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Potential role of dopamine transporter in behavioral flexibility

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Behavioral flexibility is subserved by the prefrontal cortex and the basal ganglia. Orbitofrontal cortex (OFC) and dorsomedial striatum (DMS) form a functional frontocorticostriatal circuit crucial for the mediation of flexibility during reversal learning via dopamine (DA) neurotransmission. The regulatory control in maintaining DA homeostasis and function is provided by the dopamine transporter (DAT), which therefore likely plays a significant role in controlling the influence of DA on cognitive processes. Here we used a gene knockout mouse model to investigate the role of DAT in the performance on the Attentional Set-Shifting Task (ASST) stages dependent upon the OFC and the DMS. Additionally, behavior of mice after repeated administration of selective DAT inhibitor, GBR 12909, was examined. The animals were treated with the inhibitor to elicit a compensatory DAT up-regulation following withdrawal. Learning was slower and the number of errors during reversal learning and intra-dimensional shift stages was higher in DAT+/− mutant mice than in WT mice. GBR 12909-treated mice had deficits in reversal stages of the ASST. Neuronal activation in the OFC and DMS during the ASST was examined with early growth response proteins 1 and 2 (egr-1, egr-2) immunohistochemistry. Density of egr-2 labeled cells in the OFC was lower in mutant mice than in wild-types during reversal learning and the expression of the egr-1 was lower in mutant mice in the OFC and DMS during reversal and intra-dimensional shift stages. Mice with decreased DAT levels displayed behavioral difficulties that were accompanied by a lower task-induced activation of neurons in brain regions involved in the reversal learning. Altogether, these data indicate the role of the DAT in the behavioral flexibility.

Key words: reversal, DAT+/−, early growth response genes, orbitofrontal cortex, dorsomedial striatum

INTRODUCTION

It is well known that learning and behavioral flexibility are subserved by the prefrontal cortex (PFC). Distinct regions of PFC mediate different forms of flexibility. The results of several studies point to a crucial role of the medial prefrontal cortex (mPFC) in shifting between strategies or attentional sets, while the orbitofrontal cortex (OFC) has been implicated in reversal learning (Dias et al. 1996, Ragozzino et al. 1999, Birrell and Brown 2000, McAlonan and Brown 2003, Boulougouris et al. 2007). However, flexible behavior is not solely supported by the PFC, but rather by a complex neural network, which includes the striatum (Kolb 1977, Ragozzino 2007, Clarke et al. 2008). Inactivation or lesion of the dorsomedial striatum (DMS) in rats impairs reversal learning (Ragozzino et al. 2002a, 2002b, Clarke et al. 2008, Castañe et al. 2010, Tait et al. 2017).

PFC is very sensitive to the imbalance in its neurotransmitters and even small changes in catecholamine modulation of PFC cells can have profound effects on its ability to control executive functions. The dopamine (DA) system is implicated in executive control of cognitive processes (Robbins and Roberts 2007, Ranganath and Jacob 2015), in flexible behavior (Haluk and Floresco 2009) and in synaptic plasticity in brain regions supporting reversal learning performance, i.e. PFC and striatum (Reynolds and Wickens 2002, Cagniard et al. 2006a, 2006b, Calabresi et al. 2007). The most important regulatory control of temporal and spatial activity of released DA is provided by the dopamine transporter (DAT) (Cook et al. 1995, Lohr et al. 2017). Alternations in dopaminergic modulation in frontocorticostriatal circuits are associated with schizophrenia (e.g. Ratajczak et al. 2015) and attention deficit hyperactivity disorder (ADHD) symptoms (Biederman and Faraone 2005, Arnsten 2006) and changes in DAT level in the striatum have been found in patients with ADHD (Krause 2008). The most effective treatments for ADHD are medications that enhance dopamine transmission by inhibiting DAT action (e.g. Mazei-Robinson and Blakely 2006). In spontaneously hypertensive rats (SHR), an animal model

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of ADHD, altered dopamine activity in the OFC leads to the deficits in reversal learning (Cheng et al. 2013). Based on that result it was postulated that orbitofrontal dopamine system might be an essential part in the neural pathology responsible for the dysfunction of inhibitory control observed in ADHD (Cheng et al. 2013).

The executive functions related to attention, inhibitory control and behavioral flexibility can be tested with the intra-dimensional/extra-dimensional Attentional Set-Shifting Task (ASST), which is available for humans (Owen et al. 1993), monkeys (Dias et al. 1997), rats (Birrell and Brown 2000) and mice (Bissonette et al. 2008, Young et al. 2010). That task comprises of a series of perceptual discriminations that require an animal to form an attentional set, to shift an attentional set within and between dimensions and also to alter behavior under reversal conditions (Birrell and Brown 2000). The behavioral deficits observed in DAT-KO mice are reminiscent of the deficiencies in executive functions noticeable in ADHD and it was postulated that the study of DAT-KO mice can contribute to better understanding of the molecular basis of this and other dopamine-related neuropsychiatric disorders. However, it is unlikely that complete absence of DAT functions occurs in ADHD patients, and therefore it might be more appropriate to study DAT heterozygous mice, which are less extreme case of a DAT dysfunction (Gainetdinov and Caron 2003). DAT heterozygotes are characterized by heightened levels of DA synthesis and turnover, with increased extracellular DA levels in the striatum and nucleus accumbens and reduced tissue levels of DA (Giros et al. 1996, Jones et al. 1998, Gainetdinov and Caron 2003). We have described behavioral deficits of DAT heterozygous mice in the intra- and extra-dimensional shifts of attention, accompanied by deficiencies in the task-induced activation of neurons in mPFC and posterior parietal cortex (PPC) regions (Cybulska-Klosowicz et al. submitted). The purpose of the current study was to examine whether normal expression of DAT is also required for optimal neuronal activation in brain regions supporting reversal learning performance, i.e. OFC and striatum, during training in the ASST. The performance of WT and DAT heterozygous mice in the ASST was analyzed and the task-induced expression of the early growth response proteins 1 (egr-1, Zif-268) and 2 (egr-2, Krox-20) in the OFC and DMS during ASST stages was compared. Induction of the egr-1 depends on neuronal activity and is thought to be involved in neuronal plasticity (Cole et al. 1989), learning and memory (e.g. Hall et al. 2001, Bozon et al. 2003), while the level of egr-2 expression in the prefrontal cortex (OFC and mPFC) was found to correlate with the magnitude of cognitive control involved in the task performance (DeSteno and Schmauss 2008). Beside the DAT mutant mice, we also examined the mice withdrawn from the treatment with the potent and selective DAT inhibitor, GBR 12909 (Heikkila and Manzino 1984). It has been postulated that withdrawal from GBR 12909 induces a rebound DAT over-expression and it has been demonstrated that it results in mild increases in locomotor activity and deficits in discrimination abilities in the novel object discrimination task (Hewitt et al. 2005, 2009).

