Metabolic Brain Disease (2019) 34:341–352
https://doi.org/10.1007/s11011-018-0359-3

ORIGINAL ARTICLE

Adolescent social isolation affects parvalbumin expression in the medial prefrontal cortex in the MAM-E17 model of schizophrenia

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Received: 8 August 2018 / Accepted: 28 November 2018 / Published online: 5 December 2018

Abstract

Altered parvalbumin (PV) expression is observed in the prefrontal cortex of subjects with schizophrenia. Environmental context, particularly during adolescence, might regulate PV expression. In the present study, we investigated the effect of adolescent social isolation (SI) on PV expression in the medial prefrontal cortex in a neurodevelopmental model (MAM-E17) of schizophrenia. SI exposure occurred from postnatal day 30 to 40, followed by resocialization until late adolescence or early adulthood. PV mRNA and protein levels, as well as the number of PV cells, were analysed at these ages. Moreover, epigenetic regulation of PV expression by histone methylation was examined by measuring the total and PV gene-bound H3K4me3 levels. MAM only decreased levels of the PV mRNA and protein in adulthood. Decreases in total H3K4me3 levels and its level at the PV gene were also observed at this age. In contrast, in late adolescence, SI induced a decrease in the expression of the PV mRNA in the MAM group that was related to the reduction in total and PV gene-bound H3K4me3 levels. However, at this age, SI increased the levels of the PV protein in both the control and MAM groups. In adulthood, SI did not affect PV mRNA or H3K4me3 levels but decreased levels of the PV protein in both groups. Both MAM and SI failed to change the number of PV cells at any age. The results indicate that adolescent SI accelerated epigenetic impairments of PV expression in MAM-E17 rats; however, subsequent resocialization abolished this dysfunction, but failed to prevent alterations in PV protein.

Keywords Environment · Risk factor · Schizophrenia · Epigenetics · Neurodevelopment

Introduction

Schizophrenia is a devastating mental disorder, and its neurodevelopmental characteristics have prompted ongoing debate about the causes of schizophrenia. According to epidemiological studies, both genetics and environmental factors are involved in the aetiology of schizophrenia (van Os et al. 2008). Several factors acting prenatally (infection and malnutrition) and/or during childhood/adolescence (social stress, cannabis use) increase the risk of development of schizophrenia symptoms observed in late adolescence/early adulthood (Schmitt et al. 2014; Selemon and Zecevic 2015), and according to the "two-hit" model of schizophrenia, both prenatal and adolescent risk factors might subsequently contribute to the emergence of schizophrenia (Schmitt et al. 2014). Even transient exposure to risk factors, particularly in early life, might change the normal brain development towards maladaptive trajectories and cause schizophrenia-related abnormalities (Schmitt et al. 2014).

Adolescence is usually defined as a transitional period from childhood to adulthood in which the brain and neuronal circuits involved in the regulation of motivation, emotion and cognition develop and mature (Caballero et al. 2016). The prefrontal cortex is a brain structure that continues to develop until adulthood (Selemon and Zecevic 2015), and its maturation is associated with maturity of the γ-aminobutyric acid (GABA)ergic system, resulting in increased inhibitory control of prefrontal cortical output (Caballero and Tseng 2016). The main effectors of GABAergic transmission in the prefrontal cortex are GABA-expressing interneurons that differ in phenotype, morphology, and physiology (DeFelipe et al. 2013). Parvalbumin (PV) is one of the calcium-binding proteins present in GABA neurons (DeFelipe et al. 2013), and PV interneurons have a fast-spiking phenotype and form perisomatic inhibitory synapses onto excitatory pyramidal cells (Hu et al.

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2014). PV expression is increased during the maturation of the prefrontal cortex in various species (Caballero et al. 2014; Fung et al. 2010; Hoftman et al. 2015), and only PV-positive interneurons change their intrinsic and synaptic excitability during the development of the prefrontal cortex (Caballero and Tseng 2016). Therefore, alterations in the normal course of PV development might result in long-lasting functional abnormalities (Morishita et al. 2015). Thus, adolescence appears to be a developmental window that is sensitive to changes in PV maturation and alterations in the developmental trajectory of GABAergic circuits that might influence the excitatory-inhibitory balance in the prefrontal cortex.

PV interneuron activity is essential for the generation of cortical gamma oscillations that are crucial for cognitive function (Gonzalez-Burgos et al. 2015). Therefore, alterations in PV interneurons in the prefrontal cortical circuity in patients with schizophrenia might cause the cognitive dysfunction observed in these patients (Gonzalez-Burgos et al. 2015; Lewis 2012). Lower levels of the PV mRNA and protein (Fung et al. 2010; Glausier et al. 2014; Hashimoto et al. 2008; Hashimoto et al. 2003; Hoftman et al. 2015) have been detected in the prefrontal cortex of patients with schizophrenia compared with control subjects. Generally, the number of PV-positive interneurons is unchanged in the prefrontal cortex (Beasley et al. 2002; Cotter et al. 2002; Hashimoto et al. 2003; Tooney and Chahl 2004; Woo et al. 1997); however, some evidence suggests a reduction in the number of these neurons (Beasley and Reynolds 1997; Sakai et al. 2008). Moreover, decreased levels of the PV mRNA and protein have been observed in several animal models of schizophrenia based on NMDA receptor hypofunction, perinatal immune activation or genetic ablation (Powell et al. 2012).

PV expression depends on genetic and environmental factors (Jiang et al. 2013). Both prenatal risk factors (Volk and Lewis 2013) and environmental stressors experienced during adolescence, e.g., social isolation (SI) (Buwalda et al. 2011), affect PV levels in the adult brain, including the prefrontal cortex (Ueno et al. 2017). Moreover, SI exposure throughout the postweaning period reduces PV levels in the medial prefrontal cortex (mPFC) (Gilabert-Juan et al. 2013) or the hippocampus (Gaskin et al. 2016) in neurodevelopmental models of schizophrenia based on neonatal blockade of the NMDA receptor. However, researchers have not clearly determined whether an environmental risk factor that only acts in a specific window of adolescence might influence PV expression in the prefrontal cortex of subjects who are predisposed to schizophrenia development following exposure to prenatal risk factors. Furthermore, knowledge of the mechanism by which environmental factors regulate PV expression at different stages of postnatal development is still limited.

