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Effects of intranasal administration of Pregnenolone on Acetylcholine in regions of the rat brain assessed by in-vivo microdialysis

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II. Acknowledgments

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III. Abstract

The present study determined the effects of intranasal pregnenolone (IN-PREG) on acetylcholine (ACh) levels in selected areas of the rat brain. In the first experiment, IN-PREG (5.6 and 11.2 mg/mL) or vehicle was applied bilaterally, and ACh levels were assessed in amygdala, hippocampus and frontal cortex, using in vivo microdialysis. In the second experiment, IN-PREG (11.2 mg/mL) or vehicle was applied only into one nostril in order to determine whether ACh is predominantly increased in the ipsilateral relative to the contralateral amygdala. Bilateral IN-PREG (5.6 and 11.2 mg/mL) increased frontal cortex and hippocampal ACh in a time-dependent fashion relative to both baseline and vehicle. Moreover, 11.2 mg/mL PREG increased ACh in the amygdala relative to baseline, the lower dose and vehicle. Unilateral application of IN-PREG increased ACh in the ipsilateral amygdala, whereas no effect was observed on the contralateral side. Findings suggest that PREG is effectively transported from the nostrils to the brain via the olfactory epithelial pathway but not by circulation. The present data provide additional information on the time- dependency of IN-PREG action in the cholinergic system of frontal cortex and hippocampus. This may be relevant for therapeutic IN application of PREG in neurogenerative diseases and neuropsychiatric disorders.

IV. Zusammenfassung

Die vorliegende Studie ermittelte die Auswirkungen von intranasal verabreichten Pregnenolone (IN-PREG) auf den Acetylcholin-Spiegel (ACh) in ausgewählten Regionen des Gehirns. In dem ersten Experiment wurde IN-PREG (5.6 und 11.2 mg/mL) oder Gel-Trägerstoff bilateral verabreicht, und der ACh-Spiegel wurden in der Amygdala, dem Hippokampus und dem frontalen Kortex mit in vivo Mikrodialyse gemessen. In dem zweiten Experiment wurde IN-PREG (11.2 mg/mL) oder Gel-Trägerstoff unilateral verabreicht, um festzustellen, ob ACh im ipsilateralen Wert gegenüber der kontralateralen Amygdala überwiegend erhöht ist. Bilaterales IN-PREG (5.6 und 11.2 mg/mL) erhöhte den ACh-Spiegel im frontalen Kortex und Hippokampus zeitabhängig im Verhältnis zur Grundlinie und oder Gel-Trägerstoff. 11.2 mg/mL IN-PREG erhöhte den ACh-Wert in der Amygdala im Vergleich zur Grundlinie, der niedrigeren Dosis und des Gel-Trägerstoff. Die einseitige Anwendung von IN-PREG erhöhte den ACh-Wert in der ipsilateralen Amygdala, während keine Wirkung auf der kontralateralen Seite beobachtet wurde. Die Ergebnisse deuten darauf hin, dass PREG effektiv von den Nasenlöchern zum Gehirn über den olfaktorischen Epithelweg transportiert wird, aber nicht durch Zirkulation. Die vorliegenden Daten liefern zusätzlich Informationen zur zeitlichen Abhängigkeit der IN-PREG Wirkung im cholinergen System des frontalen Kortexes und des Hippokampus. Dies kann für therapeutische IN-Anwendungen und neuropsychiatrische Erkrankungen relevant sein.

V. Abbreviations

3βHSD	3beta-hydroxysteroid dehydrogenase
A (r, l)	amygdala (right, left)
ALLO	allopregnanolone
ACh	acetylcholine
AD	Alzheimer´s disease
BBB	blood-brain barrier
BF	basal forebrain
CB1	type-1 cannabinoid
CNS	central nervous system
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulfate
F	frontal cortex
GABA	gamma-aminobutyric acid
H	hippocampus
HPLC-EC	high-performance liquid chromatography electrochemical detection
HST	cytosolic hydroxysteroid sulfotransferase
IN	intranasal administration
i.p.	intraperitoneal injection
min	minutes
NBM	nucleus basalis magnocellularis
NMDA	N-methyl-D-aspartate
P450scc	cholesterol side-chain cleavage enzyme
P450c17	cytochrome P450 17 alpha-hydroxylase
PBS	phosphate-buffered saline
PD	Parkinson’s disease
PDD	Parkinson’s disease with dementia
PREG	pregnenolone
PREGS	pregnenolone sulfate
PROG	progesterone

1. Introduction

The cholinergic system has a crucial role for intact cognitive performance, including attention and memory functioning. The basal forebrain (BF) cholinergic system regulates both cortical and subcortical structures. Cortical acetylcholine (ACh) exerts its role in top-down control over attention and stimulus discrimination (Sarter et al. 2016). Cholinergic subcortical projections to hippocampus and amygdala are involved in the regulation of specific types of memory, respectively spatial memory and emotional-involved memories (Burgess et al. 2002; Janak and Tye 2015). The cognitive decline due to impaired cholinergic system has been evidenced in neurodegenerative diseases, for instance Alzheimer´s disease (AD) and Parkinson´s disease (PD). In AD neurofibrillary tangles and amyloid plaques are responsible for neuronal loss in the basal forebrain (BF) and loss of cholinergic markers in the cortex. This neuronal degeneration leads to the impairment of memory and attention processes in the AD (Whitehouse et al. 1982; Geula and Mesulam 1989). Moreover, patients affected by PD and PD with dementia (PDD) also incur cognitive function decline. In fact, cholinergic neuronal loss in the BF has been found in PD patients and it has been proposed being induced by aggregates of alpha synuclein into Lewi bodies (Perry et al. 1995). Therefore, it is important to investigate pro-cholinergic drug treatments, which might reduce cognitive impairments.

Neurosteroids are a category of steroids which are synthetized in the central nervous system (CNS) and are involved in brain function (Baulieu and Robel 1990). In mitochondria the cholesterol side-chain cleavage enzyme (P450scc) catalyzes the synthesis of pregnenolone (PREG) upon cholesterol (Harteneck 2013). PREG is a neuroactive steroid which can be converted into different steroids such as dehydroepiandrosterone (DHEA), testosterone, progesterone, estrogen and cortisol. In contrast to the described steroid hormones transcription regulation function, named as the genomic pathway, neurosteroids are involved in non-genomic pathways, as no nuclear receptor has been so far identified (Weng and Chung 2016). The non-genomic pathways can occur at the plasma membrane level and in the cytoplasm. In the cytoplasm, neurosteroid action of PREG is exerted by sigma 1 receptor binding (Su et al. 1988; Monnet and Maurice 2006). When the sigma 1 receptor is activated, it translocates from the endoplasmic reticulum membrane to

either other organelles or to the plasma membrane, modulating neurotransmitter responses (Hayashi and Su 2001; Maurice et al. 1998). Sigma 1 receptor activation potentiates NMDA-evoked responses as shown in both in vivo and in vitro studies (Monnet et al. 1992; Monnet et al. 1990). Moreover, sigma receptor 1 receptor agonists modulate the cholinergic system (Matsuno et al. 1992; Matsuno et al. 1995). Thus, PREG may be transformed into pregnenolone sulfate (PREG-S) through sulfotransferase catalysis (Robel et al. 1995). This compound both disinhibits (through negative modulation of GABAA receptors) and activates (through positive modulation of NMDA receptors) the cholinergic neurons of the medial-septal diagonal band of Broca, which project to the hippocampus (Darnaudéry et al. 2002). This activity represents the non-genomic pathway at the plasma membrane (Rudolph et al. 2016). It has been stated that the presence of sulfate group is necessary for the GABAA receptor inhibition (Weaver et al. 2000). Moreover, the negatively charged group at the carbon C3 position of the steroid core on PREG-S is central in the potentiation of NMDA receptor function, in contrast to the uncharged PREG, which instead is considered to have a minimal effect (Weaver et al. 2000). Nevertheless, PREG indirect activation of NMDA receptors via sigma receptor modulation has been shown (Monnet et al. 1990; Nuwayhid and Werling 2003; Schverer et al. 2018). Various studies have investigated possible interactions between PREG or PREG-S and the cholinergic system. Intracerebroventricular (icv) administration of PREG-S induced memory-enhancing effect in mice, compensated scopolamine-induced learning deficits in visual discrimination tasks in rodents and improved spatial memory (Flood et al. 1992; Meziane et al. 1996; Darnaudéry et al. 2000). Similarly, when injected into the amygdala, PREG-S enhanced memory processes in mice (Flood *et al.* 1995). Moreover, PREG-S increased acetylcholine (ACh) release in the hippocampus (Darnaudéry *et al.* 2000). Likewise, intraperitoneal or bilateral intrahippocampal injection of PREG-S ameliorated memory deficits in cognitively impaired rats and stimulated ACh release in the rat hippocampus (Vallée *et al.* 1997).

Also, PREG, the precursor of PREG-S, is neurochemically active. Nuwayhid and Werling (2003) demonstrated that PREG inhibits the NMDA-stimulated dopamine release in the striatum via sigma receptors with involvement of the coupled-PKCß pathway. Therefore, PREG, acting as a sigma receptor agonist and/or after being metabolized to PREG-S, may increase ACh levels in the rat and human brain

(Darnaudéry et al. 2000; Su et al. 1988; Matsuno et al. 1992; Matsuno et al. 1995). Since intranasal administration allows circumvention of the blood brain barrier, the delivery of compounds directly from nose to brain has been established as a key alternative to oral and parenteral modes of administration. Intranasally applied PREG (IN-PREG) has also been shown to influence memory and cognitive processes. Abdel-Hafiz *et al.* (2016) reported that pre-trial IN administration of PREG facilitated long-term memory in both object-preference and object-location preference tests 48 hours post-administration in rats. Besides, the treated animals showed a superior memory for the position of the escape platform in the Morris water maze compared to controls (Abdel-Hafiz *et al.* 2016).

Ducharme *et al.* (2010) compared the brain distribution and behavioral effects of PREG after IN and intravenous administration. Intravenous administration of PREG was associated with higher stability in the blood, while IN administration was associated with higher stability in the brain. Also, PREG was degraded to a lesser extent in the brain after intranasal relative to intravenous injection (Ducharme *et al.* 2010). Intranasally applied diffusable substances, including steroids, are delivered via the olfactory epithelial pathway into the perineural spaces of the olfactory nerve in the ipsilateral olfactory bulb and into the subarachnoid space of the brain and central nervous system (CNS; (Baker and Spencer 1986; Shipley and Ennis 1996; Frey *et al.* 1997; Chen *et al.* 1998; Illum 2000). Behavioral studies have suggested links between pregnenolone administration and the cholinergic system, but neurochemistry analysis in specific brain areas is needed to understand in detail the neurochemical action of pregnenolone (Ducharme et al. 2010; Abdel-Hafiz et al. 2016). For this reason, in this study we analyzed the neurochemical and pharmacological effects of IN-PREG on the cholinergic system in rat using the in vivo microdialysis technique. In vivo microdialysis is a sampling technique to collect extracellular tissue fluids, widely used for instance in neuropharmacology to investigate the effects of drugs in neurotransmitters and neuropeptides release in the brain tissues (Bourne 2003). This technique, thus, provides important information on new drug pharmacodynamic profiles. Via a semi-permeable membrane, perfused with a physiological liquid (named the perfusion liquid), introduced in the brain tissue, molecules in the brain tissue of interest passively diffuse across a concentration gradient, Fick´s law of diffusion, then collected to be further analyzed. The advantage of this technique in neuropharmacology research is the possibility to continuously

collect dialysate in both anaesthetized animals and in awake freely moving animals before and after drug administration. The microdialysis approach has been widely used to investigate drugs effects on the cholinergic system and to investigate the role of Ach release in behavioral settings (Westerink 1995). The goal of this study was to investigate the effects of IN administered PREG on the extracellular ACh concentrations over a period of 100 min in frontal cortex, hippocampus and amygdala of the rat brain using in vivo microdialysis. Previous studies have shown that the unilateral IN administration of L-DOPA increased extracellular dopamine only in the ipsilateral neostriatum, excluding the systemic transport of the drug from the nose to the CNS (de Souza Silva *et al.* 1997a; de Souza Silva *et al.* 1997b). This prompted us to assess the circuitry of PREG action, by measuring ACh release in the ipsilateral and contralateral amygdala after unilateral IN application of PREG.

2. Methods Overview

In the following section, a brief summary of the materials and methods used in this dissertation is described. For more details, the reader can refer to the method sections of the submitted article provided in the appendix.

2.1 Surgical procedures

Animals

Between three- and four-months old male Wistar rats, (purchased from Janvier, Tierversuchanlage, University of Düsseldorf, Germany) were used. Before the microdialysis probe implantation, the animals were group-housed in Makrolon cages (Type IV; 60 x 38 x 20 cm); after the brain surgery, the rats were individually kept in cages (40 x 26 x 26 cm). Their average weight at the time of the surgery 450 g. Rats were kept in ad-libitum conditions for water and food, the room humidity (60% humidity) and temperature (20 ± 2°C) were controlled, and the light-dark cycle rhythm was reversed (lights off from 7:00 to 19:00).

No samples from the frontal cortex samples were collected in 13 animals (vehicle: n= 3, 5.6 mg/mL PREG: n=5, 11.2 mg/mL PREG: n=5). No samples from the hippocampus were collected in 12 animals (vehicle: n=5, 5.6 mg/mL PREG: n=4, 11.2 mg/mL PREG: n=3). No samples from the amygdala were collected in 13 animals (vehicle: n=4, 5.6 mg/ml PREG: n=4, 11.2 mg/mL PREG: n=5). Cannula localization was incorrect in the frontal cortex of 7 animals (vehicle: n=2, 5.6 mg/mL PREG: n=2, 11.2 mg/mL PREG: n=3), in the hippocampus of 6 animals (vehicle: n=1; 5.6 mg/mL PREG: n=3; 11.2 mg/mL PREG: n=2) and in the amygdala of 2 animals of the PREG 11.2 mg/mL group. Moreover, because of undetectable ACh concentration or low signal-noise ratio in the HPLC analyses, 8 samples of the frontal cortex (vehicle: n=2, 5.6 mg/mL PREG: n=4, 11.2 mg/mL PREG: n=29, 5 samples of the hippocampus (vehicle: n=1, 5.6 mg/mL PREG: n=3, 11.2 mg/mL PREG: n=1) and 11 samples of the amygdala (vehicle: n=1, 5.6 mg/mL: n=5,11.2 mg/mL PREG: n=5) were excluded. In experiment 2, a total of 16 animals were used. However, due to failed microdialysis probes, only in 10 animals it was possible to collect samples from both amygdalae.

Implantation guiding cannulae

To investigate the effects of IN-PREG in the release of ACh in frontal cortex, hippocampus and amygdala in experiment I, and in both amygdalae in experiment II, rats underwent stereotaxic brain surgery.

The animals were anaesthetized i.p. with a mixture of ketamine hydrochloride (90.0 mg/kg) and xylazine hydrochloride (8.0 mg/kg). After being anaesthetized, the animals were placed on a heating pad for monitoring the body temperature and their heads were fixed in the stereotactic frame (David Kopf Instruments). Subcutaneous local anesthesia of 0.1 mL Bucain (DeltaSelect, Germany) was injected prior to proceeding with skull exposure. Then stainless steel guide cannulae for the microdialysis probes were implanted (length 14 mm; 22 gauge; 30 mm diameter).

Rat brain atlas, Paxinos and Watson 1996, was used for accurate validation of the brain coordinates for: anterior-posterior (A-P), medial-lateral (M-L), and dorsal-ventral (D-V) planes, specific for each brain area. The stereotactic coordinates were previously calculated on the relative distances to the bregma point, used as a reference location.

In experiment 1, the probes were implanted unilaterally above frontal cortex and hippocampus and the amygdala, contralaterally (de Souza Silva *et al.* 2013). The coordinates were: frontal cortex [anterior-posterior (AP): + 3.7 mm; medial-lateral (ML): ± 3.0 mm; dorsal-ventral (DV): − 1.5 mm], the hippocampus (AP: − 6.0 mm; ML: ± 4.8 mm; DV: − 3.2 mm) and contralateral amygdala (AP: − 2.5 mm; ML: ± 4.6 mm; DV: − 7.2 mm) (Figure 1. A, B, C).

In experiment 2, each rat had microdialysis probe implants on the right and on the left amygdala, (AP: − 2.5 mm; ML: ± 4.6 mm; DV: − 7.2 mm) (Figure 1. D).

To provide cannulae a stable hold, two additional stainless screws were inserted in the bone. After implantation, dental cement was then used to fix the implants. Subsequently, local analgesic, Carprofen (5 mg/kg; Pfizer, Germany), carried by phosphate-buffered saline, was injected in the head-neck area. The animals were individually kept in cages at least 3 days before the microdialysis day, to fully recover from the surgery.

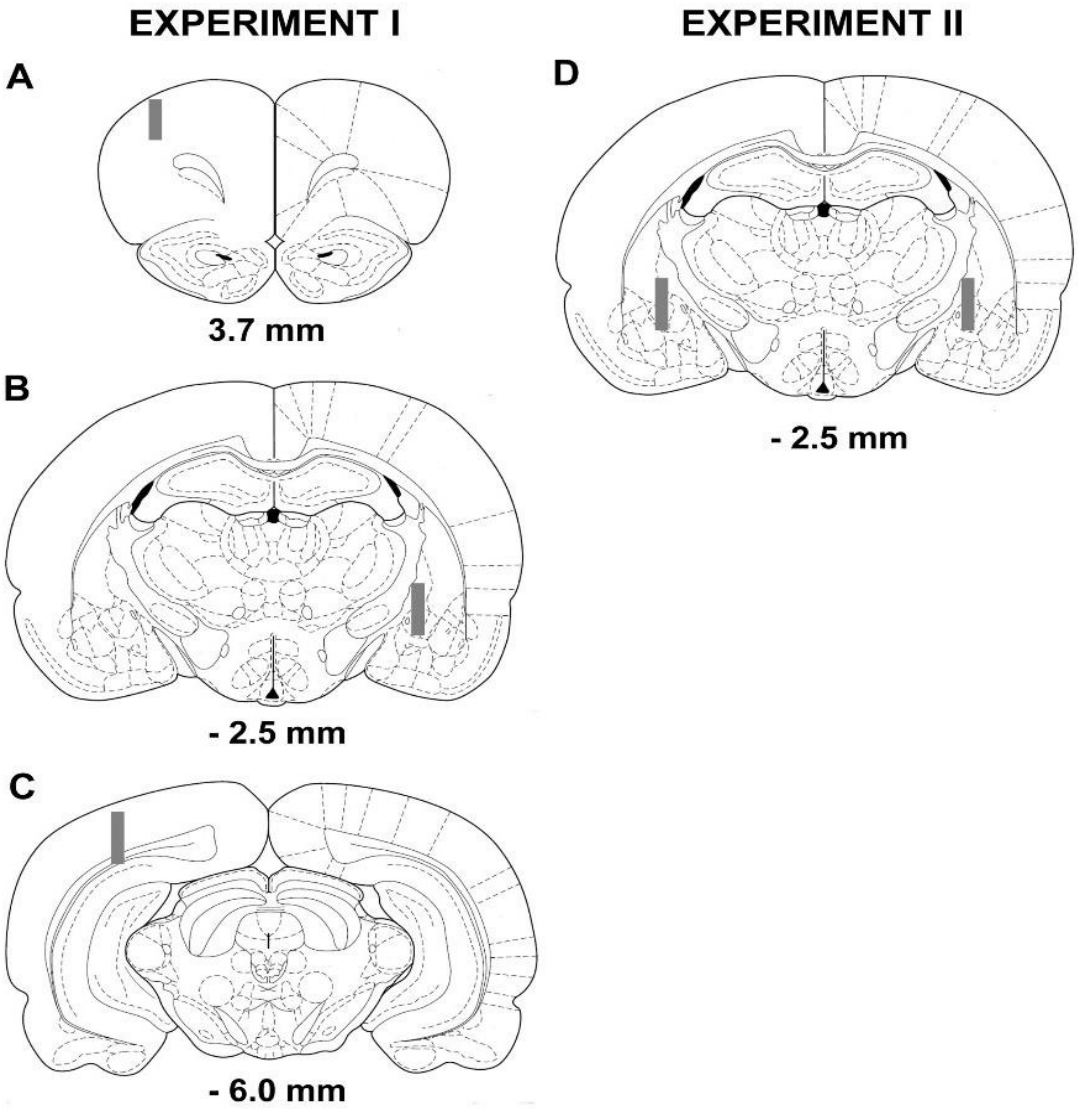

Figure 1. Schematic of microdialysis cannulae implantation. In experiment I (A, B, C), the implants were in the frontal cortex and hippocampus, and contralaterally in the amygdala. The locations were counterbalanced, to investigate both hemispheres. In experiment II (D), the rats underwent bilateral implants in the amygdalae. Grey parts represent the dialyzed areas of the brain. Numbers indicate the distance from bregma in mm (Paxinos and Watson 2007).