MATERIALS AND METHODS

Animals

DAT+/– (n=39) and wild-type (n=39) littermate female mice 10–15 weeks of age were used in this study. Homozygous DAT-KO, heterozygous DAT+/–, and WT mice were obtained by homologous recombination as previously described (Giros et al. 1996), and the two B6-DAT and D2-DAT congenic strains were maintained by consistently backcrossing for more than 12 generations onto the B6 and D2 inbred strains as previously described (Morice et al. 2007). The couples of founders for the cohort used in this study were obtained from Prof. B. Giros. The mice were bred in the Animal Facility, Faculty of Biology, University of Warsaw and weaned at 4 weeks. Then they were housed under standard conditions, on a 12 h light/dark cycle in the Animal House, Nencki Institute. The genotypes of the animals were determined by PCR analysis with the use of the primers DAT-1 (CCCGTC TACCCATGAG-TAAAA), DAT-2 (C TCCACC TTCC TAGCAC TAAC), and NEO2 (TGACCGC TTCC TCGTGC) (Carboni et al. 2001).

Experiment with DAT inhibitor, GBR 12909, was performed on 15 C57BL/6J female mice ~6 weeks of age. The animals were kept in a temperature-controlled room with a natural light/dark (12-h:12-h) cycle.

All work was conducted in accordance with the European Community Council Directive (2010/63/EU) and was approved by the Local Ethics Committee No. 1 in Warsaw.

GBR 12909 administration

GBR 12909 dihydrochloride (1-[2-[bis(4-fluorophenyl) methoxy]ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride) (Tocris, Bristol, UK) was administered (20 mg/kg) by intraperitoneal injection. Animals were treated twice a day (between the hours of 07.00–8.00 h and 16.00–17.00 h) for 4 consecutive days. Two groups of mice received either GBR 12909 (n=7) or vehicle (sterile water) (n=8) ascribed in a pseudorandom manner.

The first discrimination stage of the ASST (simple discrimination) was performed on these mice 10 days following withdrawal from the drug treatment regime.

Attentional set-shifting task (ASST)

The ASST was developed as a measure of attention and behavioral flexibility in rats (Birrell and Brown 2000). ASST is a rodent version of the intra-dimensional/extra-dimensional component of the Cambridge Neuropsychological Test Automated Battery (CANTAB) which is used to identify cognitive dysfunction in humans and non-human primates (Nagahara et al. 2010, Rock et al. 2014). In the ASST task animals learn to discriminate between two perceptual dimensions: odor and texture.

Test apparatus

The test apparatus was a wooden cage (30×38×21 cm). Additional panel divided half of the length of the cage into two "choice" sections in which the digging bowls were placed and a removable divider separated these two "choice" sections from the "start" section. The digging bowls (glass pots; interior diameter 4.5 cm, depth 5 cm) were filled to the inner rim with digging media of different textures (gravel, wool, plastic pellets, straws, pleated paper strips, coarse sand). The digging media were scented with various flavoring essences (lemon, almond, orange, vanilla, arrack, rum; Dr. Oetker®, Poland) (see Table I for examples). During testing the bowls were baited with small pieces of sweetened dry breakfast cereal (DottyChrups, Otmuchow, Poland), which were placed on the bottom of the glass pot and buried under the digging media. Only one of the bowls was baited and mice were required to learn to pay attention and respond to the relevant dimension (e.g., odor) and ignore an irrelevant dimension (e.g., digging medium).

Handling and habituation period

Before testing mice were handled for 5 days. During that time they were habituated with the bowl filled with the reinforcer in their home cage for 24h and then acclimated with the reinforcer presented in the digging bowls filled with the media and placed in the testing apparatus for 10 min per day for 2 days, or as many days as they needed to be habituated and to consume the reinforcer. The non-discriminable digging media were used and were not scented during that period and the reinforcer was placed in both digging bowls. A piece of the food reinforcer was placed on top of the digging media during the first day of habituation and covered with a thin layer of the medium on the second day. Mice were allowed to consume the retrived food revard before being returned to the home cage. When mice were habituated enough to consumed the reinforcer, the same non-discriminable media were scented, each of the bowl with different odor, and only one was baited with the reinforcer. Mice were trained to a criterion of six consecutive correct trials in that pre-test. After habituation period mice were moved to the first stage of the ASST.

Behavioral paradigm

The animals had no access to food for approximately 12 hours before testing, while water was freely available in the home cage all the time. The order of the training discriminations (odor and medium), the reinforced odor and medium and their left or right position in the test apparatus were determined pseudorandomly. Mice were allowed 3 minutes of exploration; if the response (digging) did not occur in that time, the trial was terminated and an error of omission was recorded. An error of commission was recorded if the mouse dug in the unbaited bowl and the trial was terminated. Digging was defined as active digging with both front paws or active foraging with the snout in the digging medium (Birrell and Brown 2000). Mice were trained to a criterion of six consecutive correct trials in all stages of the ASST. The number of trials to criterion was counted for each test stage.

Six discrimination phases (Birrell and Brown 2000, Glickstein et al. 2005) comprised the ASST and were performed in the following order: simple discrimination (SD), compound discrimination (CD), reversal learning (Rev), intra-dimensional shift (IDS), extra-dimensional shift (EDS) and reversal of the EDS (EDS-Rev). An example of the entire task is shown in Table II. In the SD the bowls differed along one of two dimensions and mice learned to discriminate of either two different odors or two digging media. There were three "exploratory" trials at the beginning of that stage, where the mice, after an error, were allowed to retrieve the reinforcer from the correct bowl. Responses of an animal during these "exploratory" trials were not included into analyses. The

Table I. Exemplar pairs of stimuli used in the ASST

dimension	exemplar pairs		
digging medium	gravel	wool	straws
	coarse sand	pleated paper strips	plastic pellets
odor	lemon	almond	orange
	vanilla	arrack	rum

following CD stage had the same correct (reinforced) and incorrect (non-reinforced) stimulus properties as the SD, but a new irrelevant dimension was introduced. For the Rev stage, the relevant dimension and the stimuli were unchanged, however the previously correct stimulus was now non-reinforced. The next test phase required a shift of attention within the dimension (intra-dimensional shift, IDS). In this stage both correct and incorrect stimuli changed, but the relevant dimension (either odor or medium) remained the same. In the next stage shifting of attention between dimensions was required (extra-dimensional shift of attention, EDS). In the EDS stage the correct and incorrect stimuli changed again, but the originally relevant dimension became irrelevant and the formerly irrelevant dimension became relevant. In the last, EDS-Rev stage, stimuli did not change, but the previously incorrect stimulus became a correct-reinforced one; the irrelevant dimension was still not predictive of the reinforcer.

Animals performed each of the ASST stages in a single test session (single day; one stage per day). The two-session (two consecutive days) procedure was introduced when needed in the difficult stages of the test (Rev), because in this case mice appeared satiated before the stage was completed in 1 day. Behavior was monitored by a camera and stored to be analyzed off-line if needed.

Behavioral analysis of the results of mutant animals (DAT+/– and WT mice) was accomplished for the results of the group which went through all ASST stages (DAT+/–, n=8; WT, n=8). Five mice of each genotype were used for the analyses of test-induced EGRs expression (EDS-Rev; WT, n=5; DAT+/–, n=5).