As shown in our previous study, transient SI exposure from postnatal days (P)30-P40 followed by resocialization altered schizophrenia-like behaviours in a neurodevelopmental model of schizophrenia based on prenatal (at embryonic day (E) 17) administration of the environmental risk factor methylazoxymethanol (MAM) (Kisby et al. 2013), but not in control rats. SI accelerated the appearance of some forms of cognitive dysfunction, i.e., a recognition memory deficit was already observed in late adolescence, but prevented impairments in social behaviour (Bator et al. 2018b). Moreover, depending on the age and treatment conditions, SI affects levels of the glutamic acid decarboxylase (GAD)65 and GAD67 proteins in the mPFC (Bator et al. 2018b). The chosen period of adolescence is also important for studies of PV interneuron maturation (Caballero et al. 2014). Thus, transient exposure to SI with subsequent resocialization might change the developmental trajectory of the brain that is impaired by prenatal risk factors, and the GABAergic system appears to be sensitive to the effects of SI during a defined period of adolescence. Therefore, in the present study, we used the MAM-E17 model of schizophrenia that represents the neurodevelopmental features of schizophrenia (Modinos et al. 2015), and we determined whether SI exposure from P30-P40 followed by resocialization (Bator et al. 2018b) might affect PV expression in the mPFC of normal and MAM-E17 rats. Previous findings related to PV levels in the mPFC are inconsistent, showing both decreases (Gastambide et al. 2012; Lodge et al. 2009) and no changes (Penschuck et al. 2006) in adult MAM-E17 rats, although changes in PV expression are usually observed in the hippocampus (Chen et al. 2014; Du and Grace 2016; Gill and Grace 2014; Lodge et al. 2009; Penschuck et al. 2006) and start in adolescence (Chen et al. 2014; Gill and Grace 2014). Thus, researchers have not clearly determined whether prenatal MAM administration affects the developmental trajectory of PV expression in the mPFC or only increases vulnerability to environmental factors experienced during adolescence. We investigated the effects of SI on PV mRNA and protein levels in either late adolescence or early adulthood that correspond to P46-P59 and P60-P75, respectively, in the rat to address this problem (Burke et al. 2017).

The emergence of schizophrenia might be associated with altered epigenetic regulation of gene expression, i.e., DNA methylation, histone modifications (Millan 2013). Epigenetic dysregulation has been suggested to play a crucial role in impairments in the development of the inhibitory GABAergic circuity (Morishita et al. 2015). PV interneuron maturation is also controlled by epigenetic mechanisms (Koh and Sng 2016; Nott et al. 2015), and data from a post-mortem study of subjects with schizophrenia (Fachim et al. 2018) and an animal model of schizophrenia (Fachim et al. 2016) reveal that epigenetic dysregulation at the PV gene promoter is related to DNA methylation. Our previous study showed development-related alterations in total levels of the histone H3 protein trimethylated at lysine 4 (H3K4me3) in the mPFC of MAM-E17 rats (Mackowiak et al. 2014), and these changes

are associated with a decrease in the expression of the Gad1 gene in adulthood (Bator et al. 2018a). Thus, we investigated whether H3K4me3 might be involved in the regulation of PV gene expression during mPFC maturation in the MAM-E17 model of schizophrenia to define a potential mechanism involved in the control of PV expression by environmental factors. Moreover, we determined whether SI might change the epigenetic control of PV expression at particular developmental stages.

Experimental procedures

Animals and treatments

Pregnant dams (Wistar Han rats) were obtained from an animal provider (Charles River, Germany) and housed individually in polycarbonate cages (26.5 × 18 × 42 cm). Rats were randomly assigned to different experimental groups, and at E17, pregnant females were intraperitoneally (i.p.) injected with 22 mg/kg of methylazoxymethanol acetate (MAM, MRIGlobal, USA) or vehicle (VEH, 0.9% NaCl, 1 ml/kg), as previously described (Bator et al. 2018a; Bator et al. 2018b; Mackowiak et al. 2014). Offspring were weaned 21 days after birth, and experiments were performed on males. The experimental groups consisted of animals chosen randomly from different litters to avoid litter effects and assigned to experimental groups as previously described (Bator et al. 2018b). In particular, between P21-P30, the animals were housed in groups of 4 in small cages (26.5 × 18 × 42 cm). At P30, the animals were randomly assigned to standard housing (SH) or SI rearing and the following 4 groups were formed: prenatal VEH-treated reared in SH (VEH-SH), prenatal MAM-treated reared in SH (MAM-SH), prenatal VEH-treated reared in SI (VEH-SI) and prenatal MAM-treated reared in SI (MAM-SI). Under SH conditions, rats were housed in large polycarbonate boxes (38 × 20 × 60 cm) in groups of 4. For SI conditions, rats were housed individually in small cages (26.5 × 18 × 42 cm) for 10 days during adolescence (P30-P40). All rats were housed in the same room, sharing the same controlled light, temperature and humidity. Rats reared in isolation could hear and smell other animals, but they were unable to have physical contact with them. At P41, SI rats were moved to large polycarbonate boxes and housed in groups of 4 with the same animals as before SI. From P41 to adulthood, all rats were reared under SH conditions. All rats were housed in groups of 4 with ad libitum access to food and water and an artificial 12/12-h light/dark cycle (lights on at 7 a.m.). The biochemical studies (quantitative reverse transcriptase real time polymerase chain reaction (qRT-PCR), chromatin immunoprecipitation (ChIP), Western blot analysis and immunohistochemistry) were performed during late adolescence (P46-P47) and early adulthood (P70-P71) (Burke et al. 2017). The experimental groups consisted of 6–9 animals. A separate set of animals was used for each of these experiments. The study was conducted in strict accordance with the recommendations of the European Council Guide for the Care and Use of Laboratory Animals (86/609/EEC), as adopted and promulgated by the European Communities Council Directive (2010/63/EU). The experimental protocols were approved by the Committee for Laboratory Animal Welfare and the Ethics of the Institute of Pharmacology, Polish Academy of Sciences in Kraków. All efforts were made to minimize animal suffering and to reduce the number of animals used.

