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The potential and limitations of DOV 216,303 as a triple reuptake inhibitor for the treatment of major depression: A microdialyis study in olfactory bulbectomized rats

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ABSTRACT

DOV 216,303 belongs to a new class of antidepressants, the triple reuptake inhibitors (TRIs), that blocks serotonin, norepinephrine and dopamine transporters and thereby increases extracellular brain monoamine concentrations.

The aim of the present study was to measure extracellular monoamine concentrations both in the prefrontal cortex (PFC) and dorsal hippocampus (DH) after chronic administration of DOV 216,303 in the OBX animal model of depression and to compare the effects with acute drug treatment.

OBX animals showed lower dopamine levels in PFC upon acute administration of DOV 216,303 than sham animals for up to five weeks after surgery. No such changes were observed in the DH. Unexpectedly, a DOV 216,303 challenge in chronic DOV 216,303 treated sham animals resulted in a blunted dopamine response in the PFC compared to the same challenge in vehicle treated animals. This blunted response probably reflects pharmacokinetic adaptations and/or pharmacodynamic changes, since brain and plasma concentrations of DOV 216,303 were significantly lower after chronic administration compared to acute administration.

Surprisingly, and in contrast what we have reported earlier, chronic DOV 216,303 treatment was unable to normalize the hyperactivity of the OBX animals. Interestingly, by measuring the drug plasma and brain levels, it was demonstrated that at the time of behavioral testing (24 h after last drug treatment) DOV 216,303 was not present anymore in either plasma or brain. This seems to indicate that this putative antidepressant drug has no lasting antidepressant-like behavioral effects in the absence of the drug in the brain.

1. Introduction

Selective serotonin reuptake inhibitors (SSRIs) are the first line of treatment for major depression. However, 30% of the depressed patients do not respond to these drugs (Steffens et al., 1997). Efficacy of SSRIs depends on the severity of the depression (Kirsch et al., 2008) and on the specific depressive symptoms (Katz et al., 1994). Anhedonia is one of the core symptoms of depression and characterized by loss of pleasure and motivation. It has been postulated that a hypodopaminergic state causes anhedonia (Dunlop and Nemeroff, 2007; Nestler and Carlezon, 2006). Therefore, drugs that increase dopaminergic activity may be of interest for the treatment of depression.

One such class of molecules are triple monoamine reuptake inhibitors (TRIs), such as DOV 216,303, that not only block serotonin and norepinephrine transporters, but also dopamine transporters, thereby increasing synaptic concentrations and function of all three monoamines. TRIs are believed to consist of a novel class of antidepressants because DOV 216,303 and/or its enantiomer(s) increase extracellular monoamines *in vitro* (Skolnick et al., 2006) as well as *in vivo* (Popik et al., 2006; Prins et al., 2010), and are effective in animal models of depression (Breuer et al., 2008; Skolnick et al., 2003) and in depressed patients (Skolnick et al., 2006), thereby supporting the classical monoamine theory of depression. In this theory depression is associated with decreased synaptic concentrations of the monoamines dopamine, norepinephrine and serotonin (Schildkraut, 1965). Moreover, animals subjected to chronic stress, leading to anhedonia (Willner et al., 1992, 1987), also show a reduced release of dopamine in the prefrontal cortex in response to palatable food compared to non-stressed rats. This blunted dopamine response could be restored with chronic antidepressants (Di Chiara et al., 1999). But a note of caution: it cannot be assumed that every drug that

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inhibits the three monoamine transporters *in vitro* will possess clinically efficacious antidepressant activity as illustrated by the recent studies of two putative TRIs, NS2359 and SEP 225289, which did not meet their endpoints in phase 2 depression studies.

The olfactory bulbectomized (OBX) rat is considered an animal model of depression (Song and Leonard, 2005). Removal of the olfactory bulbs results in hyperactive behavior in a novel stressful environment (Klein and Brown, 1969), which can be normalized by chronic, but not acute, antidepressant treatment (Breuer et al., 2007; Kelly et al., 1997). In a recent microdialysis study in OBX rats, animals had lower extracellular dopamine levels in the prefrontal cortex than their sham counterparts one day after bulbectomy. Furthermore, acute administration of DOV 216,303 lowered extracellular dopamine levels in OBX animals (Prins et al., 2010). Such decreased dopamine levels might cause the anhedonic state of these OBX animals as reflected by increased ICSS thresholds (Slattery et al., 2007). Moreover, the blunted response to amphetamine on ICSS thresholds and a long-lasting reduction in sucrose intake (Romeas et al., 2009), further supported the suggestion that OBX animals have dysfunctional reward circuitries. These findings make this animal model an attractive model to study new antidepressant agents and justify the use of the OBX model to investigate brain mechanisms underlying anhedonia.

Because antidepressants require several weeks before they relieve symptoms of depression in patients, animal studies should ideally run over multiple weeks. Several studies examined the behavioral effects that characterize OBX only ten till fourteen days after surgery (Giardina and Radek, 1991; Richman et al., 1972) and start antidepressant treatment after three weeks (Breuer et al., 2009b).

We aimed to assess the effect of an acute DOV 216,303 challenge and repeated drug administration on monoamine release a few weeks after OBX surgery. Monoamines were measured in two brain areas simultaneously, the medial prefrontal cortex (PFC) and the dorsal hippocampus (DH) because disturbed neural mechanisms in these brain areas are both associated with symptoms of depression (Hjorth and Auerbach, 1994; Malagie et al., 1996). The DH is involved in reward-related processes via contextual cues which are processed via the hippocampus with projections to the nucleus accumbens (Everitt and Robbins, 2005), while the PFC is involved in overall cognitive "executive" functioning that allows organisms to get things done, reinforcement, reappraisal and suppression of negative affect (Koenigs and Grafman, 2009; Koob and Volkow, 2010; Robbins and Arnsten, 2009). Moreover, the PFC and DH are both innervated by fibres from the dopaminergic ventral tegmental area (Gasbarri et al., 1997; Romanides et al., 1999) and the serotonergic raphe nuclei (Acsady et al., 1996). Furthermore, we measured the concentration of DOV 216,303 in blood plasma and whole brain after acute and chronic DOV 216,303 administration in OBX and sham animals.

