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α-Synuclein expression in the brain and blood during abstinence from chronic alcohol drinking in mice

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ABSTRACT

α-Synuclein is a presynaptic protein proposed to serve as a negative regulator of dopaminergic neurotransmission. Recent research has implicated α-synuclein in chronic neuroadaptations produced by psychostimulant and opiate use, as well as in genetically determined susceptibility to alcoholism in humans. The aim of our study was to characterize the changes in α-synuclein expression after short-term abstinence from chronic alcohol drinking in mice.

Male C57BL/6J mice were allowed to drink increasing concentrations of alcohol in the two-bottle choice procedure. Then the mice were given constant access to an 8% alcohol solution and water for 32 days, and were sacrificed 2 h, 24 h or 48 h after alcohol withdrawal. RT-PCR, in situ hybridization and Western blotting techniques were used to measure α-synuclein mRNA and protein levels in the brain and blood. α-Synuclein protein levels were elevated by up to 80% in the amygdala of mice withdrawn from alcohol for 24 h or 48 h. No changes in α-synuclein levels were found in the mesencephalon or striatum/accumbens. The levels of α-synuclein mRNA remained unchanged in all brain regions examined (the striatum, nucleus accumbens, amygdala, substantia nigra, ventral tegmental area). α-Synuclein mRNA was up-regulated in the whole blood 48 h after alcohol withdrawal.

The accumulation of α-synuclein in the amygdala, observed in this study, seems to be a common feature of alcohol and opiate abstinence. This finding suggests a role of α-synuclein in common neuroadaptations produced by long-term alcohol and drug use. Although α-synuclein expression in the blood seems unrelated to that in the brain, it may serve as a peripheral biomarker of chronic alcohol consumption.

1. Introduction

α-Synuclein is a protein abundantly expressed by neurons, and is present almost exclusively in presynaptic terminals (Iwai et al., 1995; Clayton and George, 1998, 1999). It is best known for its involvement in neuropathology, especially Parkinson's disease and other synucleinopathies, whereas its physiological roles remain unclear (Lotharius and Brundin, 2002; Marti et al., 2003; Vekrellis et al., 2004). Its proposed physiological functions include the regulation of neurotransmitter release and reuptake, presynaptic vesicle recycling and the role of a molecular chaperone (reviewed by Vekrellis et al., 2004). Reports focusing on brain dopaminergic neurons, which express α-synuclein at high levels, have suggested that the protein might negatively regulate the function of dopaminergic cells by several mechanisms. α-Synuclein was found to inhibit activity or expression of enzymes involved in dopamine synthesis (Perez et al., 2002; Baptista et al., 2003), affect function of the dopamine transporter (Lee et al., 2001;Wersinger and Sidhu, 2003) and inhibit dopamine release in response to repetitive stimulation (Abeliovich et al., 2000; Yavich et al., 2004; Larsen et al., 2006). This suggests participation of α-synuclein in the regulation of reward and reinforcement mechanisms mediated by the mesocorticolimbic dopaminergic system, a conclusion supported by the observation of an altered rate of intracranial self-stimulation of medial forebrain bundle in α-synuclein knockout mice (Oksman et al., 2006).

Several recent studies have also implicated α-synuclein in substance dependence and abuse, processes known to involve abnormalities in dopaminergic function. Elevated levels of the protein were found in the brains of cocaine addicts (Mash et al., 2003; Qin et al., 2005) and in rodents treated with psychostimulants (Brenz Verca et al., 2003; Fornai et al., 2005). Moreover, we previously reported a long-lasting accumulation of α-synuclein in the mouse striatum and amygdala following withdrawal from chronic morphine treatment (Ziolkowska et al., 2005). Taking into account the inhibitory influence of α-synuclein on dopaminergic neurotransmission, we proposed that the up-regulation of α-synuclein

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produced both by psychostimulants and opiates may contribute to reduction of dopaminergic system activity, which is characteristic of withdrawal states from various drugs of abuse (Ziolkowska et al., 2005).

Interestingly, a growing body of evidence suggests that α-synuclein might also play a role in alcohol addiction. In rats, the α-synuclein gene is localized within the quantitative trait locus for alcohol consumption, and its basal expression in the hippocampus and striatum is higher in an inbred alcohol-preferring strain than in a nonpreferring strain, apparently due to a single nucleotide difference in the gene sequence (Liang et al., 2003). α-Synuclein gene polymorphisms, which correlate with alcohol dependence and craving phenotypes, were also identified in human alcohol addicts (Bonsch et al., 2005b; Foroud et al., 2007). On the other hand, the level of α-synuclein protein and mRNA in the blood was higher in alcoholic patients during early abstinence than in healthy control subjects (Bonsch et al., 2004, 2005a). Moreover, the blood level of α-synuclein expression of those patients correlated with alcohol craving (Bonsch et al., 2004, 2005a). However, these human studies did not address the question if high blood levels of α-synuclein expression in alcoholics were constitutive or, alternatively, reflected an increase in the expression resulting from chronic alcohol consumption.