RNA extraction and qRT-PCR

Total RNA was extracted from the whole mPFC and qRT-PCR was performed as described previously (Bator et al. 2018a; Mackowiak et al. 2014). Cytoplasmic RNA was extracted using Tri-Reagent (Sigma, Poland) from combined membrane and cytosolic fractions of the mPFC (prepared as described below in the Western blot section). qRT-PCR was performed using the QuantStudio 12 K Flex Real-Time PCR System (Applied Biosystems, Poland). The gene-specific primers and probe for parvalbumin (assay Rn00574541_m1) from the TaqMan Gene Expression Assays (Applied Biosystems) were used, and 1 μl of each sample was used for cDNA amplification. Amplification was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) under the following conditions: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The expression of the glyceraldehyde-3-phosphate dehydrogenase (Gapdh) transcript (assay Rn99999916_s1) was quantified to control for variations in cDNA levels. All qRT-PCR experiments were performed in duplicate and included no-template controls. The cycle threshold values were calculated automatically. Relative quantification was performed using the comparative threshold (CT) method according to $2^{-\Delta CT}$, where ΔCT = (CT, target gene – CT, reference gene).

ChIP

Chromatin was isolated from the mPFC using a standard protocol (iDeal ChIP-qPCR, Diagenode, USA) and sheared to 100–1000-bp fragments using a Sonic Dismembrator Vibra-Cell VCX-130 (Sonics). Each sample was sonicated five times on ice for 10 s each at 20% maximum power. Chromatin was immunoprecipitated using an antibody against histone subunit H3K4me3 (1:1000, Abcam, UK) and the iDeal ChIP-qPCR kit according to the manufacturer's instructions.

Quantitative real-time PCR was performed with primers specific for the PV promoter: the forward primer was 5' ACTCCCCCATCCTTTATGGC 3′ and the reverse primer was 5' GCTGTCCGGAAAAACTTGCC 3′. The PV

promoter was investigated since H3K4me3 marks active promoters but not enhancers (Heintzman et al. 2007). Input and immunoprecipitated DNA amplification reactions were run in duplicate in the presence of SYBR Green (Real Time PCR Master Mix, Bio-Rad, Poland). All primer sets had comparable amplification efficiency. CT values were normalized to input DNA. Data are presented as percentages of the control group (VEH-SH).

Western blotting

The immunoreactivity of the PV protein was analysed in the mPFC homogenate prepared using previously described methods (Bator et al. 2018b). Cytosolic, membrane and nuclear extracts of the mPFC were also obtained using the ProteoExtract Subcellular Proteome Extraction Kit according to the manufacturer's instructions (Calbiochem/Merck, Poland) (Bator et al. 2018a; Mackowiak et al. 2014). The immunoreactivity of the H3K4me3 protein was measured in the nuclear extracts of mPFC, while membrane and cytosolic fractions were used for cytoplasmic RNA extraction (see above). Protein extracts (5 μg) were separated on 12.5% SDS-PAGE gels and transferred to nitrocellulose membranes (Bio-Rad). Blots were incubated overnight at 4 °C with the following primary antibodies: rabbit anti-parvalbumin (1:1000, Abcam), rabbit anti-H3K4me3 (1:1000, Cell Signalling Technology, USA), rabbit anti-GAPDH (1:5000, Cell Signalling Technology), and rabbit anti-histone H3 (1:25,000, Upstate/Millipore, Poland). Immune complexes were detected using a peroxidase-conjugated secondary antibody against rabbit IgG (1:1000, Roche, Poland). Bands were visualized with enhanced chemiluminescence (ECL; Lumi-Light^{PLUS} Western Blotting Kit, Roche). Chemiluminescence was recorded using a luminescent image analyser (Fujifilm LAS-1000, Fujifilm Corporation, Japan). The relative levels of immunoreactivity were quantified using ImagePro Plus software (Media Cybernetics, USA). The level of H3K4me3 was normalized to the total level of histone H3, and the level of PV was standardized to the GAPDH protein.

Immunohistochemical staining

Tissues were prepared as described previously (Bator et al. 2018a; Mackowiak et al. 2014). Free-floating sections from the mPFC were processed for PV staining. Sections were incubated with a blocking buffer [5% normal goat serum (Vector Laboratories, USA) and 0.2% Triton X-100 in 0.01 M PBS] for 1 h and then incubated (48 h at 4 °C) with the primary anti-parvalbumin rabbit antibody (1:1000, Abcam) diluted in 3% normal goat serum and 0.2% Triton X-100 in 0.01 M PBS. The reaction was visualized with a biotinylated anti-rabbit IgG (Vector Labs) and avidin-biotin-horseradish peroxidase complex (Vectastain Elite ABC Kit, Vector Labs, at the concentration recommended by the manufacturer) with a 3,3′-diaminobenzidine tetrahydrochloride solution (0.02% DAB solution and 0.025% H_2O_2 solution). Images were captured on an Aperio Scan Scope system and the final photomicrographs composed using Adobe Photoshop. The number of PV-immunopositive cells in the mPFC was estimated using unbiased stereological methods, as previously described (Mackowiak et al. 2014).

Statistics

The results are presented as group means ± standard errors of the means (SEM). Statistical evaluations were performed using Statistica software and consisted of two-way analyses of variance (ANOVA), as indicated in the figures, followed by the Newman-Keuls post hoc test. Differences were considered statistically significant at $p < 0.05$.

Results

Late adolescence

PV mRNA expression

Total PV mRNA We examined the regulation of PV expression throughout development to test whether the MAM treatment strengthened the effect of SI on PV interneurons. Although the MAM treatment did not affect the expression of the PV mRNA [treatment: $F(1, 24) = 1.73$, $p = 0.20$], SI exerted an effect [housing: $F(1, 24) = 5.04$, $p < 0.04$]. Moreover, a statistically significant interaction between MAM administration and SI rearing was observed [treatment x housing: $F(1, 24) = 9.74$, $p < 0.005$]. A significant decrease in the level of the PV mRNA was observed in the MAM-SI group ($p < 0.02$ compared with the VEH-SH and VEH-SI groups and $p < 0.005$ compared with the MAM-SH group, Fig. 1a). Our findings suggest a stronger effect of SI on the PV mRNA level in the MAM group than in the VEH group at this age.

