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Differences in basal and morphine-induced FosB/ΔFosB and pCREB immunoreactivities in dopaminergic brain regions of alcohol-preferring AA and alcohol-avoiding ANA rats

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ABSTRACT

Besides alcohol, alcohol-preferring AA and alcohol-avoiding ANA rats differ also with respect to other abused drugs. To study the molecular basis of these differences, we examined the expression of two transcription factors implicated in addiction, ΔFosB and pCREB, in brain dopaminergic regions of AA and ANA rats. The effects of morphine and nicotine were studied to relate the behavioral and molecular changes induced by these drugs. Baseline FosB/ΔFosB immunoreactivity (IR) in the nucleus accumbens core and pCREB IR in the prefrontal cortex (PFC) were elevated in AA rats. Morphine increased ΔFosB-like IR more readily in the caudate-putamen of AA rats than in ANA rats. In the PFC morphine decreased pCREB IR in AA rats, but increased it in ANA rats. In addition to enhanced locomotor response, the development of place preference to morphine was enhanced in AA rats. The enhanced nicotine-induced locomotor sensitization found in AA compared with ANA rats seems to depend in addition to dopamine and ΔFosB on other mechanisms. These findings suggest that enhanced sensitivity of AA rats to morphine is related to augmented morphine-induced expression of FosB/ΔFosB and morphine-induced reduction of pCREB levels. Moreover, altered innate expression of FosB/ΔFosB and pCREB in AA rats is likely to affect the sensitivity of these rats to abused drugs.
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1. Introduction

Progressive enhancement of psychomotor responses after repeated exposure to various drugs of abuse, termed behavioral/psychomotor sensitization, is thought to be associated with some critical aspects of drug addiction (Robinson and Berridge, 2003; Vanderschuren and Kalivas, 2000), such as drug-seeking and drug-taking (Robinson and Berridge, 1993; Vezina, 2004). Numerous investigations have shown the involvement of the brain dopaminergic systems in this process, but the molecular mechanisms underlying sensitization are still not well understood. Interesting models for studying these mechanisms are animal lines selected for their drug consumption.

Alcohol-preferring AA and alcohol-avoiding ANA rat lines have been selectively bred on the basis of their alcohol consumption (Eriksson, 1968; Sommer et al., 2006). Previous studies have shown that in addition to alcohol, AA rats consume more etonitazene and cocaine containing solutions than ANA rats (Hyyatia and Sinclair, 1993). Recently, we found that conditioned place preference (CPP) to cocaine develops more readily in AA than in ANA rats (Marttila et al., 2007). Furthermore, repeated cocaine or morphine administration sensitizes the locomotor responses of AA rats more readily than those of ANA rats (Honkanen et al., 1999b; Ojanen et al., 2003; Ojanen et al., 2007). The sensitivity of mesolimbic dopamine system appears to be a critical factor determining the different behavioral effects of cocaine between these rats. This conclusion can be reached after observing an enhanced psychomotor response in AA rats accompanied by sensitized mesolimbic dopamine release (Mikkola et al., 2001a). However, divergent sensitization of mesolimbic dopamine release does not explain the differences between AA and ANA rats in the psychomotor sensitization to morphine (Honkanen et al., 1999a; Mikkola et al., 2000; Ojanen et al., 2003).

Drugs of abuse are known to cause several neuroadaptations in dopaminergic brain areas. One such adaptation is the altered expression of transcription factors, which give rise to changes in gene expression and may possibly lead to alterations in sensitivity to drugs of abuse (Nestler et al., 2001). Several studies have specifically implicated two transcription factors, ΔFosB and CREB (cAMP response element binding protein), in addiction-related neural plasticity. Repeated stimuli, such as repeated exposure to drugs of abuse, lead to a gradual increase in ΔFosB levels, an effect that persists for a relatively long time after the cessation of drug treatment (Hope et al., 1994; Nestler et al., 2001). In contrast, CREB is a constitutively expressed transcription factor, the activity of which is tightly regulated by its phosphorylation at serine 133 (Lonze

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and Ginty, 2002). Plenty of evidence suggests that an increased expression of ΔFosB enhances locomotor-activating and rewarding effects of cocaine (Colby et al., 2003; Kelz et al., 1999; Peakman et al., 2003). Indeed, we recently found that cocaine increases the expression of ΔFosB-like proteins more in the nucleus accumbens (NAc) of AA rats than in that of ANA rats (Marttila et al., 2007). Furthermore, cocaine reward is shown to be accompanied by changes in pCREB (phosphorylated form of CREB) levels (Carlezon et al., 1998). There is also evidence for the role of these transcription factors in morphine sensitization and reward (McDaid et al., 2006; Olson et al., 2005, 2007; Zachariou et al., 2006). Therefore, it is important to find out whether the effects of morphine on these transcription factors differ between AA and ANA rats.

In the present study we examined whether differences in locomotor responses to morphine in AA and ANA rats are associated with differences in the expression of FosB/ΔFosB and pCREB. Further, CPP method was used to assess morphine reward in AA and ANA rats. In addition to those of morphine and cocaine, the addictive properties of nicotine are thought to involve the dopaminergic system. Therefore, we compared the effects of repeated nicotine administration on locomotion, dopamine release and FosB/ΔFosB expression in AA and ANA rats.

2. Materials and methods

2.1. Animals

3–4-month-old male AA and ANA rats (Department of Mental Health and Alcohol Research, National Public Health Institute, Helsinki, Finland) were used. The rats were housed in groups of 4–5 and kept under a 12:12-h light/dark cycle at an ambient temperature of 20–23 °C. Rat chow and tap water were available ad libitum. Prior to the experiments, the rats were acclimated to handling for 7 days in order to minimize stress during the experiments. All animal experiments were approved by the chief veterinarian of the county administrative board and were conducted according to "the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes".