Thus, the available data indicate that individual variations in α-synuclein gene sequence may contribute to the genetically determined susceptibility to alcoholism. They also suggest that alcohol drinking may regulate α-synuclein expression in the blood, which is related to craving in alcohol-dependent subjects. Whereas a direct causative relationship between peripheral α-synuclein expression and craving seems doubtful, the effect of alcohol drinking on α-synuclein levels in the brain has not yet been assessed. Moreover, it is not known if any relationship exists between expression of α-synuclein in the brain and blood. To further our understanding of the role of α-synuclein in alcohol addiction, it would also be interesting to compare the putative effects of alcohol on α-synuclein expression in the brain to those of opiates and psychostimulants (Brenz Verca et al., 2003; Mash et al., 2003; Fornai et al., 2005; Qin et al., 2005; Ziolkowska et al., 2005).

In order to address the above issues, we employed a mouse model of chronic voluntary alcohol drinking, in which we assessed the levels of α-synuclein mRNA and protein both in the blood and in brain regions encompassing the dopaminergic pathway, involved in the regulation of motivation and reward. In contrast to human studies, the scope of which is limited for obvious reasons, the use of this simple animal model permits a more thorough evaluation of α-synuclein expression changes produced by long-term alcohol exposure. Such an approach seems to be an essential step towards understanding the putative functional role of α-synuclein in alcoholism and other addictive disorders.

2. Methods

2.1. Animals

The experiments were performed on male C57BL/6J mice (bred in the Medical Research Center, Warsaw, Poland) weighing 25–30 g at the start of behavioral procedures. The mice were kept under standard conditions, on a 12/12 h light/dark cycle, with free access to rodent chow (Labofeed H, Kcynia, Poland) and tap water. Treatment of the mice in the present study was in full accordance with the ethical standards laid down in the respective European (Directive no. 86/609/EEC) and Polish regulations. The experimental protocol was approved by the ethics committee on animal studies.

2.2. Alcohol drinking in the two-bottle choice procedure

C57BL/6J mice present high two-bottle choice ethanol preference across a wide range of ethanol concentrations and consume pharmacologically relevant amounts of ethanol (He et al., 1997; Peirce et al., 1998). In our preliminary experiment (Bienkowski et al., unpublished), C57BL/6J mice ($n = 6$) were given free choice between 8% (v/v) ethanol and water. The animals were killed by decapitation 2 h into the dark phase, and samples of trunk blood were collected. Blood ethanol levels exceeded 50 mg% confirming that the mice consumed pharmacologically relevant amounts of ethanol.

The two-bottle choice model of oral alcohol self-administration was also used in the present study (Korkosz et al., 2004). The animals were housed singly in standard Plexiglas boxes equipped with two graduated drinking tubes. They were allowed 7 days for acclimatization, during which both drinking tubes were filled with tap water, and baseline water drinking was assessed. On the following days, one of the tubes was filled with water and the other one with an ethanol solution. The alcohol concentration was increased gradually from 2% (for 2 days) through 4% (4 days) to reach 8%. Then the mice were exposed to the 8% ethanol solution for 32 days, and had free choice between alcohol solution and water throughout the experiment; the control group received water in both tubes. The ethanol solutions were replaced daily and the drinking tubes were rotated every day to prevent position preference. Fig. 1 shows the time course of alcohol solutions vs. water consumption during the experiment. In mice exposed to 8% ethanol, the average (±S.D.) daily consumption of alcohol was 11.43 ± 1.75 g/kg. The preference for 8% ethanol, calculated according to the formula: [ethanol intake/total fluid intake] × 100%, equalled 91.03 ± 9.69%.

The bottles containing alcohol were withdrawn immediately after the end of the dark period. During abstinence, the two bottles were filled with water. The mice were randomly assigned to different experimental groups and sacrificed by decapitation 2 h, 24 h or 48 h after alcohol withdrawal. These withdrawal periods were dictated by our previous observation that the changes in α-synuclein levels resulting from chronic morphine exposure developed only after the drug withdrawal (Ziolkowska et al., 2005). The experimental groups did not differ in terms of ethanol consumption and preference (all p values > 0.05). In the preliminary experiment, no handling-induced convulsive activity, spontaneous signs of withdrawal hyperexcitability (Chan et al., 1991) or other signs of alcohol withdrawal (e.g. body tremor, piloerection) were observed after 2 h, 24 h or 48 h of abstinence.

2.3. RNA isolation from blood and reverse transcription-real-time PCR

Blood was collected after decapitation into ethylenediaminetetraacetic acid (EDTA)-containing tubes, frozen and stored at −70 °C until RNA isolation. Total RNA was extracted from the whole blood by the method of Chomczynski and Sacchi (1987), with further modifications, using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was then purified using the RNeasy Mini Kit columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quality of the total RNA was assessed based on the intensity of 28S and 18S rRNA bands after denaturing agarose gel electrophoresis followed by ethidium bromide staining, and by the spectrophotometric ratio A260/A280 (1.9–2.1). RNA concentration was measured using the fluorescent reagent RiboGreen (Molecular Probes, Eugene, Oregon, USA).