2. Experimental procedures

2.1. Animals

Forty-eight male Sprague Dawley rats (Harlan, Horst, The Netherlands) weighing between 290 and 350 g at the time of OBX or sham surgery were socially housed, two or four per cage on a 12 h light–dark cycle with lights on at 6:00 h and off at 18:00 h. Food and water were available *ad libitum*. Animals had one week to acclimate to their environment and were subjected to an open field test in week one, four or five days before receiving surgery (Fig. 1). All animal experimental procedures were carried out in accordance with the governmental guidelines and approved by the Ethical Committee for Animal Research of the Faculties of Pharmaceutical Sciences, Chemistry and Biology at Utrecht University, The Netherlands.

2.2. Open field

Locomotion was measured in grey open field chambers measuring 70×70×45 cm with use of EthoVision® 3.1 (Noldus). All testing was done during the light period, under 20 lx lighting in the open field. After a 30-minute period, during which the animals acclimated to the test room in their home cage, each animal was placed in a corner of the open field and allowed to explore for 15 min. Animals were tested four times in the open field. The first time was four or five days before OBX/sham surgery. Based on these results animals were randomized across surgery groups. The first post-surgery open field took place two weeks after surgery; based on these results animals were assigned to treatment groups. Drug treatment was initiated 21 days after surgery and animals were tested 30 min after the first injection. The final open field test took place on the fourteenth day after the start of treatment, 24 h after daily drug administration (Fig. 1).

2.3. Olfactory bulbectomy

Olfactory bulbectomy (OBX) surgery was performed in animals which were anesthetized by inhalation of isoflurane gas (2–3%), mixed with nitrous oxide and oxygen and animals were placed in a stereotaxic instrument (Kopf, David Kopf Instruments). Lidocaine

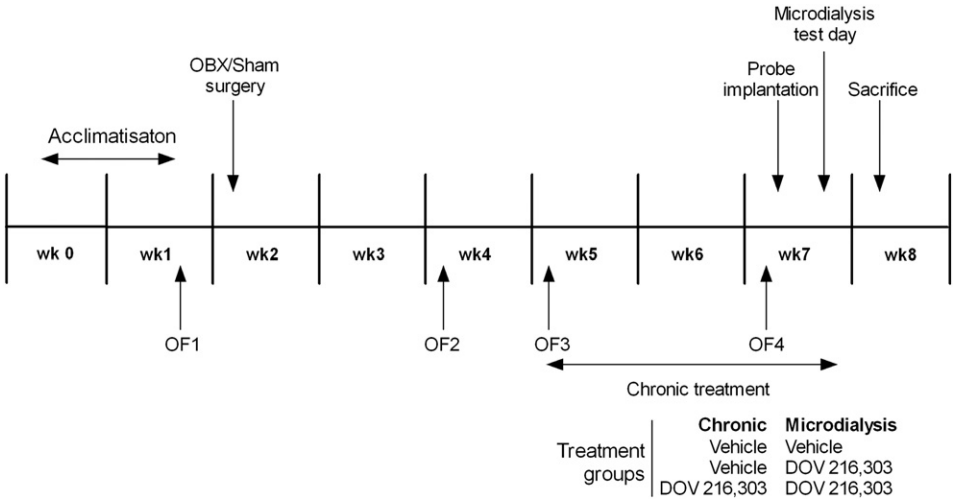

Fig. 1. Experimental design, with time of surgeries, open field tests, chronic drug administration and microdialysis, OF is open field. OF1 is a pre-surgical open field test, OF2 is post-surgery. OF3 is 30 min after start drug treatment, and OF4 is performed two weeks after start of treatment, 30 min before injection.

hydrochloride (2%) + adrenaline (0.001%) were applied in the incision as a local anesthetic. Two burr holes with a diameter of 2 mm were drilled bilaterally, 8 mm anterior to bregma and 2 mm from the midline of the frontal bone overlying the olfactory bulbs. The bulbs were aspirated with a blunt hypodermic needle attached to a vacuum pump. Burr holes were filled up with haemostatic sponge to prevent blood loss. Sham-operated animals underwent the same procedure except that their olfactory bulbs were not removed. All incisions were closed with use of 5-0 vicryl rapide suture material and animals received Rimadyl (5 mg/kg, subcutaneously) for pain relief.

2.4. Drug treatment

Drug treatment started 21 days after OBX/sham surgery. All animals received one injection every day for 17 days. All injections were given by oral gavage. Treatment groups consisted of either vehicle (sterile water) or 20 mg/kg DOV 216,303 [(±)-1-(3,4-dichlorophenyl)-3-azabicyclo-[3.1.0]hexane hydrochloride) synthesized by Sepracor Inc., Marlborough, USA], administered in a volume of 2 ml/kg.

The vehicle–vehicle group received 17 daily vehicle injections in total with the last injection on the microdialysis day. The vehicle–DOV 216,303 group received 16 vehicle injections and one DOV 216,303 (20 mg/kg, p.o.) injection during microdialysis. The DOV 216,303–DOV 216,303 group received 17 injections with the DOV 216,303 compound (20 mg/kg, p.o.), the last injection occurring during microdialysis. The experimental set up is shown in Fig. 1.

2.5. Microdialysis probe implantation

After 14 days of drug treatment, cuprofane microdialysis probes (MAB 4.7.3 CU) were implanted in the medial prefrontal cortex (PFC) and dorsal hippocampus (DH). The coordinates of the PFC were incisor bar lowered at −3.3, AP: +3.2 mm, ML: ±0.8 mm, DV: −4.0 mm from bregma and dura. For the DH, the coordinates were incisor bar lowered at −3.3, AP: −3.8 mm, ML: ±2.8 mm, DV: −4.0 mm from bregma and skull. Probes were anchored with three screws and dental cement on the skull. After microdialysis probe implantation animals were housed individually until the end of the experiment.

2.6. Microdialysis experiment

Two days after implantation, microdialysis experiments were performed in conscious freely moving animals. The system was perfused with Ringer solution (147 mM NaCl, 2.3 mM KCL, 2.3 mM $CaCl_2$, and 1 mM $MgCl_2$) with use of a KdScientific Pump 220 series (USA) at constant flow rate of 1 ml/min. Animals were connected to a dual channel swivel (type 375/D/22QM) which allowed them to move relatively unrestricted. During microdialysis, the pump rate was set at 0.09 ml/h. Two hours after connection ten 30-minute samples were manually collected in vials containing 15 µl of 0.1 M acetic acid and frozen at −80 °C until analysis with HPLC. After 2 h of baseline samples animals were injected orally with either DOV 216,303 (20 mg/kg, 2 ml/kg, p.o.) or vehicle (sterile water), and samples were collected for an additional 3 h. After a drug washout period of three days, all animals were sacrificed and their brains were removed and examined to verify complete olfactory bulb ablation and probe placement accuracy.