Immunohistochemistry

Task-induced expressions of the egr-1 and egr-2 were analyzed in mutant mice (DAT+/– and WT littermate mice). Separate groups of animals were trained to analyze the expression of the egr-1 and egr-2 after each of the ASST stages. Animals were sacrificed one hour after the end of the particular ASST training session (separate group of mice for each ASST stage; 5 mice in each WT and DAT+/– subgroup). Tissues of both genotypes, that were concurrently subjected to the same ASST stage, were processed in parallel, and then subjected separately to egr-1 and egr-2 immunolabelings. Additionally, egr-1 and egr-2 immunohistochemistry has been done in control, not-trained, naïve DAT heterozygous (n=6) and WT (n=6) animals.

Mice were deeply anesthetized with an overdose of Nembutal and perfused transcardially with ice-cold 0.9% NaCl followed by 4% paraformaldehyde in 0.1M phosphate buffer, PB, pH 7.4. After perfusion, the brains were removed and postfixed in the same fixative for 3 h and then replaced with 10% sucrose (10% sucrose in phosphate buffered saline, PBS, pH 7.4) for 1 day, followed by 20% sucrose for 1 day and finally with 30% sucrose for one day. Sections were stored in PBS containing sodium azide in 4°C. After several washes in PBS, endogenous peroxidase activity was blocked by incubation in 0.3% H_2O_2 in PBS. Then, all sections were blocked in PBS containing 2% bovine serum albumin 2% normal serum blocking solution from the same species as the secondary antibody, and 0.1% Triton X-100 at room temperature to avoid non-specific staining. The sections were incubated overnight (egr-1) or over 3 nights (egr-2) at 4°C with a primary antibody (rabbit polyclonal anti-egr-1 antibody, Santa Cruz Biotechnology, Inc; Santa Cruz, CA; sc-189; 1:1000; rabbit polyclonal anti-egr-2 antibody, Covance; Berkeley, CA; PRB-236P; 1:1000). These antibodies were used previously and evaluated for specificity (DeSteno and Schamuss 2008). Then, sections were incubated at room temperature with a biotinylated secondary antibody (anti-rabbit IgG, 1:100, Vector Laboratories, Burlingame, CA), followed by an incubation in avidin-biotin-peroxidase complex (Vectastain Elite Kit; Vector Laboratories). The reaction was developed by using DAB/urea–H_2O_2 tablets (SigmaFAST, D4293, Sigma) with the addition of nickel ammonium sulfate hexahydrate (0.02%), resulting in black nuclear staining. All sections were rinsed in PBS and

Table II. Example of a sequence of discriminations in the ASST

discriminations	dimension		combinations of exemplars			
	relevant	*irrelevant*	*reinforced*		*non-reinforced*	
simple discrimination (SD)	odor (o)	medium (m)	o1		o2	
compound discrimination (CD)	odor	medium	o1	m1, m2	o2	m1, m2
reversal (Rev)	odor	medium	o2	m1, m2	o1	m1, m2
intra-dimensional shift (IDS)	odor	medium	o3	m3, m4	o4	m3, m4
extra-dimensional shift (EDS)	medium	odor	m5	o5, o6	m6	o5, o6
extra-dimensional shift – reversal (EDS-Rev)	medium	odor	m6	o5, o6	m5	o5, o6

mounted onto gelatin-coated slides, dehydrated, cleared in xylene and embedded in DePeX.

Control experiments were conducted in which brain sections were subjected to the immunocytochemistry procedure described above, except that the primary or secondary antibodies were omitted.

Microscope pictures of the immunolabeled sections, obtained using an Eclipse E600 microscope (Nikon), were captured with a computer-assisted camera using Image-Pro Plus Version 5.0 software (Media Cybernetics, Bethesda, MD, USA). Representative examples of egr-1 and egr-2 immunolabeled sections are shown in Figs 3C, 3D and 4B.

Behavioral results revealed profound difficulties of DAT+/– mice in the Rev stage of the task (Cybulska-Klosowicz et al. submitted), therefore we decided to compare neuronal activation in WT and DAT mutant mice in two structures which are well known to be involved in the reversal learning – OFC and DMS. The expression of egr-2 and egr-1 immunoreactivity was measured in the ventrolateral part of the OFC (vlOFC) and in DMS, determined using The Mouse Brain in stereotaxic coordinates (Franklin and Paxinos 1997). Immunolabelling was analyzed in sections that were collected from +2.7 to +2.1 relative (rostral) to bregma for OFC and +1.1 to +0.5 relative (caudal) to bregma for DMS. Three to five sections from each one animal were analyzed. The vlOFC and DMS were determined and outlined in every analyzed section in both hemispheres. Image-J v1.45s software was used for automated analysis of the number of egr-1 and egr-2 positive nuclei. A person blinded to the experimental conditions performed the analysis.

In all cases the density of immunoreactive cells is presented. The counts for each region were averaged for each animal, and the obtained values were used to produce a group mean.

Statistical analyses

Kolmogorov-Smirnov normality tests were applied to all data before statistical comparisons. T-tests were applied to compare the total number of errors (both, commission and omission) between compared groups of mice (WT *vs.* DAT+/– mice and GBR 12909-treated *vs.* control mice). The non-parametric statistics were applied for all the remaining behavioral ASST data (trials to criterion), because they did not pass the normality test. Comparisons were made using Kruskal-Wallis tests and differences between GBR 12909-treated and control mice were determined with *post hoc* comparisons (Mann-Whiney U-tests, with Bonferroni correction). Two-way analysis of variance (ANOVA) was applied to separately analyze the results of egr-1 and egr-2 immunohistochemistry. For each analysis, the effects of "DAT genotype", "ASST stage" and the interaction of those 2 factors on EGRs expression were analyzed; *post hoc* comparisons were conducted using the Bonferroni statistics. GraphPad Prism 5 software was used for all statistical analyses.

RESULTS

GBR 12909-treated mice

Response accuracies (number of trials to criterion) of GBR 12909 (n=7) and control (n=8) mice on the ASST are shown on Fig. 1A. All animals, both GBR 12909-treated and control, were able to complete all of the ASST stages. The data did not pass the Kolmogorov-Smirnov normality test, therefore the non-parametric statistics were applied. Kruskal-Wallis test yielded significant effects of drug-treatment on a ASST test stage performance level (Kruskal-Wallis statistic: 40.57, $p<0.0001$). GBR 12909-treated mice needed significantly more trials to reach the criterion in Rev ($p=0.025$), and extra-dimensional shift – reversal (EDS-Rev) ($p=0.032$) stages of the ASST (Mann-Whitney U-tests with Bonferroni correction). No differences in performance between the two groups of animals were observed in all the remaining stages (SD, CD, IDS, EDS) of the ASST (Fig. 1A).