2.2 Microdialysis

Microdialysis procedure

Microdialysis is an in-vivo sampling procedure, which allows continuous measurements of substances in tissues. In our experiments we collected neurotransmitters from brain tissues.

Prior the microdialysis sampling, the animals were anaesthetized with Urethane i.p. (1.25g/kg; non-survival; Sigma Aldrich, Saint Louis, Missouri, USA) and a catheter was placed i.p., to allow 0.2 mL every 10 minutes fluid supply, to prevent animal dehydration (Ringer´s solution; B. Braun, Melsungen, Germany). During microdialysis procedure, the rat was kept in an acrylic box (45 x 25 x 22 cm) on a heating pad (CMA/150, Carnegie Medicin, Stockholm, Sweden), to provide stable body temperature of 36 ± 0.5 C° throughout the experiment duration. After attesting the correct functioning of the microdialysis probe, the probes were inserted into the brain through the cannulae. The inlet tubes were perfused with Ringer´s solution which contained Neostigmine (10μM; Sigma-Aldrich, Saint Louis, Missouri, USA), pumped at a 2μL/min flow with the microinfusion pump (CMA/100, Carnegie Medicin, Stockholm, Sweden). Neostigmine is an acetylcholine esterase inhibitor and it is used to prevent acetylcholine clearance; neostigmine in concentration of 10μM was shown to facilitate the detection of low concentration of ACh and inhibiting its clearance (Marshall & Wurtman, 1993; Vinson & Justice, 1997). After the 2h stabilization time after probe insertion, dialysate sampling of 20μL every 10 minutes were collected. 6 samples, corresponding to 6-time bins of 10 minutes, represent the individual fluctuation of neurotransmitters, which represents the baseline measurements to compare with neurotransmitter content after drug administration. At the end of the collection of the 6 baseline samples, the rat was administered vehicle or PREG into the nostrils. In experiment I, bilateral IN administration of 5μL of vehicle or low PREG dose (5.6 mg/mL PREG) or high PREG dose 11.2 mg/mL) was administered. In experiment II, each rat received 5μL of high PREG dose in one nostril and 5μL of vehicle in opposite one. In each sampling tube, 10μL of internal standard (25 μL of 125 mg/mL ethylhomocholine in 100 mL of NaOH diluent) was present and the dialysate volume was 20μL. Dialysate samples were stored at -80 °C until neurochemical analysis.

Intranasal pregnenolone administration

Drug formulation was a lipid-based gel containing either a low concentration of PREG (Bayer HealthCare Pharmaceuticals; Berlin, Germany) (5.6 mg/mL) or high concentration of PREG (11.2 mg/mL). Moreover, a vehicle lipid-based gel was used as a control. The drugs were provided by M et P Pharma AG (Emmetten, Switzerland). Considering the mean weight of the rats that received the drug via IN

was 450 g, the administered PREG dose for the low and high dose respectively was PREG 0.124 mg/kg, PREG 0.249 mg/kg. Both nostrils were given 5µL of gel each with a Microman pipette (Gilson, Villiers le Bel, France). In experiment I bilateral administration of the same drug, PREG 5.6 mg/mL or PREG 11.2 mg/mL, or vehicle, was administered to each rat. In experiment II, each rat received the high dose of PREG in one nostril and vehicle in the nostril of the opposite side.

2.3 Neurochemical and histological analysis

Histology

After the microdialysis procedure, the rats were given an overdose of Pentobarbital-Natrium (0.5 -1 mL). Then the rats were transcardially perfused. This procedure provides a rapid and uniform preservation of the brain tissue for further sectioning and staining. The perfusion procedure, with phosphate-buffered saline (PBS) and 10% formalin, was performed, by exploit of circulatory system to uniformly perfusing the fixative. The dissected brains were then stored in vials containing 10% formalin and 30% sucrose at 4°C degrees for at least 48h. Brain tissue slices, of 50µm thickness, were obtained with cryostat (Leica, Germany) and subsequently stained with cresyl violet (Sigma-Aldrich, USA). The histology analysis allowed to examine whether the probe was successfully implanted in the expected location, referring to Paxinos and Watson 1996 Rat brain atlas.

Only the brains with successful cannulae implantation were considered in the statistical analysis. In experiment 1, the brain areas were analyzed separately, since it was not always possible to collect samples from all three areas in the same animal due to the failure of microdialysis probes or HPLC issues.

HPLC-EC Assessment of Acetylcholine

The neurochemical analysis was performed via high-performance liquid chromatography with coupled electrochemical detection (HPLC-EC), to investigate variation in the extracellular level of ACh in the dialyzed brain areas after IN-PREG. A reverse-phase column of 75 mm length, packed with ChromSpher 5C18 (Merck KGaA, Darmstadt, Germany) and loaded with sodium dodecyl sulfate (Sigma–Aldrich, Saint Louis, Missouri, US) was used. Enzyme reactor (ER) was prepared and then fixed next to the column. Electrochemical detector with platinum electrode (Intro, Antec, Netherlands) was set at 0.350 mV at a 37°C and had ISAAC (in situ

Ag/AgCl) reference electrode (Antec, Leyden, Netherlands). The mobile phase composed of 1 mM tetramethylammonium chloride and 0.18 M K2HPO4 and adjusted to pH 8.0 with KH2PO4 (Merck, Darmstadt, Germany), was kept at a constant flow of 0.3µL/min in the HPLC system (de Souza Silva *et al.*, 2013).
In the experiment I, the data collected from each area of the brain was analyzed as independent variable. Whereas, in experiment II, pairwise comparisons, drug- vs vehicle-treated nostril side, on within-subject level were investigated. The within-subject mean of the 6 baseline measurements for ACh, reflecting neurotransmitter fluctuation prior IN-PREG, was defined as 100% and the after-drug administration ACh levels were normalized as a percentage of baseline mean. Data were assessed with the Chrom Perfect Software (Justice Laboratory Software, Denville, NJ, USA)

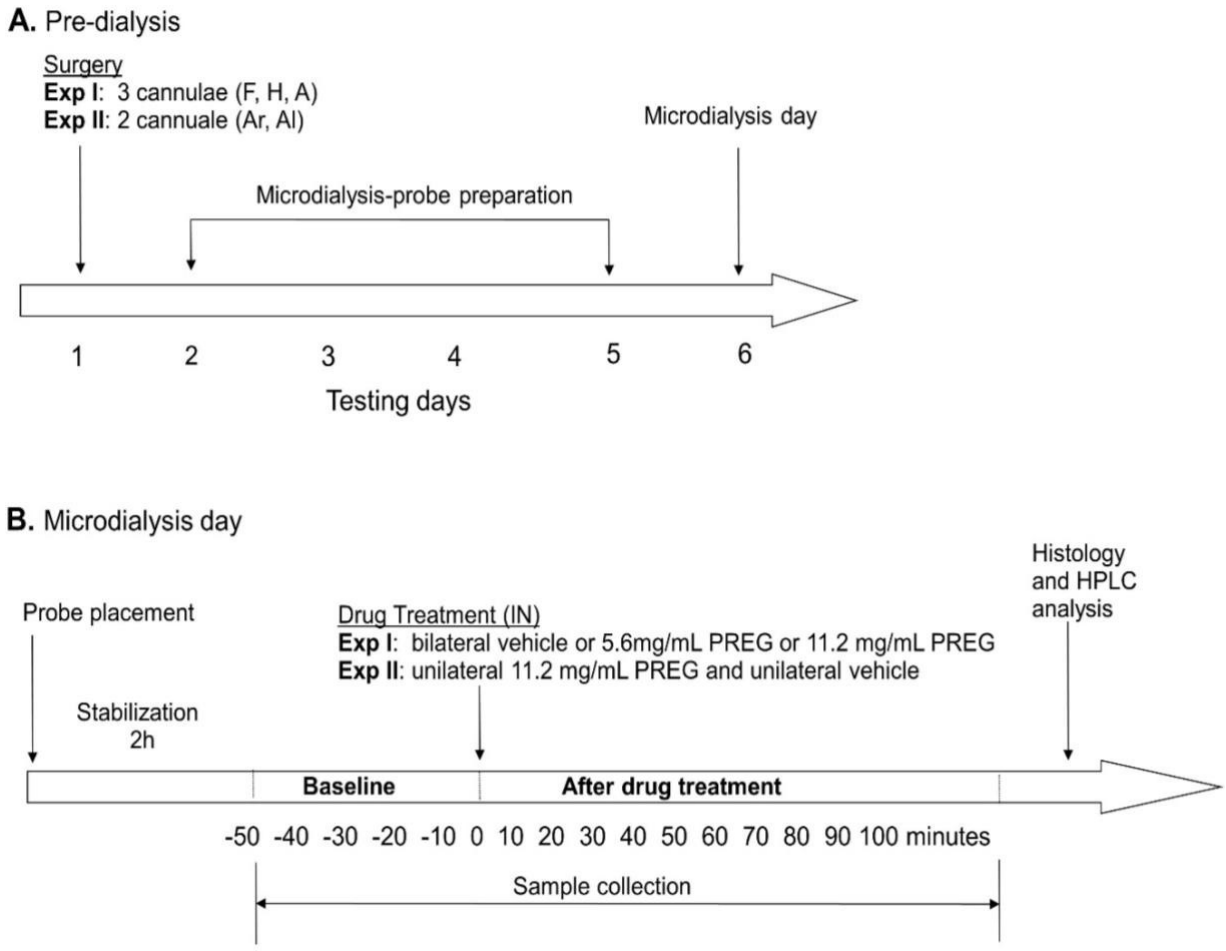

Figure 2. Outline of experimental schedule. In A. pre-dialysis steps are shown; brain surgeries in experiment I and II differed in cannulae-implantation location. The recovery time was between 4-5 days, before microdialysis. The time between the

surgery and the microdialysis day was used for microdialysis-probe preparation. In B. a detailed timeline of a typical microdialysis day is shown. After the animal is successfully anaesthetized, the microdialysis probes are inserted into the brain through the guiding cannulae. A 2h stabilization time is needed, before starting to collect dialysate. Collection period is every 10 minutes for 16 times, for a total time of dialysate collection of 160 minutes. After the collection period is completed, the microdialysis process is over and the animal is given a Pentobarbital-Natrium overdose and the brain is collected for further histology analysis and samples can be either stored at -80°C or directly tested for quantification through HPLC-EC.
Abbreviation: F, frontal cortex; H, hippocampus; A (r, l), amygdala (right, left); IN, intranasal administration; Exp, experiment.

Statistical analysis

Data from each brain area (experiment 1) were analysed separately. Different areas belonging to the same subject were considered independent. The subject-level mean of the six baseline samples (representing within-subject fluctuations of the ACh level) was set to 100%; all scores were expressed as percentage of the subject-level baseline mean. In experiment 1, for the frontal cortex, the number of animals/group were: n=9 (vehicle) n=6 (5.6 mg/mL PREG) and n=8 (11.2 mg/mL PREG; Figure 3A). For the hippocampus, the number of animals/group were: n=9 (vehicle), n=7 (5.6 mg/mL PREG) and n=12 (11.2 mg/mL PREG; (Figure 3B). For the amygdala, the number of animals/group were: n=11 (vehicle), n=8 for (5.6 mg/mL PREG) and n=6 (11.2 mg/mL PREG; Figure 3C). Moreover, the number of animals/group which received vehicle and had collected dialysate from the right hemisphere or left hemisphere were respectively n=4 and n= 5 for the frontal cortex, n=2 and n= 7 for the hippocampus, n=7 and n= 4 for the amygdala. The number of animals/group which received 5.6 mg/mL PREG and had collected dialysate from the right hemisphere or left hemisphere were respectively n=3 and n= 3 for the frontal cortex, n=4 and n= 7 for the hippocampus, n=4 and n= 4 for the amygdala. The number of animals/group which received 11.2 mg/mL PREG and had collected dialysate from the right hemisphere or left hemisphere were respectively n=4 and n= 4 for the frontal cortex, n=6 and n= 6 for the hippocampus, n=4 and n= 2 for the amygdala (Figure 4,5). In experiment 2 (bilateral amygdala, 11.2 mg/mL PREG) the sample size totaled up to n=10. For the whole data set (experiments 1 and 2), the

assumption of sphericity for repeated measurements did not hold (Mauchly's test). For main effect of time and interaction effects involving time, the Greenhouse-Geisser correction was applied.

Experiment 1. The impact of IN administration of PREG (5.6 mg/mL, 11.2 mg/mL) on ACh levels in frontal cortex, hippocampus and amygdala was assessed in a 3 (between) x 16 (within) ANOVA (sums of squares type 3: each main effect assessed after controlling for the other main effect and the interaction; Figure 3). Moreover, 3 (between) x 10 (within) ANOVA, was also separately tested for each hemisphere from where the dialysates were collected (right vs left hemisphere) (Figure 5). The within-subject effect of the individual drug doses over time was measured over 16 10-min time-bins (T; 6 in baseline and 10 post- treatment). The main effect of time and the time*dose interaction were assessed for the time curves after baseline. The main effect of dose (vs. vehicle), for the subject-level average of the time curve (after baseline), was assessed in 3 groups of animals and followed up by 2-tailed Dunnett post-hoc pairwise comparisons of both low and high PREG dose to vehicle. The within-subject effect of the individual drug doses over time effect was measured separately for each hemisphere from where the dialysates were collected (right vs left hemisphere). For each dose separately, the baseline mean (100%) was compared to each after-treatment time-bin by means of within-subject simple contrasts (non-orthogonal, meaning that the comparisons are related, as the after drug-treatment values are expressed as % of the baseline mean; Field 2009). Moreover, a comparison of estimated marginal means (method: Fisher's Least Significant Difference) was performed to compare the three groups (5.6 mg/mL PREG, 11.2 mg/mL PREG, vehicle) within every time-bin after drug treatment (10 x 10 min intervals).

Experiment 2 focused on the amygdala. The effect of the high dose of PREG (11.2 mg/mL) was assessed in a 2 x 16 within-subject design. The time course of drug effect was again measured by collecting samples from 16 10-min intervals (6 in baseline and 10 post-treatment). The effect of the individual PREG doses was compared to vehicle within each subject (Figure 6A). For the drug- and vehicle-treated nostril side separately, an overall hypothesis about time course of drug effect was tested (within-subject ANOVA: 10 time-bins and baseline mean), followed up by a simple contrast of each time-bin after treatment to the baseline mean (set to

100%). Separate pairwise estimated marginal means comparisons of drug-treated vs vehicle-treated side at every time-bin are also reported. The effect of left vs right hemisphere as a possible confounding factor was assessed in separate sets of scores (Figure 6B): (1) only vehicle-treated hemisphere (n=10, out of which localized in the left hemisphere: n=7, in the right hemisphere: n=3); (2) only PREG-treated hemisphere (n=10, out of which localized in the left hemisphere: n=3, in the right hemisphere: n=10). Only time-bins after treatment were considered. Reported are F- and p-values of between-subject ANOVA after controlling for within-subject effect of time and possible interaction of both factors. Calculations were performed with IBM SPSS Statistics 24.0 (IBM SPSS Software Germany, Ehningen, Germany).

Normality assumption

Shapiro-Wilk normality test (to assess whether $p<0.05$, data non-normally distributed) and z-test for normality (when Z score > |1.96|, absolute z-score, data non-normally distributed) were performed (Field 2009; Kim 2013). The z-test valuates if a population is normally distributed, using two parameters: the skewness, which estimates the symmetry of the distribution, and kurtosis values, which estimates the pointiness of the distribution, which means how the scores cluster at the tails of the distribution (Field 2009). The z-score is calculated for each sample by dividing the skewness value or the kurtosis value by their standard errors. In small samples ($n < 50$) the null hypothesis is rejected if the absolute z-score is higher than 1.96 (Kim 2013). Data was not normally distributed for experiment 1: in the frontal cortex, for the vehicle at the time-bins BT50 (Z score > |1.96|), BT40 (Normality test: p=0.038, Z score > |1.96|), for PREG 5.6 mg/mL at the time-bin BT30 (Normality test: p=0.007, Z score > |1.96|), for PREG 11.2 mg/mL at the time-bin T50 (Z score > |1.96|); in the hippocampus, for PREG 5.6 mg/mL at the time-bin T40 (Normality test: p=0.018, Z score > |1.96|), for PREG 11.2 mg/mL at the time-bin T10 (Normality test: p=0.0001, Z score > |1.96|), T50 (Z score > |1.96|), T80 (Normality test: p=0.001, Z score > |1.96|), T90 (Normality test: p=0.013, Z score > |1.96|); in the amygdala, for the vehicle at the time-bin BT30 (Z score > |1.96|), BT10 (Normality test: p=0.015, Z score > |1.96|), T20 (Z score > |1.96|), T80 (Normality test: p=0.005, Z score > |1.96|); for PREG 5.6 mg/mL at the time-bin BT50 (Z score > |1.96|), BT10 (Z score > |1.96|), for PREG 11.2 mg/mL at the time-bin BT10 (Normality test: p=0.011, Z score

> |1.96|), T10 (Normality test: p=0.004, Z score > |1.96|), T20 (Normality test: p=0.028, Z score > |1.96|), T80 (Z score > |1.96|). In the right hemisphere, the frontal cortex showed non-normal distributed data for the vehicle at the time-bins T60 (Normality test: p=0.003, Z score > |1.96|), for PREG 11.2 mg/mL group at the time-bin BT10 (Normality test: p=0.041, Z score > |1.96|); in the hippocampus on the right hemisphere, for PREG 11.2 mg/mL at the time-bin T40 (Normality test: p=0.022), T80 (Normality test: p=0.008, Z score > |1.96|), T90 (Z score > |1.96|); in the amygdala on the right hemisphere in the vehicle group at the time-bins BT 10 (Normality test: p=0.027, Z score > |1.96|), T 70 (Normality test: p=0.040, Z score > |1.96|), T 80 (Normality test: p=0.018), T 100 (Normality test: p=0.035, Z score > |1.96|), for PREG 5.6 mg/mL at the time bin BT10 (Normality test: p=0.039, Z score > |1.96|), for PREG 11.2 mg/mL at the time bin BT50 (Z score > |1.96|), BT30 (Z score > |1.96|). In the left hemisphere, the frontal cortex showed non-normal distributed data for the vehicle at the time-bins T40 (Normality test: p=0.050), for PREG 5.6 mg/mL group at the time-bin BT30 (Normality test: p=0.033), for PREG 11.2 mg/mL group at the time-bin T40 (Normality test: p=0.013); in the hippocampus on the left hemisphere, in the PREG 5.6 mg/mL group at the time-bin BT60 (Normality test: p=0.016), in the PREG 11.2 mg/mL at the time-bin T10 (Normality test: p=0.003, Z score > |1.96|), T90 (Normality test: p=0.008, Z score > |1.96|); in the amygdala on the left hemisphere in the PREG 5.6 mg/mL group at the time-bins T 30 (Normality test: p=0.006), T 80 (Normality test: p=0.035, Z score > |1.96|), T 100 (Normality test: p=0.029). In experiment 2, in the vehicle at the time-bin T20 (Normality test: p=0.018), for PREG 11.2 mg/mL at the time-bin BT10 (Normality test: p=0.011, Z score > |1.96|), T40 (Normality test: p=0.037, Z score > |1.96|), T80 (Normality test: p=0.002, Z score > |1.96|), T100 (Normality test: p=0.021, Z score > |1.96|). In the right hemisphere, the PREG 11.2 mg/mL group showed not normally distributed data at the time-bin BT20 (Normality test: p=0.002, Z score > |1.96|), BT10 (Normality test: p=0.0001, Z score > |1.96|), T20 (Normality test: p=0.031, Z score > |1.96|), T40 (Z score > |1.96|), T80 (Normality test: p=0.011, Z score > |1.96|). In the left hemisphere, the vehicle group showed not normally distributed data at the time-bin T20 (Normality test: p=0.050, Z score > |1.96|).