Cytoplasmic PV mRNA We analysed the levels of the PV mRNA in the cytoplasm to investigate whether the MAM treatment enhanced the SI-mediated regulation of PV expression in cell compartments. The statistical analysis revealed an effect of MAM [treatment: $F(1, 28) = 11.95$, $p < 0.002$] but not SI on the cytoplasmic mRNA level [housing: $F(1, 28) = 0.44$, $p = 0.51$]. No interaction between the analysed factors was observed [treatment x housing: $F(1, 28) = 2.52$, $p = 0.12$]. The lowest level of the PV mRNA was detected in the MAM-SI group (Fig. 1b); however, a statistically significant decrease in the MAM-SI group was only observed when this group was compared to the VEH-SI group ($p < 0.007$), but not to the VEH-SH ($p = 0.14$) or MAM-SH ($p = 0.52$) groups. Thus, although differences between groups did not reach

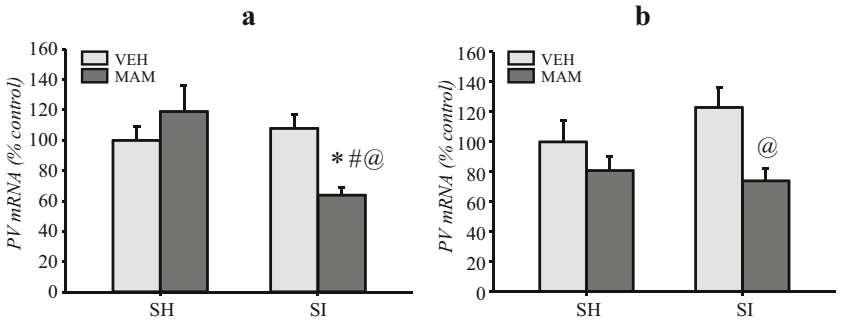

Fig. 1 The effects of adolescent social isolation (SI) on PV mRNA expression in the mPFC in late adolescence. Rats were exposed to SI (P30-P40), followed by resocialization in standard housing (SH). **a**. Total and **b.** Cytoplasmic levels of the PV mRNA. The data are presented as percentages of the levels in the VEH-SH group. Each data point represents a mean ± SEM; $n = 7$ (**a**), and $n = 8$ (**b**) animals per group; * $p < 0.05$ compared with the VEH-SH group, # $p < 0.05$ compared with the MAM-SH group, @ $p < 0.05$ compared with the VEH-SI group (two-way ANOVA followed by the Newman-Keuls test)

statistical significance, the pattern of changes in cytoplasmic mRNA levels was similar to changes observed in total mRNA levels, and the MAM group appeared to be more vulnerable to SI exposure than the VEH group.

Histone methylation

H3K4me3 bound to the PV DNA To address epigenetic control of PV expression, the regulation of the PV gene promoter by histone modification was analysed using the H3K4me3 antibody to immunoprecipitate the PV DNA. We found that MAM administration affected the amount of PV DNA bound to H3K4me3 [treatment: $F(1, 24) = 9.38$, $p < 0.006$]; however, no effect of housing conditions was observed [housing: $F(1, 24) = 1.92$, $p = 0.18$]. Additionally, no interaction between the MAM treatment and exposure to SI was observed [treatment x housing: $F(1, 24) = 0.41$, $p = 0.84$]. However, a significant decrease in the amount of PV DNA immunoprecipitated by H3K4me3 was only detected in the MAM-SI group ($p < 0.03$ compared with the VEH-SH group, Fig. 2a), indicating that SI exerted the strongest effect on the MAM group, similar to the results obtained for PV mRNA levels.

Total H3K4me3 protein We measured the levels of the H3K4me3 protein to investigate whether the observed alterations in the PV transcript were related to total histone methylation. We noticed that MAM administration did not alter the total H3K4me3 level [treatment: $F(1, 28) = 0.88$, $p = 0.36$]; however, an effect of SI was observed [housing: $F(1, 28) = 7.10$, $p < 0.02$]. A trend towards an interaction between MAM administration and housing conditions was observed [treatment x housing: $F(1, 28) = 3.96$, $p = 0.056$]. A statistically significant decrease in the H3K4me3 level was only observed in the MAM-SI group ($p < 0.05$ compared with the VEH-SH group and $p < 0.02$ compared with the MAM-SH group, Fig. 2b). Based on our findings, SI rearing affected both the levels of total and H3K4me3 specifically bound to PV DNA, but only in the MAM group.

PV protein

Total level of the PV protein We assessed PV levels to determine whether the effect of MAM on SI-induced alterations in the PV transcript would be followed by changes in protein levels. The MAM treatment did not affect PV protein levels [treatment: $F(1, 20) = 2.39$, $p = 0.14$]; however, an effect of SI rearing was observed [housing: $F(1, 20) = 24.03$, $p < 0.00009$]. No interaction between MAM administration and housing conditions was observed [treatment x housing: $F(1, 20) = 1.59$, $p = 0.23$]. Exposure to SI induced a robust increase in the levels of the PV protein in both the VEH- ($p < 0.001$ compared with the VEH-SH group) and MAM-treated groups ($p < 0.002$ compared with the VEH-SH group and $p < 0.05$ compared with the MAM-SH group, Fig. 3a). A trend towards increased levels of the PV protein was also noticed in the MAM-SH group ($p = 0.062$ compared with the VEH-SH group, Fig. 3a). Thus, SI was a main factor affecting the PV protein level, and the MAM treatment did not change the effect of SI on the PV protein.

The number of PV cells We also determined the number of PV-expressing neurons to exclude the possibility that MAM or SI affected some cell populations. Neither the MAM treatment [treatment: $F(1, 28) = 3.83$, $p = 0.06$] nor SI [housing: $F(1, 28) = 0.0022$, $p = 0.96$] altered the number of PV-immunopositive cells. Additionally, an interaction between these factors was not observed [treatment x housing: $F(1, 28) = 0.49$, $p = 0.49$, Fig. 3b]. Based on our findings, changes in the total level of the PV protein were not related to the number of cells expressing PV.