Reverse transcription was performed using Omniscript reverse transcriptase (Qiagen) and oligo(dT_{16}) primer at 37 °C for 60 min. Quantitative real-time RT-PCR reactions were performed, according to the manufacturer's protocol, using the α-synuclein TaqMan Gene Expression Assay (Mm00447333_m1) from Applied Biosystems (Foster, CA, USA). The reactions were run on the iCycler device (BioRad, Hercules, CA, USA) with the 3.0a software version. Amplification efficiency for the assay was determined by running a standard dilution curve. Expression of hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*) transcript was quantified to control for variation in cDNA amounts. The threshold cycle values were calculated automatically by iCycler IQ 3.0a software with default parameters. Abundance of RNA was calculated as $2^{-(\text{threshold cycle})}$.

2.4. In situ hybridization and image analysis

After sacrifice, the brains were removed from the skulls, frozen on dry ice and stored at −70°C. Then they were cut into 12-μm thick coronal sections on a cryostat

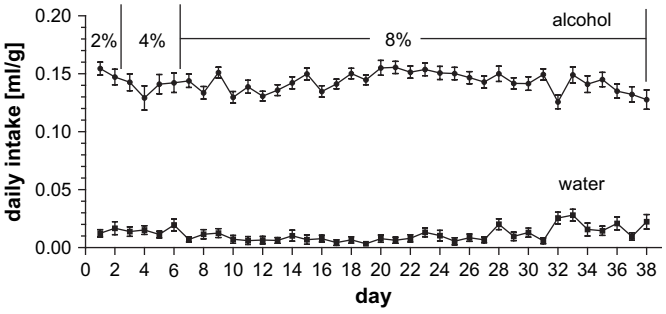

Fig. 1. Daily intake of alcohol solution and water by C57BL/6J mice during the course of the experiment (mean ± S.E.M., $n = 24$). The intake is expressed in milliliters of liquid per gram of body weight. Alcohol concentrations on consecutive days of the experiment are indicated above the upper curve.

microtome (CM 3050 S, Leica Microsystems, Nussloch, Germany), the sections were thaw-mounted on gelatin-chrom-alum-coated slides and processed for in situ hybridization according to the method of Young et al. (1986). Briefly, the sections were fixed with 4% paraformaldehyde, washed with PBS and acetylated by incubation with 0.25% acetic anhydrite (in 0.1 M triethanolamine and 0.9% sodium chloride). The sections were then dehydrated using increasing concentrations of ethanol (70–100%), treated with chloroform for 5 min, and rehydrated with decreasing concentrations of ethanol.

The sections were hybridized for approximately 15 h at 37 °C with an oligonucleotide probe complementary to nucleotides 329–377 of the mouse α-synuclein cDNA (Hong et al., 1998) (5′-TGTCTTCTGAGCGACTGCTGTCACACCAGTCACCACTG CTCCTCCAACA-3′). The hybridization buffer contained 50% formamide, 4× SSC (i.e. 0.6 M sodium chloride and 0.06 M sodium citrate), 1× Denhardt's solution, 0.25 mg/ml yeast tRNA, 0.5 mg/ml salmon sperm DNA, 50 mg/ml dextran sulphate and 10 mM dithiothreitol. The probe was labeled with ^{35}S-dATP by the 3′-tailing reaction using terminal transferase (Roche Diagnostics, Mannheim, Germany) and applied to the slides in the concentration of approximately 16 000 dpm/μl.

After hybridization, the slices were washed three times for 20 min with 1× SSC/50% formamide at 40 °C, and twice for 50 min with 1× SSC at room temperature. The slices were then dried and exposed to Fujifilm (Tokyo, Japan) phosphorimager imaging plates for 4 days. The hybridization signal was digitized using the Fujifilm BAS-5000 phosphorimager and the Image Reader software.

The in situ hybridization signal was analyzed using the MCID Elite system (Imaging Research, St. Catharines, Ontario, Canada). Mean signal density, expressed in photostimulated luminescence units/mm^2, was measured in selected brain regions in the Fujifilm BAS-5000 images. The regions included: the substantia nigra (pars compacta), ventral tegmental area (VTA), dorsal striatum, nucleus accumbens core and shell, lateral and basolateral nuclei of amygdala (see Fig. 2 for the regions definition in the autoradiograms). The two latter amygdaloid nuclei were chosen for the analysis because of a particularly high α-synuclein expression; other amygdaloid nuclei cannot be delineated in the autoradiograms because of a much lower and homogenous α-synuclein mRNA signal, which does not exceed markedly that in the surrounding tissue (Fig. 2). For each brain structure, data were collected from at least four sections per animal, bilaterally. Background signal was measured over the white matter (corpus callosum) and was subtracted from the hybridization signal in the regions of interest.

2.5. Brain dissection

After decapitation, brains were removed from the skulls and dissected rapidly. We collected samples containing (1) the rostral part of caudate/putamen plus nucleus accumbens (referred to as striatum/accumbens), (2) amygdala (the tissue about 0.5 mm below rhinal fissure including the majority of the amygdalar nuclei located between the piriform cortex on the one side, and the optic tract and ventral part of lateral ventricles, substantia innominata and ventral border of the caudate-putamen on the other), and (3) mesencephalon. Tissue samples were frozen on dry ice immediately after dissection and stored at −70 °C until protein extraction.