2.2. Morphine-induced locomotor activity

On the first experimental day, all rats were given saline (0.9% NaCl, 2 ml/kg, s.c.), and on the following day the pretreatment period was started. During that period, the rats were treated with saline or increasing doses of morphine (5, 10, 10, 15, 15 mg/kg; every other day). Fifteen minutes before and immediately after the injections, the rats were placed in locomotor activity boxes. To study the acute locomotor-activating effects of morphine 5 mg/kg, horizontal locomotor activity was measured for 4 h on the first day of the pretreatment period. The locomotor activity of the rats was registered in Plexiglas boxes (43 cm × 43 cm × 30 cm; MED Associates ENV-515, Vermont, GA, USA), and a computer registered the interruptions of infrared photo beams. After the pretreatment period, the rats were abstained from morphine for 72 h prior to the challenge. On the challenge day, all rats received morphine injections. In the first experiment, the rats first received 1 mg/kg of morphine as the challenge, and 48 h after that, a second morphine challenge was given at a dose of 3 mg/kg. In the second experiment, the challenge dose was 5 mg/kg. Locomotor activity was measured for 4 h. 24–29 h after the measurement of locomotor activity, the rats were perfused for FosB/ΔFosB immunohistochemistry. In addition, a separate control group was similarly treated with saline (both pretreated and challenged with saline) and perfused for FosB/ΔFosB immunohistochemistry.

2.3. Morphine-induced conditioned place preference (CPP)

Two place conditioning experiments were carried out with two doses of morphine as the conditioning dose (0.5 mg/kg and 1.0 mg/kg, 2 ml/kg, s.c.). The CPP experiment consisted of three distinct phases on consecutive days: two habituation days, two conditioning days, test day 1 (postconditioning), two conditioning days, test day 2, three conditioning days, and test day 3. Eight rats could be tested simultaneously. The CPP apparatus consisted of two equally sized compartments (41 cm × 21 cm × 28 cm) that were separated by a black wall with a guillotine door (MED Associates ENV-515, Vermont, GA, USA). The compartments had differently colored walls (black or white) and distinct floor textures (a rod floor in the black compartment and a wire mesh floor in the white compartment). White noise was used to cover possible background noise. Computer-registered interruptions of infrared photo beams were used to determine the position of the rat in the apparatus. During the habituation phase, the guillotine door was open, allowing free access to both compartments for 45 min. The time that the rat spent in the non-preferred compartment during the first 20 min on the habituation day 2 was used as the initial preference level (preconditioning time). During the conditioning phase all rats received a saline injection (2 ml/kg s.c.) in the morning before being confined to the vehicle-paired black-walled compartment for 45 min. After an interval of 3–4 h, each rat received a saline or morphine injection immediately before being placed in the drug-paired white-walled compartment for 45 min. Each trial included four AA rats and four ANA rats, with two rats from both lines receiving saline in both compartments and the other two receiving saline in one and morphine in the other compartment. On the postconditioning day, the guillotine door separating the two compartments was open, and the time spent by the rats in either compartment was recorded for 20 min. The change of preference was calculated as the difference in seconds between the times spent in the drug-paired compartment on the postconditioning day and the preconditioning day.

2.4. FosB/ΔFosB and pCREB-immunohistochemistry

24–29 h (FosB/ΔFosB study) or 20 min (pCREB study) after the last injection, the rats were deeply anaesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The assay time points were based on previous studies showing that 24 h after the stimulus, the observed FosB/ΔFosB IR represents almost solely ΔFosB (Chen et al., 1997; Nestler, 2004; Perrotti et al., 2008; Ulery et al., 2006), and that a peak in the level of pCREB is seen 20 min after the stimulus (Mattson et al., 2005). After perfusion, the brains were post-fixed for 4 h with the same fixative and stored in sodium phosphate buffer containing 20% sucrose until coronal sections (40 μm) were cut on a cryostat. The sections were stored at −20 °C until they were used. Free-floating sections from selected brain areas were stained on 24-well plates. After rinsing the sections in PBS for 3 × 10 min, sections were treated with 0.5% H_2O_2 in PBS for 10 min and rinsed again in PBS 3 × 10 min. Sections were then placed in a blocking solution containing 3% normal goat serum (NGS) and 0.5% Tween 20 in PBS for 1 h. After that, the sections were incubated for 22 h in primary FosB/ΔFosB (1:500 Santa Cruz Biotechnology) or $pCREB^{133}$ (1:5000; Upstate Biotechnologies) antibody diluted in PBS containing 3% NGS, 0.5% Tween 20, and 0.1% sodium azide. Subsequently, the sections were washed in PBS for 3 × 10 min, and incubated for 2 h with biotinylated anti-rabbit antibody (Vectastain® Elite ABC Kit PK-6101, Vector Laboratories, CA, USA) in PBS containing 1.5% NGS. Standard avidin–biotin procedure was then performed using the Vectastain® Elite ABC Peroxidase Kit following the protocol suggested by the manufacturer. After washing the sections in PBS for 3 × 10 min, the immunoreactivity was revealed using 0.06% 3,3-diaminobenzidine and 0.1% H_2O_2 diluted in PBS, followed by washing with phosphate buffer 3 × 5 min. All washes and incubations were carried out at room temperature under gentle shaking. The sections were mounted on gelatin/chrome-alume-coated slides, air-dried and dehydrated through graded ethanols to HistoClear® (National diagnostics, GA, USA), and coverslipped with DePex® (BDH Laboratory supplies, Poole, England).

FosB/ΔFosB immunostaining was quantified using a computerized image-analysis system including CoolSNAP-Pro digital camera, Nikon Eclipse TE300microscope, and a computer with Image-Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD, USA). The counting system detected stained nuclei that were above a preset intensity, i.e., moderately to highly stained nuclei. In the pCREB study, digitized images from brain regions of interest were taken with an Optronics digital camera, and optical densities were measured using Image Pro-Plus. Background signal was measured over the white matter regions and was subtracted from the signal in the region of interest. The quantifier was unaware of the nature of the sample being counted. FosB/ΔFosB and pCREB immunoreativities for each brain area of interest were determined from both hemispheres of three coronal sections/brain at +2.20 for prefrontal cortex (PFC), +1.70 for nucleus accumbens (NAc) core and shell, +0.70 for caudate-putamen (CPu, dorsomedial part), and −4.80 for ventral tegmental area (VTA, rostral part) and substantia nigra (SN, pars compacta), relative to the Bregma. The Atlas of Paxinos and Watson (1986) was used to identify the different brain regions that were studied.