Early adulthood

PV mRNA expression

Total PV mRNA We examined PV expression in adulthood to determine whether the effects of MAM or SI on PV

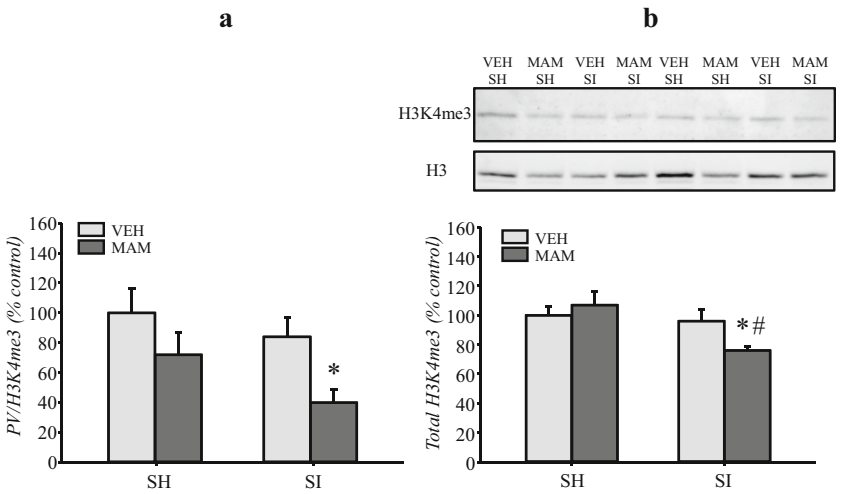

Fig. 2 Effects of adolescent social isolation (SI) on histone methylation in the mPFC in late adolescence. Rats were exposed to SI (P30-P40), followed by resocialization in standard housing (SH). **a**. The PV DNA was immunoprecipitated with the H3K4me3 antibody. **b**. Total levels of the H3K4me3 protein. Photomicrographs show representative immunoblots for H3K4me3 and total histone H3. The data are presented as percentages of the levels in the VEH-SH group. Each data point represents a mean ± SEM; n = 7 animals per group in **a** and n = 8 animals per group in **b**; * $p < 0.05$ compared with the VEH-SH group and # $p < 0.05$ compared with the MAM-SH group (two-way ANOVA followed by the Newman-Keuls test)

interneurons were maintained in the mature brain. MAM administration did not affect the PV mRNA level [treatment: $F(1, 28) = 0.38$, $p = 0.54$]. However, an effect of housing conditions was observed [housing: $F(1, 28) = 4.83$, $p < 0.04$], and an interaction between the factors was found [treatment x housing: $F(1, 28) = 11.58$, $p < 0.002$]. A statistically significant decrease in PV mRNA expression was only detected in the MAM-SH group ($p < 0.03$ compared with the VEH-SH group, Fig. 4a]. Therefore, SI rearing abolished the reduction in total PV mRNA expression in the adult MAM group.

Cytoplasmic PV mRNA We analysed the cytoplasmic levels of the PV mRNA to investigate whether the MAM treatment continued to support the effects of SI on PV expression in cell

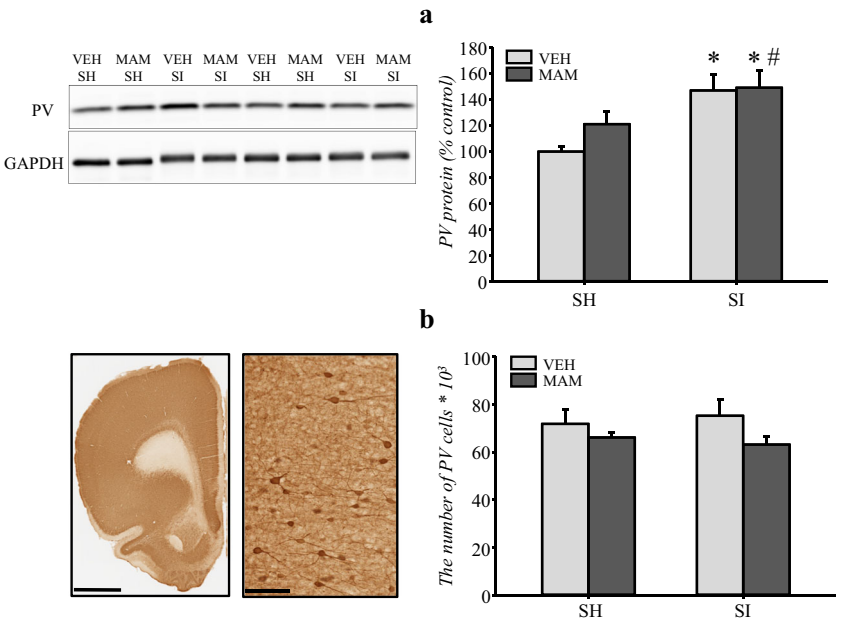

Fig. 3 The effects of adolescent social isolation (SI) on levels of the PV protein in the mPFC in late adolescence. Rats were exposed to SI (P30-P40), followed by resocialization in standard housing (SH). **a**. Levels of the PV protein; the data are presented as percentages of the levels in the VEH-SH group. **b**. The number of PV-immunopositive cells. Photomicrographs (**a**) show representative immunoblots probed with PV and GAPDH antibodies. Representative photomicrographs (**b**) showing PV immunostaining. The scale bars represent 1 and 0.1 mm. Each data point represents a mean ± SEM; $n = 6$ animals per group in **a** and n = 8 animals per group in **b**; * $p < 0.05$ compared with the VEH-SH group and # $p < 0.05$ compared with the MAM-SH group (two-way ANOVA followed by the Newman-Keuls test)

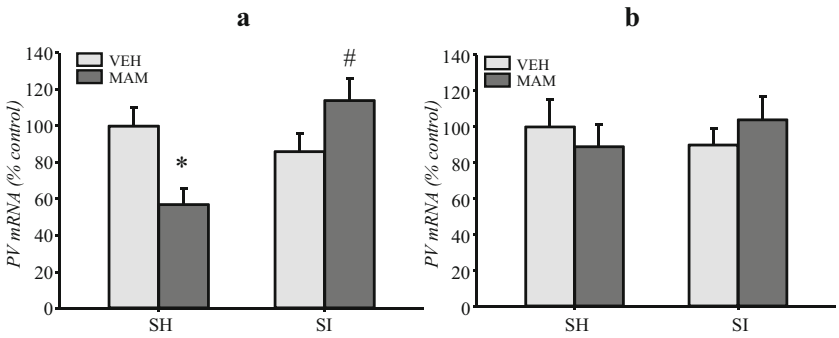

Fig. 4 The effects of adolescent social isolation (SI) on PV mRNA expression in the mPFC in early adulthood. Rats were exposed to SI (P30-P40), followed by resocialization in standard housing (SH). **a.** Total and **b**. Cytoplasmic levels of the PV mRNA. The data are presented as percentages of the levels in the VEH-SH group. Each data point represents a mean ± SEM; n = 8 animals per group; * $p < 0.05$ compared with the VEH-SH group and # $p < 0.05$ compared with the MAM-SH group (two-way ANOVA followed by the Newman-Keuls test)

compartments. Neither MAM administration [treatment: $F(1, 28) = 0.01$, $p = 0.91$] nor SI rearing [housing: $F(1, 28) = 0.002$, $p = 0.97$] affected the mRNA level. No interaction between prenatal and adolescent factors was observed [treatment x housing: $F(1, 28) = 0.63$, $p = 0.43$, Fig. 4b]. In contrast to the total mRNA level, statistically significant differences in cytoplasmic mRNA levels were not observed between groups. Our findings showed no effects of MAM and SI on the cytoplasmic mRNA level, although the lowest level of the PV mRNA in the cytoplasm was observed in the MAM-SH and VEH-SI groups similar to the pattern of changes in total mRNA levels.