2.6. Western blot

The dissected tissue samples were homogenized in hot 2% sodium dodecyl sulphate (SDS), boiled for 8 min and cleared by centrifugation (20 800*g* for 30 min). Protein concentration in the supernatant was determined using the BCA Protein Assay Kit (Sigma–Aldrich, St. Louis, MO, USA). Samples (containing 65 μg of protein) were heated for 10 min at 95 °C in loading buffer (50 mM Tris–HCl, 2% SDS, 2% ß-mercaptoethanol, 8% glycerol, 0.1% bromophenol blue) and resolved by SDS-PAGE on 12% polyacrylamide gels. The amount of protein per lane was optimized in pilot studies, in which Western blots were performed using different amounts of protein per sample (20–150 μg) extracted from each experimental brain region. For quantitative analysis of α-synuclein, the amount of total protein was chosen, for which twofold differences in protein content were linearly reflected by the assay. After the gel electrophoresis, proteins were electrophoretically transferred to nitrocellulose membranes (Trans-Blot; BioRad). To control equal gel loading, gels were stained with Coomassie Brilliant Blue R250 after protein transfer, and intensity of staining was compared between lanes. The blots were blocked using 5% albumin (Sigma–Aldrich) in Tris-buffered saline (TBS) for 1 h and incubated overnight at 4 °C with the sheep anti-α-synuclein polyclonal antibody (dilution 1:500; Abcam, Cambridge, UK). The blots were then incubated with a peroxidase-conjugated secondary antibody (donkey anti-Sheep IgG, Abcam) at a dilution of 1:3000 for 1 h at room temperature. After three 15 min washes in TBS and 0.1% Tween-20, and one wash in TBS, immunocomplexes were detected using a buffered solution of 250 mM luminol sodium salt and 30% hydrogen peroxide in 1 M Tris–HCl (pH 8.5) containing 90 mM *p*-coumaric acid. The signal was visualized applying the Fujifilm LAS-1000 fluorimager system. Relative levels of immunoreactivity were quantified using Fujifilm software (Image Gauge). As another control for protein quantity in the samples, some of the membranes were re-probed with a mouse anti-β-actin antibody (1:15 000 dilution, Sigma–Aldrich) after three 10-min washes in TBS and 0.1% Tween-20.

2.7. Statistical analysis

The results were statistically analyzed by one-way analysis of variance (ANOVA). The Newman–Keuls post-test was used to locate significant differences between experimental groups.

3. Results

3.1. Influence of alcohol drinking and withdrawal on α-synuclein mRNA levels in the brain

α-Synuclein mRNA expression in the brain was studied by in situ hybridization. The obtained signal distribution and relative expression levels in discrete brain regions (Fig. 2) were consistent with those reported previously by us and other authors (Maroteaux and Scheller, 1991; Nakajo et al., 1994; Hong et al., 1998; Ziolkowska et al., 2005). α-Synuclein mRNA signal densities were quantified in the dorsal striatum, nucleus accumbens core and shell, basolateral amygdala, substantia nigra and ventral tegmental area. No significant changes in α-synuclein mRNA levels were found in any brain region examined at any time point tested (2 h, 24 h or 48 h after alcohol withdrawal) (Fig. 2).

3.2. Influence of alcohol drinking and withdrawal on α-synuclein protein levels in the brain

α-Synuclein protein levels were measured by Western blotting in homogenates of the striatum/accumbens, amygdala and mesencephalon of mice withdrawn for 2 h, 24 h or 48 h from chronic alcohol drinking. No changes were observed in the striatum/accumbens and mesencephalon at any time point tested (Fig. 3). In the amygdala, a significant increase by about 80% was found after 24-h abstinence, and the α-synuclein levels remained elevated after 48-h abstinence (Fig. 3).

3.3. Influence of alcohol drinking and withdrawal on α-synuclein mRNA levels in the blood

The level of α-synuclein mRNA in the whole blood was measured by quantitative RT-real-time PCR. An increase in α-synuclein expression by about 40% was observed in mice withdrawn for 48 h from chronic alcohol exposure (Fig. 4). No changes were detected after 24 h of withdrawal (Fig. 4). Expression of the control gene *Hprt1* did not differ significantly between the experimental groups (data not shown).

4. Discussion

Previous studies in selectively bred alcohol-preferring rats and in alcohol-dependent humans have implicated the α-synuclein gene in genetically determined susceptibility to alcoholism. Associations of several polymorphic variants of the α-synuclein gene (SNPs or the polymorphic repeat NACP-REP1) with alcohol dependence, craving or preference were found in humans and rats (Liang et al., 2003; Bonsch et al., 2004, 2005b; Foroud et al., 2007). Moreover, the sequence variants associated with alcohol-drinking-prone phenotypes were shown to confer higher α-synuclein expression in the brain or blood (Liang et al., 2003; Bonsch et al., 2005b).