3. Aims

The aim of the study is to investigate the effects on the extracellular ACh release in the rat brain after intranasal administration of pregnenolone. Behavioral studies pointed out the link between pregnenolone administration and cholinergic system interaction, but neurochemistry analysis in specific brain areas are needed to understand in detail the onset of the activity of pregnenolone (Abdel-Hafiz et al. 2016; Ducharme et al. 2010). IN-PREG studies showed that PREG reached rat brain regions via the intranasal route (Ducharme et al. 2010).

We hypothesized that: Experiment 1: bilateral administration of IN-PREG would induce an increase of ACh release in frontal cortex, hippocampus and amygdala. Experiment 2: due to anatomical separation of the rat nostrils by the septum and ethmoid bone (Shipley and Reyes 1991), unilateral IN-PREG, through epithelial pathway, would result in a lateralized effect on the ipsilateral hemisphere of the brain, specifically, in the release of ACh in the amygdala. In both experiments, dialysate of the selected brain regions was achieved by in-vivo microdialysis technique and the ACh extracellular content was assessed by HPLC analysis. In Abdel-Hafiz et al. 2016, the doses used in pregnenolone intranasal administration showed an effect on memory restoration in a water maze experiment, which is mediated by acetylcholinergic circuits (Abdel-Hafiz et al., 2016). Moreover, performance in the Morris water maze was found to be correlated with hippocampal levels of PREG-S (Vallée et al., 1997). For these reasons we expected effects on ACh content in the hippocampus by pregnenolone intranasal administration. Changes in hippocampal ACh concentrations were expected, as pregnenolone has been proposed to be involved in the processing of emotions, particularly in depression-related behaviors (Zorumski et al. 2013). Moreover, an effect on the amygdala was expected, as direct PREG-S injection in the amygdala showed an impact as a memory enhancement in Flood et al. (1995).

4. Results

4.1 Experiment 1: Effects of bilateral IN-PREG in the ACh release in frontal cortex, hippocampus and amygdala

In this study, bilateral IN-PREG administration in healthy anaesthetized rats was performed to investigate PREG nasal delivery in time- and location-specificity effects on the ACh release in the rat brain. Two PREG doses were incorporated into a lipophilic gel formulation: low dose, 5.6 mg/mL PREG (0.124 mg/kg), and high dose, 11.2 mg/mL PREG (0.249 mg/kg). Extracellular ACh content through HPLC analysis of brain dialysates, continuously collected before (for 60 minutes) and after IN-PREG (for 100 minutes), was measured. Comparisons of extracellular ACh concentration after IN-PREG between the treated-groups were performed. In addition, within-subject ACh-content variation before and after IN-PREG was also measured.

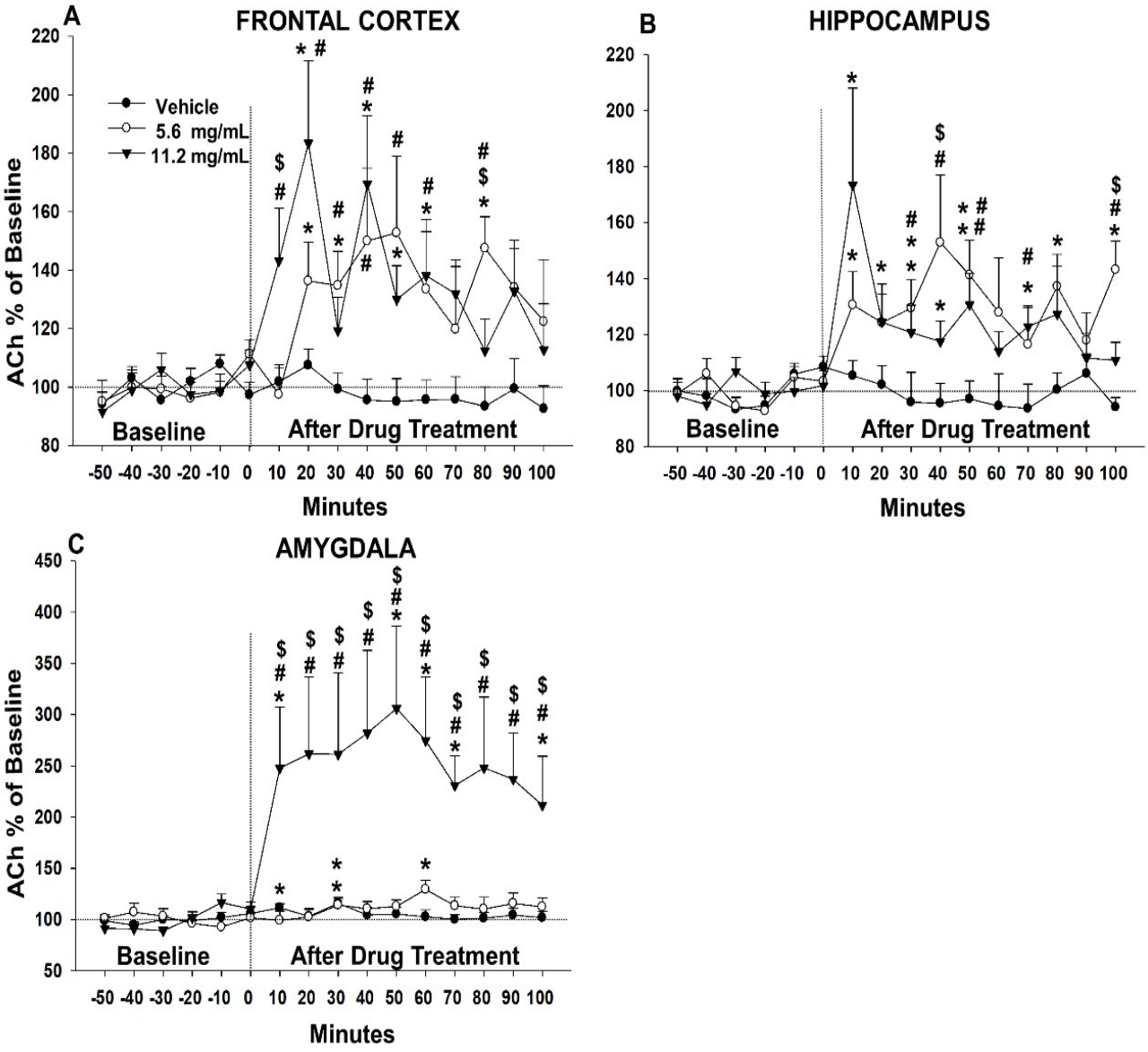

Figure 3. Effects of intranasal (bilateral) administration of pregnenolone on extracellular acetylcholine (ACh) levels in frontal cortex (**A**), hippocampus (**B**) and amygdala (**C**), measured with in-vivo microdialysis in anesthetized rats. Samples were taken every 10 minutes. Values (mean + standard errors of the mean [SE]) are presented as % of baseline, with six baseline samples taken as 100%. Pregnenolone 5.6 mg/mL, 11.2 mg/mL, and vehicle were administrated intranasally into both nostrils, 5µL each, at T0 minute time point. Sample size: (A) vehicle (n=9), 5.6 mg/mL (n=6), 11.2 mg/mL (n=8); (B) vehicle (n=9), 5.6 mg/mL (n=7), 11.2 mg/mL (n=12); (C) vehicle (n=11), 5.6 mg/mL (n=8), 11.2 mg/mL (n=6). * $p < 0.050$,

compared with baseline; # $p < 0.050$ compared with vehicle, $ $p < 0.050$ comparison PREG doses (5.6 mg/mL vs 11.2 mg/mL).

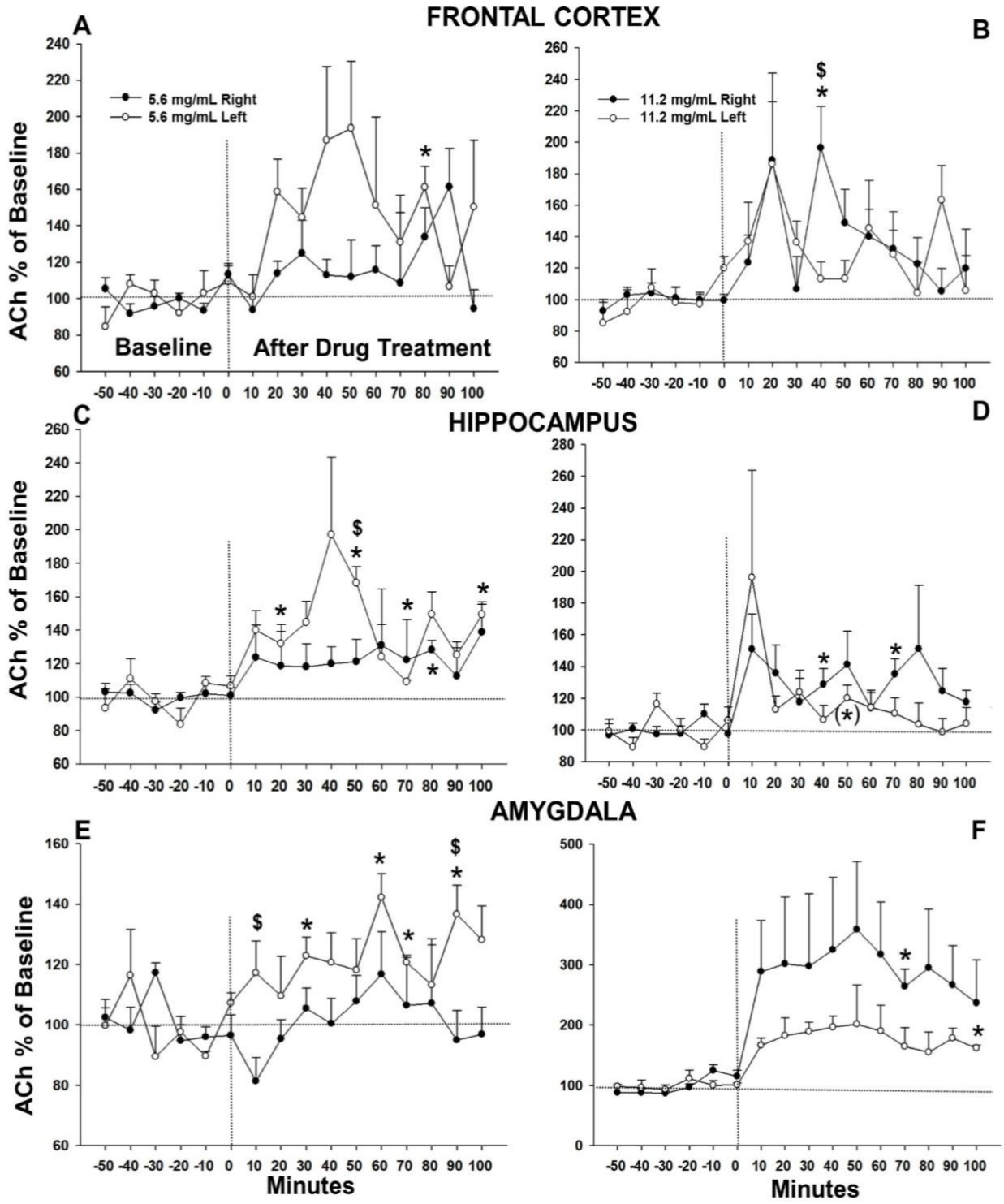

Figure 4. Comparison of the effect on ACh of bilateral IN-PREG administration (5.6 mg/mL, 11.2 mg/mL) in the right vs left hemisphere. Each rat had samples collected either from a right or left hemisphere from each brain region. The graph shows the level of ACh in both hemispheres (right and left) of the frontal cortex (A), hippocampus (C) and amygdala (E) before and after administration of PREG 5.6

mg/mL. Moreover, the graph shows the level of ACh in both hemispheres of the frontal cortex (B), hippocampus (D) and amygdala (F) before and after administration of PREG 11.2 mg/mL. Extracellular ACh was measured with in-vivo microdialysis in anesthetized rats. Samples were taken every 10 minutes. Values (mean + standard errors of the mean [SE]) are presented as % of baseline, with six baseline samples taken as 100%. Pregnenolone 5.6 mg/mL, 11.2 mg/mL, and vehicle were administrated intranasally into both nostrils, 5µL each, at T0 minute time point. * $p<0.050$, compared with baseline mean. Sample size: (A) left (n=3), right (n=3), (B) left (n=4), right (n=4), (C) left (n=3), right (n=4), (D) left (n=6), right (n=6), (E) left (n=4), right (n=4), (F) left (n=2), right (n=4). * $p < 0.050$, compared with baseline mean; $ $p < 0.050$ comparison of both hemispheres at the same time points.

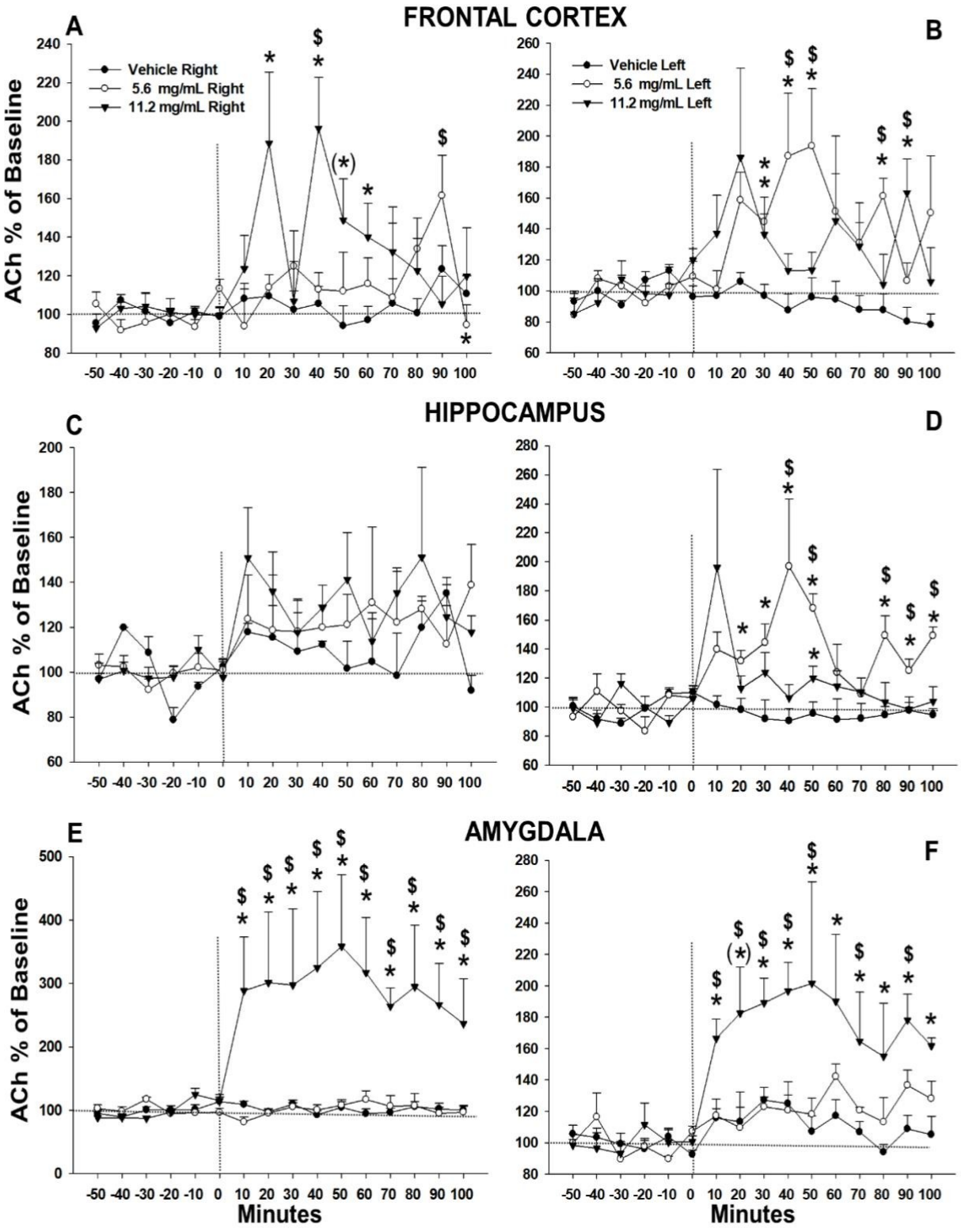

Figure 5. Comparison of the effect on ACh of bilateral IN-PREG administration (5.6 mg/mL, 11.2 mg/mL) and vehicle in the same hemisphere (right or left hemisphere). Each rat had IN-PREG administration (5.6 mg/mL or 11.2mg/mL) or vehicle into both nostrils, 5µL each, at T0 minute time point. The graph shows the level of ACh in the

right hemisphere of the frontal cortex (A), hippocampus (C) and amygdala (E) before and after intranasal administration. Moreover, the graph shows the level of ACh in the left hemisphere of the frontal cortex (B), hippocampus (D) and amygdala (F) before and after intranasal administration. Extracellular ACh was measured with in-vivo microdialysis in anesthetized rats. Samples were taken every 10 minutes. Values (mean + standard errors of the mean [SE]) are presented as % of baseline, with the mean of six baseline samples taken as 100%. * p<0.050, compared with baseline mean. Sample size: (A) vehicle (n=4), 5.6 mg/mL (n=3), 11.2 mg/mL (n=4); (B) vehicle (n=2), 5.6 mg/mL (n=4), 11.2 mg/mL (n=6); (C) vehicle (n=7), 5.6 mg/mL (n=4), 11.2 mg/mL (n=4); (D) vehicle (n=5), 5.6 mg/mL (n=3), 11.2 mg/mL (n=4); (E) vehicle (n=7), 5.6 mg/mL (n=3), 11.2 mg/mL (n=6); (F) vehicle (n=4), 5.6 mg/mL (n=4), 11.2 mg/mL (n=2). * p < 0.050, compared with vehicle at the same time point; $ p < 0.050 comparison of PREG doses (5.6 mg/mL vs 11.2 mg/mL) at the same time points.

Frontal cortex

The sums of squares type 3 ANOVA showed no significant main effect of “time” (for 10 measurements after baseline) on the pooled set of subjects (n=23; F(4.59, 91.73)=2.14, p=.74). The interaction between “time” and “dose” was also not statistically significant (F(9.17, 91.73)=1.39, p=.201). The main effect of “dose” on all time-bins after baseline was statistically significant (F(2,20)=7.08, p=.005). Equality of error variances (Levene’s test) cannot be assumed for several time-bins: T10-30, T50. A 2-tailed post-hoc Dunnett comparison between (1) 11.2 mg/mL PREG and vehicle (p=.005), and (2) 5.6 mg/mL PREG and vehicle (p=.017) confirmed the effect of IN-treatment. There were, therefore, no distinguishable peaks or drops, and both PREG doses differed from vehicle (vehicle time curve remains flat after drug application, Figure 3A).

Focused comparisons

For the vehicle-treated animals, simple contrasts of each time-bin from T10 to T100 were not statistically significant. For the 5.6 mg/mL PREG group, simple contrasts yielded statistically significant differences between baseline and T20 (F(1,5)=7.6, p=.040), T30 (F(1,5)=8.79, p=.031), and T80 (F(1,5)=19.68, p=.007). For the 11.2

mg/mL PREG group, simple contrasts were statistically significant between baseline and T20 (F(1,7)=7.57, p=.028), T40 (F(1,7)=7.1, p=.032), T50 (F(1,7)=5.62, p=.05), and T60 (F(1,7)=6.83, p=.035). These comparisons confirmed the effect previously shown by the ANOVA (flat time curve for vehicle vs. positive slope and complex curve form after IN-treatment for both PREG doses; Figure 3A). Furthermore, the relative rise in the ACh level ranged from +19% (T70) and +52% (T50) for the low dose (after an initial drop by ca. -2.6% at T10) and from +12% (T100) to +54% (T40) for the high dose.

Pairwise comparisons of estimated marginal means at each time-bin produced significant differences between: (1) vehicle and 5.6 mg/mL PREG at T30 (p=.025), T40 (p=.044), T50 (p=.016), and T80 (p=.001); (2) vehicle and 11.2 mg/mL PREG at T10 (p=.052, marginally sig.), T20 (p=.009), T40 (p=.019), and T60 (p=.033); (3) 5.6 mg/mL PREG and 11.2 mg/mL PREG at T10 (p=.049) and T80 (p=.032). The onset of drug effect was earlier after the higher dose (Figure 3A).