2.7. HPLC-ECD

Microdialysis samples were stored at −80 °C until analysis. The following neurotransmitters and metabolites were measured: norepinephrine (NE), dopamine (DA) and serotonin (5-HT) and 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) were detected simultaneously by HPLC with electrochemical detection using an Alexys 100 LC-EC system (Antec Leyden, The Netherlands) (Korte-Bouws et al., 1996; Verhagen et al., 2009). The system consisted of two pumps, one autosampler with a 10 port injection valve, two columns and two detector cells. Column 1 (ALF 105 C18 1×50×mm, 3 µm particle size) in combination with detector cell 1, separated and detected DA and 5-HT. Column 2 (ALF 115 C18 1×150 mm, 3 µm particle size) in combination with detector cell 2, separated and detected NE and the metabolites. The mobile phase for column 1 consisted of 50 mM phosphoric acid, 8 mM KCL, 0.1 mM EDTA (pH 6.0), 12% Methanol and 500 mg/L 1-Octanesulfonic acid, sodium salt (OSA). The mobile phase for column 2 consisted of 50 mM phosphoric acid, 50 mM citric acid, 8 mM KCl, 0.1 mM EDTA (pH 3.2), 10% methanol and 500 mg/L OSA. Both mobile phases were pumped at 50 µl/min. Samples were kept at 8 °C during analysis. From each microdialysis sample 5 µl was injected simultaneously onto each column. The neurotransmitters were detected electrochemically using µVT-03 flow cells (Antec Leyden, The Netherlands) with glassy carbon working electrodes. Potential settings were for DA and 5-HT +0.30 V versus Ag/AgCl and for NE and metabolites +0.59 V versus Ag/AgCl. The columns and detector cells were kept at 35 °C in a column oven. The chromatogram was recorded and analyzed using the Alexys data system (Antec Leyden, The Netherlands). The limit of detection was 0.03 nM (S/N ratio 3:1).

2.8. Histology

All animals were sacrificed three days after the microdialysis test day. Brains were quickly frozen in isopentane and stored at −80 °C until verification of probe track. Data were discarded if olfactory bulbs were not completely ablated, or if the microdialysis probe was not in the PFC or DH.

2.9. Determination of DOV 216,303 in blood plasma and whole brain

A separate group of twenty-four animals was subjected to the same procedures as described above; including open field studies, OBX/sham surgery and drug treatment, except for the microdialysis experiment. Two weeks after OBX/sham surgery, animals were distributed across three different groups ($n=8$ each) and received either DOV 216,303 (20 mg/kg, p.o) or vehicle (sterile water). One group was treated with vehicle for 14 days and injected with DOV 216,303 on the last day 30 min before sacrifice. One group was treated with DOV 216,303 for 14 days and received a last DOV 216,303 injection on the 15th day, 30 min before sacrifice. A third group of animals was treated with DOV 216,303 for 14 days and sacrificed on the last day, 24 h after the final injection. 5 ml of trunk blood of each animal was collected in a tube containing 280 µl 0.21 M EDTA. After collection tubes were shaken and kept on ice until centrifugation for 15 min at 4 °C at 3000 RPM. Plasma was collected for measurement of DOV 216,303 and brains were extracted and quickly frozen in isopentane and stored at −80 °C until further use.

2.10. Measurement of DOV 216,303 concentrations in plasma and whole brain

In order to determine plasma and brain concentrations, brain aliquots (~200 mg) were diluted to 40 mg/ml with 95:5 water: acetonitrile containing 0.1% formic acid. The mixture was then homogenized using an ultrasonic homogenizer (Sonics model VCX 130; Newtown, CT) for 1 min. A 60 µl aliquot of plasma or homogenized rat brain was placed into a 96 deep-well plate and 200 µl of acetonitrile was added to precipitate the proteins. Samples were vortexed for 1 min, and centrifuged at 4500 rpm for 15 min at 4 °C. 200 µl aliquots of supernatant were transferred to a 96 deep-well plate, and evaporated to dryness. The samples were then reconstituted

in 150 µl 90:10 5 water:acetonitrile with 0.1% formic acid, and centrifuged at 4500 rpm for 15 min at 4 °C. A 45 µl aliquot from the supernatant was injected into the LC/MS/MS instrument. The LC/MS/MS analysis was performed using an Applied Biosystems MDS Sciex API-2000 (Concord, ON, Canada) triple quadruple mass spectrometer using a Turboion® spray source. The MS was coupled to an Agilent 1200 series HPLC system (Santa Clara CA) and a Leap HTS PAL autosampler (Carrboro, NC). DOV 216,303 was optimized by infusion of the 1.0 µg/ml solutions in positive ion mode. Multiple reaction monitoring was used for quantitation using a dwell time of 150 ms. Nitrogen was used as the collision gas at a setting of 5.0 for the collision-induced dissociation (CID). The source temperature was 500 °C. Chromatographic separation was accomplished using an ACE C-8 column (50 mm × 2.1 mm, i.d.; 5 µm; Mac Mod, Chadds Ford, PA). Mobile phases A and B consisted of HPLC grade water or acetonitrile with 0.1% formic acid added, respectively.

2.11. Statistical analysis

Pre- and post surgical open field data were analyzed by a one-way ANOVA with 'surgery' as between factor. Open field data after acute (OF3) and chronic (OF4) treatment were analyzed by a multivariate analysis of variance with locomotion data of OF3 and OF4 as dependent variables and 'treatment' and 'surgery' as fixed factors.

The area under the curve of the dopamine response was calculated using the trapezoid algorithm. To detect significant differences in area under the curve data analysis of variance (one-way ANOVA) was used with Tukey post-hoc comparisons.

Baseline microdialysis data were analyzed with use of multivariate analysis of variance, and in case of significant effects followed by one-way ANOVA with post-hoc Tukey tests. Microdialysis data were analyzed with use of repeated measures ANOVA with 'time' as within and 'surgery' and 'treatment' as between factors. When a significant surgery × treatment interaction was found, each timepoint was analyzed by one-way ANOVA, in case of significant ANOVA effects, post-hoc Tukey tests were performed. Where appropriate, all reported results were corrected by the Greenhouse–Geisser procedure.