Whereas potential mechanisms of α-synuclein involvement in alcohol dependence have so far mainly been linked to the influence of α-synuclein on the brain dopaminergic system and other limbic regions, expression data in primates are limited to measurements of peripheral blood α-synuclein protein or mRNA levels (Bonsch et al., 2004, 2005b; Walker and Grant, 2006). Interpretation of these results is confounded by a lack of knowledge of the

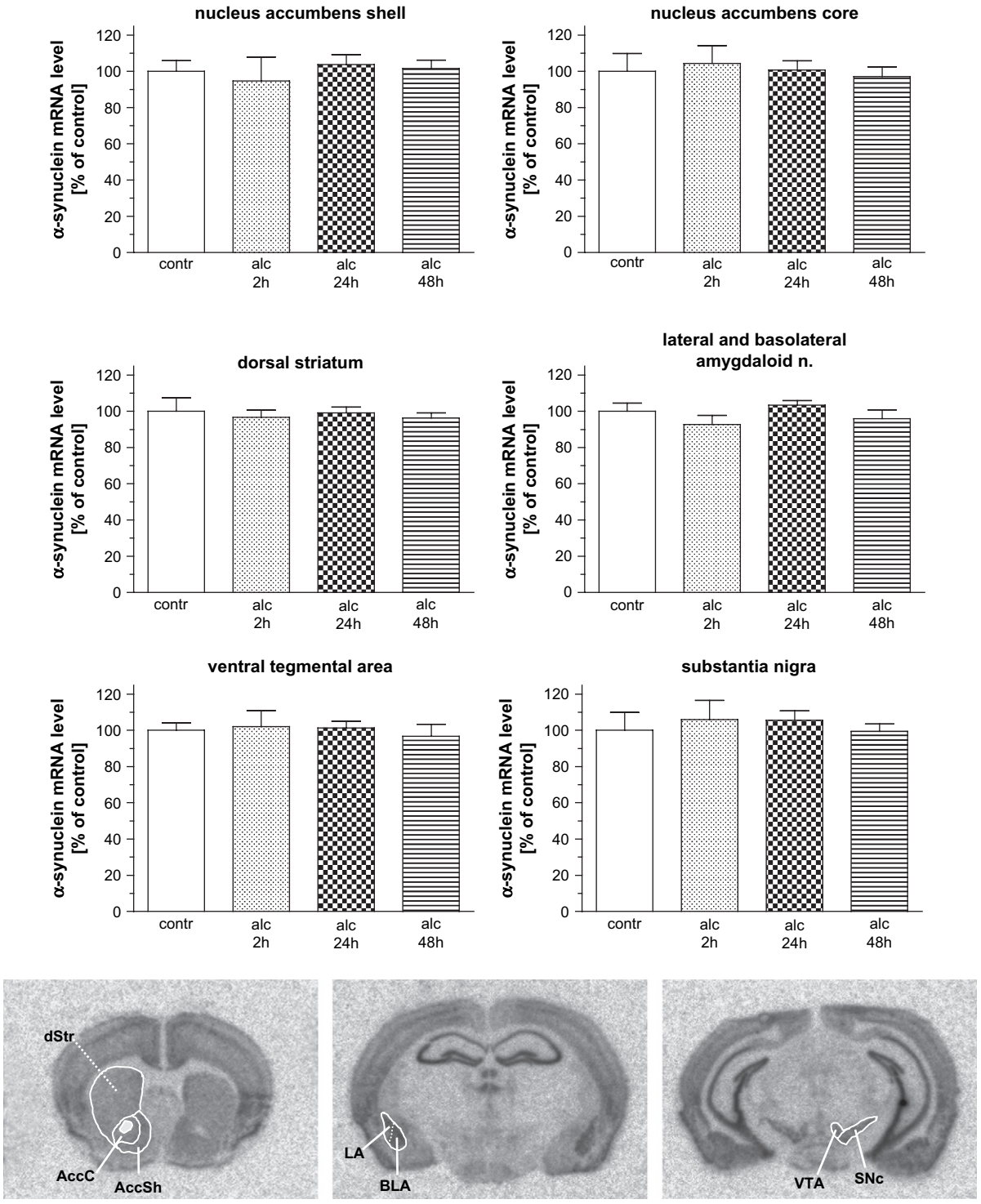

Fig. 2. The influence of alcohol drinking and withdrawal on α-synuclein mRNA in situ hybridization signal densities in subregions of the mouse striatum/accumbens, amygdala and ventral mesencephalon. The mice drank 8% (v/v) alcohol for 32 days in the two-bottle choice procedure and were withdrawn from alcohol for the indicated periods of time (alc 2 h, alc 24 h, alc 48 h). The control group (contr) had no access to alcohol at any time. The results are presented as the mean ± S.E.M. (expressed as % of control) of five to eight animals per group. One-way ANOVA did not reveal statistically significant differences between the groups. Representative autoradiograms of brain sections hybridized with the α-synuclein probe are shown at the bottom. The outlines show the regions of interest analyzed in the study. dStr – dorsal striatum; AccC – nucleus accumbens core; AccSh – nucleus accumbens shell; LA – lateral amygdaloid nucleus; BLA – basolateral amygdaloid nucleus; VTA – ventral tegmental area; SNc – substantia nigra pars compacta.