To further analyze differences between GBR 12909-tretaed and control groups of mice in the reversal learning, the number of both commission and omission errors in both Rev and EDS-Rev stages was analyzed. GBR 12909-treated mice committed significantly more commission errors than control mice in the Rev ($p=0.014$) and EDS-Rev ($p=0.018$) stages of the task (Mann-Whitney U-tests with Bonferroni correction) (Fig. 1D). No differences between both groups of mice in the Rev (control mice – 1.38+/–0.92; GBR 12909-treated mice – 1.14+/–1.46) and EDS-Rev (control mice – 0.13+/–0.35; GBR 12909-treated mice – 0) stages were found for omission errors ($p>0.05$; Mann-Whitney U-tests with Bonferroni correction). The total number of commission errors was significantly higher in GBR 12909-treated mice in comparison with control mice ($t=3.605$; $df=13$; $p=0.003$; t-test) (Fig. 1B). The total number of omission errors was low in both group of mice (control mice – 1.85+/–1.36; GBR 12909-treated mice – 2+/–2.16) and there was no significant difference between the two groups of animals ($t=0.1362$; $df=13$; $p=0.89$; t-test) (Fig. 1C).

DAT+/– and littermate WT mice

Attentional set-shifting task

All animals, both DAT+/– and WT, were able to complete all of the ASST stages. We have previously shown that

DAT+/– mice required significantly more trials than WT mice to reach the criterion in Rev, IDS and EDS-Rev stages of the task (Cybulska-Klosowicz et al. submitted). Moreover, contrary to WT mice, there were no difference in the performance between IDS and EDS phases in DAT+/– mice. Analysis of the data revealed that WT mice could form and shift attentional-set successfully, while it was difficult for the DAT+/– mice to acquire and form the attentional set (Cybulska-Klosowicz et al. submitted).

Further analyses of the data, focused on the reversal learning, revealed more specific deficits of DAT+/– mice and are presented in the current study.

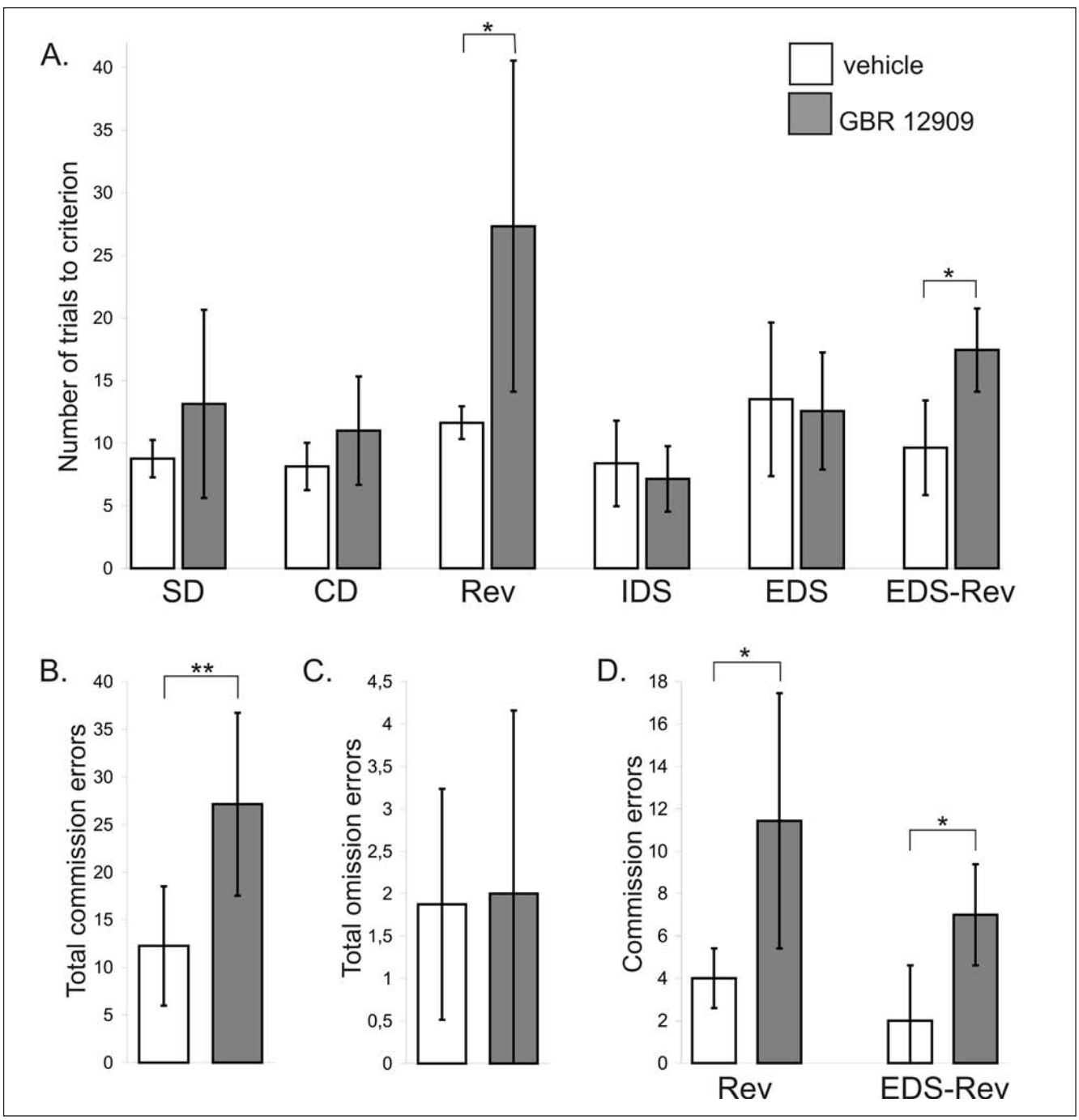

Fig. 1. ASST performance of GBR 12909-treated and control mice. (A) The individual stages of the ASST are indicated. (B) Total number of commission errors committed to criterion in all stages of the ASST. (C) Total number of omission errors committed to criterion in all stages of the ASST. (D) Number of commission errors in the Rev and EDS-Rev stages. For each stage, data are means ±SD. * p<0.05, Kruskal-Wallis followed by *post hoc* (Mann-Whitney U-tests with Bonferroni correction) for A and D; ** p<0.01, t-test for B and C.

In order to more thoroughly understand behavioral deficits of DAT heterozygous mice in the Rev, IDS and EDS-Rev stages of the ASST task, the numbers of both commission errors and omissions to the criterion were analyzed in those three stages of the task. Statistically significant differences between WT and