Blood and brain concentrations of DOV 216,303 were analyzed by multivariate analysis of variance with 'surgery' and 'treatment' as between factors, in case of significant effects post-hoc Tukey tests were performed. All statistical analyses were carried out with the Statistical Package SPSS 16.0. The level of significance in all tests was set *a priori* at $p<0.05$.

3. Results

3.1. Locomotion in open field (Fig. 2)

No animals were excluded based on incomplete olfactory bulb ablation. Olfactory bulbectomy increased locomotion compared to sham-operated controls two weeks after surgery ($F(1, 42)=32.5$, $p<0.001$) (Fig. 2A). The OBX animals remained hyperactive during the complete duration of the experiment. After acute treatment, vehicle treated OBX animals were more active than vehicle treated shams ($F(1, 44)=6.791$, $p<0.05$). No effect of acute DOV 216,303 administration could be observed on locomotion in the OBX animals compared to vehicle treated OBX rats (Fig. 2B). Two weeks after treatment, vehicle treated OBX animals were more active than their vehicle treated shams ($F(1, 44)=15.298$, $p<0.001$). After 14 days of treatment no difference could be observed in locomotion in the DOV 216,303 treated OBX animals when compared with vehicle treated OBX rats (Fig. 2B), suggesting that DOV 216,303 administration, both acute and chronic, did not reduce hyperactivity.

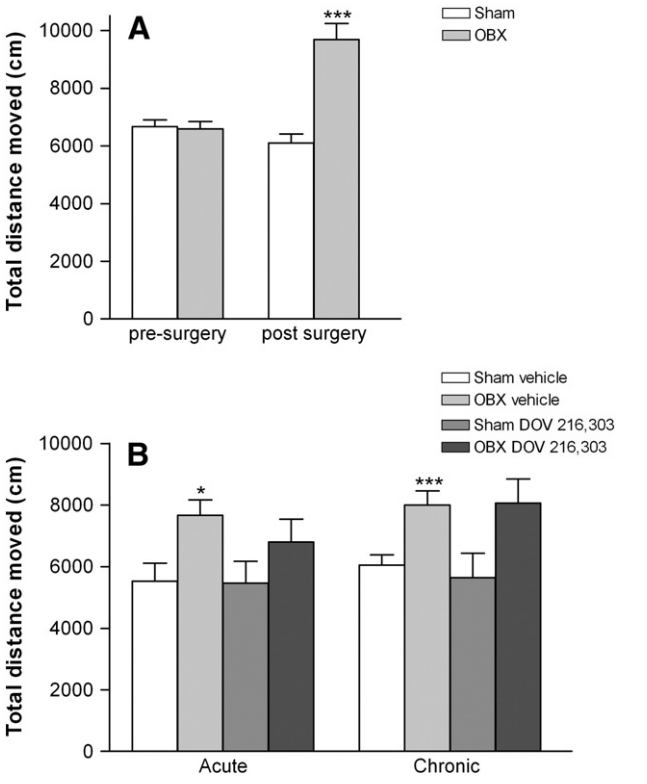

Fig. 2. Total distance travelled in an open field in 15 min. A. Locomotion before and after OBX/sham surgery. B. Locomotion 30 min after DOV 216,303 injection (acute) and 24 h after the 14th injection in chronically treated animals (chronic). ***$p<0.001$; *$p<0.05$ compared to their sham counterparts.

3.2. Baseline monoamine levels in prefrontal cortex and dorsal hippocampus (Fig. 3)

Baseline monoamine levels in PFC were not different between treatment groups. In the DH a significant surgery × treatment interaction was found for baseline serotonin levels ($F(1, 32)=$

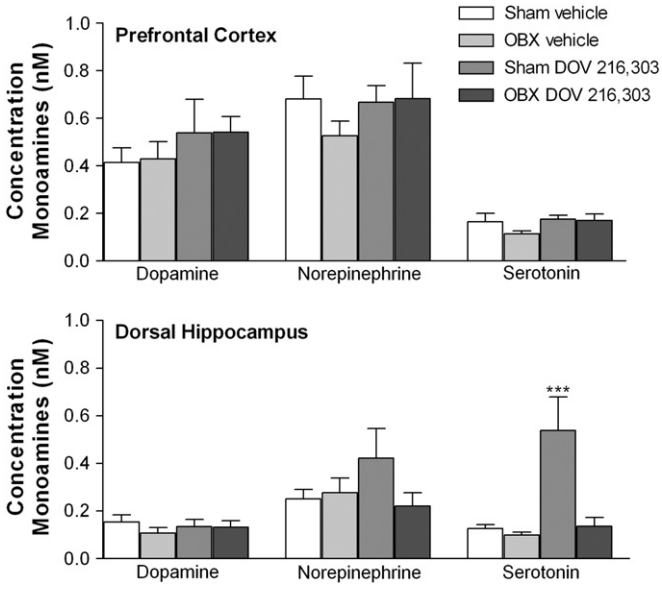

Fig. 3. Absolute baseline concentrations in prefrontal cortex (upper panel) and dorsal hippocampus (lower panel) in sham and OBX animals chronically treated with either vehicle or DOV 216,303. ***$p<0.001$ compared to DOV 216,3030-treated OBX and vehicle treated groups.

10.798, $p<0.01$), while baseline levels for dopamine and norepinephrine were not affected. (Fig. 3). One-way ANOVA with post-hoc Tukey tests revealed significantly higher baseline serotonin levels in the DH of chronic DOV 216,303-treated shams compared to the vehicle treated sham and OBX and the DOV 216,303-treated OBX ($F(3, 38) = 12.612$, $p<0.001$).

3.3. Extracellular monoamine levels in prefrontal cortex after drug challenge (Fig. 4)

3.3.1. Histology

Some animals were excluded from analysis based on several reasons. Either on incorrect microdialyis probe placement, or because of technical problems during microdialysis sampling or because of drop-out earlier in the experiment, twelve animals in the PFC group and eleven animals in the DH group were excluded. The number of animals used in each treatment group was sham vehicle–vehicle (PFC, $n=7$; DH, $n=8$), OBX vehicle–vehicle (PFC, $n=6$; DH, $n=6$), sham vehicle–DOV 216,303 (PFC, $n=5$, DH, $n=7$), OBX vehicle–DOV 216,303 (PFC, $n=5$; DH, $n=5$), sham DOV 216,303–DOV 216,303 (PFC, $n=7$; DH, $n=6$), OBX DOV 216,303–DOV 216,303 (PFC, $n=6$; DH, $n=5$).