2.5. Nicotine-induced locomotor activity

Rats were placed in the locomotor activity boxes 15 min prior to the injections. On the first experimental day, all rats were given saline (2 ml/kg, s.c.), and placed for 1 h in the locomotor activity boxes. Thereafter, on the five consecutive days the rats received saline or nicotine (0.4 mg/kg, 2 ml/kg, s.c., pH adjusted to 7) injections. The locomotor activity was measured as described above for 1 h.

2.6. Microdialysis

Immediately before the surgery, rats were given buprenorphine (0.05 mg/kg s.c.) to relieve pain. The rats were under general isoflurane anesthesia as microdialysis guide cannulae (BAS MD-2250, BAS Bioanalytical Systems Inc., IN, USA) were implanted using a stereotaxic device (Stoelting, IL, USA). The coordinates for guide cannulae were calculated relative to the Bregma, and guide cannulae were aimed at a point above NAc (A/P + 1.7, L/M + 1.4, D/V − 6.3) according to the Atlas of Paxinos and Watson (1986). The cannula was fastened to the skull with three stainless steel screws and dental cement (Aqualox, Voco, Cuxhaven, Germany). After the surgery, rats were placed into individual test cages and allowed to recover for 6–8 days before the microdialysis experiment. 6–12 h after the operation the rats were given another dose of buprenorphine. In addition, after the surgery and on the two following days the rats received carprofen (5 mg/kg) for pain relief. Half of the rats was given nicotine (0.4 mg/kg s.c.) and the other half was given saline for four consecutive days before the experiment. After the injections, the test cages were surrounded with black walls for 1 h to associate the effect of nicotine/saline with an environmental cue, so that the experimental set-up would be similar to that used in the locomotor activity measurements.

On the morning of the experimental day, a microdialysis probe (BAS MD-2200 BR-2, 2 mm polyacrylonitrile membrane, OD 0.32 mm) was inserted into the guide cannula, and the probe was perfused with a modified Ringer solution (147 mM NaCl, 1.2 mM $CaCl_2$, 2.7 mM KCl, 1.0 mM $MgCl_2$, and 0.04 mM ascorbic acid) at a flow rate of 2.5 µl/min. After stabilizing for 3–4 h, the collection of actual microdialysis samples (every 15 min, 37.5 µl/sample) was started. The concentrations of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were determined as described in Piepponen et al. (2002). The chromatograms were processed and integrated with Azur Chromatography Software and Data Acquisition System (version 4.0, Datalys, Theix, France). The average concentration of 3–4 consecutive stable samples was defined as 100%. After baseline collection, the rats received nicotine (0.4 mg/kg s.c.), and samples were collected for 3 h after the injection. The effects of nicotine on the monoamine output are presented relative to the baseline. After the completion of the experiments, the positions of the probes were verified histologically by fixing coronal brain sections on gelatin/chrome-alume-coated slides.

2.7. Statistical analysis

Data were analyzed with Student's *t*-test (baseline IR) or 2-way analysis of variance (ANOVA), with the factors of treatment (saline or morphine/nicotine) and rat line (AA or ANA). Post-hoc comparisons were conducted using contrast analysis when line × treatment interaction was $p<0.15$. Nicotine locomotor activity and microdialysis data were analyzed by 2-way ANOVA for repeated measurements. Results were considered significant when $p<0.05$.

3. Results

3.1. Morphine-induced locomotor activity and conditioned place preference (CPP)

The acute locomotor-activating effect of morphine (5 mg/kg) was significantly greater in AA rats (ambulatory counts: AAsal: 331 ± 92; AAmor: 2825 ± 698) compared with ANA rats (ambulatory counts: ANAsal: 314 ± 43; ANAmor: 307 ± 112) [line effect $F(1,28)=12.608$, $p<0.01$; treatment effect $F(1,28)=12.147$, $p<0.01$; line × treatment interaction $F(1,28)=12.272$, $p<0.01$]. Morphine pretreatment significantly increased the locomotor-activating effect of morphine challenges (1 mg/kg and 3 mg/kg) [pretreatment effect 0–60 min: 1 mg/kg: $F(1,25)=17.871$, $p<0.001$; 3 mg/kg: $F(1,25)=18.937$, $p<0.001$], but there were no significant differences between the lines [line × treatment interaction $F(1,25)=1.740$, $p=0.199$; $F(1,25)=0.260$, $p=0.614$, respectively]. With the challenge dose of 5 mg/kg ANOVA revealed a significant effect of rat line [0–60 min: $F(1,26)=4.994$, $p<0.05$] and pretreatment [$F(1,26)=17.885$, $p<0.001$]. In addition, there was a tendency of the locomotor response to morphine to be more augmented in AA rats compared with ANA rats [line × treatment interaction $F(1,26)=2.772$, $p=0.108$; Fig. 1]. Contrast analysis showed that morphine pretreatment significantly increased the locomotor-activating effect of the morphine challenge only in AA rats.

Morphine (0.5 mg/kg) did not induce significant place preference in rats of either line after two conditioning sessions. After four conditioning sessions ANOVA showed a significant effect of treatment [$F(1,36)=6.597$, $p<0.05$] but no line × treatment interaction [$F(1,36)=1.712$, $p=0.20$]. After seven conditionings the ANOVA showed a significant effect of treatment [$F(1,34)=6.224$, $p<0.05$; Fig. 2A] and a strong trend for the line × treatment interaction [$F(1,34)=3.929$, $p=0.056$]. Contrast analysis revealed a significant shift in preference in the morphine-treated AA rats, but not in ANA rats.

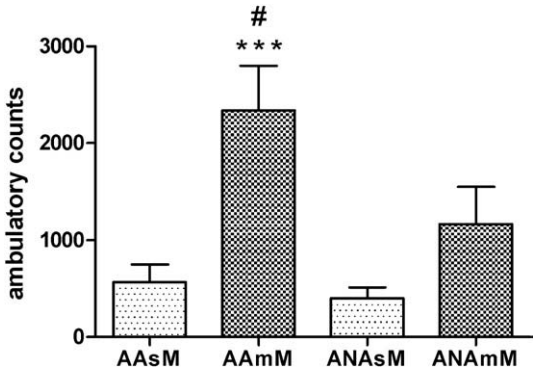

Fig. 1. Locomotor activity (0–60 min) after morphine challenge (5 mg/kg) in AA and ANA rats pretreated repeatedly with saline or morphine. sM=saline pretreatment + morphine challenge; mM=morphine pretreatment + morphine challenge, $n=7$–8. $^{\#}p<0.05$ compared with corresponding ANA rats, $^{***}p<0.001$ compared with corresponding saline-treated rats.