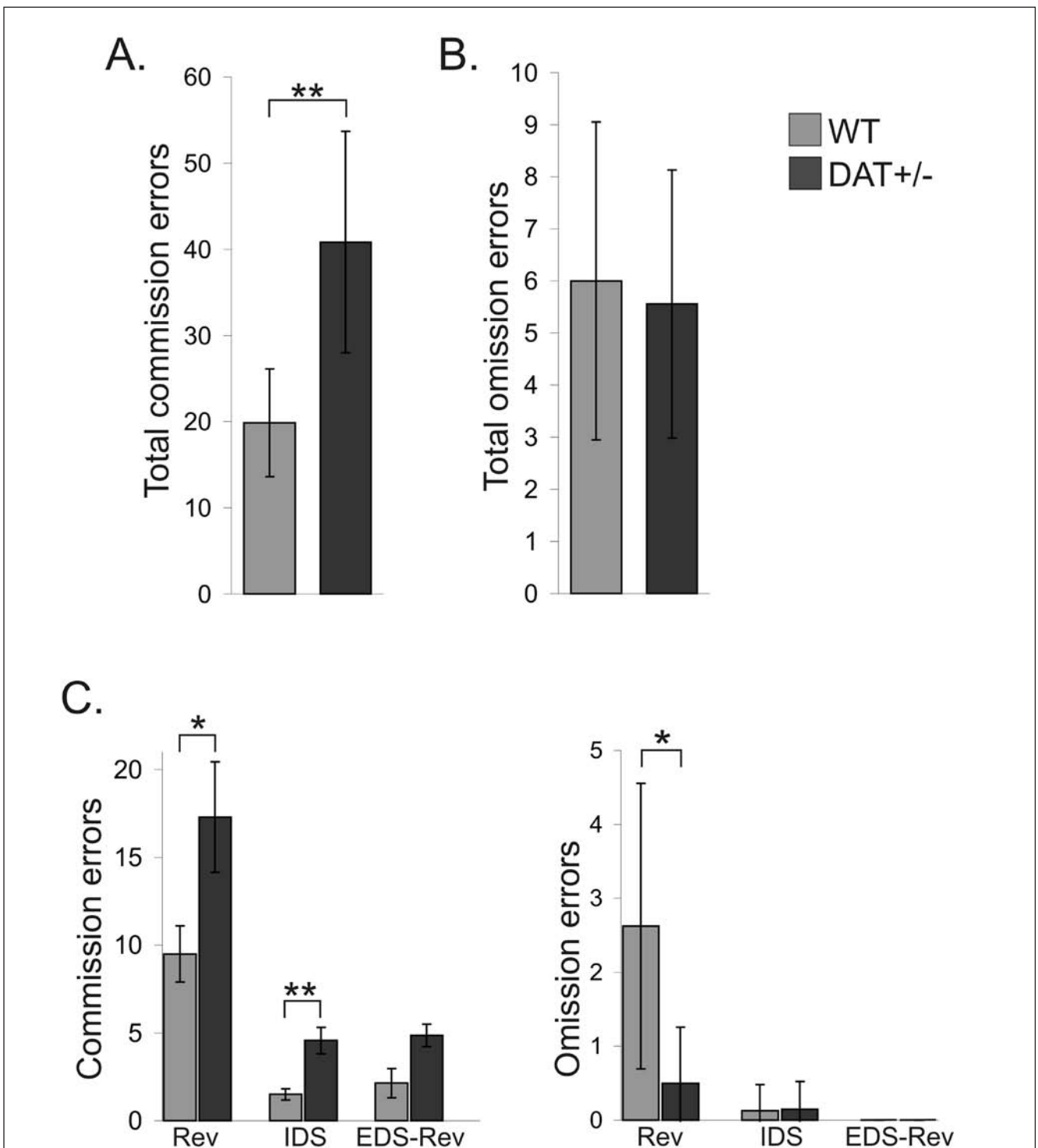

Fig. 2. ASST performance of DAT heterozygotes (DAT+/−) and wild type (WT) mice. (A) The total number of commission errors. (B) The total number of omission errors, ** $p < 0.01$, t-test. (C) The number of commission errors to criterion and D. the number of omission errors to the criterion in Rev, IS and EDS-Rev stages of the ASST. Individual test stages of the ASST are indicated. For each stage, data are means ±SD of trials to criterion obtained from 5-6 animals per genotype. * $p<0.05$ Kruskal-Wallis followed by *post hoc* (Mann-Whitney U-tests with Bonferroni correction).

DAT+/– mice were shown for both commission errors (Kruskal-Wallis statistic: 31.30; p<0.0001) and for omissions (Kruskal-Wallis statistic: 52.54; p<0.0001). DAT+/– mice committed significantly more commission errors than WT mice in the Rev (p=0.042) and IDS (p=0.006) stages of the task (Mann-Whitney U-tests with Bonferroni correction) (Fig. 2C). The number of omissions was significantly lower in DAT+/– mice when compared with WT mice (p=0.0459; Mann-Whitney U-tests with Bonferroni correction) (Fig. 2D). The Rev stage was the most difficult stage of the ASST task; both DAT+/– and WT mice needed significantly more trials to reach the criterion and committed significantly more commission errors in Rev than in other stages (p<0.05 for each of the comparisons; Mann-Whitney U-tests with Bonferroni correction). The total number of commission errors was significantly higher in DAT mutant mice in comparison with WT mice (t=3.838; df=12; p=0.003; t-test) (Fig. 2A). There was no difference between the two groups of mice in the total number of omission errors (t=0.2839; df=12; p=0.78; t-test) (Fig. 2B).

egr-1 expression

No differences in egr-1 expression in neither OFC (t=0.1990, df=8) nor DMS (t=0.5137, df=8) between naïve WT and naïve DAT+/– mice were detected (t-tests, p>0.05; Figs 3A, 3B).

The data for the egr-1 expression in both OFC and DMS after all stages of the ASST training in WT and DAT+/– mice passed the Kolmogorov-Smirnov normality test. Two-way ANOVA indicated that there was a significant interaction between the effects of DAT genotype and the ASST stage on the density of the egr-1 immunoreactive cells in the OFC ($F_{(5,48)}$=3.976, p=0.0043). There were significant differences in the egr-1 expression between DAT genotypes ($F_{(1,48)}$=12.77, p=0.0008) and between ASST stages (OFC: $F_{(5,48)}$=4.915, p=0.0010). Within the ASST stage factor *post hoc* comparisons showed that egr-1 expression was significantly lower in DAT+/– mice than in WT mice in OFC after completion of the IDS stage (p<0.001) and EDS-Rev stage (p<0.05) (Figs 4A, 4C).

For the DMS there was a significant interaction between the effects of DAT genotype and ASST stage on egr-1 expression level ($F_{(5,47)}$=4.834, p=0.0012). The egr-1 density was significantly affected by the ASST stage ($F_{(5,47)}$=12.62, p<0.0001), and by the DAT genotype ($F_{(1,47)}$=18.45, p<0.0001). Within the ASST stage factor *post hoc* comparisons showed that egr-1 expression in the DMS was significantly lower in mutant mice than in WT mice after completion the Rev stage (p<0.05) and IDS stage (p<0.001) (Figs 4B, 4D).

egr-2 expression

As shown in Fig. 3C, no difference between naïve WT and naïve DAT heterozygous animals in egr-2 expression in the OFC was detected (t-test: t=0.2279, df=8, p>0.05).