3.3.2. Dopamine in PFC

An acute challenge of DOV 216,303 increased extracellular dopamine levels in the prefrontal cortex over time ($F(4, 54) = 9.503$, $p<0.001$, $\varepsilon = 0.215$) (Fig. 3A, B). A significant surgery x treatment interaction was found ($F(2, 28) = 4.853$, $p<0.05$). One-way ANOVA analysis per time point with Tukey post-hoc analysis revealed significantly lower dopamine levels at T 60 min and T 90 min in the acute DOV 216,303 treated OBX animals compared to their sham counterparts ($p<0.001$) (Fig. 4A). Analysis also revealed significantly lower extracellular dopamine concentrations in the chronically DOV 216,303 treated shams with DOV 216,303 challenge when compared to the sham animals with an acute DOV 216,303 challenge at timepoints T 30 min, T 60 min, and T 90 min ($p<0.01$). (Fig. 4B).

Sham animals, acutely treated with DOV 216,303 showed significant increased area under the curve (AUC) data for dopamine when compared to OBX animals after acute treatment with DOV 216,303 ($F(5, 30) = 11.858$, $p<0.05$) (Fig. 5). A trend was observed

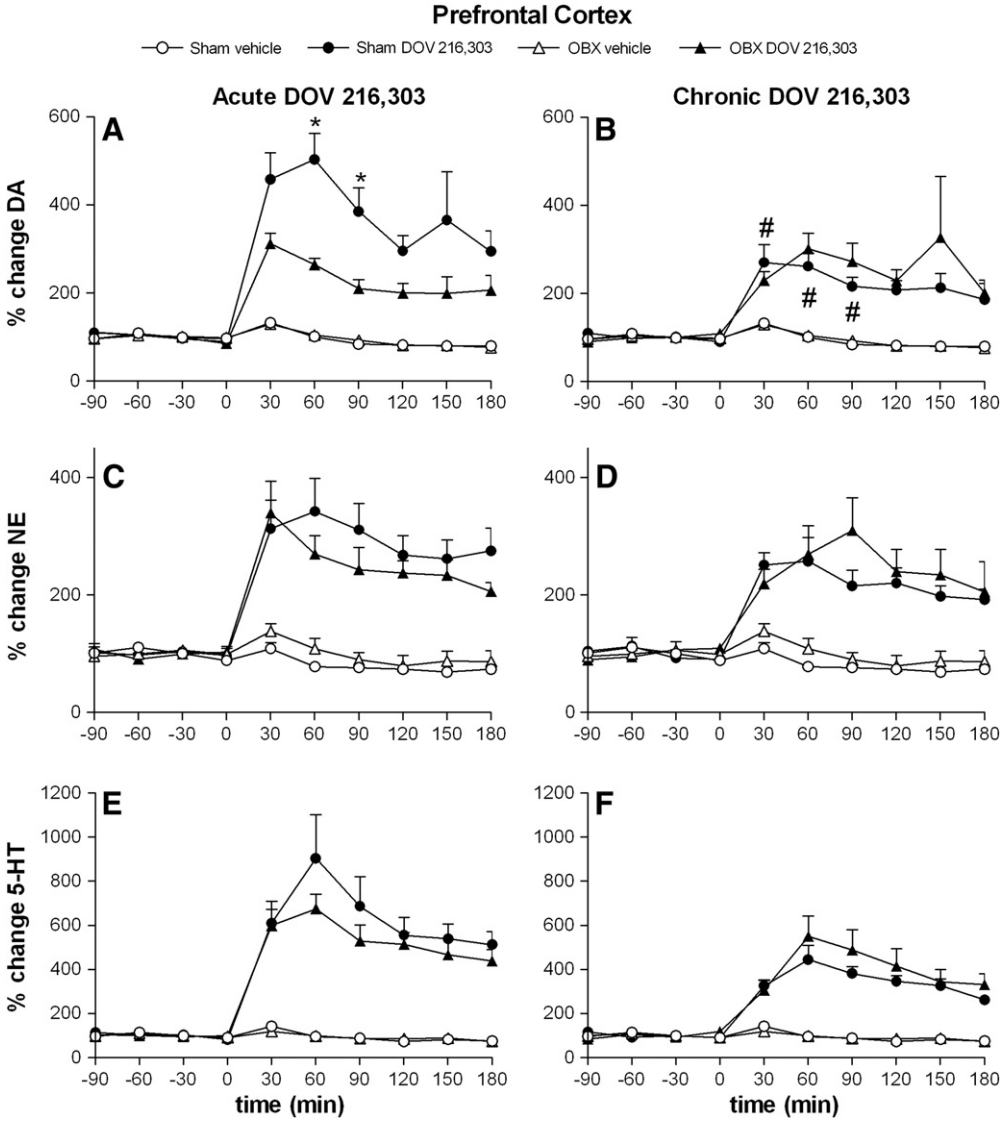

Fig. 4. Extracellular monoamine concentrations in prefrontal cortex five weeks after OBX/sham surgery, after an acute challenge of DOV 216,303 in chronic vehicle treated animals (A, C and E). And after a DOV 216,303-challenge in animals chronically treated with DOV 216,303 (B, D and F). Timepoints −90 till 0 min represents baseline measurements. At T = 0 a single injection with either DOV 216,303 or vehicle was given. Data is represented as percentage of baseline. *$p<0.01$, sham vehicle–DOV 216,303 compared to OBX vehicle–DOV 216,303. #$p<0.01$ sham DOV 216,303–DOV 216,303 compared to sham vehicle–DOV 216,303.

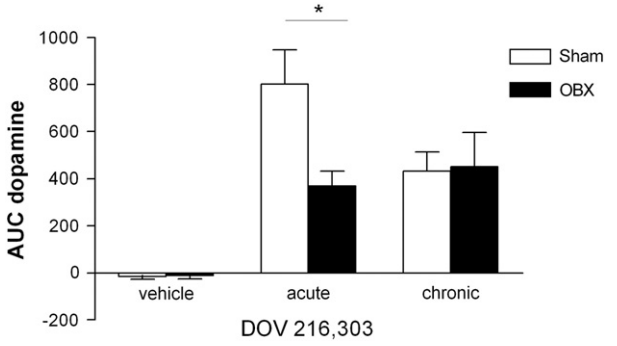

Fig. 5. Area under the curve (AUC) data from the dopamine response in prefrontal cortex of OBX and sham animals after treatment with vehicle–vehicle, vehicle–DOV 216,303 (acute) or DOV 216,303–DOV 216,303 (chronic). $^{*}p<0.05$.

towards a decreased AUC for dopamine in chronic DOV 216,303 treated shams, compared to an acute DOV 216,303 administration in sham animals ($p=0.85$) (Fig. 5).