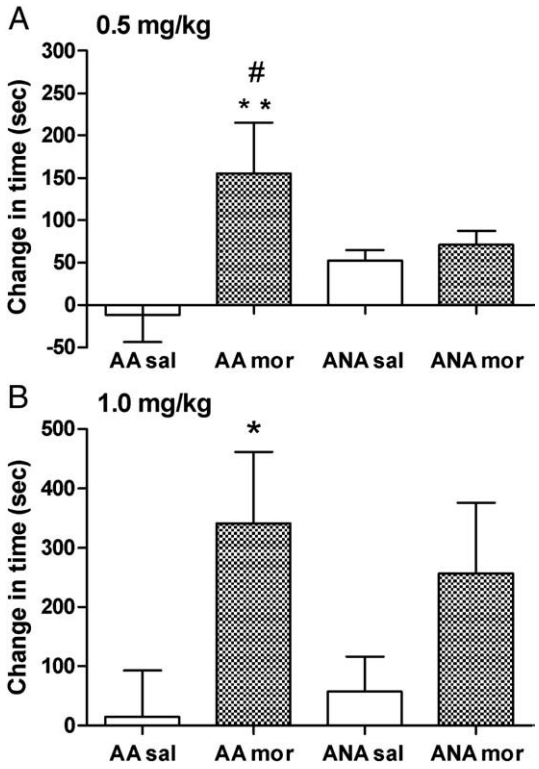

Fig. 2. Morphine-induced conditioned place preference (CPP) in AA and ANA rats. The figure presents the changes in the postconditioning (after 7 conditioning sessions) vs. preconditioning times (s) spent in the morphine-paired compartment during 0–20 min. A) Morphine 0.5 mg/kg, $n=9$–10. B) Morphine 1.0 mg/kg, $n=7$–8. $^{\#}p<0.05$ compared with corresponding ANA rats, $^{*}p<0.05$, $^{**}p<0.01$ compared with corresponding saline-treated rats.

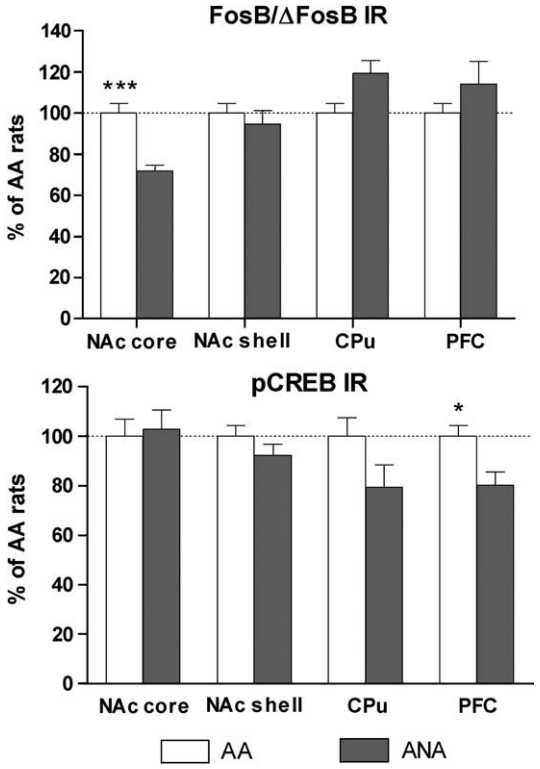

Fig. 3. Baseline FosB/ΔFosB and pCREB levels in AA and ANA rats. The results are expressed as % of AA rats, $n=8$–10. NAc, nucleus accumbens; CPu, caudate-putamen; PFC, prefrontal cortex. $^{*}p<0.05$, $^{***}p<0.001$.

Morphine (1.0 mg/kg) induced a significant place preference in both AA and ANA rats after only two conditioning sessions [treatment effect $F(1,26)=7.362$, $p<0.05$]. A significant effect of morphine was also seen after four and seven conditioning sessions [treatment effect: four conditionings: $F(1,26)=22.475$, $p<0.0001$; seven conditionings: $F(1,26)=6.676$, $p<0.05$; Fig. 2B], but the effect was similar in rats of both lines.

3.2. Baseline FosB/ΔFosB and pCREB levels

Fig. 3 shows the FosB/ΔFosB and pCREB IR in AA and ANA rats that were repeatedly injected with saline (s.c.). FosB/ΔFosB expression was higher in the NAc core of AA rats than of ANA rats ($p<0.001$, Student's *t*-test) and the pCREB levels were higher in the PFC of AA rats compared with ANA rats ($p<0.05$). Furthermore, as shown in the Table 1, pCREB levels in the SN of ANA rats were higher than those of AA rats ($p<0.05$).

3.3. Morphine-induced FosB/ΔFosB and pCREB levels

At 24–29 h after the last morphine challenge the number of FosB/ΔFosB positive nuclei was significantly increased in the CPu [treatment effect $F(1,31)=4.812$, $p<0.05$; line × treatment interaction $F(1,31)=2.329$, $p=0.137$; Fig. 4]. Contrast analysis revealed a significant increase in the number of FosB/ΔFosB positive nuclei in the saline-pretreated, morphine-challenged AA rats (sM) compared with saline-treated AA rats (sS). Such an increase was not seen in ANA rats (sM vs. sS). Repeated morphine treatment similarly increased FosB/ΔFosB IR in the CPu of rats of both lines [treatment effect $F(1,29)=12.715$, $p<0.01$; line × treatment interaction $F(1,29)=0.232$, $p=0.634$]. In addition, in the NAc shell there was a significant effect of morphine in morphine-challenged rats [sS vs. sM: treatment effect $F(1,30)=5.879$, $p<0.05$], and this effect did not differ between AA and ANA rats [line × treatment interaction $F(1,30)=0.895$, $p=0.352$]. In the NAc core, the FosB/ΔFosB IR was higher in AA rats than in ANA rats [line effect $F(1,30)=21.740$, $p<0.001$], but morphine did not significantly increase FosB/ΔFosB IR either after the two challenge doses or with repeated treatment [treatment effect: $F(1,30)=2.252$, $p=0.144$; $F(1,29)=2.877$, $p=0.101$, respectively]. No differences were found in the PFC.