Control of confounding variable "left vs right hemisphere" in frontal cortex

Vehicle-treated and PREG-treated targets were analyzed separately (three independent samples). In each sample (vehicle, 5.6 11.2 mg/mL PREG), the possible interaction between localization (left or right) and time course of drug effect was assessed in a mixed design (Figure 4A, 4B). For the IN-VEH targets, out of 9 subjects, n=4 received vehicle into the right nostril; n=5 received vehicle into the left nostril. For the IN-PREG targets, dose 5.6 mg/mL, out of 6 subjects, n=3 PREG into the left nostril; n=3 received PREG into the right nostril (Figure 4A). For the IN-PREG targets, dose 11.2 mg/mL, out of 8 subjects, n=4 received PREG into the left nostril; n=4 received PREG into the right nostril (Figure 4B). For the IN-PREG treated targets, dose 5.6 mg/mL, no statistical significant interaction between time (10 time bins after drug treatment) and left vs. right side was found (F(2.78,11.11)=1.58, p=.249). Furthermore, a between-subject comparison of the subject-average of time curves of PREG*left vs PREG*right groups was performed. The main effect of left vs right hemisphere was not statistically significant (F(1,4)=2.71, p=.175) (Figure 4A).

For the IN-PREG treated targets, dose 11.2 mg/mL, no statistically significant interaction between time (10 time bins after drug treatment) and left vs. right side was found (F(2.31,13.85)=1.60, p=.237). Furthermore, a between-subject comparison of the subject-average of time curves of PREG*left vs PREG*right

groups was performed. The main effect of left vs right hemisphere was not statistically significant (F(1,6)=0.64, p=.808). (Figure 4B). For the IN-VEH treated targets, no statistically significant interaction between time (10 time bins after drug treatment) and left vs. right side was found (F(3.55,24.85)=2.23, p=.101). Furthermore, a between-subject comparison of the subject-average of time curves of VEH*left vs VEH*right groups was performed. The main effect of left vs right hemisphere was not statistically significant (F(1,7)=2.05, p=.195).

Moreover, the effect over time after drug application was compared to the baseline mean for PREG*left and PREG*right treated subjects, separately. For 5.6 mg/mL PREG*left (n=3), the overall effect of time after drug treatment (baseline mean and T10-T100 = 11 time bins) was not statistically significant F(1.66,3.32)=1.83, p=.285); the overall effect of time after the drug treatment (T10-T100 = 10 time bins) was not statistically significant (F(1.632,3.264)=1.522, p=.330).

The lack of the overall effect of time over 10 and 11 time bins could mean that the curve representing the mean of the values over the 10 and 11 time points has no discernible positive or negative inclination due to its complexity. Statistically significant contrast to baseline mean (100%) was found at T80 (F(1,2)=28.54, p=.033). For 5.6 mg/mL PREG*right (n=3), the overall effect of time after drug treatment (baseline mean and T10-T100 = 11 time bins) was not statistically significant (F(1.31,2.62)=1.18, p=.394); the overall effect of time after drug treatment (T10-T100 = 10 time bins) was not statistically significant (F(1.315,2629)=1.114, p=.407). No statistically significant contrast to baseline mean (100%) was found. For 11.2 mg/mL PREG*left (n=4), the overall effect of time after drug treatment (baseline mean and T10-T100 = 11 time bins) was not statistically significant (F(1.62,4.85)=1.29, p=.343); the overall effect of time after drug treatment (T10-T100 = 10 time bins) was not statistically significant (F(1.598,4.794)=1.135, p=.378). No statistically significant contrast to baseline mean (100%) was found. For 11.2 mg/mL PREG*right (n=4), the overall effect of time after drug treatment (baseline mean and T10-T100 = 11 time bins) was not statistically significant (F(2.10,6.30)=3.68, p=.086); the overall effect of time after drug treatment (T10-T100 = 10 time bins) was not statistically significant (F(1.903,5.708)=3.511 p=.102). Statistically significant contrast to baseline mean (100%) was found at T40 (F(1,3)=13.36, p=.035), with T40 value being significantly higher than 100%, namely, +96.381%. For VEH*left (n=5), the overall effect of time after drug treatment (vehicle) (baseline mean and T10-T100 =

11 time bins) was not statistically significant (F(3.05,12.20)=2.03, p=.163); the overall effect of time after drug treatment (T10-T100 = 10 time bins) was not statistically significant (F(3.200,12.799)=2.113, p=.146). No statistically significant contrast to baseline mean (100%) was found. For VEH*right (n=4), the overall effect of time after drug treatment (baseline mean and T10-T100 = 11 time bins) was not statistically significant (F(2.22,6.68)=1.10, p=.394); the overall effect of time after drug (vehicle) treatment (T10-T100 = 10 time bins) was not statistically significant (F(2.092,6.276)=1.129, p=.384). No statistically significant contrast to baseline mean (100%) was found. Pairwise comparisons of estimated marginal means at each time-bin produced statistically significant differences between: (1) 11.2 mg/mL PREG*right and 11.2 mg/mL PREG*left at T40 (p=.027), with PREG*right > PREG*left.

Drug dose comparison in the frontal cortex right hemisphere targets only

For the frontal cortex right hemisphere, a statistically significant interaction between time (10 time bins after drug treatment) and drug treatment (vehicle n=4; 5.6 mg/mL PREG, n=3; 11.2 mg/mL PREG, n=4) was found F(7.160,28.641)=2.556, p=0.035 (Figure 5A). This comparison confirmed the effect of the drug previously shown in the total pooled samples (right and left hemisphere pooled) (flat time-curve for vehicle vs. positive slope and complex curve after IN-treatment for both PREG doses; Figure 5A). Furthermore, a between-subject comparison of the subject-average of time curves of right hemisphere groups was performed. The main effect of drug was not statistically significant (F(2,8)=2.1442, p=.180). Pairwise comparisons of estimated marginal means at each time-bin in the right hemisphere produced statistically significant differences between: (1) vehicle and 11.2 mg/mL PREG at: T20 (p=.046), T40 (p=.006), T50 (nearly significant p=.053), T60 (p=.047), with 11.2 mg/mL PREG respectively +80%, +91%, +55%, +43% (as mean % of the difference) higher than vehicle (2) 5.6 mg/mL PREG and 11.2 mg/mL PREG at the time-bins: T40 (p=.014), T90 (p=.037), with 11.2 mg/mL PREG respectively +83% higher and -56% lower than 5.6 mg/mL PREG. Furthermore, the relative rise in the ACh level after the drug treatment ranged from 9% (T70) and +61% (T90) for the low dose (after an initial drop by ca. -6% at T10), in contrast to the baseline variation (between -8% at BT50 and +13% at BT10) and from +5% (T90) to +96% (T40) for the high dose, in contrast to the baseline variation (between -7% at BT60 and +4% at BT40).

Drug dose comparison in the frontal cortex left hemisphere targets only

For the frontal cortex left hemisphere, a statistically significant interaction between time (10 time bins after drug treatment) and drug treatment (vehicle n=5; 5.6 mg/mL PREG, n=3; 11.2 mg/mL PREG, n=4) was not found F(5.590,25.153)=1.654, p=0.177 (Figure 5B). This might be influenced by the small sample size. Furthermore, a between-subject comparison of the subject-average of time curves of left hemisphere groups was performed. The main effect of drug was statistically significant (F(2,9)=7.271, p=.013). A 2-tailed post-hoc Dunnett comparison between (1) 11.2 mg/mL PREG and vehicle (p=.012), and (2) 5.6 mg/mL PREG and vehicle (p=.038) confirmed the effect of IN-treatment, with marginal means of PREG doses > compared to the marginal mean of the vehicle. Pairwise comparisons of estimated marginal means at each time-bin in the left hemisphere produced statistically significant differences between: (1) vehicle and 5.6 mg/mL PREG at: T30 (p=.019), T40 (p=.006), T50 (p=.006), T80 (p=.007), T100 (p=.039), with 5.6 mg/mL PREG respectively +48%, +100%, +98%, +74%, +72% (as mean % of the difference) higher than vehicle (2) vehicle and 11.2 mg/mL PREG at: T30 (p=.030), T90 (p=.003) with 11.2 mg/mL PREG respectively +40%, +83% (as mean % of the difference) higher than vehicle, (3) 5.6 mg/mL PREG and 11.2 mg/mL PREG at the time-bins: T40 (p=.033), T50 (p=.021), T80 (p=.028), T90 (p=.037), with 11.2 mg/mL PREG respectively +80%, +91%, +55%, +43% (as mean % of the difference) higher than vehicle. Furthermore, the relative rise in the ACh level ranged after the drug treatment from 1% (T10) and +84% (T50) for the low dose, in contrast to the baseline variation (between -15% at BT60 and +9% at BT10), and from +6% (T100) to +86% (T20) for the high dose, in contrast to the baseline variation (between -15% at BT60 and +19% at BT10).

Hippocampus

The mixed ANOVA showed no significant effect of “time” (after baseline) in the pooled set of subjects (n=28; F(2.69, 67.36)=0.99, p=.396). The interaction between “time” and “dose” was also not statistically significant (F(5.39, 67.36)=0.921, p=.478). The main effect of “dose” was statistically significant (F(2,25)=9.7, p=.001). The assumption of equality of error variances (Levene’s test) was met for all time-bins. A 2-tailed post-hoc Dunnett comparison between (1) high dose and vehicle (p=.002), and (2) low dose and vehicle (p=.001) confirmed the effect of IN-treatment. Figure

3B depicts the groups' time curves: while in the vehicle group the curve after IN-treatment remains flat and fluctuating around the 100% mark, the curves for both PREG doses are clearly above.

Focused comparisons

For the vehicle group (n=9), simple contrasts of each time-bin from T10 to T100 to baseline mean (100%) were not statistically significant. For the 5.6 mg/mL PREG group (n=7), the simple contrasts yielded statistically significant differences between baseline and T10 ($F(1,6)=6.65$, $p=.042$), T30 ($F(1,6)=8.16$, $p=.029$), T50 ($F(1,6)=10.88$, $p=.016$), T80 ($F(1,6)=25.47$, $p=.002$), and T100 ($F(1,6)=17.98$, $p=.005$). For the 11.2 mg/mL PREG group (n=12), simple contrasts were statistically significant between baseline and T10 ($F(1,11)=4.51$, $p=.057$, marginally sig.), T20 ($F(1,11)=5.98$, $p=.032$), T30 ($F(1,11)=4.51$, $p=.057$, marginally sig.), T40 ($F(1,11)=5.8$, $p=.035$), T50 ($F(1,11)=7.5$, $p=.019$), and T70 ($F(1,11)=9.29$, $p=.011$). Focused comparisons confirm the effect previously shown by the ANOVA (lack of effect for vehicle vs positive change after IN-treatment with both PREG doses; Figure 3B). The range of effect for the low dose was from +17% (T90) to +52 (T40). For the high dose it was larger, namely from +10% (T100) to +73% (T10).

Pairwise comparisons of estimated marginal means at each time-bin produced significant differences between: (1) vehicle and low dose at T30 ($p=.046$), T40 ($p=.005$), T50 ($p=.012$), T100 ($p<.001$); (2) vehicle and high dose at T50 ($p=.026$) and T70 ($p=.028$); (3) low dose and high dose at T40 ($p=.058$, marginally sig.) and T100 ($p=.003$). Figure 3B shows the same onset of effect for both low and high dose; however, the separate comparisons evidence that the effect of the high dose was less consistent across subjects (large peaks in some animals and smaller in others).

Control of confounding variable "left vs right hemisphere" hippocampus

Vehicle-treated and PREG-treated targets were analyzed separately (three independent samples). In each sample (vehicle, 5.6 11.2 mg/mL PREG), the possible interaction between localization (left or right) and time course of drug effect was assessed in a mixed design (Figure 4C, 4D). For the IN-VEH targets, out of 9 subjects, n=2 received vehicle into the right nostril; n=7 received vehicle into the left nostril. For the IN-PREG targets, dose 5.6 mg/mL, out of 7 subjects, n= 3 PREG into the left nostril; n= 4 received PREG into the right nostril (Figure 4C). For the IN-

PREG targets, dose 11.2 mg/mL, out of 12 subjects, n= 6 received PREG into the left nostril; n= 6 received PREG into the right nostril (Figure 4D). For the IN-PREG treated targets, dose 5.6 mg/ml, no statistically significant interaction between time (10 time bins after drug treatment) and left vs. right side was found ($F(3.23,16.17)=1.11$, $p=.377$). Furthermore, a between-subject comparison of the subject-average of time curves of PREG*left vs PREG*right groups was performed. The main effect of left vs right hemisphere was not statistically significant ($F(1,5)=1.89$, $p=.228$) (Figure 4C). For the IN-PREG treated targets, dose 11.2 mg/mL, no statistically significant interaction between time (10 time bins after drug treatment) and left vs. right side was found ($F(1.81,18.09)=.664$, $p=.512$). Furthermore, a between-subject comparison of the subject-average of time curves of PREG*left vs PREG*right groups was performed. The main effect of left vs right hemisphere was not statistically significant ($F(1,10)=2.64$, $p=.135$) (Figure 4D). For the IN-VEH treated targets, no statistically significant interaction between time (10 time bins after drug treatment) and left vs. right side was found ($F(4.02,28.14)=.653$, $p=.630$). Furthermore, a between-subject comparison of the subject-average of time curves of VEH*left vs VEH*right groups was performed. The main effect of left vs right hemisphere was not statistically significant ($F(1,7)=1.49$, $p=.261$). Moreover, the effect over time after drug application was compared to the baseline mean for PREG*left and PREG*right treated subjects, separately. For 5.6 mg/mL PREG*left (n=3), the overall effect of time after drug treatment (baseline mean and T10-T100 = 11 time bins) was not statistically significant ($F(1.24,2.48)=2.36$, $p=.250$); the overall effect of time after drug treatment (T10-T100 = 10 time bins) was not statistically significant ($F(1.240,2.480)=1.810$ $p=.302$). Statistical significant contrast to baseline mean (100%) was found at T20 ($F(1,2)=18.89$, $p=.049$), T50 ($F(1,2)=47.61$, $p=.020$). T70 ($F(1,2)=59.96$, $p=.016$), T100 ($F(1,2)=62.86$, $p=.016$), having all the values higher than the baseline mean, respectively +32%, +68%, +10%, +50%. For 5.6 mg/mL PREG*right (n=4), the overall effect of time after drug treatment (baseline mean and T10-T100 = 11 time bins) was not statistically significant ($F(2.56,7.67)=.402$, $p=.728$); the overall effect of time after drug treatment (T10-T100 = 10 time bins) was not statistically significant ($F(2.551,7.652)=.214$, $p=.857$). Statistically significant contrast to baseline mean (100%) was found at T80 ($F(1,3)=23.784$, $p=.016$), +28% higher compared to baseline mean. For 11.2 mg/mL PREG*left (n=6), the overall effect of time after drug treatment (baseline mean and

T10-T100 = 11 time bins) was not statistically significant (F(1.10,5.48)=1.38, p=.295); the overall effect of time after drug treatment (T10-T100 = 10 time bins) was not statistically significant (F(1.092,5.459)=1.321 p=.304). Nearly statistically significant contrast to baseline mean (100%) was found at T50 (F(1,5)=6.05, p=.057), +20% compared to baseline mean. For 11.2 mg/mL PREG*right (n=6), the overall effect of time after drug treatment (baseline mean and T10-T100 = 11 time bins) was not statistically significant (F(1.81,9.07)=.795, p=.469); the overall effect of time after drug treatment (T10-T100 = 10 time bins) was not statistically significant (F(1.806,9.030)=.518 p=.595). Statistically significant contrast to baseline mean (100%) was found at T40 (F(1,5)=8.22, p=.035), T70 (F(1,5)=13.69, p=.014), respectively +29% and +35% higher than the baseline mean. For VEH*left (n=7), the overall effect of time after drug treatment (baseline mean and T10-T100 = 11 time bins) was not statistically significant (F(3.95,23.71)=3.13, p=.865); the overall effect of time after drug treatment (T10-T100 = 10 time bins) was not statistically significant (F(3.955,23.728)=.282 p=.885). No statistically significant contrast to baseline mean (100%) was found. For VEH*right (n=2), the overall effect of time after drug treatment (baseline mean and T10-T100 = 11 time bins) was not statistically significant(F(1,1)=1.19, p=.473); the overall effect of time after drug treatment (T10-T100 = 10 time bins) was not statistically significant (F(1,1)=1.150, p=.478). No statistically significant contrast to baseline mean (100%) was found. Pairwise comparisons of estimated marginal means at each time-bin produced statistically significant differences between: (1) 5.6 mg/mL PREG*right and 5.6 mg/mL PREG*left at T50 (p=.048), with PREG*right < PREG*left.

Drug dose comparison in the hippocampus right hemisphere targets only

For the hippocampus right hemisphere, a statistically significant interaction between time (10 time bins after drug treatment) and drug treatment (vehicle n=2; 5.6 mg/mL PREG, n=4; 11.2 mg/mL PREG, n=6) was not found F(5.640,25.379)=.323, p=0.911 (Figure 5C). Furthermore, a between-subject comparison of the subject-average of time curves of right hemisphere groups was performed. The main effect of drug was not statistically significant (F(2,9)=.940, p=.426). Pairwise comparisons of estimated marginal means at each time-bin in the right hemisphere produced no statistically significant differences. Furthermore, the relative rise in the ACh level, after the drug treatment, ranged from 12% (T90) and +39% (T100) for the low dose, in contrast to

the baseline variation (between -8% at BT40 and +3% at BT60), and from +13% (T60) to +51% (T80) for the high dose, in contrast to the baseline variation (between -3% at BT60 and +10% at BT20).

Drug dose comparison in the hippocampus left hemisphere targets only

For the hippocampus left hemisphere, a statistically significant interaction between time (10 time bins after drug treatment) and drug treatment (vehicle n=7; 5.6 mg/mL PREG, n=3; 11.2 mg/mL PREG, n=6) was not found F(3.072,19.968)=1.290, p=0.305 (Figure 5D). Furthermore, a between-subject comparison of the subject-average of time curves of left hemisphere groups was performed. The main effect of drug was statistically significant (F(2,13)=15.890, p=.001). A 2-tailed post-hoc Dunnett comparison between (1) 11.2 mg/mL PREG and vehicle (p=.010), and (2) 5.6 mg/mL PREG and vehicle (p=.0001) confirmed the effect of IN-treatment, with marginal means of PREG doses > compared to the marginal mean of the vehicle.. Pairwise comparisons of estimated marginal means at each time-bin in the left hemisphere produced statistically significant differences between: (1) vehicle and 5.6 mg/mL PREG at: T20 (p=.027), T30 (p=.036), T40 (p=.001), T50 (p=.001), T80 (p=.007), T90 (p=.038), T100 (p=.001) with 5.6 mg/mL PREG respectively +33%, +52%, +106%, +72%, +55%, +27% +54% (as mean % of the difference) higher than vehicle, (2) vehicle and 11.2 mg/mL PREG at: T50 (p=.047) with 11.2 mg/mL PREG respectively +24% (as mean % of the difference) higher than vehicle, (3) 5.6 mg/mL PREG and 11.2 mg/mL PREG at the time-bins: T40 (p=.033), T50 (p=.005), T80 (p=.020), T90 (p=.048), T100 (p=.003) with 11.2 mg/mL PREG respectively -91%, -48%, -46%, -27%, -45% (as mean % of the difference) lower compared to 5.6 mg/mL PREG. Furthermore, the relative rise in the ACh level after the drug treatment ranged from 9% (T70) and +87% (T40) for the low dose, in contrast to the baseline variation (between -16% at BT30 and +10% at BT50), and from +3% (T80) to +96% (T10) for the high dose, in contrast to the baseline variation (between -11% at BT50 and +16% at BT40).

Amygdala

The mixed ANOVA showed no significant effect of “time” (after baseline) in the pooled set of subjects (n=25): F(2.88,63.44)=1.75, p=.168. The interaction between “time” and “dose” was also not statistically significant (F(5.77,63.44)=1.32, p=.261;

Figure 3C) The main effect of “dose” on all time-bins after baseline was statistically significant ($F(1,22)=10.26$, $p=.001$). Equality of error variances (Levene’s test) cannot be assumed for any of the 10 time-bins. A 2-tailed post-hoc Dunnett comparison between (1) high dose and vehicle ($p=.001$), and (2) low dose and vehicle ($p=.969$) showed a high effect of the high dose of PREG, whereas the effect of the low dose was indistinguishable from vehicle. The effect of the high PREG dose in the amygdala was higher relative to the other groups (Figure 3 C vs A, B). In this case, the lack of equality of error variances (Levene’s test) confirms the effect: higher effect goes along with higher variation. In contrast to the frontal cortex and the hippocampus, the low dose did not produce any sizable effect on the time curve relative to vehicle.