3.3.3. Norepinephrine in PFC

Administration of DOV 216,303 during microdialysis resulted in significantly elevated extracellular norepinephrine levels over time ($F\ (7, 91)=15.450$, $p<0.001$, $\varepsilon=0.363$) (Fig. 4C, D). No effect of surgery ($F\ (1, 28)=0.001$, $p=0.975$) or surgery × treatment interaction could be observed ($F\ (2, 28)=1.006$, $p=0.358$).

3.3.4. Serotonin in PFC

DOV 216,303-challenge in vehicle treated animals as well as in chronic DOV 216,303-treated animals resulted in significantly elevated extracellular serotonin levels over time ($F\ (3, 48=30.902$, $p<0.001$, $\varepsilon=0.180$) (Fig. 4E, 4 F). No effect of surgery ($F\ (1, 30)=0.106$, $p=0.747$) or surgery × treatment interaction could be found ($F\ (2, 30)=1.146$, $p=0.332$).

3.4. Extracellular monoamine levels in dorsal hippocampus after drug challenge (Fig. 6)

3.4.1. Dopamine in DH

Administration of DOV 216,303 in vehicle treated animals as well as in chronic DOV 216,303 treated animals resulted in significantly

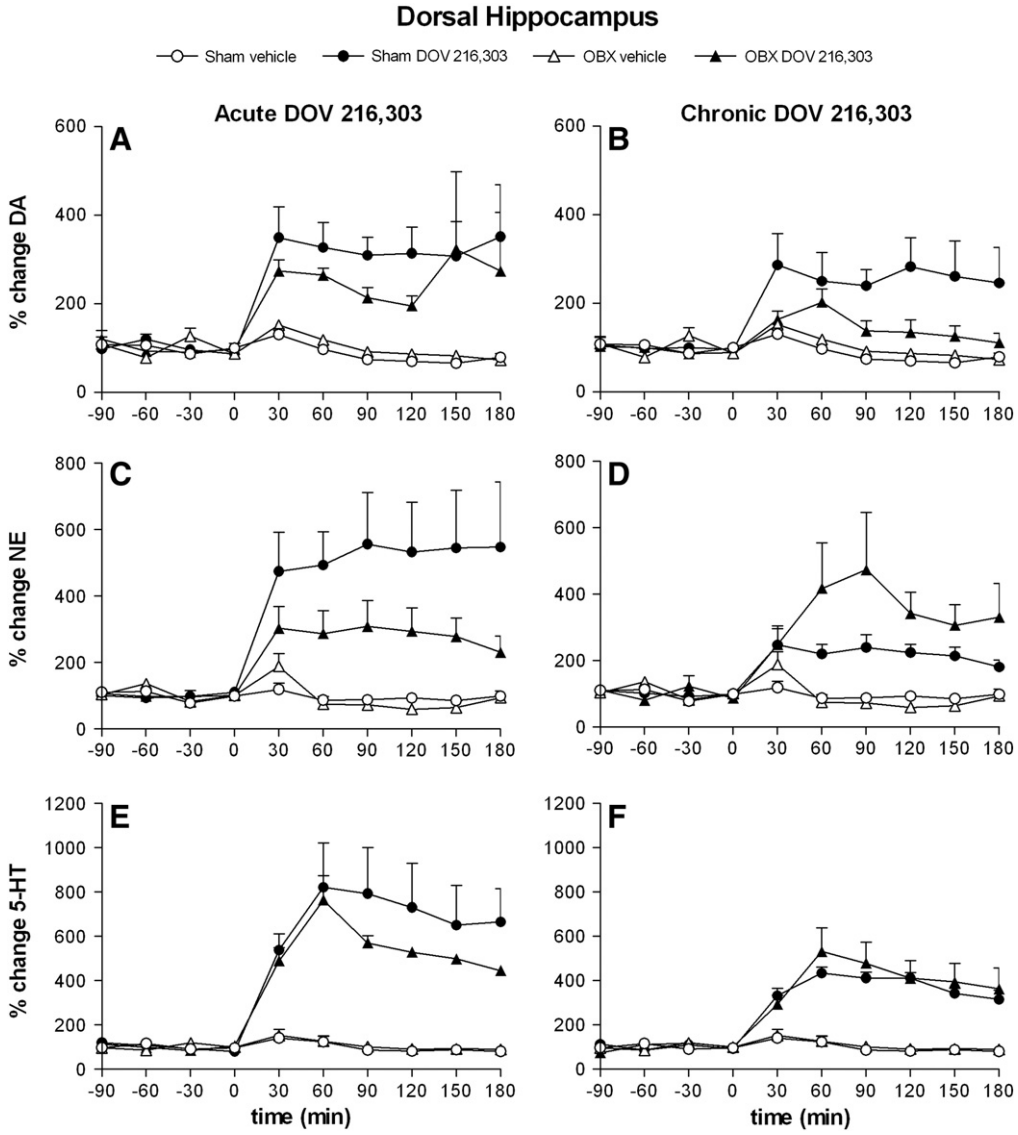

Fig. 6. Extracellular monoamine concentrations in dorsal hippocampus five weeks after OBX/sham surgery, after an acute challenge of DOV 216,303 in chronic vehicle treated animals (A, C and E). And after a DOV 216,303-challenge in animals chronically treated with DOV 216,303 (B, D and F). Timepoints −90 till 0 min represents baseline measurements. At T = 0 a single injection with either DOV 216,303 or vehicle was given. Data is represented as percentage of baseline.

elevated extracellular dopamine levels in the dorsal hippocampus over time ($F(4,54) = 3.884$, $p<0.01$, $\varepsilon = 0.215$) (Fig. 6A, B). Neither a significant effect of surgery ($F(1, 28) = 3.636$, $p = 0.067$), nor surgery × treatment interaction could be observed ($F(2, 28) = 1.724$, $p = 0.197$).

3.4.2. Norepinephrine in DH

Administration of DOV 216,303 during microdialysis resulted in significantly elevated extracellular norepinephrine levels over time ($F(4, 57) = 6.752$, $P<0.001$, $\varepsilon = 0.218$) (Fig. 6C, D). No effect of surgery ($F(1,29) = 0.368$, $p = 0.549$) and a trend towards a surgery × treatment interaction could be observed ($F(2,2) = 3.236$, $p = 0.054$).