The effect of morphine on pCREB levels in the four dopaminergic brain regions and in the cell body areas of the dopaminergic neurons is presented in Fig. 5 and Table 1. There was a significant interaction for the effect of morphine in the PFC both acutely and after repeated morphine pretreatment [line × treatment interaction $F(1,28)=18.131$, $p<0.001$; $F(1,28)=11.763$, $p<0.01$, respectively]. Contrast analysis revealed a significant decrease in the pCREB IR in morphine-treated AA rats compared with saline-treated AA rats both after acute and after chronic morphine treatment, and a significant increase in ANA rats after acute treatment. The increase in the pCREB IR in ANA rats after repeated morphine treatment did not reach significance. In the CPu, there was a tendency for both acute and repeated morphine to alter the pCREB IR in

Table 1
Morphine-induced pCREB levels in the ventral tegmental area (VTA) and substantia nigra (SN) of AA and ANA rats.

		sS	sM	mM
VTA	AA	100 ± 9.1	111.4 ± 7.0	114.8 ± 7.9
	ANA	99.3 ± 12.9	87.3 ± 9.1	98.5 ± 10.0
SN	AA	100 ± 8.7	90.7 ± 6.7	103.2 ± 9.3
	ANA	128.2 ± 9.7*	122.1 ± 4.3	124.1 ± 3.9

sS=saline pretreatment + saline challenge, sM=saline pretreatment + morphine challenge, mM=morphine pretreatment + morphine challenge.
The results are expressed as % of AA rats treated with saline (sS) (mean ± SEM; $n=8$).
$^{*}p<0.05$ compared with the corresponding AA rats.

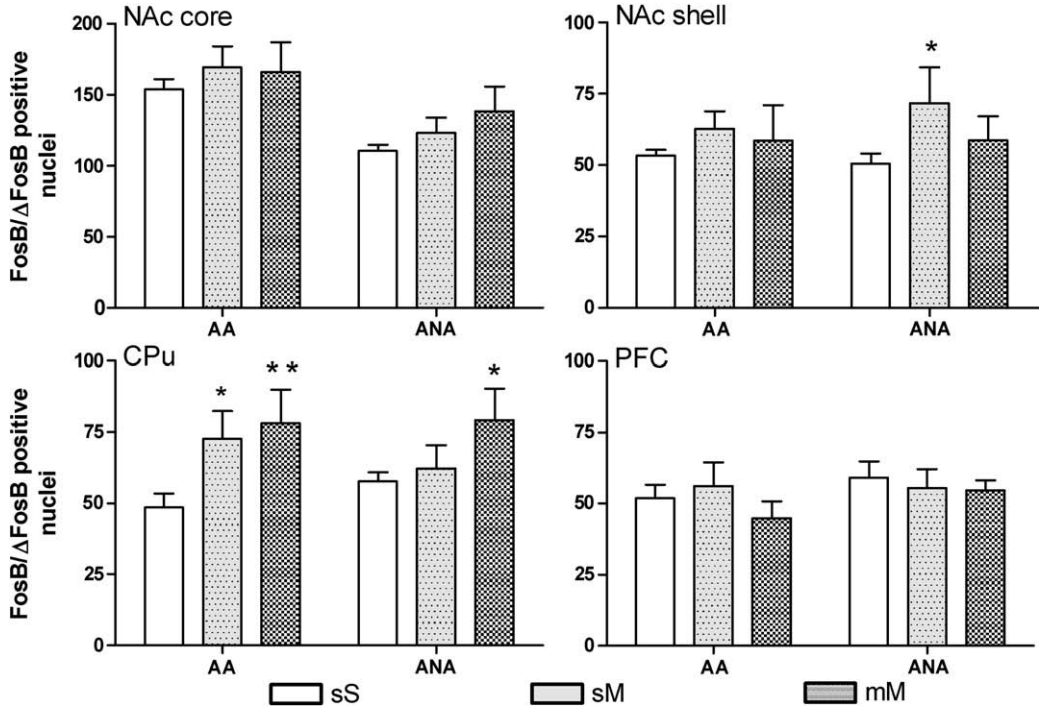

Fig. 4. Morphine-induced FosB/ΔFosB IR in AA and ANA rats. The rats were treated with saline or increasing doses of morphine (5, 10, 10, 15, 15 mg/kg, every other day). 72 h after the last injection, the rats were given a morphine challenge (1 mg/kg) followed by a second challenge (3 mg/kg) 48 h later. Some of the saline-pretreated rats were given a saline injection instead of morphine challenges. After 24–29 h, the rats were perfused for FosB/ΔFosB immunohistochemistry. sS=saline pretreatment + saline challenge, sM=saline pretreatment + morphine challenge, mM=morphine pretreatment + morphine challenge, $n = 6$–10. Note that the scale of the *y*-axis in the NAc core panel differs from that in the other panels. NAc, nucleus accumbens; CPu, caudate-putamen; PFC, prefrontal cortex. $^{*}p<0.05$, $^{**}p<0.01$ compared with corresponding saline-treated rats.

AA and ANA rats in different ways [line × treatment interaction: $F(1,28) = 2.630$, $p = 0.116$; $F(1,28) = 2.639$, $p = 0.115$, respectively], but contrast analysis did not show significant differences in the morphine effects. No other significant differences were found in the brain regions studied.