The data obtained for the egr-2 expression in OFC after ASST in both experimental groups passed the Kolmogorov-Smirnov normality test. Two-way ANOVA indicated that there was a significant interaction between the effects of DAT genotype and the ASST stage on the density of the egr-2 immunoreactive cells in the OFC ($F_{(5,49)}$=2.468, p=0.0451) and there were significant differences in the egr-2 expression between ASST stages ($F_{(5,49)}$=3.237, p=0.0133). Within the ASST stage factor

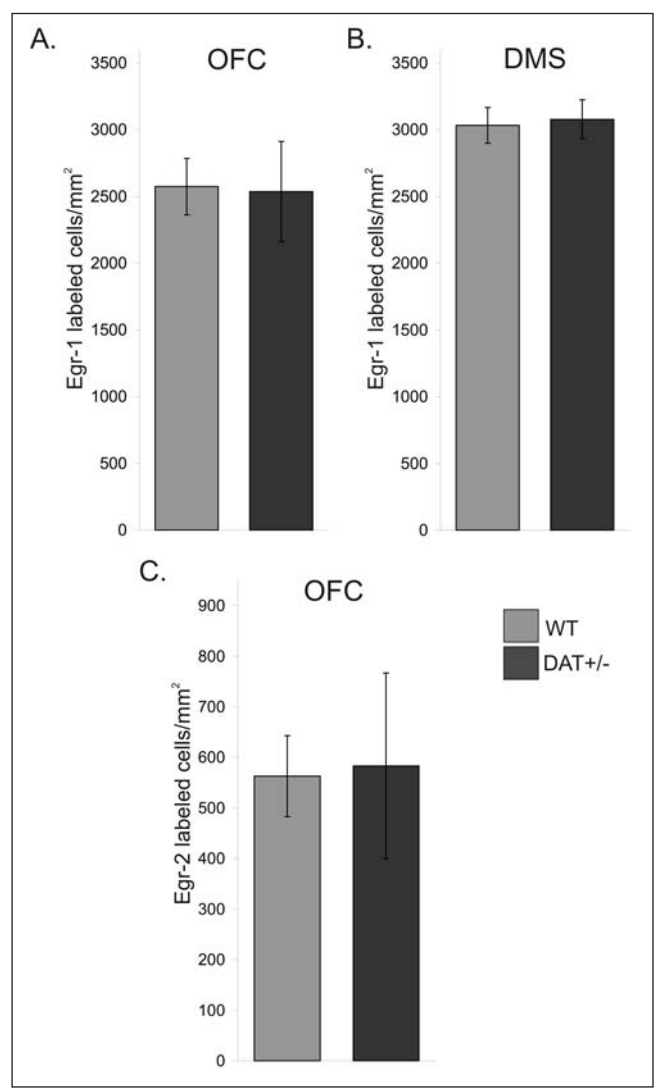

Fig. 3. Comparison of neuronal activity in OFC and DMS of control, naïve DAT mutants (DAT+/–) and wild-type mice (WT). egr-1 expression level in the OFC (A) and DMS (B) of DAT+/– and WT mice. (C) egr-2 expression level in the OFC of DAT+/– and WT mice. Data represent means ±SD. No statistically significant differences were observed between the two groups of mice (p>0.05; t-tests).

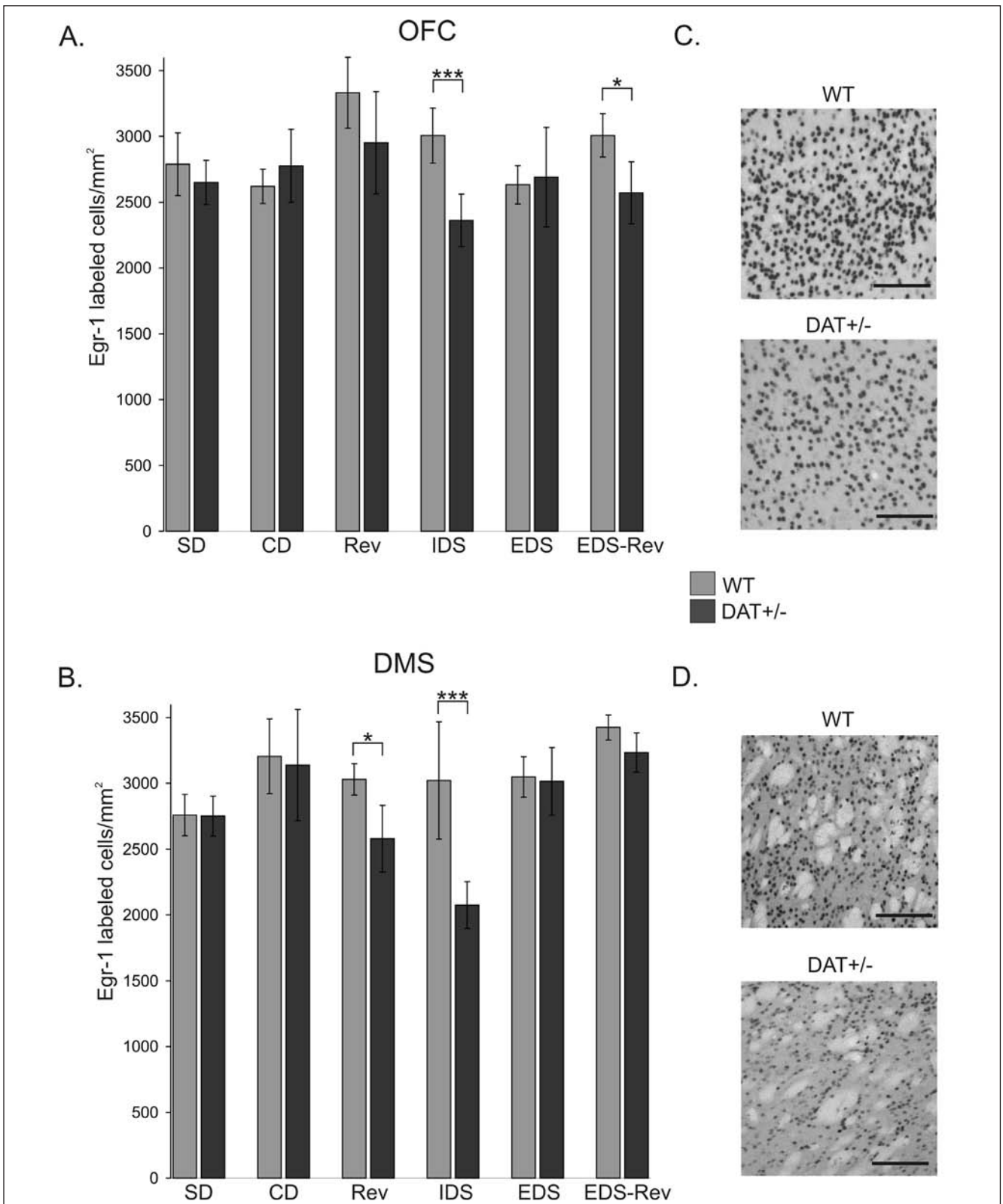

Fig. 4. (A, B) Comparison of the densities of egr-1-labeled cells in the OFC (A) and DMS (B) of DAT mutants (DAT+/−) and wild-type mice (WT) after completion of each particular stage (SD, CD, IDS, Rev, EDS, EDS-Rev) of the ASST. Data represent means ±SD. Statistical differences revealed by Two-way ANOVA were resolved *post hoc* (Bonferroni statistics), * $p<0.05$, ** $p<0.01$. (C, D) Representative examples of egr-1-labeled sections comprising the OFC (C) and DMS (D) of WT and DAT+/− mice after IDS testing. Scale bar=100 μm.

post hoc comparisons showed that egr-2 expression was significantly lower in DAT+/– mice than in WT mice after completion the Rev stage in the OFC ($p<0.05$) (Fig. 5). The density of the egr-2 labeled cells was lower in DAT+/– mice in comparison with WT mice after EDS-Rec stage, too, however the difference was not statistically significant.