Focused comparisons

For the vehicle group, simple contrasts of each time-bin produced two statistically significant results between baseline mean and T10 ($F(1,10)=9.16$, $p=.013$) as well as T30 ($F(1,10)=8.34$, $p=.016$). For 5.6 mg/mL PREG, simple contrasts yielded statistically significant differences between baseline and T30 ($F(1,7)=6.89$, $p=.034$) as well as T60 ($F(1,7)=10.95$, $p=.013$). For 11.2 mg/mL PREG, simple contrasts were statistically significant between baseline and T10 ($F(1,5)=6.18$, $p=.055$, marginally sig.), T50 ($F(1,5)=6.56$, $p=.051$, marginally sig.), T60 ($F(1,5)=7.89$, $p=.038$), T70 ($F(1,5)=20.41$, $p=.006$), and T90 ($F(1,5)=9.1$, $p=.03$). Unlike frontal cortex and hippocampus, the vehicle treatment produced an effect in the amygdala, similar (although smaller) in size to the effect of the low dose: vehicle T10: +11%, vehicle T30: +15%, low dose T30: +14%, low dose T60: +29%. The time-curve after treatment with the high dose shows a relative rise of ACh between +111% (T100) and +206% (T50).

Pairwise comparisons of estimated marginal means at each time-bin produced significant differences between: (1) vehicle and 11.2 mg/mL PREG at all time-bins: T10 ($p=.001$), T20 ($p=.002$), T30 ($p=.006$), T40 ($p=.002$), T50 ($p<.001$), T60 ($p<.001$), T70 ($p<.001$), T80 ($p=.002$), T90 ($p<.001$), and T100 ($p=.001$); (2) 5.6 mg/mL PREG and 11.2 mg/mL PREG also at all time-bins: T10 ($p=.001$), T20 ($p=.003$), T30 ($p=.008$), T40 ($p=.003$), T50 ($p=.001$), T60 ($p=.002$), T70 ($p<.001$), T80 ($p=.006$), T90 ($p=.001$), and T100 ($p=.005$). The low dose, 5.6 mg/mL PREG, did not differ from the vehicle treated group in any time-bin after treatment. Pairwise comparisons of doses

per time-bin confirmed the results obtained after comparisons between baseline means and time-bins post-treatment (see also Figure 3C).

Control of confounding variable "left vs right hemisphere" amygdala

Vehicle-treated and PREG-treated targets were analyzed separately (three independent samples). In each sample (vehicle, 5.6 11.2 mg/mL PREG), the possible interaction between localization (left or right) and time course of drug effect was assessed in a mixed design (Figure 4E, 4F). For the IN-VEH targets, out of 11 subjects, n=7 received vehicle into the right nostril; n=4 received vehicle into the left nostril. For the IN-PREG targets, dose 5.6 mg/mL, out of 8 subjects, n= 4 PREG received into the left nostril; n= 4 received PREG into the right nostril (Figure 4E). For the IN-PREG targets, dose 11.2 mg/mL, out of 6 subjects, n= 2 received PREG into the left nostril; n= 4 received PREG into the right nostril (Figure 4F). For the IN-PREG treated targets, dose 5.6 mg/mL, no statistically significant interaction between time (10 time bins after drug treatment) and left vs. right side was found ($F(2.02,12.13)=.602$, $p=.565$). Furthermore, a between-subject comparison of the subject-average of time curves of PREG*left vs PREG*right groups was performed. The main effect of left vs right hemisphere was statistically significant ($F(1,6)=12.90$, $p=.011$) (Figure 4E). For the IN-PREG treated targets, dose 11.2 mg/ml, no statistically significant interaction between time (10 time bins after drug treatment) and left vs. right side was found ($F(2.14,8.58)=.133$, $p=.890$). Furthermore, a between-subject comparison of the subject-average of time curves of PREG*left vs PREG*right groups was performed. The main effect of left vs right hemisphere was not statistically significant ($F(1,4)=.833$, $p=.413$). (Figure 4F). For the IN-VEH treated targets, no statistically significant interaction between time (10 time bins after drug treatment) and left vs. right side was found ($F(3.63,32.686)=1.205$, $p=.326$). Furthermore, a between-subject comparison of the subject-average of time curves of VEH*left vs VEH*right groups was performed. The main effect of left vs right hemisphere was not statistically significant ($F(1,9)=2.41$, $p=.155$). Moreover, the effect over time after drug application was compared to the baseline mean for PREG*left and PREG*right treated subjects, separately. For 5.6 mg/mL PREG*left (n=4), the overall effect of time after drug treatment (baseline mean and T10-T100 = 11 time bins) was not statistically significant ($F(1.56,4.67)=1.707$, $p=.270$); the overall effect of time after drug treatment (T10-T100 = 10 time bins) was not statistically

significant (F(1.524,4.573)=1.1559, p=.371). Statistically significant contrast to baseline mean (100%) was found at T30 (F(1,3)=13.07, p=.036), T60 (F(1,3)=28.506, p=.013), T70 (F(1,3)=132.345, p=.001), T90 (F(1,3)=14.345, p=.032), respectively +23%, +42%, +21%, +37% higher to the baseline mean (100%). For 5.6 mg/mL PREG*right (n=4), the overall effect of time after drug treatment (baseline mean and T10-T100 = 11 time bins) was not statistically significant (F(1.69,5.06)=.697, p=.517); the overall effect of time after drug treatment (T10-T100 = 10 time bins) was not statistically significant (F(1.694,5.081)=.702, p=.516). No statistically significant contrasts to baseline mean (100%) were found. For 11.2 mg/mL PREG*left (n=2), the overall effect of time after drug treatment (baseline mean and T10-T100 = 11 time bins) was not statistically significant (F(1,1)=1.32, p=.456); the overall effect of time after drug treatment (T10-T100 = 10 time bins) was not statistically significant (F(1,1)=.413, p=.636). Furthermore, the sample size in the analysis is very small (n=2). Nearly statistically significant contrast to baseline mean (100%) was found at T100 (F(1,1)=147.736, p=.052), +161% higher than baseline mean. For 11.2 mg/mL PREG*right (n=4), the overall effect of time after drug treatment (baseline mean and T10-T100 = 11 time bins) was not statistically significant (F(1.77,5.30)=1.93, p=.233); the overall effect of time after drug treatment (T10-T100 = 10 time bins) was not statistically significant (F(1.974,5.922)=.630, p=.563 Statistically significant contrast to baseline mean (100%) was found at T70 (F(1,3)=32.26, p=.011), +264% higher than the baseline mean. For VEH*left (n=4), the overall effect of time after drug treatment (baseline mean and T10-T100 = 11 time bins) was not statistically significant (F(1.62,4.85)=1.18, p=.368); the overall effect of time after drug treatment (T10-T100 = 10 time bins) was not statistically significant (F(1.603,4,809)=1.060, p=.397). No statistically significant contrast to baseline mean (100%) was found. For VEH*right (n=7), the overall effect of time after drug treatment (baseline mean and T10-T100 = 11 time bins) was not statistically significant (F(2.82,16.92)=8.58, p=.476); the overall effect of time after drug treatment (T10-T100 = 10 time bins) was not statistically significant (F(2.652,15.909)=.891 p=.456). No statistically significant contrast to baseline mean (100%) was found. Pairwise comparisons of estimated marginal means at each time-bin produced statistically significant differences between: (1) 5.6 mg/mL PREG*right and 5.6 mg/mL PREG*left at T10 (p=.035) with PREG*right < PREG*left, T90 (p=.023) with PREG*right < PREG*left.

Drug dose comparison in the amygdala right hemisphere targets only

For the amygdala right hemisphere, a statistically significant interaction between time (10 time bins after drug treatment) and drug treatment (vehicle n=7; 5.6 mg/mL PREG, n=4; 11.2 mg/mL PREG, n=4) was not found F(4.712,28.272)=.863, p=0.512 (Figure 5E). Furthermore, a between-subject comparison of the subject-average of time curves of right hemisphere groups was performed. The main effect of drug was statistically significant (F(2,12)=7.626, p=.007). A 2-tailed post-hoc Dunnett comparison between 11.2 mg/mL PREG and vehicle was statistically significant (p=.007) with marginal mean of 11.2 mg/mL PREG dose > compared to the marginal mean of the vehicle.. Pairwise comparisons of estimated marginal means at each time-bin in the right hemisphere produced statistically significant differences between: (1) vehicle and 11.2 mg/mL PREG at all the time-bins: T10 (p=.006), T20 (p=.013), T30 (p=.029), T40 (p=.010), T50 (p=.004), T60 (p=.002), T70 (p=.0001), T80 (p=.011), T90 (p=.002), T100 (p=.012), with 11.2 mg/mL PREG respectively +179%, +204%, +188%, +232%, +254%, +223%,+167%, +189%, +164%, +136% (as mean % of the difference) higher than vehicle, (2) 5.6 mg/mL PREG and 11.2 mg/mL PREG at all the time-bins: T10 (p=.005), T20 (p=.023), T30 (p=.044), T40 (p=.022), T50 (p=.009), T60 (p=.008), T70 (p=.0001), T80 (p=.022), T90 (p=.004), T100 (p=.020) with 11.2 mg/mL PREG respectively +207%, +206%, +192%, +224%, +250%, +200%, +158%, +188%, +171%, +140% (as mean % of the difference) higher than 5.6 mg/mL PREG. Furthermore, the relative rise in the ACh level after the drug treatment ranged from -19% (T10) and +8% (T50) for the low dose, in contrast to the baseline variation (between -5% at BT30 and +17% at BT40), and from +136% (T70) to +258% (T50) for the high dose, in contrast to the baseline variation (between -13% at BT60 and +24% at BT20).

Drug dose comparison in the amygdala left hemisphere targets only

For the amygdala left hemisphere, a statistically significant interaction between time (10 time bins after drug treatment) and drug treatment (vehicle n=4; 5.6 mg/mL PREG, n=4; 11.2 mg/mL PREG, n=2) was not found F(5.085,17.799)=.642, p=.673 (Figure 5F). Furthermore, a between-subject comparison of the subject-average of time curves of left hemisphere groups was performed. The main effect of drug was statistically significant (F(2,7)=13.543, p=.004). A 2-tailed post-hoc Dunnett comparison between 11.2 mg/mL PREG and vehicle was found statistically

significant (p=.003) with marginal means of 11.2 mg/mL PREG dose > compared to the marginal mean of the vehicle.. Pairwise comparisons of estimated marginal means at each time-bin in the left hemisphere produced statistically significant differences between: (1) vehicle and 11.2 mg/mL PREG at all the time-bins: T10 (p=.012), T20 (nearly significant, p=.053), T30 (p=.003), T40 (p=.011), T50 (p=.028), T60 (p=.021), T70 (p=.010), T80 (p=.040), T90 (p=.004), T100 (p=.019) with 11.2 mg/mL PREG respectively +51%, +69%, +62%, +72%, +94%, +73% +58%, +61%, +69%, +56% (as mean % of the difference) higher than vehicle, (2) 5.6 mg/mL PREG and 11.2 mg/mL PREG at: T10 (p=.014), T20 (p=.044), T30 (p=.002), T40 (p=.009), T50 (p=.045), T70 (p=.033), T90 (p=.040) with 11.2 mg/mL PREG respectively +49%, +73%, +66%, +76%, +83%, +48% +44%,+42%, +42%, +33% (as mean % of the difference) higher than 5.6 mg/mL PREG. Furthermore, the relative rise in the ACh level after the drug administration ranged from 9% (T20) and +42% (T60) for the low dose, in contrast to the baseline variation (between -11% at BT40 and +16% at BT50), and from +55% (T80) to +101% (T50) for the high dose, in contrast to the baseline variation (between -7% at BT40 and +11% at BT30).

4.2 Experiment 2: Effects of unilateral IN-PREG on ACh release in the amygdala

In the previous experiment, the results of the high dose IN-PREG administration in ACh content in the amygdala were used as the starting point of experiment II.

Our interest was consequently to focus on the mechanism behind PREG nasal delivery and cholinergic modulation in the rat brain. We hypothesized that, unilateral IN-PREG administration would selectively activate cholinergic system in the ipsilateral hemisphere, due to epithelial pathway. Instead, if no differences between the two hemispheres in ACh release had been found, it would have meant circulatory delivery.

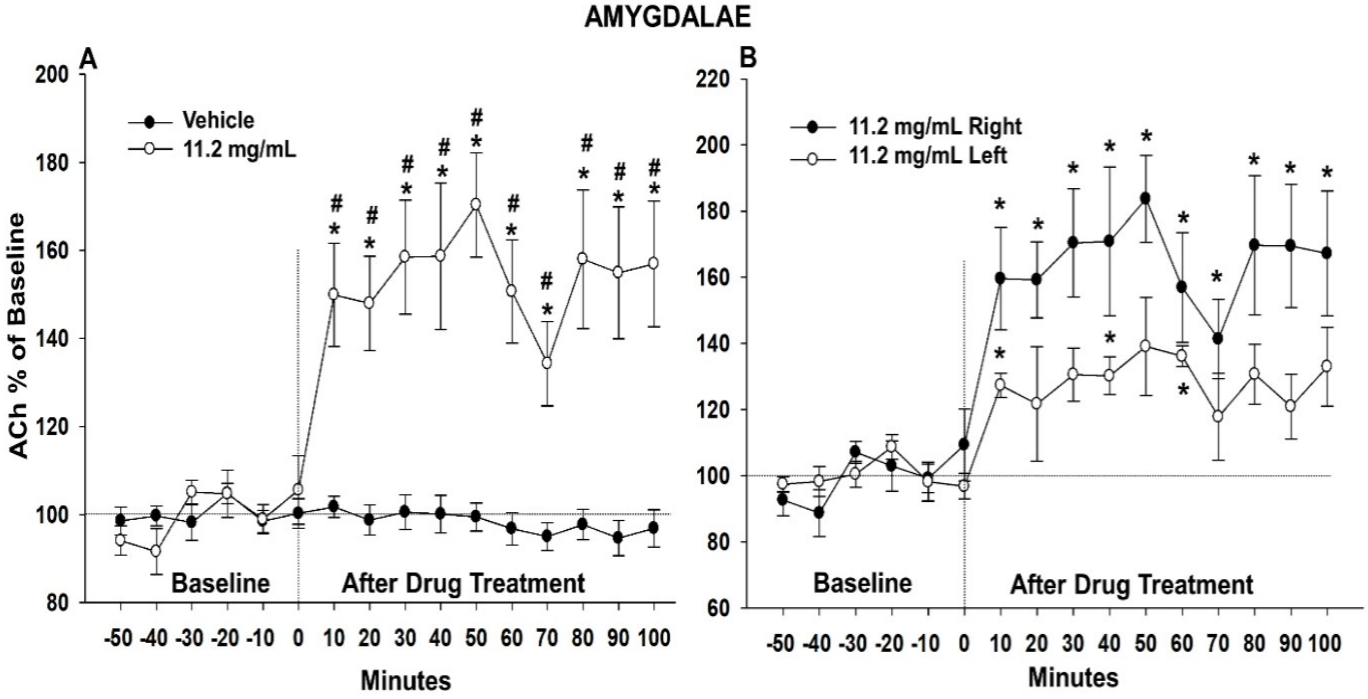

Figure 6. A. Lateralized effects of unilateral intranasal administration of 11.2 mg/mL PREG on ACh in the amygdala. The intranasal administration was performed at T0. Each rat received 5µL of 11.2 mg/mL PREG into one nostril and 5µL of vehicle into the other. # $p<0.050$ comparison of ACh in the amygdala ipsilateral to the nostril, which had received PREG to ACh in contralateral amygdala (ipsilateral to nostril, which had received vehicle). * $p<0.050$, compared with baseline mean. Sample size n=10. **B.** Comparison of the effect on ACh in the ipsilateral amygdala after PREG administration in the right vs left nostril. The graph shows the level of ACh in the

ipsilateral amygdalae (right and left) before and after administration. * p<0.050, compared with baseline mean. Sample size: right (n=7), left (n=3). Values are presented as % of baseline with six baseline samples taken as 100% (mean + SE).

IN-PREG vs. vehicle (within-subject)

A 2-factor within-subject ANOVA was performed on all scores from the entire sample of n=10. PREG- and vehicle-treated side are considered as dependent data (as well as the time-bins). An interaction between time course of drug effect (10 time-bins after treatment) and drug effect (11.2 mg/mL PREG vs vehicle) was found to be statistically significant ($F(2.56,23.012)=4.51$, $p=.016$). Also the main effect of PREG vs vehicle for the hemisphere-average of time-curves was highly significant ($F(1,9)=40.2$, $p<.001$). Figure 6A depicts a flat curve for the vehicle-treated amygdala and a rise in ACh after IN-PREG treatment in the PREG-treated amygdala.

Focused hypotheses

The baseline mean (100%) was compared to all time-bins after treatment with PREG and vehicle by means of simple contrast. For vehicle-treated animals, none of the separate comparisons between time-bins and baseline mean were statistically significant. The change of ACh levels relative to baseline ranged from -5% (T70) to +1.7% (T10). For PREG-treated targets, all contrasts between baseline mean and time-bins were statistically significant (T10 ($F(1,9)=18.11$, $p=.002$), T20 ($F(1,9)=20.07$, $p=.002$), T30 ($F(1,9)=20.47$, $p=.001$), T40 ($F(1,9)=12.44$, $p=.006$), T50 ($F(1,9)=35.09$, $p<.001$), T60 ($F(1,9)=18.47$, $p=.002$), T70 ($F(1,9)=12.88$, $p=.006$), T80 ($F(1,9)=13.55$, $p=.005$), T90 ($F(1,9)=13.5$, $p=.005$), T100 ($F(1,9)=15.89$, $p=.003$)). The relative rise of ACh levels ranged from +34% (T70) to +70% (T50). The effect of PREG vs vehicle assessed at separate time bins yielded the following statistically significant results: T10 ($F(1,9)=16.14$, $p=.003$), T20 ($F(1,9)=27.56$, $p=.001$), T30 ($F(1,9)=31.13$, $p<.001$), T40 ($F(1,9)=15.27$, $p=.004$), T50 ($F(1,9)=39.74$, $p<.001$), T60 ($F(1,9)=24.62$, $p=.001$), T70 ($F(1,9)=13.9$, $p=.005$), T80 ($F(1,9)=18.6$, $p=.002$), T90 ($F(1,9)=16.93$, $p=.003$), T100 ($F(1,9)=23.21$, $p=.001$). The effect of unilateral application reflects the effects observed in experiment 1.

Control of confounding variable "left vs right hemisphere"

Vehicle-treated and PREG-treated targets were analysed separately (two independent samples of n=10). In each sample (vehicle, 11.2 mg/mL PREG), the possible interaction between localization (left or right) and time course of drug effect was assessed in a mixed design (out of 10 subjects, n=3 received vehicle into the right and PREG into the left nostril; n=7 received vehicle into the left and PREG into the right nostril) (Figure 6B). For the IN-PREG treated targets, no significant interaction between time and left vs. right side was found ($F(2.35,18.81)=0.15$, $p=.89$). Furthermore, a between-subject comparison of the subject-average of time curves of PREG*left vs PREG*right groups was performed. The main effect of left vs right hemisphere was not statistically significant ($F(1,8)=4.17$, $p=.075$). For the IN-vehicle treated targets, no significant interaction between time and left vs. right side was found ($F(4.21,33.69)=0.55$, $p=.712$). The between-subject effect of vehicle*left vs vehicle*right was found to be not statistically significant ($F(1,8)=0.03$, $p=.869$). Moreover, the effect over time after drug application was compared to the baseline for PREG*left (n=3) and PREG*right (n=7) treated subjects, separately. For PREG*left, the main effect of time was not significant ($F(1.64,3.28)=1.2$, $p=.391$). Significant contrasts to baseline mean (100%) were found at T10 ($F(1,2)=54.26$, $p=.018$), T40 ($F(1,2)=27.57$, $p=.034$), and T60 ($F(1,2)=134.94$, $p=.007$). For PREG*right, the overall effect of time was not significant ($F(2.17,13.04)=2.81$, $p=.094$). Significant contrasts to baseline mean (100%) were found at all time-bins: T10 ($F(1,6)=14.76$, $p=.009$), T20 ($F(1,6)=26.47$, $p=.002$), T30 ($F(1,6)=18.41$, $p=.005$), T40 ($F(1,6)=9.93$, $p=.02$), T50 ($F(1,6)=40.95$, $p=.001$), T60 ($F(1,6)=11.78$, $p=.014$), T70 ($F(1,6)=11.93$, $p=.014$), T80 ($F(1,6)=10.94$, $p=.016$), T90 ($F(1,6)=13.91$, $p=.01$), T100 ($F(1,6)=12.62$, $p=.012$). The average rise of ACh relative to the baseline mean ranged from +17% (T70) to +39%(T50) in the PREG*right group and from +41% (T70) to +83% (T50) in the PREG*left group. Figure 6B also shows a similar time curve with an initial peak at T10, a positive slope until T50 and a drop at T70, followed by a renewed new rise (resp. plateau).

