hsp70 induced by a single morphine exposure was attenuated by an opioid receptor antagonist naloxone (Ammon-Treiber et al. 2004). These findings together suggest that Hsp70 is highly associated with drug-induced behavioural plasticity.

It is worth noting that, while Hsp70 is involved in the protein synthesis-dependent labile phase in the development of behavioural sensitization, Hsp70 itself cannot essentially explain the most remarkable feature of the single morphine-induced behavioural sensitization: the persistence of the behavioural changes involved. The transient activation of Hsp70 seen after a single morphine exposure reverts to normal levels after 4 h, indicating that it is highly probable that other proteins, such as ΔFosB (Nestler, 2001) and cyclin-dependent kinase 5 (Hawasli & Bibb, 2007; Hawasli et al. 2007; Lai & Ip, 2009), are involved in the protein synthesis-dependent labile phase after expression of Hsp70. The complementary roles played by Hsp70 and other molecules suggest that the labile phase is dynamic and may involve both simultaneous and sequential activation of different signalling cascades. Hsp70 herein might be an initiator of the following process or functions as a molecular chaperone assisting protein folding and/or refolding.
In summary, our study for the first time demonstrates that the development of behavioural sensitization to a single morphine exposure consists of two important phases, namely, a protein synthesis-dependent labile phase and a protein synthesis-independent stable phase. Additionally, the protein expression of Hsp70 in the labile phase correlates positively with the behavioural sensitization induced by a single morphine exposure. Furthermore, Hsp70 is functionally involved in the labile phase. Therefore, except for playing a protective role against cell stress, Hsp70 is discovered to be a regulator protein of the single morphine-induced behavioural sensitization. Together with the previous and the present study, we propose that behavioural sensitization to a single morphine exposure involves not only protein synthesis, but also protein folding and assembly.

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Statement of Interest

None.

References