3.4. Nicotine-induced locomotor activity and accumbal dopamine release

Acute nicotine induced similar increased locomotor activity in rats of both lines [treatment effect $F(1,27) = 28.200$, $p<0.0001$; line × treatment

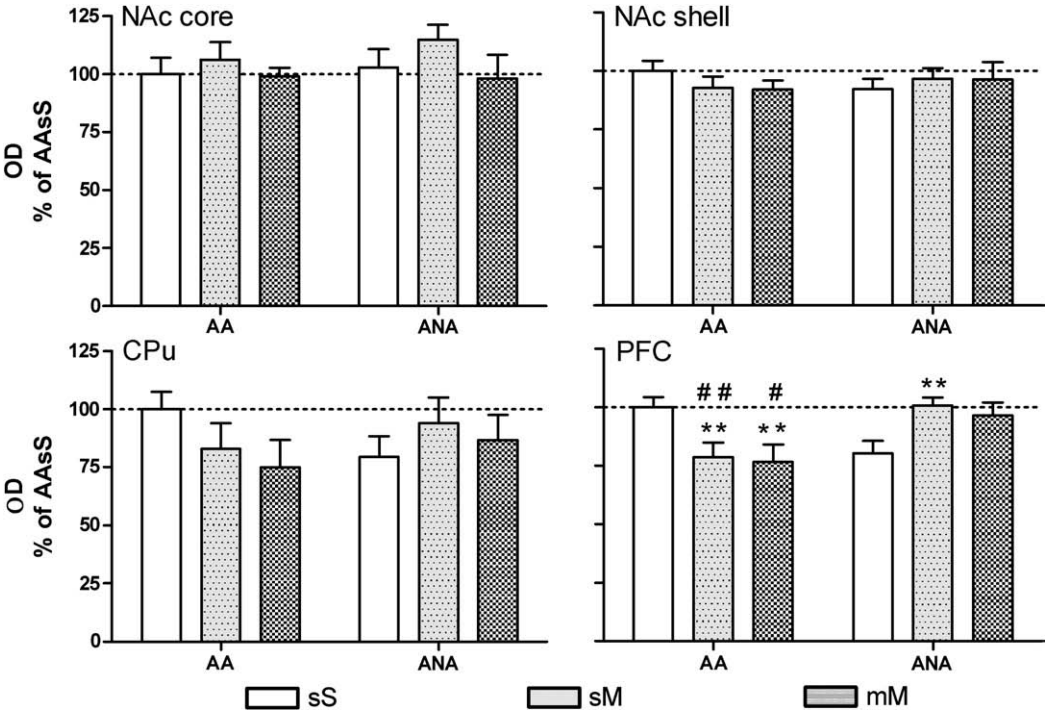

Fig. 5. Morphine-induced pCREB IR in AA and ANA rats. The rats were treated with saline or increasing doses of morphine (5, 10, 10, 15, 15 mg/kg, every other day). 72 h after the last injection, the rats were challenged with morphine (5 mg/kg) or saline and perfused 20 min thereafter. The results are expressed as % of AA rats (sS), $n = 8$. sS=saline pretreatment + saline challenge, sM=saline pretreatment + morphine challenge, mM=morphine pretreatment + morphine challenge. NAc, nucleus accumbens; CPu, caudate-putamen; PFC, prefrontal cortex. $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with corresponding ANA rats, $^{**}p<0.01$ compared with corresponding saline-treated rats.

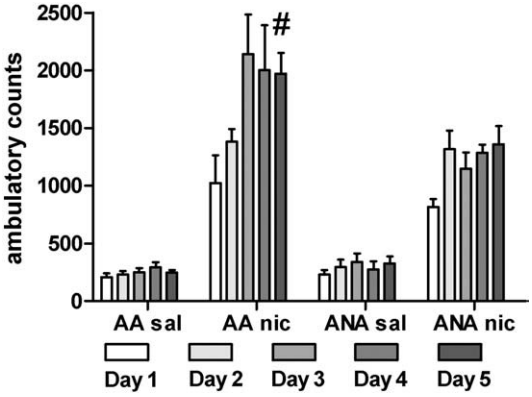

Fig. 6. Nicotine-induced locomotor activity in AA and ANA rats. The rats were treated with saline or nicotine (0.4 mg/kg) on five consecutive days. The locomotor activity was measured for 1 h. $n=7$–8 (days 1, 2 and 5); $n=5$–6 (days 3 and 4). $^{\#}p<0.05$ compared with ANA rats.

interaction $F(1,27)=0.720$, $p=0.40$]. In addition, repeated nicotine treatment significantly increased locomotor activity in both AA and ANA rats [treatment effect $F(1,27)=78.418$, $p<0.0001$, Fig. 6], but after repeated treatment nicotine-induced locomotor activity was significantly more enhanced in AA rats compared with ANA rats [day × line × treatment interaction $F(1,27)=4.212$, $p<0.05$].

To study the role of accumbal dopamine release in the differential locomotor sensitization to nicotine in AA and ANA rats, we used *in vivo* microdialysis. The basal extracellular concentrations (nM) of dopamine (AAsal: 0.94 ± 0.12; AAnic: 0.74 ± 0.12; ANAsal: 1.25 ± 0.2; ANAnic: 0.96 ± 0.13) and its metabolites DOPAC (AAsal: 839.7 ± 150.2; AAnic: 881.7 ± 111.8; ANAsal: 1122.1 ± 132.6; ANAnic: 812.7 ± 105.3) and HVA (AAsal: 273.6 ± 61.4; AAnic: 241.6 ± 38.6; ANAsal: 276.9 ± 30.7; ANAnic: 185.7 ± 24.6) were slightly, but not significantly, reduced in nicotine-pretreated rats of both lines compared with controls 24 h after the last nicotine injection [pretreatment effects: dopamine: $F(1,20)=2.410$, $p=0.136$; DOPAC: $F(1,20)=1.100$, $p=0.307$; HVA: $F(1,20)=2.793$, $p=0.110$]. After four days of treatment with either nicotine or saline, all rats were given a nicotine injection. Nicotine elevated extracellular dopamine concentrations significantly more in nicotine-pretreated rats compared with the saline-pretreated controls [0–180 min: pretreatment effect $F(1,20)=6.271$, $p<0.05$; line effect $F(1,20)=0.135$, $p=0.717$; Fig. 7]. The enhancement was not significantly different between the rat lines. No significant differences between the lines or pretreatments were in seen in nicotine-induced extracellular DOPAC or HVA concentrations (data not shown).