5. General Discussion

In the present study, we investigated the effect of IN-PREG (5.6 mg/mL or 11.2 mg/mL) and vehicle on ACh release in frontal cortex, hippocampus and amygdala. We present first-time evidence that intranasal administration of PREG in either dose increased frontal and hippocampal ACh in a time-dependent fashion relative to both baseline and vehicle. Moreover, 5.6 mg/ml PREG increased ACh release in the amygdala relative to baseline, the lower dose and vehicle. In a next step, we investigated the effect of PREG (11.2 mg/mL) administered into one nostril in ACh levels in the ipsi- and contralateral amygdala. An increase of extracellular ACh was observed in the ipsilateral amygdala, whereas no effect was visible in the amygdala of the contralateral hemisphere. This result demonstrates an effective and selective modulation of cholinergic system by PREG. This finding is in line with a previous study, which showed an effect of the PREG derivative PREG-S on ACh release (Darnaudéry *et al.* 1998).

5.1 IN pathway

The greatest increase of ACh in the amygdala was observed within the first ten minutes after administration of 11.2 mg/mL PREG, while a second increase, although smaller, could be seen at T80. The extent to which the drug is transported and reaches the brain is highly dependent on the physicochemical properties of the drug in question, mainly lipophilicity, molecular weight and degree of dissociation (Sakane *et al.* 1991). PREG is a neutral lipophilic molecule with a molecular weight of 316 D and, like small, lipophilic drugs with a molecular weight up to 600 Da, can easily enter the blood stream and cross the BBB (Pardridge 1991). The most likely way of delivery is passive diffusion across the epithelium, since it can pass membranes readily and to be retained in the brain (Qaiser et al. 2017). A smaller amount could also have been directly absorbed by the capillaries at the lamina propria of the olfactory epithelium and, after a time of systemic circulation, cross the BBB, which is in line with the increase in ACh 70 minutes after drug administration. Thus, we propose that the first major increase corresponds to the direct olfactory epithelial transport, while the second slighter increase may be due to a smaller amount of drug crossing the blood-brain barrier after having entered the bloodstream. This is

consistent with previous studies, in which epithelial transport was proven to be rapid, with drugs like dihydroergotamine (Wang *et al.* 1998), lidocaine (Chou and Donovan 1998), and cefalexin (Sakane *et al.* 1991) appearing in the CFS and brain only a few minutes after administration. Moreover, we have previously shown that nasally administrated cocaine is transported within minutes from the nasal cavities into the brain via the olfactory epithelial pathway (de Souza Silva *et al.* 1997a).

5.2 IN-PREG effects in the cholinergic system in the rat brain

PREG acts as a sigma 1 receptor agonist (Nuwayhid and Werling 2003). Sigma 1 receptors modulate NMDA receptors (Monnet *et al.* 1990; Pabba *et al.* 2014). Activated NMDA receptors, in turn, might activate cholinergic neurons in the basal forebrain, which project to frontal cortex, hippocampus and amygdala. Sigma1 receptors are highly expressed in the rat olfactory bulb, amygdaloid complex, hippocampus and hypothalamus (Alonso *et al.* 2000). In-vivo microdialysis studies in freely moving rats as well as in-vitro studies in hippocampal and frontal rat brain slices showed significant increases of extracellular ACh in hippocampus and frontal cortex after systemic administration of a sigma 1 receptor agonists (Matsuno *et al.* 1992; Kobayashi *et al.* 1996; Horan *et al.* 2002). The authors propose that the stimulation of ACh release is either triggered by cholinergic nerve terminals expressing sigma1 receptors or by the activation of other neurons, which express this receptor subtype and, upon their activation, modulate cholinergic transmission.
As previously proposed by Giovannini *et al.* (1997), the glutamatergic-GABAergic-cholinergic circuitry in the basal forebrain is be responsible for the regulation of ACh levels in various areas of the brain, including hippocampus, frontal cortex and amygdala. A low affinity of PREG for GABAA receptors and NMDA receptors has been described, while its sulfated form, PREG-S, is considered a strong modulator of these receptors (Weaver *et al.* 1997; Park-Chung *et al.* 1999). It is not clear as of yet, whether the release of ACh is induced via modulation of sigma receptors by PREG, or via activation of GABAA receptors and/or NMDA receptors by PREG or PREG-S. Darnaudéry *et al.* (2002) have shown that infusion of PREG-S into the medial septum nucleus increased hippocampal ACh. In the basal forebrain, the medial septal nucleus and the vertical limb of the diagonal band of Broca (MSDB) send cholinergic efferents to the hippocampus (Woolf *et al.* 1984; Melander *et al.* 1985; Wainer *et al.* 1985). Moreover, 50% of the neurons involved in the septo-

hippocampal pathway are GABAergic and glutamatergic neurons of the MSDB (Amaral and Kurz 1985). The modulation of GABAA receptors or NMDA receptors on either cholinergic and GABAergic neurons in the MSDB enhanced hippocampal efflux of ACh (Gorman *et al.* 1994; Darnaudéry *et al.* 2002; Elvander-Tottie *et al.* 2009; Roland *et al.* 2014). Hence, also in the present study, IN-PREG might have modulated the release of ACh in the hippocampus by modulating GABAA and/or NMDA receptors (possibly via sigma1 receptor activation) on neurons in the MSDB. Based on this hypothesis, the memory enhancement observed in a previous study after IN-PREG administration (Abdel-Hafiz *et al.* 2016) could be explained as the result of hippocampal theta oscillations induced by the activation of the cholinergic septo-hippocampal pathway (Keita *et al.* 2000).

Unilateral IN-PREG showed a significant increase of extracellular ACh in the ipsilateral amygdala, but not in the contralateral one. Similarly, unilateral intranasal application of L-DOPA induced an elevation of dopamine levels only in the ipsilateral neostriatum. This either implies that L-DOPA entered brain and neostriatum via the nasal mucosa or that it entered a brain site more proximal to the nasal epithelium, and, by the action on this brain site, indirectly affected dopamine activity in the neostriatum (de Souza Silva *et al.* 1997a). The lateralized effects of PREG observed in the present study support the hypothesis that IN-PREG entered the brain via the epithelial pathway. The lateralized diffusion of PREG from one nostril to the amygdala of the ipsilateral hemisphere can be accounted for by the anatomical separation of the right and left nostril by the septum and ethmoid bone (Shipley and Reyes 1991). In the contralateral amygdala, a direct nasal-brain transport was not expected. However, due to PREG lipophilicity, an effect on ACh release also in the contralateral amygdala at T70, resulting from the entry of PREG into systemic circulation, could have been expected. In the Ducharme et al. (2010) study, 23% of PREG after bilateral IN administration entered the circulation. Nevertheless, the lack of ACh release in the contralateral amygdala, implies that the drug did not reach the dose threshold in the contralateral amygdala to trigger the cholinergic activity via the sigma 1 receptor (Matsuno et al. 1992).

By activation of NMDA receptors, PREG may increase glutamate-induced depolarization (Shipley and Ennis 1996; Alonso *et al.* 2000; Monnet and Maurice 2006; Lethbridge *et al.* 2012). Through unilateral afferent glutamate projections from the olfactory bulb, cholinergic neurons of the basal forebrain are activated

(Giovannini *et al.* 1997), which in turn, stimulate the release of ACh in the amygdala (Dani and Bertrand 2007). Specifically, the nucleus basalis magnocellularis-cortical pathway could be involved in the release of extracellular ACh in the amygdala after PREG administration. The rat nucleus basalis magnocellularis–substantia innominata complex sends cholinergic efferents to the dorsolateral frontal and parietal cortex and to the basolateral amygdala (Heckers and Mesulam 1994; Mesulam 1995). PREG-S infusion into the nucleus basalis has been shown to increase ACh release in frontal cortex and amygdala, with a subsequent improvement of spatial and emotional memory (Pallarés *et al.* 1998; Gold 2003; Tinsley *et al.* 2004). In the present study, no significant difference was found between administration into the right and the left nostril on ACh release in the ipsilateral amygdala. This result is in line with the findings of Parthasarathy and Bhalla (2013), who found no difference in symmetry and laterality of information flow in the nostrils of rats. Moreover, no significant activity on the contralateral side was observed after unilateral presentation of odors, indicating that contralateral odorant input has no effect on the response at the glomerular level of the ipsilateral olfactory bulb. The higher ACh efflux in the amygdala compared to the other investigated regions induced by the higher IN-PREG dose (11.2 mg/mL) might be related to the higher density of cholinergic terminals in the basolateral and lateral amygdala (Nagai *et al.* 1982; Woolf and Butcher 2011). Besides, the differences in ACh release might be due to tissue-specific differences in cholinergic regulation, which depends on the expression and density of specific cholinergic receptors subtypes such as the presynaptic muscarinic m2 and m4 receptor, the muscarinic m2 and m4 autoreceptor (Fitzgerald 2009), and the postsynaptic alpha-7 nicotinic receptor (Arroyo et al. 2014). Continuous cholinergic modulation has been shown to be decreased when regulated by postsynaptic alpha-7 nicotinic receptors, due to their fast desensitization to ACh, in contrast to non-alpha-7 nicotinic receptors arroyo (Arroyo et al. 2014). Muscarinic receptors, m2 and m4, autoreceptors, presynaptically expressed, have inhibitory feedback mechanism (Fitzgerald 2009).

5.3 PREG role in the behavior

There is extensive proof that the amygdala is involved in emotional memory and that emotional arousal stimulates the amygdala resulting in the modulation of long-term memory storage in other brain regions (McGaugh *et al.* 1996). Experimental studies

in animals and humans suggest that highly arousing experiences will be remembered more precisely, more easily and over a longer period of time compared to less arousing experiences (Cahill *et al.* 1996; McGaugh *et al.* 1996; Schafe *et al.* 2001). More specifically, amygdala and hippocampus act synergistically to form long-term memories of emotional events (Phelps 2004). It seems, therefore, reasonable to speculate that PREG, by increasing ACh release in the amygdala, can stimulate memory and learning. This is in line with previous findings, showing IN-PREG enhances memory and cognitive processes (Abdel-Hafiz *et al.* 2016). As a consequence, this neurosteroid may be relevant for the maintenance of cognitive function in conditions which are characterized by a deficiency of ACh, such as early dementia or manifest Alzheimer´s disease.

5.4 PREG as therapeutic candidate in brain disorders

The IN-PREG approach might be useful for brain-related disorders, as impaired cholinergic system leads to defective cognitive functions. Sigma 1 receptor agonists, such as PREG, are considered to ameliorate memory impairments resulting from cholinergic system damage in pathological conditions (Meyer et al. 2002). In AD the loss of cholinergic markers in the cortex and hippocampus and neuronal loss in the basal forebrain (BF), due to neurofibrillary tangles and amyloid plaques, has been found. This neuronal degeneration leads to the impairment of memory and attention processes in the AD (Whitehouse et al. 1982; Geula and Mesulam 1989). Moreover, patients affected by PD and PD with dementia (PDD) also incur decline of cognitive functions. In fact, cholinergic neuronal loss in the BF has been found in PD patients and it has been proposed as being induced by aggregates of alpha synuclein into Lewi bodies (Perry et al. 1995). However, difficulties in learning, working memory and attention are not only limited to AD and PD, but also appear in schizophrenia. In mouse model of schizophrenia PREG treatment provided enhanced memory and attention deficits (Wong et al. 2012). The same cognitive improvements were also found when PREG was orally administrated as antipsychotic monotherapy or as anti-inflammatory adjuvant therapy for patients with schizophrenia (Marx et al. 2009; Kreinin et al. 2017; Cho et al. 2019). Furthermore, PREG treatments reduced negative symptoms, namely, anxiety and depression (Marx et al. 2009; Wong et al. 2012; Brown et al. 2014). Cholinergic neurotransmission in septohippocampal brain regions has an important role in controlling anxiety (File et al. 2000). The results

obtained in the clinical studies for patients with schizophrenia suggested that PREG has been metabolized into PREG-S and consequently improved NMDA receptor hypofunction in schizophrenia or PREG activates sigma receptor modulation of NMDA receptor. Behavioral evidence showed PREG involvement in the regulation of anxiety mechanisms in mice (Melchior and Ritzmann 1994). Interestingly, IN-PREG in healthy rats showed no anxiety relief in the elevated plus-maze (Abdel-Hafiz et al. 2016). Additionally, IN-PREG could be considered as new therapeutic approach for drug abuse. PREG has been found acting as a negative allosteric modulator for the type-1 cannabinoid (CB1) receptor. Vallée et al. (2014) showed that PREG reduces cannabinoid THC-related effects at behavioral level, impairment of memory consolidation and THC-induced food-intake, and at the neurobiological level by limiting agonist efficacy on the CB1 receptor. Together with the improvements on cognitive functions after PREG administration, the neuroprotective function of PREG provides an additional prospective on the therapeutic effects. Thus, PREG might be relevant for preventing the progression of the AD at early stages by blocking Abeta toxicity. In vitro study demonstrated PREG neuroprotective effects as it attenuated inflammatory toxicity against glutamate and amyloid beta protein neurotoxicity, which were previously shown to induce to neuronal cell death (Cardounel et al. 1999; Gursoy et al. 2001). This might be related to the modulation of the sigma receptor, as their activation facilitates cognition in AD by preventing Abeta-associated NMDA receptor neurotoxicity (Yang et al. 2012). However, PREG has further neuroprotection properties against oxidative stress damage, resulting in cell death, via modulation of cytoskeleton dynamics by binding with high affinity to the microtubule-associated protein 2 (MAP2) (Murakami et al. 2000; Fontaine-Lenoir et al. 2006). MAP2 is localized in neuronal cell bodies, dendrites and dendritic spines and has a role in assembling and maintaining stability of microtubules, having an important role in neuronal plasticity (Conde and Cáceres 2009). The neuronal plasticity is affected by stress, aging and depression. In the above clinical studies, oral administration of PREG showed no serious adverse events and benefited the improvement of negative and cognitive symptoms (Marx et al. 2009; Marx et al. 2014; Brown et al. 2014; Ritsner et al. 2014). Moreover, the MAPREG start-up (Baulieu et al. 2004) has established a 3-methoxy analog of PREG (MAP4343) currently in Phase 2 Clinical trials, aiming to treat resistant depression. However, conventional drug delivery might be not efficient enough in targeting the brain. High

doses of drugs are often needed when orally administrated to induce a biological effect and may lead to side effects. The IN way of administration provides an efficient drug delivery through the brain, minimizing systemic exposure and consequently leading to minor side effects (Banks et al. 2009).

6. Conclusions

The present study yields first-time evidence that IN-PREG increases frontal and limbic ACh in a dose- and time-dependent fashion. Application into one nostril increased ACh levels in the ipsilateral amygdala, whereas no effect was observed in the amygdala of the contralateral hemisphere. This implies that IN-PREG reached the brain mainly via the direct olfactory epithelial pathway. Results demonstrate an effective modulation of the cholinergic system by IN-PREG, suggesting a therapeutic value in age-related neurodegenerative diseases linked to memory disturbances and depressed ACh activity, such as in Alzheimer's disease.

7. References

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8. Original Research Articles

8.1 Publication in support of the focus of this dissertation

1. **Benedetta Fazari**, Cvetana Ilieva Decheva, Victoria González García, Laila Abdel-Hafiz, Susanne Nikolaus, Cornelis P. Hollenberg, Joseph P. Huston, Maria A. de Souza Silva, Claudia Mattern, Intranasal pregnenolone increases acetylcholine in frontal cortex, hippocampus and amygdala - preferentially in the hemisphere ipsilateral to the injected nostril (in submission).

8.2 Other pubblications by the author

1. A.L. Wang, **B. Fazari**, O.Y. Chao, S. Nikolaus, S.V. Trossbach, C. Korth, F.J. Sialana, G. Lubec, J.P. Huston, C. Mattern, M.A. de Souza Silva, Intra-nasal dopamine alleviates cognitive deficits in tgDISC1 rats which overexpress the human *DISC1* gene, Neurobiology of Learning and Memory 146 (2017) 12-20.
2. F.J. Sialana, A.L. Wang, **B. Fazari**, M. Kristofova, R. Smidak, S.V. Trossbach, C. Korth, J.P. Huston, M.A. de Souza Silva, G. Lubec, Quantitative Proteomics of Synaptosomal Fractions in a Rat Overexpressing Human *DISC1* Gene Indicates Profound Synaptic Dysregulation in the Dorsal Striatum, Frontiers in Molecular Neuroscience 11(26) (2018).
3. Abdel-Hafiz, L., Müller-Schiffmann, A., Korth, C., **Fazari, B**., Chao, O.Y., Nikolaus, S., Schäble, S., Herring, A., Keyvani, K., Lamounier-Zepter, V., Huston, J.P., de Souza Silva, M.A., (2018). Abeta induce behavioral and neurochemical deficits of relevance to early Alzheimer´s disease. Neurobiology of Aging, 69 (1-9).

9. Appendix

Intranasal pregnenolone increases acetylcholine in frontal cortex, hippocampus and amygdala - preferentially in the hemisphere ipsilateral to the injected nostril

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Intranasal pregnenolone increases acetylcholine in frontal cortex, hippocampus and amygdala - preferentially in the hemisphere ipsilateral to the injected nostril

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Short title: Intranasal pregnenolone effects on acetylcholine

Abstract

The present study determined the effects of intranasal pregnenolone (IN-PREG) on acetylcholine (ACh) levels in selected areas of the rat brain. In the first experiment, IN-PREG (5.6 and 11.2 mg/mL) or vehicle was applied bilaterally, and ACh levels were assessed in amygdala, hippocampus and frontal cortex, using in vivo microdialysis. In the second experiment, IN-PREG (11.2 mg/mL) or vehicle was applied only into one nostril in order to determine whether ACh is predominantly increased in the ipsilateral relative to the contralateral amygdala. Bilateral IN-PREG (5.6 and 11.2 mg/mL) increased frontal cortex and hippocampal ACh in a time-dependent fashion relative to both baseline and vehicle. Moreover, 11.2 mg/mL PREG increased ACh in the amygdala relative to baseline, the lower dose and vehicle. Unilateral application of IN-PREG increased ACh in the ipsilateral amygdala, whereas no effect was observed on the contralateral side. Findings suggest that PREG is effectively transported from the nostrils to the brain via the olfactory epithelial pathway but not by circulation. The present data provide additional information on the time-dependency of IN-PREG action in the cholinergic system of amygdala and hippocampus. This may be relevant for therapeutic IN application of PREG in neurogenerative diseases and neuropsychiatric disorders.

1. Introduction

The neuroactive steroid pregnenolone (PREG) is synthesized from cholesterol (Harteneck, 2013) and can be converted into different steroids such as dehydroepiandrosterone (DHEA), testosterone, progesterone, estrogen and cortisol. PREG may be transformed into pregnenolone sulfate (PREG-S; Robel *et al.* 1995). This compound both disinhibits (through negative modulation of $GABA_A$ receptors) and activates (through positive modulation of NMDA receptors) the cholinergic neurons of the medial-septal diagonal band of Broca, which project to the hippocampus (Darnaudéry *et al.* 2002). Also, PREG, the precursor of PREG-S, is neurochemically active. Nuwayhid and Werling (2003) demonstrated that PREG inhibits the NMDA-stimulated dopamine release in the striatum via sigma receptors with involvement of the coupled-PKCß pathway.

Cholinergic transmission is known to be involved in memory processes, and various studies have shown promnesic effects of this neurosteroid. Flood *et al.* (1992) reported a memory-enhancing effect in mice by immediate post-training intracerebroventricular (icv) administration of PREG-S. Similarly, when injected into the amygdala, PREG-S enhanced memory processes in mice (Flood *et al.* 1995). Icv injection of PREG-S in rodents compensated scopolamine-induced learning deficits in visual discrimination tasks (Meziane *et al.* 1996) and improved spatial memory (Darnaudéry *et al.* 2000). Moreover, PRE-S increased acetylcholine (ACh) release in the hippocampus (Darnaudéry *et al.* 2000). Likewise, intraperitoneal or bilateral intrahippocampal injection of PREG-S ameliorated memory deficits in cognitively impaired rats and stimulated ACh release in the rat hippocampus (Vallée *et al.* 1997).