Histone methylation

H3K4me3 bound to the PV DNA We examined the regulation of the PV gene promoter by histone methylation to determine whether MAM administration influenced the effect of SI on the epigenetic control of PV expression. Neither MAM [treatment: $F(1, 28) = 1.41$, $p = 0.24$] nor SI [housing: $F(1, 28) = 0.12$, $p = 0.73$] affected the amount of PV DNA bound to H3K4me3. However, an interaction between these two factors was detected [treatment x housing: $F(1, 28) = 7.22$, $p < 0.02$]. A statistically significant decrease in the amount of PV DNA immunoprecipitated by H3K4me3 was only detected in the MAM-SH group ($p < 0.04$ compared with the VEH-SH group, Fig. 5a). Based on these findings, SI exposure abolishes the MAM-induced changes in adulthood.

Total H3K4me3 protein We analysed the levels of the H3K4me3 protein to determine whether the MAM treatment potentiated the effect of SI on histone methylation. Neither MAM [treatment: $F(1, 28) = 3.45$, $p = 0.074$] nor SI [housing: $F(1, 28) = 0.19$, $p = 0.67$] affected H3K4me3 levels. However, an interaction between MAM administration and exposure to SI was observed [treatment x housing: $F(1, 28) = 6.94$, $p < 0.02$]. A statistically significant decrease in the total H3K4me3 level was only detected in the MAM-SH group ($p < 0.02$ compared with the VEH-SH groups, Fig. 5b) similar to alterations in H3K4me3 levels bound to the PV DNA. Thus, SI rearing abolishes the reduction in histone methylation in MAM-treated animals.

PV protein

Total level of the PV protein We examined the PV protein to investigate whether the MAM treatment enhanced the effect of SI on PV interneurons. Both MAM administration [treatment: $F(1, 32) = 5.16$, $p < 0.03$] and SI rearing [housing: $F(1, 32) = 7.17$, $p < 0.02$] affected PV protein levels. However, an interaction between MAM and SI was not observed [treatment x housing: $F(1, 32) = 0.71$, $p = 0.40$]. A significant decrease in the PV protein level was detected in the MAM-SH ($p < 0.03$ compared with the VEH-SH group), VEH-SI ($p < 0.05$ compared with the VEH-SH group) and MAM-SI groups ($p < 0.006$ compared with the VEH-SH group, Fig. 6a) suggesting that levels of the PV protein were altered in all experimental groups.

The number of PV cells We also determined the number of PV-expressing cells to extend the study of whether the MAM treatment modulated the effects of SI on PV interneurons. We did not observe any effect of MAM administration on the number of PV-immunopositive cells [treatment: $F(1, 28) = 2.35$, $p = 0.14$]. Housing conditions also did not influence the number of PV-immunopositive cells [housing: $F(1, 28) = 0.84$, $p = 0.37$]. An interaction between these factors was not observed [treatment x housing: $F(1, 28) = 0.008$, $p = 0.93$, Fig. 6b], indicating a lack of correlation between changes in total PV protein levels and the number of PV cells.

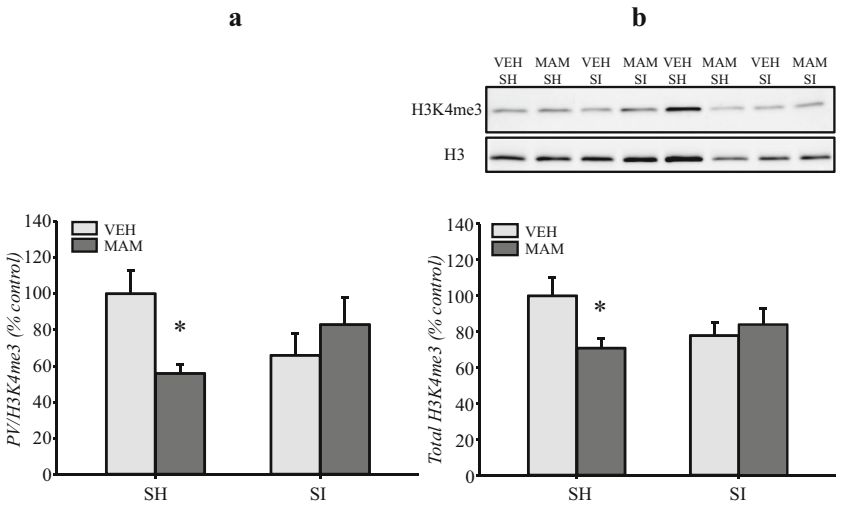

Fig. 5 Effects of adolescent social isolation (SI) on histone methylation in the mPFC in early adulthood. Rats were exposed to SI (P30-P40), followed by resocialization in standard housing (SH). **a**. The PV DNA was immunoprecipitated with the H3K4me3 antibody. **b**. Total levels of the H3K4me3 protein. Photomicrographs show representative immunoblots of H3K4me3 and total histone H3. The data are presented as percentages of the levels in the VEH-SH group. Each data point represents a mean ± SEM; n = 8 animals per group; * $p < 0.05$ compared with the VEH-SH group (two-way ANOVA followed by the Newman-Keuls test)

Discussion

Based on the results of the present study, transient SI rearing during a defined period of adolescence and subsequent resocialization changed the dynamic pattern of PV expression in the mPFC of both prenatal VEH- and MAM-treated rats.