relationship between α-synuclein expression in the brain and in the blood. Moreover, the only study in which α-synuclein expression was assessed in the brain with reference to alcohol use described only basal expression of the gene in inbred alcohol-preferring and -nonpreferring rat strains, without considering effects of exposure to alcohol (Liang et al., 2003). On the other hand, human studies in which elevated blood levels of α-synuclein protein and mRNA were demonstrated in alcoholics did not make a distinction between basal α-synuclein expression (possibly related to the α-synuclein gene polymorphisms) and its possible up-regulation resulting from

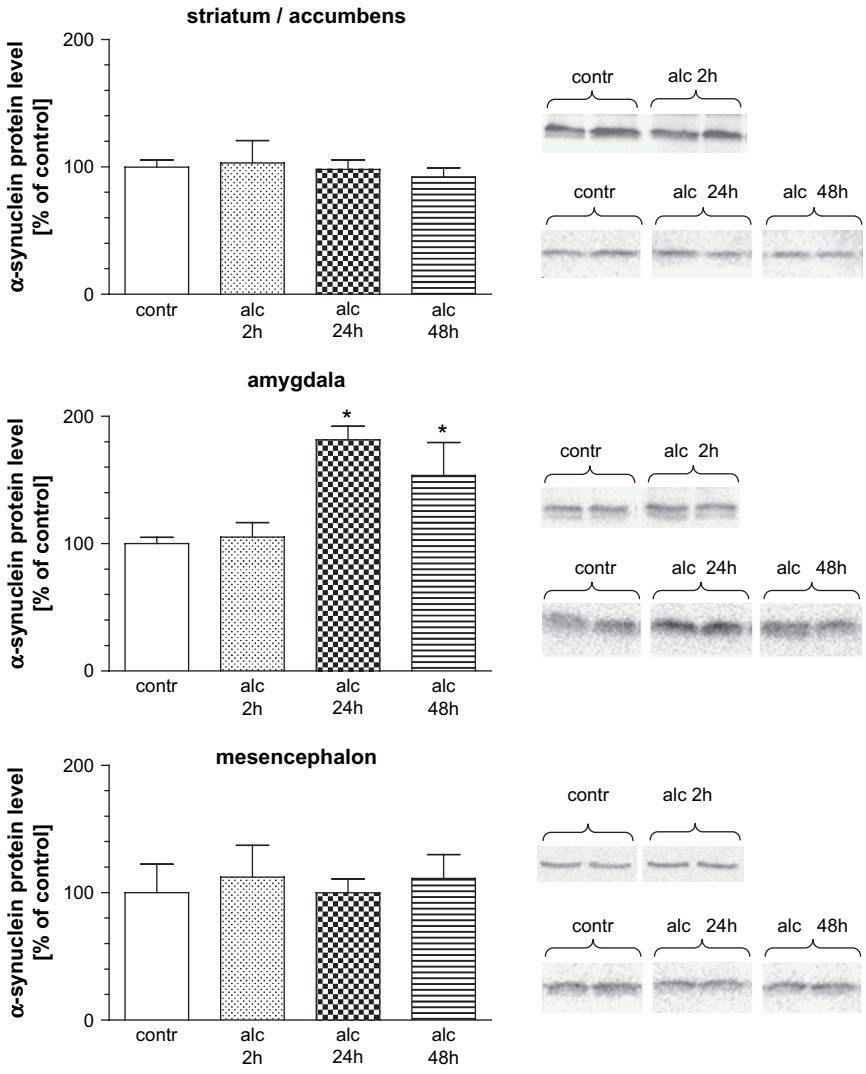

Fig. 3. The influence of alcohol drinking and withdrawal on α-synuclein protein levels in the mouse striatum/accumbens, amygdala and mesencephalon, as measured by Western blot analysis. The mice drank 8% (v/v) alcohol for 32 days in the two-bottle choice procedure and were withdrawn from alcohol for the indicated periods of time (2 h, 24 h, 48 h). The control group (contr) had no access to alcohol at any time. The results are presented as the mean ± S.E.M. (expressed as % of control) of four to six animals per group and are representative of several blots for each brain region. *Statistically significant ($p < 0.05$) vs. the control group, one-way ANOVA followed by Newman–Keuls test. Representative Western blot α-synuclein bands are shown on the right panels.

long-term alcohol drinking (Bonsch et al., 2004, 2005a). Altogether, despite the fairly convincing evidence of α-synuclein involvement in some aspects of alcohol abuse, the actual role of α-synuclein remains obscure, whereas the existing data are difficult to compare and integrate due to heterogeneity of research approaches.