Since intranasal administration allows circumvention of the blood brain barrier, the delivery of compounds directly from nose to brain has been established as a key alternative to oral

and parenteral modes of administration. Intranasally applied PREG (IN-PREG) has also been shown to influence memory and cognitive processes. Abdel-Hafiz *et al.* (2016) reported that pre-trial IN administration of PREG facilitated long-term memory in both object-preference and object-location preference tests 48 hours post-administration in rats. Besides, the treated animals showed a superior memory for the position of the escape platform in the Morris water maze compared to controls (Abdel-Hafiz et al., 2016).

Ducharme *et al.* (2010) compared brain distribution and behavioral effects of PREG after IN and intravenous administration. Intravenous administration of PREG was associated with higher stability in the blood, while IN administration was associated with higher stability in the brain. Also, PREG was degraded to a lesser extent in the brain after intranasal relative to intravenous injection (Ducharme *et al.* 2010).

Intranasally applied diffusable substances, including steroids, are delivered via the olfactory epithelial pathway into the perineural spaces of the olfactory nerve in the ipsilateral olfactory bulb and into the subarachnoid space of the brain and central nervous system (CNS; Baker and Spencer 1986; Chen *et al.* 1998; Frey *et al.* 1997; Illum 2000; Shipley and Reyes 1991).

The goal of this study was to investigate the effects of IN administered PREG on the extracellular ACh concentrations over a period of 100 min in frontal cortex, hippocampus and amygdala of the rat brain using *in vivo* microdialysis. Previous studies have shown that the unilateral IN administration of L-DOPA increased extracellular dopamine only in the ipsilateral neostriatum, excluding the systemic transport of the drug from the nose to the CNS (de Souza Silva *et al.* 1997b; de Souza Silva *et al.* 1997a). This prompted us to assess the circuitry of PREG action, by measuring ACh release in the ipsilateral and contralateral amygdala after unilateral IN application of PREG.

2. Materials and methods

2.1. Subjects

A total of 41 Wistar rats (age: 3 to 4 months) weighing between 400 and 500 g at the time of surgery, were obtained from the local animal facility (ZETT, Heinrich-Heine University of Düsseldorf, Germany). Rats were maintained in standard macrolon cages (590 x 380 x 200 mm; 4 animals per cage) under a reversed light-dark cycle (lights off from 7 a.m. to 7 p.m.), with free access to food and water. After surgery, animals were kept individually (cage size: 40 x 26 x 26 cm). The room temperature was 20 ± 2 °C with controlled humidity. After an adaptation time of 2 weeks, rats were implanted with microdialysis probes. All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) on animal welfare and the German Law on the Protection of Animals. The protocol was approved by the regional authority (Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen, Recklinghausen, Germany).

2.2. Drugs

PREG (Bayer HealthCare Pharmaceuticals, Berlin, Germany; year of purchase: 2016) was dissolved in a lipid-based gel formulation for IN administration (provided by M et P Pharma AG, Emmetten, Switzerland) and administered in concentrations of 5.6 mg/L or 11.2 mg/mL. A lipid-based gel vehicle was used as control. With a Microman pipette (Gilson, Villiers le Bel, France), 5 µL of the formulation was carefully applied into each nostril. Since the average weight of the animals at the time of surgery was 450 g, the mean lower dose was 0.124 mg/kg (= a total of 0.056 mg per rat) and the mean higher dose was 0.249 mg/kg (= a total of 0.112 mg per rat).

2.2. Surgery

After anesthesia with a mixture of ketamine hydrochloride (90.0 mg/kg; Pfizer GmbH, Berlin, Germany, catalogue number: 61879226.00.00, year of purchase: 2016) and xylazine hydrochloride (8.0 mg/kg; Bayer Vital GmbH, Leverkusen, Germany, catalogue number: 6293841.00.00, year of purchase: 2016), for surgical level of anesthesia, rats were placed into a stereotaxic frame (David Kopf Instruments, Tujunga, California, USA). Bupivacaine (Bucain, Actavis Group GmbH, Munich, Germany; 2.5 mg/mL, injection volume: 0.1 mL, catalogue number:1435402004, year of purchase: 2016) was applied above the skull as a local anesthetic.

In Experiment I, three stainless steel guide cannulae with a thread in the top (length: 14 mm; diameter: 30 mm diameter, 22 gauge) for the insertion of the microdialysis probes were implanted unilaterally above the ipsilateral frontal cortex [anterior-posterior (AP): + 3.7 mm; medial-lateral (ML): ± 3.0 mm; dorsal-ventral (DV): − 1.5 mm], the ipsilateral hippocampus (AP: − 6.0 mm; ML: ± 4.8 mm; DV: − 3.2 mm) and the contralateral amygdala (AP: − 2.5 mm; ML: ± 4.6 mm; DV: − 7.2 mm relative to Bregma (Paxinos and Watson, 1986; *Figure 2A,2B,2C)*. In Experiment II, two guide cannulae (length: 14 mm; diameter: 30 mm diameter, 22 gauge) were implanted in the right and left amygdala (AP: − 2.5 mm; ML: ± 4.6 mm; DV: − 7.2 mm relative to Bregma (Paxinos and Watson, 1986; *Figure 2D)*.For additional fixation of the implant, two stainless steel screws (length: 2.6 mm) were fastened to the skull. In order to reduce postoperative pain, Carprofen (Rimadyl, Pfizer, GmbH, Berlin, Germany 5 mg/kg, catalogue number: 4006884.00.00, year of purchase: 2016) carried by phosphate-buffered saline (PBS; Dulbecco´s PBS, Life Technologies Ltd, Grand Island, New York, USA, catalogue number: 14190-094, year of purchase: 2016), volume of 1 mL/kg (0.1 mL/kg

Carprofen and 0.9 mL/kg PBS), was injected into the head-neck area. The animals were allowed to recover from surgery for 3 to 6 days prior to the microdialysis study (*Figure 1)*. Surgeries were performed between 10:00 and 18:00 h.

2.3. Microdialysis probes

Custom-made microdialysis probes (Boix *et al.* 1995) consisted of a semipermeable membrane (Cuprophan, Akzo, Faser AG, Germany) (o.d: 250µm, pore size: 6kDa), glued with 2-Ton Epoxy glue (ITW Devcon, Danvers, MA, US) to a gauge stainless steel cannula (Small Parts Inc, Logansport, Indiana, USA; length: 20 mm; 22 gauge), which was connected to a medline tubing (Tygon, Saint-Gobain PPL Corporation, Paris, France; i.d. 0.020 IN, o.d. 0.060 IN). Inside the probe, a fused silica capillary tube (Cluzeau Info Labo, Cil, Sainte-Foy-La-Grande, France; i.d. 75µm) operated as outlet. The length of the cannula (14 mm as implanted guide cannula in the rat brain) was defined with a metal wire ferrule (TRU Components, Conrad Electronic SE, Hirschau, Germany; dimensions:1.5 mm^2 x 7 mm). A metal nut secured the microdialysis probe to the implanted guide cannula. The semipermeable membrane had a length of 2.0 mm for frontal cortex and amygdala and 4.0 mm for hippocampus. The tip of the semipermeable membrane was sealed with 2Ton-Epoxy glue.

2.4. Microdialysis

Prior to microdialysis, the animals were anesthetized with Urethane i.p. (1.25 g/kg, Sigma Aldrich, St Louis, Min, US; de Souza Silva *et al.* 1997a, catalogue number: U2500-250G, year of purchase: 2016), for profound and long-lasting (6-10 h) anesthesia in non-recovery procedures. To prevent dehydration, a catheter was inserted into the i.p. cavity, and

0.2 mL of Ringer's solution (B. Braun, Melsungen, Germany, catalogue number: 6737462.00.01, year of purchase: 2016) was injected every 10 min. Animals were placed into an acrylic box (45 × 25 × 22 cm), where body temperature was maintained at 36.5 ± 0.5 °C, using a temperature controller (CMA/150 Carnegie Medicin, Stockholm, Sweden) and a heating pad. The inlet tubes were connected to a microinfusion pump (CMA/100) and perfused with Ringer's solution containing the choline esterase inhibitor neostigmine bromide (Sigma-Aldrich, St. Louis, Missouri, USA, 10 µmol, catalogue number: N2001-1G, year of purchase: 2016) with a flow rate of 2 µL/min. After a stabilization period of 2 h, six baseline samples were collected every 10 min in sampling tubes containing 10 µL of internal standard (25 µL of 125 mg/mL ethylhomocholine in 100 mL of NaOH diluent) (MuseChem, Fairfield, New Jersey 07004, USA, catalogue number: M017127, year of purchase: 2016) (J.T. Baker, Mallinckrodt Baker B.V., Deventer, Holland, catalogue number: 0414650019, year of purchase: 2016). Microdialysis was performed between 10:00 and 18:00 h.

2.5. Experiment I. Effects of bilateral IN-Preg on cerebral ACh.

Thirty-one adult male Wistar rats were used. The animals were divided into three groups. After the sixth baseline sample, 5 µL of PREG (5.6 mg/mL or 11.2 mg/mL) or vehicle was injected into each nostril. After the treatment, another 10 samples were collected at 10-min intervals for a total of 100 min (T10 to T100). The volume of each sample was 20 µL. The number of animals/group were for the frontal cortex: n=9 (vehicle), n=6 (5.6 mg/mL PREG) and n=8 (11.2 mg/mL PREG); for the hippocampus: n=9 (vehicle), n=7 (5.6 mg/mL PREG) and n=12 (11.2 mg/mL PREG); and for amygdala: n=11 (vehicle), n=8 for (5.6 mg/mL PREG) and n=6 (11.2 mg/mL PREG).

2.6. Experiment II. **Effects of unilateral IN-Preg on ACh.**

A total of 10 adult male Wistar rats were used. After the sixth baseline sample, 5 µL of 11.2 mg/mL PREG was injected into one nostril (ipsilateral) and 5µL of vehicle lipid gel into the opposite nostril (contralateral). Half of the animals were administered IN-PREG into the right and half into the left nostril in order to determine hemispheric differences in the effects on ACh. After the treatment, another 10 samples were collected at 10-min intervals. The volume collected for each sample was 20 µL.

2.7. ACh assay

ACh concentrations were determined with high-pressure liquid chromatography (HPLC) with electrochemical detection (EC; de Souza Silva *et al.* 2000). The individual components of the sample were separated with a reverse-phase column (length: 75 mm) filled with ChromSpher 5C18 (Merck KGaA, Darmstadt, Germany, catalogue number: R00PK201D5, year of purchase: 2016) and loaded with sodiumdodecylsulfate (Sigma–Aldrich, Saint Louis, Missouri, US, catalogue number: 74255-250G, year of purchase: 2016). The enzyme reactor coupled to the column was filled with LiChrosorb-NH2 (Merck, Darmstadt, Germany, catalogue number: 1093330010, year of purchase: 2016), activated by glutaraldehyde (Merck, Darmstadt, Germany, catalogue number: G5882-100ML, year of purchase: 2016), and then loaded with acetylcholine esterase (Sigma–Aldrich, Saint Louis, Missouri, USA, catalogue number: C3389-10KU, year of purchase: 2016) and choline oxidase (Sigma–Aldrich, Saint Louis, Missouri, USA, catalogue number: C5896-1KU, year of purchase: 2016). EC was performed with a platinum electrode set at a potential of 0.350 mV. An ISAAC (in situ Ag/AgCl; Antec, Fremont, California, USA) was used as reference electrode. The mobile phase was composed of 1 mM tetramethylammonium chloride (Sigma–Aldrich, Saint Louis,

Missouri, USA, catalogue number: 8221560250, year of purchase: 2016) and 0.18 M K_2HPO_4 (Carl Roth GmbH, Karlsruhe, Germany, catalogue number:6878.1, year of purchase: 2016) and adjusted to pH 8.0 with KH2PO4 (Merck, Darmstadt, Germany, catalogue number: 1048711000; year of purchase: 2016; de Souza Silva *et al.* 2013). The flow rate of the HPLC pump (Merck, Darmstadt, Germany) was 0.3 µL/min. The experimenter was unaware of the animal´s group during data analysis.

Data were evaluated with the Chrom Perfect Software (Justice Laboratory Software, Denville, New Jersey, USA).

2.8 Histology

After microdialysis, rats were applied an overdose of Pentobarbital-Natrium (0.5 – 1 mL) (Narcoren, Merial GmbH, Hallbergmoos, Germany, catalogue number: 6088986.00.00; year of purchase: 2016;) and perfused with PBS and 10% formalin (Formaldehyde 37-38% stabilized with methanol (USP, BP, Ph. Eur.) pure, pharma grade; Applichem GmbH, Germany, catalogue number: 141328.1211, year of purchase: 2016). The brain was removed and placed into a vial with 10% formalin and 30% sucrose (D(+)-Saccharose > 99% Ph.Eur., Carl Roth GmbH, Karlsruhe, Germany, catalogue number: 4661.1, year of purchase: 2016) and stored at 4 °C. Except for the cerebellum, brains were sliced with a cryostat (Leica CM1900, Leica Biosystems Nussloch GmbH, Nußloch, Germany) and stained with cresyl violet (acetate) (Sigma- Aldrich, St Louis, Missouri, USA, catalogue number: 1052350025, year of purchase: 2016). The correct locations of the microdialysis probes were ascertained using a rat brain atlas (Paxinos and Watson 1986). Only the brains with successful cannulae implantation were considered in the statistical analysis. In experiment 1, the brain areas were analysed separately, since it was not always possible to collect samples from all three areas in the same animal due to the failure of microdialysis probes or HPLC issues. The total

number of animals was 51 with n=16 (vehicle), n=17 (5.6 mg/mL PREG) and n=18 (11.2 mg/mL PREG).

No samples from the frontal cortex were collected from 13 animals in the frontal cortex (vehicle: n= 3, 5.6 mg/mL PREG: n=5, 11.2 mg/mL PREG: n=5), from 12 animals in the hippocampus (vehicle: n=5, 5.6 mg/mL PREG: n=4, 11.2 mg/mL PREG: n=3) and from 13 animals in the amygdala (vehicle: n=4, 5.6 mg/mL PREG: n=4, 11.2 mg/mL PREG: n=5). Cannula localization was incorrect in the frontal cortex of 7 animals (vehicle: n=2, 5.6 mg/mL PREG: n=2, 11.2 mg/mL PREG: n=3), in the hippocampus of 6 animals (vehicle: n=1; 5.6 mg/mL PREG: n=3; 11.2 mg/mL PREG: n=2) and in the amygdala of 2 animals of the PREG 11.2 mg/mL group. Moreover, because of undetectable ACh concentration or low signal-noise ratio in the HPLC analyses 8 samples of the frontal cortex (vehicle: n=2, 5.6 mg/mL PREG: n=4, 11.2 mg/mL PREG: n=29, 5 samples of the hippocampus (vehicle: n=1, 5.6 mg/mL PREG: n=3, 11.2 mg/mL PREG: n=1) and 11 samples of the amygdala (vehicle: n=1, 5.6 mg/mL: n=5,11.2 mg/mL PREG: n=5) were excluded. In experiment 2, a total of 16 animals were used. However, due to failed microdialysis probes, samples from both amygdalae could only be collected in 10 animals.

2.9. Statistical analysis

Data from each brain area (experiment 1) were analysed separately. Different areas belonging to the same subject were considered independent. The subject-level mean of the six baseline samples (representing within-subject fluctuations of the ACh level) was set to 100%; all scores were expressed as percentage of the subject-level baseline mean.

In experiment 1, for the frontal cortex, the number of animals/group were: n=9 (vehicle), n=6 (5.6 mg/mL PREG) and n=8 (11.2 mg/mL PREG; Figure 3A). For the

hippocampus, the number of animals/group were: n=9 (vehicle), n=7 (5.6 mg/mL PREG) and n=12 (11.2 mg/mL PREG; (Figure 3B). For the amygdala, the number of animals/group were: n=11 (vehicle), n=8 for (5.6 mg/mL PREG) and n=6 (11.2 mg/mL PREG; Figure 3C). In experiment 2 (bilateral amygdala, 11.2 mg/mL PREG) the sample size totalled up to n=10.

For the whole data set (experiments 1 and 2), the assumption of sphericity for repeated measurements did not hold (Mauchly's test). For main effect of time and interaction effects involving time, the Greenhouse-Geisser correction was applied.

Shapiro-Wilk normality test (to assess whether $p<0.05$, data non-normally distributed) and z-test for normality (when Z score >1.96, data non-normally distributed) were performed (Kim 2013). Data was not normally distributed for experiment 1: in the frontal cortex, for the vehicle at the time-bins BT50 (Z score > 1.96), BT40 (Normality test: p=0.038, Z score > 1.96), for PREG 5.6 mg/mL at the time-bin BT30 (Normality test: p=0.007, Z score > 1.96), for PREG 11.2 mg/mL at the time-bin T50 (Z score > 1.96); in the hippocampus, for PREG 5.6 mg/mL at the time-bin T40 (Normality test: p=0.018, Z score > 1.96), for PREG 11.2 mg/mL at the time-bin T10 (Normality test: p=0.0001, Z score > 1.96), T50 (Z score > 1.96), T80 (Normality test: p=0.001, Z score > 1.96), T90 (Normality test: p=0.013, Z score > 1.96); in the amygdala, for the vehicle at the time-bin BT30 (Z score > 1.96), BT10 (Normality test: p=0.015, Z score > 1.96), T20 (Z score > 1.96), T80 (Normality test: p=0.005, Z score > 1.96); for PREG 5.6 mg/mL at the time-bin BT50 (Z score > 1.96), BT10 (Z score > 1.96), for PREG 11.2 mg/mL at the time-bin BT10 (Normality test: p=0.011, Z score > 1.96), T10 (Normality test: p=0.004, Z score > 1.96), T20 (Normality test: p=0.028, Z score > 1.96), T80 (Z score > 1.96). In experiment 2, in the vehicle at the time-bin T20 (Normality test: p=0.018), for 11.2 mg/mL at the time-bin BT10 (Normality test: p=0.011, Z score > 1.96), T40 (Normality test: p=0.037, Z score > 1.96), T80 (Normality test: p=0.002, Z score > 1.96), T100 (Normality test:

p=0.021, Z score > 1.96).

Experiment 1. The impact of IN administration of PREG (5.6 mg/mL, 11.2 mg/mL) on ACh levels in frontal cortex, hippocampus and amygdala was assessed in a 3 (between) x 16 (within) ANOVA (sums of squares type 3: each main effect assessed after controlling for the other main effect and the interaction; Figure 3). The within-subject effect of the individual drug doses over time was measured over 16 10-min time-bins (T; 6 in baseline and 10 post-treatment). The main effect of time and the time*dose interaction were assessed for the time curves after baseline. The main effect of dose (vs. vehicle), for the subject-level average of the time curve (after baseline), was assessed in 3 groups of animals and followed up by 2-tailed Dunnett post-hoc pairwise comparisons of both low and high PREG dose to vehicle.

For each dose separately, the baseline mean (100%) was compared to each after-treatment time-bin by means of within-subject simple contrasts (non-orthogonal). Moreover, a comparison of estimated marginal means (method: Fisher's Least Significant Difference) was performed to compare the three groups (5.6 mg/mL PREG, 11.2 mg/mL PREG, vehicle) within every time-bin after drug treatment (10 x 10 min intervals).

Experiment 2 focused on the amygdala. The effect of the high dose of PREG (11.2 mg/mL) was assessed in a 2 x 16 within-subject design. The time course of drug effect was again measured by collecting samples from 16 10-min intervals (6 in baseline and 10 post-treatment). The effect of the individual PREG doses was compared to vehicle within each subject (Figure 4A). For the drug- and vehicle-treated nostril side separately, an overall hypothesis about time course of drug effect was tested (within-subject ANOVA: 10 time-bins and baseline mean), followed up by a simple contrast of each time-bin after treatment to the

baseline mean (set to 100%). Separate pairwise estimated marginal means comparisons of drug-treated vs vehicle-treated side at every time-bin are also reported.

The effect of left vs right hemisphere as a possible confounding factor was assessed between-animal comparison of the ACh concentration (expressed as % of baseline mean) in the time bins after drug treatment (Figure 4B): (1) only vehicle-treated hemisphere (n=10, out of which localized in the left hemisphere: n=7, in the right hemisphere: n=3); (2) only PREG-treated hemisphere (n=10, out of which localized in the left hemisphere: n=3, in the right hemisphere: n=10). Only time-bins after treatment were considered. Reported are F- and p-values of between-subject ANOVA after controlling for within-subject effect of time and possible interaction of both factors.