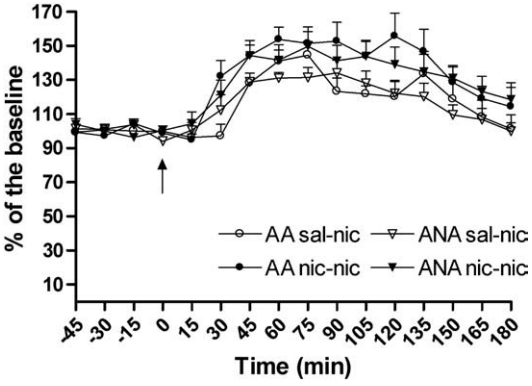

Fig. 7. The effect of nicotine (0.4 mg/kg) on extracellular levels of dopamine in the NAc. The rats were pretreated with saline or nicotine on four consecutive days. On the 5th day, all rats were given nicotine at the time indicated by the arrow. The results are expressed as % of the baseline values. AA sal-nic $n=4$, AA nic-nic $n=6$, ANA sal-nic $n=7$, ANA nic-nic $n=7$.

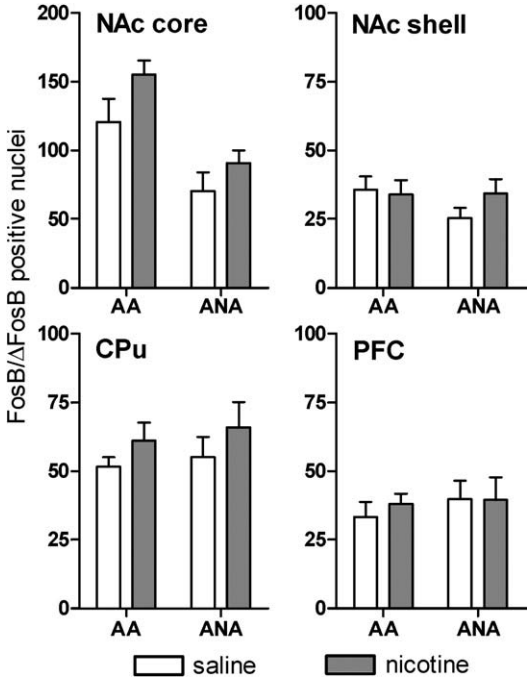

Fig. 8. Nicotine-induced FosB/ΔFosB levels in AA and ANA rats. The rats were perfused 24–29 h after the last injection of the 5-day nicotine treatment, $n=6$–8. NAc, nucleus accumbens; CPu, caudate-putamen; PFC, prefrontal cortex. Note that the scale of *y*-axis in the NAc core panel differs from that in the other panels.

3.5. Nicotine-induced FosB/ΔFosB IR

Repeated nicotine treatment increased FosB/ΔFosB IR in the NAc core to a similar extent in rats of both lines [treatment effect $F(1,25)=4.587$, $p<0.05$; line effect $F(1,25)=19.751$, $p<0.001$; line × treatment interaction $F(1,25)=0.275$, $p=0.604$; Fig. 8.], but no significant effects of nicotine were seen in the three other brain regions studied.

4. Discussion

In the present study we found differences in the expression of transcription factors ΔFosB and pCREB in dopaminergic brain areas between alcohol-preferring AA and alcohol-avoiding ANA rats. AA rats were found to have higher ΔFosB-like and pCREB immunoreactivity (IR) in the NAc core and the PFC, respectively, whereas ANA rats had higher pCREB IR in the SN. Morphine more readily increased the ΔFosB-like IR in the CPu of AA rats compared with ANA rats. Also, pCREB IR in the PFC was differentially altered by morphine in the two rat lines, as the level was decreased in AA rats but increased in ANA rats. Nicotine elevated ΔFosB-like IR in the NAc core to a similar extent in both rat lines. In association with these molecular changes, the most evident behavioral difference between AA and ANA rats was the enhanced locomotor activation of AA rats by acute morphine. Repeated morphine and nicotine treatment paradigms sensitized the locomotion somewhat more in AA rats. Moreover, the conditioned place preference (CPP) experiment suggested greater susceptibility of AA rats to the rewarding effects of morphine. Thus, our findings suggest that differences in the expression of pCREB and ΔFosB-like proteins are involved in differential behavioral responses of AA and ANA rats to morphine.

ΔFosB and CREB are transcription factors that are most often implicated in addiction. In the present study, we found differences between the rat lines in the basal levels of both pCREB and FosB/ΔFosB IR. In agreement with our previous study (Marttila et al., 2007) the FosB/ΔFosB-IR was greater in the NAc of AA rats, and this difference was now found to be localized to the NAc core. Studies with gene-

modified animals and viral vectors have shown that an overexpression of ΔFosB in certain brain regions, e.g., in the NAc, creates a behavioral phenotype with increased sensitivity to drugs of abuse (Colby et al., 2003; Kelz et al., 1999; Peakman et al., 2003; Zachariou et al., 2006). Present findings provide further support for the role of increased NAc FosB/ΔFosB levels in addiction-prone phenotypes such as AA rats.

Contrary to ΔFosB, increased CREB activity in the NAc appears to counteract the rewarding effects of drugs of abuse (Barrot et al., 2002; Carlezon et al., 1998; Pliakas et al., 2001). We hypothesized that the pCREB levels would be greater in the NAc of alcohol-avoiding ANA rats compared with AA rats, but this was not the case. Thus, basal pCREB levels in the NAc do not seem to contribute to the differences between these rats. However, we found significantly greater pCREB levels in the SN of ANA rats compared with that of AA rats. Correspondingly with the smaller pCREB level in the SN of AA rats, we have recently found that after cessation of chronic nicotine treatment the pCREB IR in the SN of nicotine-treated mice is about 80% of that in controls (Unpublished results). At that time, the nicotine-treated mice are more sensitive to the behavioral and neurochemical effects of morphine (Vihavainen et al., 2006, 2008a,b). Thus, the smaller pCREB levels in the SN of AA rats may be involved in the significantly greater acute morphine-induced locomotor activation.