No clear egr-2 expression, that could be reliably assessed, was observed in the DMS.

DISCUSSION

Behavioral deficits we found in DAT+/– mice imply behavioral inflexibility and are accompanied by lower task-induced activation of neurons in regions involved in reversal learning, the OFC and DMS.

Reversal learning is the ability to adjust and inhibit a previously learned response and switch responding to originally not reinforced stimulus within a particular dimension (Birrell and Brown 2000); reversal paradigms are among the most widely used tests of behavioral and cognitive flexibility (Izquierdo et al. 2017). Reversal has been shown to require intact serotonergic innervation of the forebrain neocortex (Clarke et al. 2007); however, dopaminergic mechanisms in mediating reversal learning have also been implicated in several pharmacological studies (Cools et al. 2007, Lee et al. 2007, Boulougouris et al. 2009, Haluk and Floresco 2009, for a review see: Izquierdo et al. 2017). Although it has been postulated that the DA effect is mediated mainly at the level of the striatum (Dodds et al. 2008), there is also evidence for a role of orbitofrontal dopamine D1 and D2 receptors in the reversal learning (Calaminus and Hauber 2008, Jocham et al. 2009), and of OFC and mPFC D1 and D2 receptors in behavioral flexibility (Winter et al. 2009). The results of our study reveal deficits of DAT mutant mice and in mice withdrawn from the treatment with DAT inhibitor in the reversal learning of ASST (both Rev and EDS-Rev). DAT heterozygotes and GBR 12909-treated mice were able to learn, but learning was significantly slower (more trials to criterion) than in WT and control mice and they committed more commission errors to the criterion in the Rev stage (and IDS) than WT and control mice. Difficulties in the reversal task have been previously shown in DAT+/– mice when tested in the H-maze (Del'Guidice et al. 2014) and have also been produced by reductions in striatal dopamine transporters evoked by methamphetamine administration (Izquierdo et al. 2010). Treatment of mice with the potent and selective

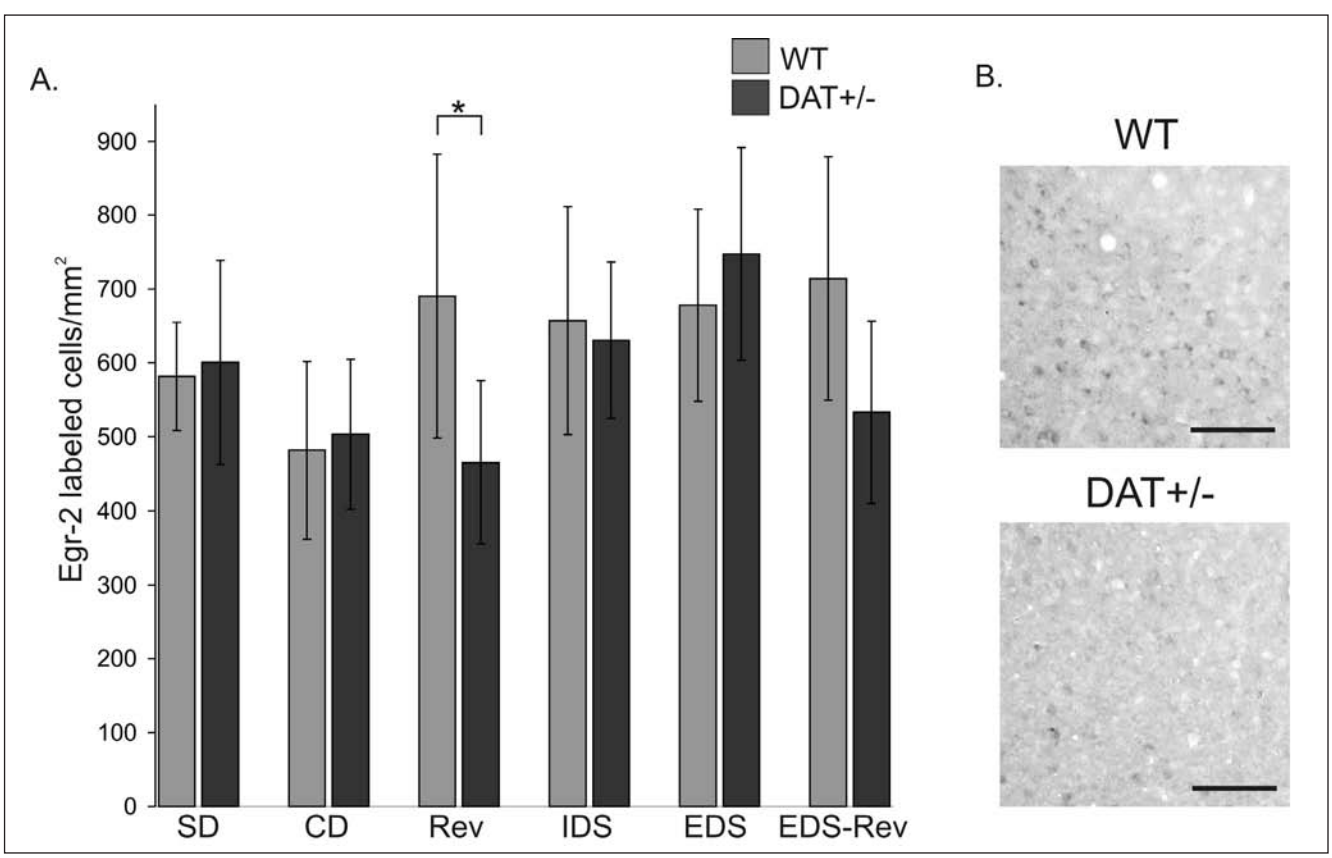

Fig. 5. (A) Comparison of the egr-2 expression in the OFC of DAT mutants (DAT+/−) and wild-types (WT) at all stages of the ASST. Data represent means ±SD. Statistical differences revealed by Two-way ANOVA were resolved *post hoc* (Bonferroni statistics), ** $p<0.01$, *** $p<0.001$. (B) Representative examples of egr-2-labeled sections comprising the OFC of WT and DAT+/− mice after Rev testing. Scale bar=100 μm.

DAT inhibitor GBR 12909 (van der Zee et al. 1980, Heikkila and Manzino 1984, Andersen 1989) would be expected initially to reduce DAT activity, but subsequently upon drug withdrawal, induce a rebound DAT over-expression, as has been previously postulated (Hewitt et al. 2005, 2009). Based on both excessive and deficient DA transmission in clinical and experimental studies, an inverted "U-shaped" relationship between DA levels and cognitive performance has been postulated (Cools 2006) and our results are in agreement with that assumption. It has been shown, that 10, 20 and 30 days withdrawal from GBR 12909 pre-treatment results in disability in discrimination a familiar over a novel object in the novel object discrimination task, indicating impaired learning and memory (Hewitt et al. 2009). Our study, to the best of our knowledge, is the first showing impaired reversal learning and deficits in behavioral flexibility after withdrawal from GBR 12909.