3.4.3. Serotonin in DH

One single injection of DOV 216,303 in vehicle treated animals as well as in chronic DOV 216,303-treated animals resulted in significantly elevated extracellular serotonin levels over time ($F(3,46) = 18.341$, $p<0.001$, $\varepsilon = 0.171$) (Fig. 6E, F). No effect of surgery ($F(1,30) = 0.318$, $p = 0.577$) or surgery × treatment interaction could be found ($F(2,30) = 0.871$, $p = 0.429$).

3.5. DOV 216,303 plasma and whole brain levels (Fig. 7)

A highly significant effect was found of treatment on DOV 216,303 concentrations in brain ($F(2,21) = 119{,}972$, $p<0.001$) (Fig. 7A) and plasma ($F(2,21) = 66.173$, $p<0.001$) (Fig. 7B). DOV 216,303 brain and plasma levels, measured 30 min after drug administration, were significantly lower in the group that received chronic DOV 216,303 when compared to the group that received chronic vehicle. No difference between sham and OBX could be observed. Post-hoc analysis revealed significant differences in DOV 216,303 concentrations between all treatment groups ($p<0.001$).

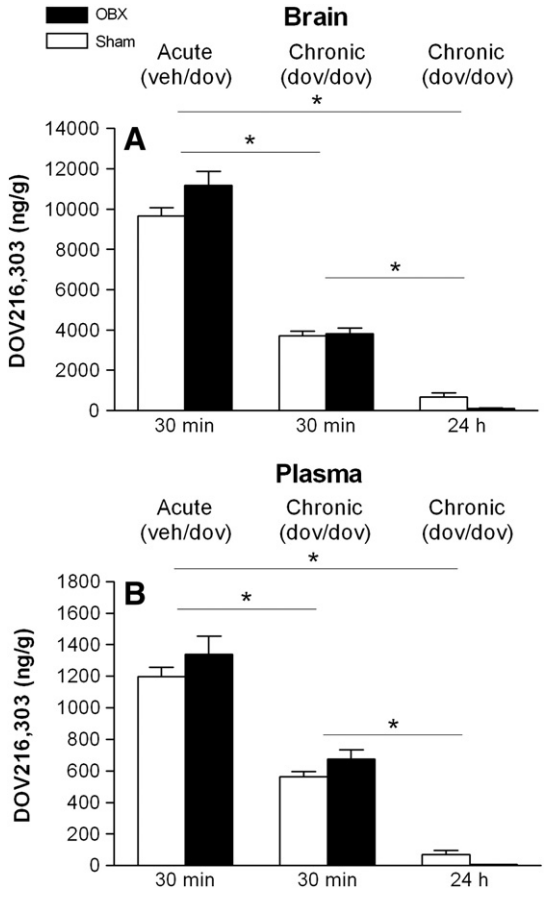

Fig. 7. Concentration of DOV 216,303 in whole brain (A) and blood plasma (B) of OBX and sham animals. 30 min are animals treated chronic with vehicle (veh/dov) or DOV 216,303 (dov/dov) and received a DOV 216,303 challenge 30 min before sacrifice. 24 h are animals chronic treated with DOV 216,303 (dov/dov) and sacrificed 24 h after the last injection of DOV 216,303. *$p<0.001$.

3.6. Body weights

The weights of the different treatment groups (mean ± S.E.M.) at the start and the end of treatment were as followed: Sham–vehicle 375 ± 5 g and 394 ± 5 g, Sham–DOV 216,303 376 ± 4 g and 380 ± 7 g, OBX–vehicle 362 ± 4 g and 381 ± 5 g, OBX–DOV 216,303 356 ± 6 g and 360 ± 4 g. At the start of treatment, three weeks after surgery, sham animals significantly weighed more that OBX animals ($F(1,41) = 7.894$, $p<0.01$). All animals increased in weight during treatment, however chronic DOV 216,303-treated OBX animals gained less weight than vehicle treated OBX animals ($F(1, 19) = 6.945$, $p<0.05$).

4. Discussion

Acute administration of the triple reuptake inhibitor (TRI) DOV 216,303 in olfactory bulbectomized (OBX) rats, five weeks after surgery, results in an increase in dopamine (DA), norepinephrine (NE) and serotonin (5-HT) in the prefrontal cortex (PFC) and dorsal hippocampus (DH). Only for DA in the PFC this increase was lower in OBX than sham animals. For the other monoamines (NE and 5-HT) no difference was found between sham and OBX. Thus, a hypodopaminergic state may be responsible for the long term behavioral depressive-like effects in the OBX model. The same challenge with DOV 216,303 in chronic DOV 216,303 treated animals also leads to a significant increase in the same three neurotransmitters. In the chronically treated animals no differences were found between sham and OBX on monoamine release. However, the same challenge of DOV 216,303 elicits higher increases in DA levels in the PFC of sham animals when given for the first time than in shams chronically treated with DOV 216,303 at the same time point after surgery.

Our microdialysis data are in accordance with our previous results which showed that an acute DOV 216,303 challenge leads to a lower DA response in the prefrontal cortex of OBX rats when compared to sham animals (Prins et al., 2010). However, in our previous study, we measured this DA release one day after the OBX surgery. It appears that the same DOV 216,303 challenge five weeks after surgery leads to a similar blunted response in these OBX animals. A recent study found that three weeks after OBX, amphetamine-induced lowered ICSS thresholds were more profound in sham animals than in OBX animals. They also showed that OBX animals had a long-lasting reduction in sucrose intake, suggesting that these animals have a dysfunctional reward circuitry and respond less to normal pleasurable stimuli (Romeas et al., 2009). Our finding that OBX animals can release less DA than their sham counterparts five weeks after surgery is a possible explanation for this blunted response to rewarding stimuli. Another study reported increased ICSS thresholds in rats following OBX surgery, suggesting an anhedonic state of the animals (Slattery et al., 2007). Remarkably, this effect only lasted for eight days, therefore it may have been due to a surgery effect rather than a disrupted reward circuitry. Nevertheless, in our previous microdialysis study we found lower baseline DA levels one day after OBX, which is consistent with the data from Slattery and co-workers, assuming that lower DA levels in the prefrontal cortex correlate with higher ICSS thresholds and thus an anhedonic state.