The baseline levels of pCREB were significantly greater in the PFC of AA rats compared with ANA rats. Surprisingly, morphine differentially altered pCREB IR in the PFC. It can be speculated that, provided a baseline difference in the pCREB level, the observed response may be dependent on the activation state before the stimulus. The effects of pCREB are, to a great extent, dependent on the brain region involved. The PFC, which is part of the mesocorticolimbic dopamine system, is a component of the motivation circuit involved in reward-oriented behaviors. Furthermore, alterations in dopamine transmission within the mesocortical brain circuit have been suggested to play a key role in the development of sensitization to at least psychostimulants (Steketee, 2003). To date, not much data have existed on the role of pCREB in the PFC. However, if it is assumed that all differences found between the rat lines are related to the selection criteria (i.e., alcohol consumption), then our results suggest that this role may be important.

ΔFosB has been associated with locomotor responses to drugs of abuse, but the exact role played by ΔFosB in neuroadaptations that contribute to the development of behavioral sensitization is not clear (Hiroi et al., 1997; Kelz et al., 1999; Peakman et al., 2003). The morphine pretreatment regimen used sensitizes the locomotor response somewhat more in AA compared with ANA rats. After morphine pretreatment the ΔFosB-like IR was increased in the CPu of rats of both lines. Despite the fact that the NAc has long been considered a key element for locomotor activation and sensitization (Robinson and Berridge, 2003), no significant morphine-induced changes were discovered in the NAc in either line. Thus, unlike the cocaine sensitization in these rats (Marttila et al., 2007), the locomotor sensitization to morphine does not appear to be associated with changes in ΔFosB IR in the NAc. In agreement with this notion, differences in the psychomotor sensitization to morphine between the rat lines were not accompanied by the sensitization of mesolimbic dopamine transmission (Mikkola et al., 2000, 2001b; Ojanen et al., 2003). In parallel with the "presensitized" locomotor response to morphine, the morphine-induced increase of ΔFosB-like IR occurred more readily in the CPu of AA rats. This result is in agreement with the enhanced sensitivity of the nigrostriatal dopamine system of AA rats in response to acute morphine (Honkanen et al., 1999a; Mikkola et al., 2000, 2001b).

The AA rats have been previously shown to be more sensitive to the locomotor sensitizing effect of morphine compared with ANA rats (Honkanen et al., 1999b; Ojanen et al., 2007). As morphine has both stimulatory and sedative actions on the locomotion of rats, we further characterized this sensitization by using different challenge doses to emphasize the stimulatory actions of morphine. In our experiments, repeated morphine pretreatment sensitized the locomotor responses in both AA and ANA rats, and the magnitude of sensitization was greater in AA rats. Thus, the mechanisms that underlie psychomotor sensitization also exist in ANA rats, but however, based on the present and previous findings (see above) they appear to be comparatively enhanced in AA rats. The degree of sensitization may be modified by several factors including the time the rats are abstained from morphine. It is also likely that higher challenge doses may be associated with greater observed differences between the lines. The most robust difference in the morphine-induced locomotion between the rat lines appears to be the greater locomotor activation of AA rats induced by acute morphine. Consistent with previous findings (Kiianmaa et al., 2000), we found that acute nicotine administration in contrast to morphine, increases locomotor activity to a similar extent in both rat lines. The finding that the locomotor response of AA rats to acute morphine is "presensitized" replicates the observation made in our previous study (Honkanen et al., 1999b). However, this effect was not seen with either acute cocaine (Honkanen et al., 1999b), or acute nicotine (present study), supporting the hypothesis that these rat lines have an inherent difference in their opioidergic system (Sommer et al., 2006).

Similarly to the locomotor-activating effects, we found that the rewarding effects of morphine were enhanced in AA rats compared with ANA rats. In the CPP experiment with morphine 0.5 mg/kg, only AA rats developed significant place preference for the morphine-paired environment. The larger dose of morphine (1 mg/kg) induced CPP in rats of both lines, suggesting that there are no differences in learning and memory between the rats that could account for the difference in the development of CPP. There is some previous evidence of enhanced reinforcing effects of opioids in AA rats compared with ANA rats. In addition to alcohol, AA rats consume more etonitazene (an opioid receptor agonist), when it is offered in a two-bottle choice paradigm (Hyytia and Sinclair, 1993). However, in an i.v. self-administration model of heroin, AA rats responded to heroin in the first session to a greater extent than do ANA rats, but once stable self-administration had been established, there were no differences between the lines (Hyytia et al., 1996). Our new CPP data demonstrate that smaller doses of morphine are reinforcing in AA rats compared with ANA rats. This finding is in line with an enhanced psychomotor response to morphine.

Another interesting finding in our experiments was that in addition to repeated cocaine and morphine treatment, AA rats are also more sensitive to the locomotor-sensitizing effect of repeated nicotine treatment. Thus, enhanced psychomotor sensitization to these three addictive compounds is a common feature of alcohol-preferring AA rats compared with alcohol-avoiding ANA rats. In agreement with our findings in Wistar rats (Marttila et al., 2006), repeated nicotine administration primarily affected ΔFosB-like IR in the NAc of both rat lines. Despite the difference in the magnitude of locomotor sensitization, no difference was found between the rat lines in the effects of nicotine on ΔFosB-like proteins. In parallel with the FosB/ΔFosB IR results, the accumbal dopamine release was similarly sensitized in rats of both lines. Thus, the enhancement of nicotine-induced locomotor sensitization found in AA compared with ANA rats seems to depend in addition to dopamine and ΔFosB on other mechanisms.

In conclusion, we found significant differences in innate expression of the transcription factors ΔFosB and pCREB in dopaminergic brain regions of AA and ANA rats. In addition to enhanced locomotor response, the development of CPP to morphine was found to be enhanced in AA rats. The enhanced sensitivity of AA rats to morphine is most likely related to augmented morphine-induced expression of FosB/ΔFosB in the CPu and morphine-induced reduction of pCREB levels in the PFC. On the other hand, the enhanced nicotine-induced locomotor sensitization in AA rats seems to depend in addition to dopamine and ΔFosB on other mechanisms. Taken together, our findings suggest that one mediator of pronounced susceptibility of AA rats to addiction-related behaviors, including alcohol consumption, could be the differential innate expression and induction of pCREB and *fosB*-derived proteins.

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