Less omission errors together with more commission errors during reversal learning in DAT heterozygous mice than in WT mice, suggests impulsive action in mutant mice. The most effective treatments for impulse control disorders, such as ADHD, are agents that target DAT and enhance dopamine transmission (Ritz and Kuhar 1989, Volkow et al. 1998). Acute GBR 12909 increased impulsive choice and action in rats (Evenden and Ryan 1996, van Gaalen et al. 2006, Baarendse and Vanderschuren 2012), therefore it has been postulated that DAT inhibition mediates impulsivity. Indeed, the results of the recent study, showing that that impulsive behavior in cued go/no-go task is associated with inherent variation in DAT level (decreased DAT function) in OFC (Yates et al. 2016), confirm that assumption. Our results, showing more commission errors in GBR 12909-treated in comparison with control mice but no differences between these groups of mice in the number of omission errors, fortify the assumption indicating that DAT inhibition, rather than up-regulation, influences impulsive behavior.

A large body of experiments has identified a crucial role for both OFC and DMS in mediating flexible behavior and it has been shown that reversal learning is sensitive to lesions of these two brain regions (Divac et al. 1967, Dias et al. 1996, Schoenbaum et al. 2002, Ragozzino 2002a, 2002b, Ragozzino et al. 2002, McAlonan and Brown 2003, Stefani and Moghaddam 2006, Ragozzino 2007, Clarke et al. 2008, McDonald et al. 2008, Castañe et al. 2010, Baker and Ragozzino 2014, Tait et al. 2017). It has been postulated that OFC and DMS form a functional frontocorticostriatal circuit crucial for mediating behavioral flexibility during reversal learning (Castañe et al. 2010). DAT mutant mice have persistent hyperdopaminergic tone in the striatum (Gainetdinov et al. 1999). This may give rise to striatum dysfunction, leading to learning deficits. In heterozygotes the neurochemical adaptations in the striatum are all intermediate, between that of wild-type and homozygote animals (Jones et al. 1998), and the extracellular dopamine levels in the prefrontal cortex are normal (Shen et al. 2004). However, since prefrontal cortex has dense connections with the dorsomedial striatum (Berendse et al. 1992), subtle changes in the corticostriatal dopaminergic circuitry balance undoubtedly occur and influence behavioral flexibility which arises from that corticostriatal circuit (Pennartz et al. 2009, Kehagia et al. 2010). Indeed, decreased spine density of pyramidal neurons in the mPFC in DAT-KO mice (Kasahara et al. 2015) and the lack of long-term potentiation (LTP) in the prefrontal cortex of these mice (Xu et al. 2009) has been reported. This could cause hypofunction of the region and contribute to the behavioral abnormalities, including cognitive deficits (Giros et al. 1996, Morice et al. 2007). It can be assumed that in heterozygous mice these deficiencies are not as explicit as in DAT-KO mice and indeed, normal basic behavior, learning and memory in DAT heterozygous mice have been reported in previous studies (Zhuang et al. 2001, Rodriguiz et al. 2004, Morice et al. 2007, Li et al. 2010). However, our study revealed that they have deficits in more complex tasks requiring executive functions and behavioral flexibility. Besides behavioral difficulties, DAT+/– mice had also deficits in neuronal activation of regions involved in tasks assessing behavioral flexibility – OFC and DMS. In OFC our data revealed a lower test-induced egr-2 expression in the reversal learning stage of the ASST task and also lower egr-1 expression in extra-dimensional shift-reversal. In the DMS egr-1 expression was lower in reversal stage in mutant mice than in WT mice. The results of control experiments, showing no differences in the level of egr-1 and egr-2 in neither OFC nor DMS of control, naïve DAT+/– and WT mice, confirm that the lower level of EGRs in OFC and DMS indeed represents deficits in neuronal activation in these regions of DAT mutant mice brains during reversal learning and intra-dimensional shift.

Behavioral pattern observed in DAT mutant mice suggests that DAT+/– mice suffer from different cognitive deficiencies in comparison with mice with lesions or inactivation of specific prefrontal cortex subregions. The results also show that the impairment might have more to do with perturbations in behavioral flexibility and inhibitory control and less to do with deficits in learning and memory processes per se (no deficits in SD and CD learning stages of the ASST). Moreover, in previous studies it has been shown that DAT-KO mice exhibit impairments in spatial memory and reversal learning in the Morris water-maze (Morice et al. 2007) and impairments in response inhibition in eight arms radial maze (Gainetdinov et al. 1999). Taken together, this suggests that DAT heterozygous mice may suffer from cognitive deficits involving not only the OFC and striatum, but possibly also other frontal cortex subregions. Indeed, in DAT heterozygous mice slower learning (more trials to criterion) was also found during IDS stage of the ASST in the present study accompanied by the lowered task-induced

neuronal activation in the OFC. It has been shown previously, that OFC-lesioned rats failed to form an attentional set and it has been proven that the set-formation impairment in these rats was not caused by the impaired preceding reversal, but rather it was an impairment in its own right (Chase et al. 2012). During IDS stage we have observed lower level of task-induced egr-1 expression, but not egr-2 expression. It has been previously postulated, that egr-2 rather than egr-1 is a highly sensitive molecular tool to identify specific neuronal populations that support distinct domains of cognitive control, and a functional link between egr-2 expression levels in the OFC and mPFC and optimal ASST performance has been shown (DeSteno and Schmauss 2008). Reduced egr-2 expression in the OFC detected only after completion of the Rev stage, in which mice exhibited deficits, clearly point to the specific deficiency of neuronal activation of that region in mice with a decreased DAT level. Therefore, behavioral inflexibility and impaired reversal learning in these mice may be partly due to the decreased task-induced neuronal activation found in OFC and DMS.

The current results demonstrate that lowered DAT function in OFC and DMS is linked to deficiencies in behavioral flexibility. Both direct and indirect effects of the DAT reduction could contribute to the altered performance of DAT+/– mice in the ASST. Although extracellular dopamine levels in the prefrontal cortex of DAT-KO mice are normal (Shen et al. 2004), their frontal cortical dopamine content is approximately 50% of wildtype levels (Sora et al. 1998), which may indicate changes in synaptic DA content. It has also been shown that serotonin levels are increased in DAT heterozygotes and DAT-KO mice in prefrontal cortex (Fox et al. 2013), which might be of special interest in the face of evidence implicating serotonin in the OFC in the ability of animals to adapt their responding to changes in reward contingencies in the environment. Further studies are needed to determine whether there are postsynaptic differences in dopaminergic function which might contribute to deficits in DAT heterozygous mice that are reported here, to specify changes in prefrontal areas and to explain mechanisms by which this contribute to the behavioral/cognitive abnormalities observed in these mice.

CONCLUSIONS

Data presented here reveal impairments in executive functions in mice with altered dopamine transporter levels. The ASST, complex behavioral paradigm used in training of DAT mutant mice and in mouse model of DAT up-regulation, allowed identification of changes in the level of behavioral flexibility. The results of this study validate usefulness of DAT heterozygotes and ASST for investigation of the neurobiological and physiological mechanism underlying dysexecutive syndromes related to imbalance in the dopaminergic system, such as ADHD and schizophrenia. The study provides evidence that altered DAT function, specifically in OFC and DMS, underlies deficits in behavioral flexibility.

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