In the present study, we employed a simple rodent model of chronic alcohol drinking to assess, in a more systematic way, the influence of long-term voluntary alcohol intake on α-synuclein expression both in the brain and the blood. We used the C57BL/6J mice, i.e., the inbred strain showing high preference for alcohol (He et al., 1997; Peirce et al., 1998). We have demonstrated that withdrawal from chronic alcohol drinking in the two-bottle choice procedure results in an increase in α-synuclein protein levels in the amygdala (but not in the striatum/accumbens or mesencephalon). No corresponding elevation of α-synuclein mRNA levels was found in the amygdala or any other brain region tested (including the dorsal striatum, nucleus accumbens and dopaminergic cell body fields in the mesencephalon), which suggests that the increase in α-synuclein protein levels was not underlain by transcriptional regulation of its gene. Since α-synuclein is a presynaptic protein, its up-regulation in the amygdala may have resulted from protein

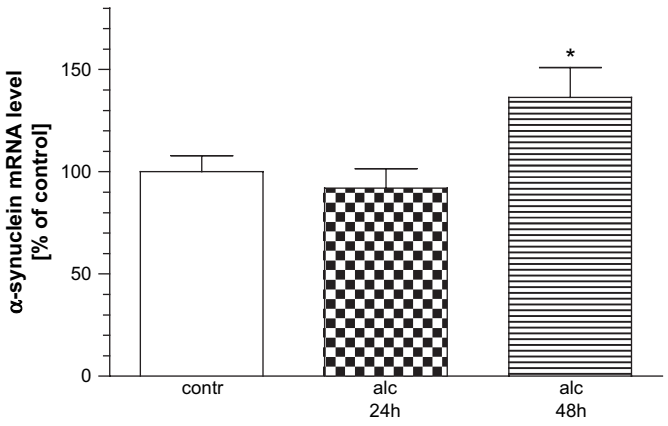

Fig. 4. The influence of alcohol drinking and withdrawal on the levels of α-synuclein mRNA in the mouse whole blood. The mice drank 8% (v/v) alcohol for 32 days in the two-bottle choice procedure and were withdrawn from alcohol for the indicated periods of time (alc 24 h, alc 48 h). The control group (contr) had no access to alcohol at any time. The results are presented as the mean ± S.E.M. (expressed as % of control) of seven to nine animals per group. *Statistically significant ($p < 0.05$) vs. the control group, one-way ANOVA followed by Newman–Keuls test.

accumulation in afferent axon terminals due to alterations of the protein transport or its degradation rate rather than regulation of the gene expression. On the other hand, a moderate increase in α-synuclein mRNA levels was found in the blood of mice withdrawn from chronic alcohol for 48 h, but not 24 h.

The latter result is consistent with the observation of elevated levels of blood α-synuclein mRNA (and protein) in alcohol-dependent humans during early abstinence (Bonsch et al., 2004, 2005a). In contrast to our data, which suggest that the increase in blood α-synuclein expression in mice develops only after some time of alcohol abstinence, Walker and Grant (2006) reported a pronounced elevation of blood α-synuclein mRNA levels in monkeys immediately after cessation of a 14-month period of access to 4% alcohol. This discrepancy between the mouse and primate models may have resulted from the difference in the time period of alcohol consumption (1 month vs. 14 months, respectively), although both models may be considered as chronic when the lifespan of the animal species involved is taken into account. Whereas further studies would be required to elucidate the discrepancy in time-courses of the α-synuclein expression changes, data from both the mouse and monkey models clearly indicate that high blood levels of α-synuclein expression may result from previous long-term alcohol consumption rather than, or in addition to, its high basal expression in alcoholism-prone individuals. It can be concluded that the elevation of blood α-synuclein mRNA levels is common to human alcoholism as well as primate and rodent models of alcohol abuse. Thus, our results further support the previous suggestion by Walker and Grant (2006) that α-synuclein may serve as a peripheral biomarker of chronic alcohol consumption both in humans and laboratory animals.

On the other hand, on the basis of the present results, α-synuclein expression in blood seems unrelated to its expression in the brain. The increase in blood α-synuclein mRNA level was not mirrored by any such change in its concentration in several brain regions under investigation. Although we did detect an elevation of α-synuclein protein level in the amygdala, this was not accompanied by a rise in mRNA level. Because of the α-synuclein protein transport into axon terminals, which might be distant from the mRNA-containing cell bodies, we cannot completely exclude that the increase in α-synuclein protein resulted from up-regulation of its mRNA in some brain region that we overlooked. Nevertheless, there was no concurrence between the changes in the blood and the brain: blood α-synuclein mRNA levels peaked 48 h after alcohol withdrawal, whereas marked elevation of α-synuclein protein levels in the amygdala was already seen 24 h earlier. Therefore, α-synuclein expression seems to be regulated independently in the blood and the brain. This suggests that peripheral changes in α-synuclein expression cannot be interpreted in terms of α-synuclein function within the brain.

To the best of our knowledge, this is the first report demonstrating that chronic alcohol drinking affects α-synuclein levels in the brain. However, the changes in α-synuclein mRNA and/or protein levels in discrete brain areas were previously reported after treatment with other drugs of abuse. Studies in cocaine addicts demonstrated up-regulation of α-synuclein mRNA and protein in dopaminergic neurons of the substantia nigra and VTA, and elevated α-synuclein protein levels in the striatum (Mash et al., 2003; Qin et al., 2005). In animal models, increased α-synuclein protein levels in the substantia nigra and up-regulation of α-synuclein gene expression in the tegmentum, striatum and hippocampus were demonstrated after administration of amphetamines (Brenz Verca et al., 2003; Fornai et al., 2005). These results suggest that psychostimulants elevate α-synuclein levels by stimulating its transcription, and these effects seem to be particularly pronounced in dopaminergic neurons. This is in contrast to the effects of chronic alcohol exposure reported above, which did not affect α-synuclein mRNA levels in the dopaminergic cells or elsewhere in the brain.