MAM-induced alterations in PV expression in the mPFC were not observed until early adulthood. Prenatal MAM administration did not alter levels of the PV mRNA and protein or the number of PV-positive cell in late adolescence. In contrast, in adulthood, a decrease in total, but not in cytoplasmic, PV mRNA levels was detected in the mPFC of MAM-E17

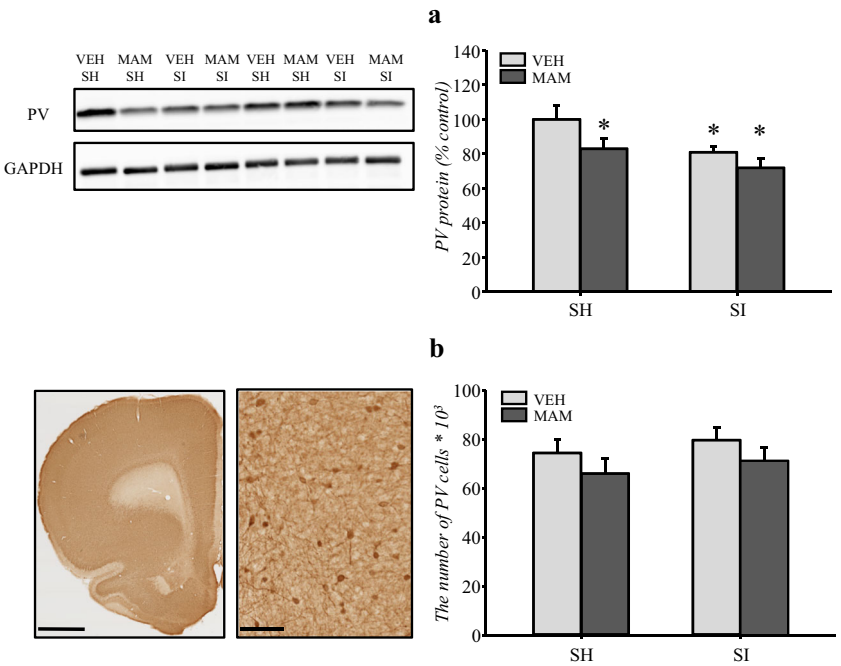

Fig. 6 Effects of adolescent social isolation (SI) on levels of the PV protein in the mPFC in early adulthood. Rats were exposed to SI (P30-P40), followed by resocialization in standard housing (SH). **a**. Levels of the PV protein; the data are presented as percentages of the levels in the VEH-SH group. **b**. The number of PV-immunopositive cells. Photomicrographs (**a**) show representative immunoblots probed with the PV and GAPDH antibodies. Representative photomicrographs (**b**) showing PV immunostaining. The scale bars represent 1 and 0.1 mm. Each data point represents a mean ± SEM; $n = 9$ animals per group in a and n = 8 animals per group in b; * $p < 0.05$ compared with the VEH-SH group (two-way ANOVA followed by the Newman-Keuls test)

rats. The observed discrepancies in total and cytoplasmic mRNA levels might be due to the use of different tissue preparation methods for mRNA extraction (whole tissue vs. mPFC fractions) or depended on mRNA export from the nucleus to the cytoplasmic compartment (Hieronymus and Silver 2003). Moreover, a MAM-induced decrease in the level of the PV protein was observed; however, it was not related to a change in the number of PV-positive cells, which was comparable to the control group. The available literature has reported either a reduction in the density of PV neurons (Gastambide et al. 2012; Lodge et al. 2009) or no changes in PV neuron numbers (Penschuck et al. 2006) in the adult mPFC following treatment with MAM. Moreover, MAM-induced impairments in PV expression in the mPFC exhibited different dynamics than in the hippocampus, where a decrease in PV immunoreactivity was already observed in adolescence (Chen et al. 2014; Gill and Grace 2014). Our findings from the MAM-E17 model reflect a feature of PV dysfunction in patients with schizophrenia, who show decreased levels of the PV mRNA and protein in the prefrontal cortex (Fung et al. 2010; Glausier et al. 2014; Hashimoto et al. 2008; Hashimoto et al. 2003; Hoftman et al. 2015), but the numbers of PV interneurons are usually unchanged (Beasley et al. 2002; Cotter et al. 2002; Hashimoto et al. 2003; Tooney and Chahl 2004; Woo et al. 1997). Moreover, a decrease in levels of the PV protein in the prefrontal cortex of subjects with schizophrenia was observed mainly in the axon terminals of PV-positive cells (Glausier et al. 2014), which might reduce inhibitory inputs onto pyramidal cells and affect gamma oscillation power (Gonzalez-Burgos et al. 2015). Diminished oscillatory activity related to lower PV levels in the mPFC in the MAM-E17 model has been reported previously (Lodge et al. 2009). Thus, changes in PV expression might have induced the cortical dysfunction and cognitive deficits observed in that model (Modinos et al. 2015). In addition, the direction of changes in PV expression was similar to the alterations in GAD67 expression in the mPFC of MAM-E17 rats described in our previous studies (Bator et al. 2018a; Mackowiak et al. 2014). The comparable pattern of the aforementioned impairments in the cortical GABAergic system has also been observed in patients with schizophrenia (Hashimoto et al. 2003) and might suggest a dysfunction of inhibitory networks in the adult mPFC induced by exposure to prenatal risk factors.

Exposure to adolescent SI altered PV expression in the mPFC in a manner dependent on the prenatal treatment and age; however, a correlation between the direction of changes in the protein level and total PV mRNA expression or the mRNA level in cytoplasmic fraction that is directly involved in translation, was not observed (Solnestam et al. 2012). A decrease in total and cytoplasmic PV mRNA levels induced by SI was observed only in the MAM-E17 rats in late adolescence, but at this age, SI increased levels of the PV protein in both VEH- and MAM-treated rats without altering the number of PV cells. In contrast, in adulthood, SI rearing did not affect total and cytoplasmic mRNA levels in any group. However, it induced a decrease in levels of the PV protein in the VEH group and did not affect the MAM-induced PV reduction. SI also did not alter the number of PV-expressing cells in any groups. The last observation is consistent with findings showing that postweaning SI does not alter the number of PV neurons in the adult mPFC (Kaalund et al. 2013; Ueno et al. 2017), although a decrease has been observed in some mPFC regions (infralimbic cortex)(Gilabert-Juan et al. 2013). However, changes in total or even cytoplasmic mRNA levels did not reflect the decrease in PV immunoreactivity in SI groups, an effect that has also been noted by other researchers (Gilabert-Juan et al. 2013). Therefore, other mRNA-independent mechanisms might be involved in the SI-mediated regulation of the PV protein levels, e.g., post-translational modifications of proteins (stability and degradation). In particular, Schiavone et al. observed changes in the levels of the PV protein induced by SI in response to oxidative stress (Schiavone et al. 2009). The patterns of disruption in PV protein levels were similar to those reported in our previous study showing changes in GAD65 levels in the mPFC (Bator et al. 2018b). Thus, SI might mainly alter levels of the PV protein in axon terminals, where GAD65 is predominantly located (Kaufman et al. 1991). Moreover, a transient increase in the levels of the PV protein in late adolescence might compensate for the effect of isolation rearing, but subsequent re-socialization did not abolish the decreased levels of the PV protein, whereas it affected the decrease in total PV mRNA levels in early adulthood.