In our previous study a challenge with DOV 216,303 seemed to result in lower extracellular monoamine concentrations in chronically DOV 216,303 treated animals compared to the same challenge in untreated animals (Prins et al., 2010). However, a definitive conclusion could not be made at the time, due to limitations in the experimental design and the absence of specific controls. In the current experiment we reproduced our previous findings. A single

administration of DOV 216,303 in chronic DOV 216,303 treated animals led again to a blunted response on DA release compared to the same challenge given to chronic vehicle treated shams. Only the DA release in the prefrontal cortex was significant. For the other monoamines and in the dorsal hippocampus this blunted response to a DOV 216,303 challenge was also marginally present in sham animals, albeit not significant. One possible explanation could be that tolerance to the effects of the drug has developed (Cohen and Baldessarini, 1985) or that less drug is available in chronic treated animals, due to an increased drug metabolism in chronic treated animals (Brosen and Naranjo, 2001). Our results contrast with a previous study in which Van der Stelt and co-workers showing that OBX animals had permanent deficits in serotonergic function; OBX rats showed lower 5-HT levels in the basolateral amygdala (BLA) and dorsal hippocampus two weeks and five months after surgery. When they blocked the 5-HT transporter (5-HTT) with fluvoxamine, the increase in 5-HT was smaller in OBX animals than in shams (van der Stelt et al., 2005). We did not see such a difference between OBX and sham animals on extracellular 5-HT concentrations in DH and PFC after blockade of the 5-HTT with DOV 216,303. However, this difference could be explained by different binding occupancies of the 5-HTT by fluvoxamine (Suhara et al., 2003) and DOV 216,303 (Chen and Skolnick, 2007; Micheli et al., 2010).

To further explore why chronically treated animals display a blunted monoamine response, the effects of chronic and acute administration of DOV 216,303 on the concentrations of this compound in blood plasma and whole brain were examined. A major finding of the present study is that 30 min after DOV 216,303 administration, concentrations of DOV 216,303 in whole brain and plasma were significantly reduced in chronic treated DOV 216,303 animals, when compared to rats that were presented with DOV 216,303 for the first time. Moreover, very little DOV 216,303 was still present in the brain or plasma 24 h after an injection with DOV 216,303 in chronic DOV 216,303 treated animals. Therefore we conclude that in chronic treated animals, DOV 216,303 is cleared more rapidly from the body than when given for the first time. However, this finding may be species specific, In a human study, DOV 216,303 was given once and daily for ten days and the plasma concentrations did not differ between day one and ten (Beer et al., 2004). The mean elimination half-time after one single dose was 3.3 to 4.4 h for six different doses, which means that after 24 h the compound should have been cleared from the body. This is confirmed by our data that 24 h after injection almost no DOV 216,303 is present in chronic treated animals. A reason for the increased elimination of DOV 216,303 after chronic use could be changes in the pharmacokinetic properties of the drug such as liver enzymes upregulation leading to faster rates of elimination from the body (Brosen and Naranjo, 2001), a phenomenon which can occur after treatment with SSRIs or other antidepressants (DeSanty and Amabile, 2007; Solomons et al., 2005). The consequence of these findings in patients is that DOV 216,303 should be given twice or three times a day in order to be clinically effective (Beer et al., 2004; Skolnick et al., 2006). However, these are only speculations; based on the present study we cannot conclude anything about an induction of liver enzymes. Further experiments are needed to confirm this hypothesis.

An increased elimination of DOV 216,303 might also explain an apparent discrepancy between the present behavioral data and previous work from our lab. Here, we also looked at the effect of DOV 216,303 on OBX-induced hyperactive locomotion in the open field test. Neither acute, nor chronic treatment with DOV 216,303 could normalize OBX-induced hyperactivity. In a previous study in our lab chronic drug treatment, but not acute administration of DOV 216,303, had antidepressant effects in the OBX animal model of depression (Breuer et al., 2008). An important difference between our study and the study from Breuer and co-workers is that in the present study the chronic open field was performed 24 h after the last injection, while in the study from Breuer and colleagues, the animals had received the last injection 30 min before the chronic open field. These behavioral data are completely in line with the DOV 216,303 concentrations we measured in blood and brain of chronically DOV 216,303 treated animals. Thirty min after the DOV 216,303 injection DOV 216,303 was still systemically available, while 24 h after the DOV 216,303 injection no DOV 216,303 was present any longer.

Although, in our hands DOV 216,303 does not seem to be the perfect alternative candidate for SSRI antidepressant treatment, the important role of dopaminergic function in the pathophysiology of major depressive disorder should not be underestimated (D'Aquila et al., 2000; Guiard et al., 2009; Naranjo et al., 2001). Recently it has been shown that the D_2/D_3 receptor agonist pramipexole acted by augmenting the antidepressant effect while given together with regular antidepressant pharmacological treatment (Goldberg et al., 2004; Gupta et al., 2006). Chronic administration of pramipexole showed antidepressant effects in the OBX animal model (Breuer et al., 2009a). Furthermore, pramipexole seemed to be effective in reducing anhedonia in Parkinson's patients as an additive treatment to L-dopa (Lemke et al., 2006) giving further evidence for a role for DA in anhedonia. Several antidepressants, each acting on one or more monoamines, are involved in the therapeutic effect of different behavioral aspects of depression (Katz et al., 2004). Drug-induced changes in the serotonergic system seem to be associated with anxiolytic and antidepressant effects, while changes in NE are primarily involved in reducing psychomotor symptoms in humans diagnosed for depression (Katz et al., 1994). In animals, the same distinction can be found in that SNRIs increase climbing behavior without affecting swimming in the forced swim test, whereas SSRIs increased swimming without affecting climbing (Lucki, 1997).

In summary, the present study showed that even five weeks after surgery, OBX animals showed lower DA levels upon acute administration with the TRI DOV 216,303. This is consistent with the notion that this animal model possesses an anhedonia component. Furthermore, chronic treatment with DOV 216,303 resulted in a blunted extracellular monoamine response in sham animals after a DOV 216,303 challenge, most likely due to the lower brain and plasma drug levels following repeated treatment. Surprisingly, chronic DOV 216,303 treatment did not have antidepressant behavioral effects in the present OBX animals. Interestingly, by measuring the drug plasma and brain levels, it was demonstrated that at the time of behavioral testing (24 h after last drug treatment) DOV 216,303 was not present anymore in either plasma or brain. This seems to indicate that this putative antidepressant drug has no lasting antidepressant-like behavioral effects in the absence of the drug in the brain.

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