On the other hand, the effects of withdrawal from chronic alcohol show striking similarity to the regulation of α-synuclein during opiate abstinence. We previously demonstrated that the α-synuclein protein level was increased in the mouse amygdala at 48 h after cessation of chronic morphine treatment and remained elevated for at least 2 weeks (Ziolkowska et al., 2005). As in the case of alcohol withdrawal, no up-regulation of α-synuclein mRNA was found in the brain of mice withdrawn from chronic morphine. Moreover, both in alcohol and morphine abstinence, the accumulation of α-synuclein protein in the amygdala occurred only after some time of withdrawal (24–48 h), whereas it was not detected at short periods (2 h or 4 h) after cessation of the treatment. This indicates that the increase in α-synuclein levels may be a characteristic feature of alcohol and morphine abstinence.

The similarity between the effects of alcohol and morphine, drugs that act by widely different pharmacological mechanisms, suggests that accumulation of α-synuclein in the amygdala may contribute to psychological and/or autonomic changes associated with drug abstinence. Actually, negative affective states, including dysphoria, anxiety, anhedonia and craving, are common symptoms of withdrawal from various addictive substances (Markou et al., 1998), and amygdala has been strongly implicated in generation of these symptoms (Pandey, 2003; Aston-Jones and Harris, 2004). They are ascribed mainly to activation of the central amygdaloid nucleus (CeA), which is observed during withdrawal from chronic alcohol and opiates, as well as nicotine and cannabinoids (Couceyro and Douglass, 1995; Rodriguez de Fonseca et al., 1997; Knapp et al., 1998; Panagis et al., 2000; Gracy et al., 2001; Frenois et al., 2002). Alcohol withdrawal also activates the basolateral amygdaloid nucleus (BLA) (Knapp et al., 1998; Borlikova et al., 2006), the nucleus otherwise strongly implicated in conditioned-stimulus (cue)-induced craving for alcohol and drugs in animal models and human addicts (Childress et al., 1999; Ciccocioppo et al., 2001; Schneider et al., 2001; Bonson et al., 2002; Shalev et al., 2002).

Being a presynaptic protein, α-synuclein could participate in the regulation of CeA and/or BLA activity by influencing neurotransmitter release from their afferent dopaminergic or glutamatergic fibers. However, understanding the mechanism of α-synuclein action within the amygdaloid complex is precluded at the moment by the lack of knowledge of the protein distribution in distinct axonal populations innervating the amygdala. Whereas we were previously able to demonstrate an abundant innervation of the BLA output neurons by α-synuclein-containing nerve terminals (Ziolkowska et al., 2005), the origin of these axons remains unknown. On the other hand, the α-synuclein-containing input to the CeA can only be inferred from the fact that this nucleus receives afferents from two neuronal populations both expressing α-synuclein mRNA at high levels: dopaminergic neurons of the VTA and glutamatergic cells of the BLA (Swanson, 1982; McDonald, 1991; Freedman and Cassell, 1994; Zhu and Pan, 2004). Thus, further immunolabelling studies are warranted to define the nature of α-synuclein-containing input to distinct amygdaloid nuclei. Nevertheless, the accumulation of α-synuclein in the amygdala occurring selectively during alcohol and opioid abstinence puts these changes in a position to regulate the emotional states typical of alcohol and drug abstinence such as negative affect, anxiety and craving, the generation of which involves the amygdala.

The putative role of α-synuclein in drug use disorders has so far been considered mainly in the context of its propensity to negatively affect dopaminergic neurotransmission in the mesostriatal pathway (Abeliovich et al., 2000; Perez et al., 2002; Baptista et al., 2003; Yavich et al., 2004). Actually, our previous study demonstrated a prolonged elevation of α-synuclein protein level in the striatum/accumbens during withdrawal from chronic morphine

(Ziolkowska et al., 2005). However, in the present experiment, no such changes were observed in the striatum/accumbens of mice withdrawn from chronic alcohol. Thus, α-synuclein does not seem to contribute to the decline in the activity of the mesostriatal projection associated with alcohol abstinence (Acquas et al., 1991; Diana et al., 1993), which does not exclude its involvement in striatal neuroadaptations to chronic use of opiates and psychostimulants (Mash et al., 2003; Qin et al., 2005; Ziolkowska et al., 2005).

In conclusion, we have demonstrated that α-synuclein mRNA level in the blood is significantly elevated during alcohol abstinence in the mouse, like in humans and monkeys, but seems unrelated to α-synuclein expression in the brain. The main effect of prolonged alcohol drinking on brain α-synuclein consisted of accumulation of α-synuclein protein in the amygdala after ethanol withdrawal. Since this phenomenon is common to alcohol and opiate abstinence, our observations suggest a role of amygdalar α-synuclein in common neuroadaptations produced by long-term drug and alcohol use.

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