PV expression is regulated by epigenetic mechanisms, e.g., histone deacetylases (Koh and Sng 2016; Nott et al. 2015). Moreover, hypermethylation of the PV gene has been observed in post-mortem tissues from subjects with schizophrenia (Fachim et al. 2018), as well as in animal model of schizophrenia based on blockade of the NMDA receptor (Fachim et al. 2016). The present study has added new information related to the epigenetic regulation of PV expression in schizophrenia and showed that PV expression in the mPFC of MAM-E17 animals is regulated by the histone modification H3K4me3 that marks active gene promoters and increases transcriptional activity (Heintzman et al. 2007). A decrease in the level of the H3K4me3 protein bound to the PV gene promoter was correlated with a lower level of total H3K4me3 in the mPFC of MAM-E17 rats and evoked a reduction in total PV mRNA expression; however, this effect was not observed until adulthood. Our previous study only showed a decrease in total levels of the H3K4me3 protein in the mPFC of MAM-treated adult rats (Mackowiak et al. 2014) that correlated with low levels of H3K4me3 bound to the Gad1 gene and reduced expression of the GAD67 mRNA (Bator et al. 2018a). Thus, this specific histone modification might mediate a decrease in the expression of both the GAD67 and PV genes in the mPFC of the

MAM-E17 model. Although the involvement of H3K4me3 in the mechanism regulating the expression of the GAD1 gene in the prefrontal cortex of subjects with schizophrenia has been suggested (Huang et al. 2007), evidence showing the same epigenetic regulation of the PV gene is currently lacking. In addition, exposure to the environmental risk factor SI during a specific period of adolescence accelerated the decrease in the total levels of H3K4me3 and reduced the amount of H3K4me3 bound to the PV gene, resulting in lower levels of the PV mRNA in MAM-E17 rats in late adolescence, which is earlier than the reduction observed in the MAM-SH group. However, subsequent resocialization appeared to normalize those deficits, and no alterations in H3K4me3-mediated regulation of PV expression were observed in the mPFC of MAM-treated adult rats exposed to SI, in contrast to the socially housed MAM group. Environmental context appears to be crucial for the epigenetic regulation of gene expression in the mPFC, as our previous study revealed that environmental enrichment during adolescence prevented the decreases in H3K4me3 levels bound to the Gad1 promoter and levels of the GAD67 mRNA (Bator et al. 2018a). Thus, environmental factors acting during adolescence might affect the regulation of H3K4 methylation and influence epigenetic dysregulation and the subsequent expression of genes related to schizophrenia development, i.e., Gad1 and PV.

Based on the results from the present study, environmental risk factors might alter the dynamics of PV maturation, depending on when exposure occurred (prenatal and/or adolescent periods). Prenatal exposure to the environmental risk factor MAM affected PV mRNA and protein levels only in the adult mPFC, and these changes might be associated with the dysfunction of epigenetic regulatory mechanisms. Adolescent exposure to the environmental risk factor SI induced a transient impairment in epigenetic regulation of PV mRNA expression only in the MAM-E17 mPFC in late adolescence; however, subsequent resocialization appeared to exert beneficial effects on the epigenetic dysregulation of PV expression in the adult mPFC. At the same age, SI decreased levels of the PV protein in both the VEH and MAM groups, which might have been caused by mechanisms other than impairments in epigenetic mechanisms and alterations in mRNA expression. Thus, the particular period of SI exposure seems to be critical for PV development in both the VEH and MAM groups, but exposure to SI clearly affected the epigenetic regulation of PV expression in MAM-treated animals. Our observations might be relevant in the context of human development and suggest that even transient impairments in social contact during a specific stage of adolescence (P30-P40 in rats corresponds to 14–16 years of age in humans) (Burke et al. 2017) might affect brain development, particularly in groups at high risk of developing schizophrenia. Interestingly, while SI affected PV expression in the developing brain, the effects of MAM were delayed until the brain had completely matured. This latency would allow early environmental events, i.e., SI, to initiate some compensatory mechanism, as observed in our behavioural studies of the MAM-E17 model of schizophrenia, where SI prevented the development of impairments in social ability (Bator et al. 2018b). Thus, we hypothesized that although SI during postnatal development usually leads to schizophrenia-related deficits (Schmitt et al. 2014), adolescent SI at a specific window of development might also exert a beneficial effect and induce resilience to the emergence of some behavioural and neurochemical abnormalities associated with schizophrenia.

Acknowledgements The study was supported by grant 2014/13/B/NZ4/00218 funded by the National Science Centre, Poland and statutory activity of Institute of Pharmacology, Polish Academy of Sciences.

Funding The study was supported by grant 2014/13/B/NZ4/00218 from the National Science Center, Poland and statutory activity of Institute of Pharmacology, Polish Academy of Sciences.

Compliance with ethical standards

Ethical approval All applicable international, national and institutional guidelines for the care and use of animals were followed. All procedures performed in the studies involving animals were conducted in accordance with the ethical standards of the institution or practice at which the studies were conducted. The study was performed in strict accordance with the recommendations of the European Council Guide for the Care and Use of Laboratory Animals (86/609/EEC), as adopted and promulgated by the European Communities Council Directive (2010/63/EU). The protocols were approved by the Committee for Laboratory Animal Welfare and the Ethics of the Institute of Pharmacology, Polish Academy of Sciences in Kraków. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Conflict of interest The authors have no conflicts of interest to declare.

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