No predetermined sample size calculation was performed.

Calculations were performed with IBM SPSS Statistics 24.0 (IBM SPSS Software Germany, Ehningen, Germany).

3. Results

Experiment I. Effects of bilateral IN-PREG on ACh

Frontal cortex

The sums of squares type 3 ANOVA showed no significant main effect of "time" (for 10 measurements after baseline) on the pooled set of subjects (n=23; $F(4.59, 91.73)=2.14$, $p=.74$). The interaction between "time" and "dose" was also not statistically significant ($F(9.17, 91.73)=1.39$, $p=.201$). The main effect of "dose" on all time-bins after baseline was statistically significant ($F(2,20)=7.08$, $p=.005$). Equality of error variances (Levene's test) cannot be assumed for several time-bins: T10-30, T50. A 2-tailed post-hoc Dunnett comparison between (1) 11.2 mg/mL PREG and vehicle ($p=.005$), and (2) 5.6 mg/mL PREG and vehicle ($p=.017$) confirmed the effect of IN-treatment. There were, therefore, no distinguishable peaks or drops, and both PREG doses differed from vehicle (vehicle time curve remains flat after drug application, Figure 3A).

Focused comparisons

For the vehicle-treated animals, simple contrasts of each time-bin from T10 to T100 were not statistically significant. For the 5.6 mg/mL PREG group, simple contrasts yielded statistically significant differences between baseline and T20 ($F(1,5)=7.6$, $p=.040$), T30 ($F(1,5)=8.79$, $p=.031$), and T80 ($F(1,5)=19.68$, $p=.007$). For the 11.2 mg/mL PREG group, simple contrasts were statistically significant between baseline and T20 ($F(1,7)=7.57$, $p=.028$), T40 ($F(1,7)=7.1$, $p=.032$), T50 ($F(1,7)=5.62$, $p=.05$), and T60 ($F(1,7)=6.83$, $p=.035$). These comparisons confirmed the effect previously shown by the ANOVA (flat time curve for vehicle vs. positive slope and complex curve form after IN-treatment for both PREG doses;

Figure 3A). Furthermore, the relative rise in the ACh level ranged from +19% (T70) and +52% (T50) for the low dose (after an initial drop by ca. -2.6% at T10) and from +12% (T100) to +54% (T40) for the high dose.

Pairwise comparisons of estimated marginal means at each time-bin produced significant differences between: (1) vehicle and 5.6 mg/mL PREG at T30 (p=.025), T40 (p=.044), T50 (p=.016), and T80 (p=.001); (2) vehicle and 11.2 mg/mL PREG at T10 (p=.052, marginally sig.), T20 (p=.009), T40 (p=.019), and T60 (p=.033); (3) 5.6 mg/mL PREG and 11.2 mg/mL PREG at T10 (p=.049) and T80 (p=.032). The onset of drug effect was earlier after the higher dose (Figure 3A).

Hippocampus

The mixed ANOVA showed no significant effect of "time" (after baseline) in the pooled set of subjects (n=28; $F(2.69, 67.36)=0.99$, p=.396). The interaction between "time" and "dose" was also not statistically significant ($F(5.39, 67.36)=0.921$, p=.478). The main effect of "dose" was statistically significant ($F(2,25)=9.7$, p=.001). The assumption of equality of error variances (Levene's test) was met for all time-bins. A 2-tailed post-hoc Dunnett comparison between (1) high dose and vehicle (p=.002), and (2) low dose and vehicle (p=.001) confirmed the effect of IN-treatment. Figure 3B depicts the groups' time curves: while in the vehicle group the curve after IN-treatment remains flat and fluctuating around the 100% mark, the curves for both PREG doses are clearly above.

Focused comparisons

For the vehicle group (n=9), simple contrasts of each time-bin from T10 to T100 to baseline mean (100%) were not statistically significant. For the 5.6 mg/mL PREG group

(n=7), the simple contrasts yielded statistically significant differences between baseline and T10 (F(1,6)=6.65, p=.042), T30 (F(1,6)=8.16, p=.029), T50 (F(1,6)=10.88, p=.016), T80 (F(1,6)=25.47, p=.002), and T100 (F(1,6)=17.98, p=.005). For the 11.2 mg/mL PREG group (n=12), simple contrasts were statistically significant between baseline and T10 (F(1,11)=4.51, p=.057, marginally sig.), T20 (F(1,11)=5.98, p=.032), T30 (F(1,11)=4.51, p=.057, marginally sig.), T40 (F(1,11)=5.8, p=.035), T50 (F(1,11)=7.5, p=.019), and T70 (F(1,11)=9.29, p=.011). Focused comparisons confirm the effect previously shown by the ANOVA (lack of effect for vehicle vs positive change after IN-treatment with both PREG doses; Figure 3B). The range of effect for the low dose was from +17% (T90) to +52 (T40). For the high dose it was larger, namely from +10% (T100) to +73% (T10).

Pairwise comparisons of estimated marginal means at each time-bin produced significant differences between: (1) vehicle and low dose at T30 (p=.046), T40 (p=.005), T50 (p=.012), T100 (p<.001); (2) vehicle and high dose at T50 (p=.026) and T70 (p=.028); (3) low dose and high dose at T40 (p=.058, marginally sig.) and T100 (p=.003). Figure 3B shows the same onset of effect for both low and high dose; however, the separate comparisons evidence that the effect of the high dose was less consistent across subjects (large peaks in some animals and smaller in others).

Amygdala

The mixed ANOVA showed no significant effect of “time” (after baseline) in the pooled set of subjects (n=25): F(2.88,63.44)=1.75, p=.168. The interaction between “time” and “dose” was also not statistically significant (F(5.77,63.44)=1.32, p=.261; Figure 3C) The main effect of “dose” on all time-bins after baseline was statistically significant (F(1,22)=10.26, p=.001). Equality of error variances (Levene’s test) cannot be assumed for any of the 10 time-bins. A

2-tailed post-hoc Dunnett comparison between (1) high dose and vehicle (p=.001), and (2) low dose and vehicle (p=.969) showed a high effect of the high dose of PREG, whereas the effect of the low dose was indistinguishable from vehicle. The effect of the high PREG dose in the amygdala was higher relative to the other groups (Figure 3 C vs A, B). In this case, the lack of equality of error variances (Levene's test) confirms the effect: higher effect goes along with higher variation. In contrast to the frontal cortex and the hippocampus, the low dose did not produce any sizable effect on the time curve relative to vehicle.

Focused comparisons

For the vehicle group, simple contrasts of each time-bin produced two statistically significant results between baseline mean and T10 (F(1,10)=9.16, p=.013) as well as T30 (F(1,10)=8.34, p=.016). For 5.6 mg/mL PREG, simple contrasts yielded statistically significant differences between baseline and T30 (F(1,7)=6.89, p=.034) as well as T60 (F(1,7)=10.95, p=.013). For 11.2 mg/mL PREG, simple contrasts were statistically significant between baseline and T10 (F(1,5)=6.18, p=.055, marginally sig.), T50 (F(1,5)=6.56, p=.051, marginally sig.), T60 (F(1,5)=7.89, p=.038), T70 (F(1,5)=20.41, p=.006), and T90 (F(1,5)=9.1, p=.03). Unlike frontal cortex and hippocampus, the vehicle treatment produced an effect in the amygdala, similar (although smaller) in size to the effect of the low dose: vehicle T10: +11%, vehicle T30: +15%, low dose T30: +14%, low dose T60: +29%. The time-curve after treatment with the high dose shows a relative rise of ACh between +111% (T100) and +206% (T50).

Pairwise comparisons of estimated marginal means at each time-bin produced significant differences between: (1) vehicle and 11.2 mg/mL PREG at all time-bins: T10 (p=.001), T20 (p=.002), T30 (p=.006), T40 (p=.002), T50 (p<.001), T60 (p<.001), T70

(p<.001), T80 (p=.002), T90 (p<.001), and T100 (p=.001); (2) 5.6 mg/mL PREG and 11.2 mg/mL PREG also at all time-bins: T10 (p=.001), T20 (p=.003), T30 (p=.008), T40 (p=.003), T50 (p=.001), T60 (p=.002), T70 (p<.001), T80 (p=.006), T90 (p=.001), and T100 (p=.005). The low dose, 5.6 mg/mL PREG, did not differ from the vehicle treated group in any time-bin after treatment. Pairwise comparisons of doses per time-bin confirmed the results obtained after comparisons between baseline means and time-bins post-treatment (see also Figure 3C).

Experiment II. **Effects of unilateral IN-Preg on acetylcholine levels in ipsi- vs contralateral amygdala.**

IN-PREG vs. vehicle (within-subject)

A 2-factor within-subject ANOVA was performed on all ACh concentrations (expressed as % of baseline mean) in the time bins after drug treatment from the entire sample of n=10. PREG- and vehicle-treated side are considered as dependent data (as well as the time-bins).

An interaction between time course of drug effect (10 time-bins after treatment) and drug effect (11.2 mg/mL PREG vs vehicle) was found to be statistically significant ($F(2.56,23.012)=4.51$, $p=.016$). Also the main effect of PREG vs vehicle for the hemisphere-average of time-curves was highly significant ($F(1,9)=40.2$, $p<.001$). Figure 4A depicts a flat curve for the vehicle-treated amygdala and a rise in ACh after IN-PREG treatment in the PREG-treated amygdala.

Focused hypotheses

The baseline mean (100%) was compared to all time-bins after treatment with PREG and vehicle by means of simple contrast. For vehicle-treated animals, none of the separate

comparisons between time-bins and baseline mean were statistically significant. The change of ACh levels relative to baseline ranged from -5% (T70) to +1.7% (T10). For PREG-treated targets, all contrasts between baseline mean and time-bins were statistically significant (T10 ($F(1,9)=18.11$, $p=.002$), T20 ($F(1,9)=20.07$, $p=.002$), T30 ($F(1,9)=20.47$, $p=.001$), T40 ($F(1,9)=12.44$, $p=.006$), T50 ($F(1,9)=35.09$, $p<.001$), T60 ($F(1,9)=18.47$, $p=.002$), T70 ($F(1,9)=12.88$, $p=.006$), T80 ($F(1,9)=13.55$, $p=.005$), T90 ($F(1,9)=13.5$, $p=.005$), T100 ($F(1,9)=15.89$, $p=.003$)). The relative rise of ACh levels ranged from +34% (T70) to +70% (T50).

The effect of PREG vs vehicle assessed at separate time bins yielded the following statistically significant results: T10 ($F(1,9)=16.14$, $p=.003$), T20 ($F(1,9)=27.56$, $p=.001$), T30 ($F(1,9)=31.13$, $p<.001$), T40 ($F(1,9)=15.27$, $p=.004$), T50 ($F(1,9)=39.74$, $p<.001$), T60 ($F(1,9)=24.62$, $p=.001$), T70 ($F(1,9)=13.9$, $p=.005$), T80 ($F(1,9)=18.6$, $p=.002$), T90 ($F(1,9)=16.93$, $p=.003$), T100 ($F(1,9)=23.21$, $p=.001$). The effect of unilateral application reflects the effects observed in experiment 1.

Control of confounding variable "left vs right hemisphere"

Vehicle-treated and PREG-treated targets were analysed separately (two independent samples of n=10). In each sample (vehicle, 11.2 mg/mL PREG), the possible interaction between localization (left or right) and time course of drug effect was assessed in a mixed design (out of 10 subjects, n=3 received vehicle into the right and PREG into the left nostril; n=7 received vehicle into the left and PREG into the right nostril) (Figure 4B).

For the IN-PREG treated targets, no significant interaction between time and left vs. right side was found ($F(2.35,18.81)=0.15$, $p=.89$). Furthermore, a between-subject comparison of the subject-average of time curves of PREG*left vs PREG*right groups was

performed. The main effect of left vs right hemisphere was not statistically significant ($F(1,8)=4.17$, $p=.075$).

For the IN-vehicle treated targets, no significant interaction between time and left vs. right side was found ($F(4.21,33.69)=0.55$, $p=.712$). The between-subject effect of vehicle*left vs vehicle*right was found to be not statistically significant ($F(1,8)=0.03$, $p=.869$).
Moreover, the effect over time after drug application was compared to the baseline for PREG*left (n=3) and PREG*right (n=7) treated subjects, separately. For PREG*left, the main effect of time was not significant ($F(1.64,3.28)=1.2$, $p=.391$). Significant contrasts to baseline mean (100%) were found at T10 ($F(1,2)=54.26$, $p=.018$), T40 ($F(1,2)=27.57$, $p=.034$), and T60 ($F(1,2)=134.94$, $p=.007$). For PREG*right, the overall effect of time was not significant ($F(2.17,13.04)=2.81$, $p=.094$). Significant contrasts to baseline mean (100%) were found at all time-bins: T10 ($F(1,6)=14.76$, $p=.009$), T20 ($F(1,6)=26.47$, $p=.002$), T30 ($F(1,6)=18.41$, $p=.005$), T40 ($F(1,6)=9.93$, $p=.02$), T50 ($F(1,6)=40.95$, $p=.001$), T60 ($F(1,6)=11.78$, $p=.014$), T70 ($F(1,6)=11.93$, $p=.014$), T80 ($F(1,6)=10.94$, $p=.016$), T90 ($F(1,6)=13.91$, $p=.01$), T100 ($F(1,6)=12.62$, $p=.012$). The average rise of ACh relative to the baseline mean ranged from +17% (T70) to +39%(T50) in the PREG*right group and from +41% (T70) to +83% (T50) in the PREG*left group. Figure 4B also shows a similar time curve with an initial peak at T10, a positive slope until T50 and a drop at T70, followed by a renewed rise (resp. plateau).

4. Discussion

In the present study, we investigated the effect of IN-PREG (5.6 mg/mL or 11.2 mg/mL) and vehicle on ACh release in frontal cortex, hippocampus and amygdala. We present first-time evidence that intranasal administration of PREG in either dose increased frontal and hippocampal ACh in a time-dependent fashion relative to both baseline and vehicle. Moreover, 5.6 mg/ml PREG increased ACh release in the amygdala relative to baseline, the lower dose and vehicle. In a next step, we investigated the effect of PREG (11.2 mg/mL) administered into one nostril in ACh levels in the ipsi- and contralateral amygdala. An increase of extracellular ACh was observed in the ipsilateral amygdala, whereas no effect was visible in the amygdala of the contralateral hemisphere. This result demonstrates an effective and selective modulation of cholinergic system by PREG. This finding is in line with a previous study, which showed an effect of the PREG derivative PREG-S on ACh release (Darnaudéry *et al.* 1998).

The greatest increase of ACh in the amygdala was observed within the first ten minutes after administration of 11.2 mg/mL PREG, while a second increase, although smaller, could be seen at T80. The extent to which the drug is transported and reaches the brain is highly dependent on the physicochemical properties of the drug in question, mainly lipophilicity, molecular weight and degree of dissociation (Sakane *et al.* 1991). PREG is a neutral lipophilic molecule with a molecular weight of 316 D and, like small, lipophilic drugs with a molecular weight up to 600 Da, can easily enter the blood stream and cross the BBB (Pardridge 1991). The most likely way of delivery is passive diffusion across the epithelium, since it can pass membranes readily. A smaller amount could also have been directly absorbed by the capillaries at the lamina propria of the olfactory epithelium and, after a time of systemic circulation, cross the BBB, which is in line with the increase in ACh 70 minutes

after drug administration. Thus, we suggest that the first major increase corresponds to the direct olfactory epithelial transport, while the second slighter increase may be due to a smaller amount of drug crossing the blood-brain barrier after having entered the bloodstream. This is consistent with previous studies, in which epithelial transport was proven to be rapid, with drugs like dihydroergotamine (Wang *et al.* 1998), lidocaine (Chou and Donovan 1998) and cefalexin (Sakane *et al.* 1991) appearing in the CFS and brain only a few minutes after administration. Also, we have previously shown that nasally administrated cocaine is transported within minutes from the nasal cavities into the brain via the olfactory epithelial pathway (de Souza Silva et al., 1997).

PREG acts as a sigma 1 receptor agonist (Nuwayhid and Werling 2003). Sigma 1 receptors modulate NMDA receptors (Monnet *et al.* 1990). Activated NMDA receptors, in turn, might activate cholinergic neurons in the basal forebrain, which project to frontal cortex, hippocampus and amygdala. Sigma1 receptors are highly expressed in the rat olfactory bulb, amygdaloid complex, hippocampus and hypothalamus (Alonso *et al.* 2000). In-vivo microdialysis studies in freely moving rats as well as in-vitro studies in hippocampal and frontal rat brain slices showed significant increases of extracellular ACh in hippocampus and frontal cortex after systemic administration of a sigma 1 receptor agonists (Horan *et al.* 2002; Kobayashi *et al.* 1996; Matsuno *et al.* 1992). The authors propose that the stimulation of ACh release is either triggered by cholinergic nerve terminals expressing sigma1 receptors or by the activation of other neurons, which express this receptor subtype and, upon their activation, modulate cholinergic transmission.

As proposed by Giovannini *et al.* (1997) , the glutamatergic-GABAergic-cholinergic circuitry in the basal forebrain is responsible for the regulation of ACh levels in various areas of the brain, including hippocampus, frontal cortex and amygdala. A low affinity of PREG for GABAA

receptors and NMDA receptors has been described, while its sulfated form, PREG-S, is considered a strong modulator of these receptors (Park-Chung *et al.* 1999; Weaver *et al.* 1997). It is not clear as of yet, whether the release of ACh is induced via modulation of sigma receptors by PREG, or via activation of GABAA receptors and/or NMDA receptors by PREG or PREG-S.

Darnaudéry *et al.* (2002) have shown that infusion of PREG-S into the medial septum nucleus increased hippocampal ACh. In the basal forebrain, the medial septal nucleus and the vertical limb of the diagonal band of Broca (MSDB) send cholinergic efferents to the hippocampus (Melander *et al.* 1985; Wainer *et al.* 1985; Woolf *et al.* 1984). Moreover, 50% of the neurons involved in the septo-hippocampal pathway are GABAergic and glutamatergic neurons of the MSDB (Amaral and Kurz 1985). The modulation of GABAA receptors or NMDA receptors on either cholinergic and GABAergic neurons in the MSDB enhanced hippocampal efflux of ACh (Darnaudéry *et al.* 2002; Elvander-Tottie *et al.* 2009; Gorman *et al.* 1994; Roland *et al.* 2014). Hence, also in the present study, IN-PREG might have modulated the release of ACh in the hippocampus by modulating GABAA and/or NMDA receptors (possibly via sigma1 receptor activation) on neurons in the MSDB. Based on this hypothesis, the memory enhancement observed in a previous study after IN-PREG administration (Abdel-Hafiz *et al.* 2016) could be explained as the result of hippocampal theta oscillations induced by the activation of the cholinergic septo-hippocampal pathway (Keita *et al.* 2000).

Unilateral IN-PREG showed a significant increase of extracellular ACh in the ipsilateral amygdala, but not in the contralateral one. Similarly, unilateral intranasal application of L-DOPA induced an elevation of dopamine levels only in the ipsilateral neostriatum. This either implies that L-DOPA entered brain and neostriatum via the nasal mucosa or that it entered a brain site more proximal to the nasal epithelium, and, by the action on this brain site, indirectly

affected dopamine activity in the neostriatum (de Souza Silva *et al.* 1997a). The lateralized effects of PREG observed in the present study support the hypothesis that IN-PREG entered the brain via the epithelial pathway. The lateralized diffusion of PREG from one nostril to the amygdala of the ipsilateral hemisphere can be accounted for by the anatomical separation of the right and left nostril by the septum and ethmoid bone (Shipley and Reyes 1991).

By activation of NMDA receptors, PREG may increase glutamate-induced depolarization (Alonso *et al.* 2000; Cheng *et al.* 2009; Lethbridge *et al.* 2012; Monnet and Maurice 2006; Shipley and Ennis 1996). Through unilateral afferent glutamate projections from the olfactory bulb, cholinergic neurons of the basal forebrain are activated (Giovannini *et al.* 1997), which in turn, stimulate the release of ACh in the amygdala (Dani and Bertrand 2007). Specifically, the nucleus basalis magnocellularis-cortical pathway could be involved in the release of extracellular ACh in the amygdala after PREG administration. The rat nucleus basalis magnocellularis–substantia innominata complex sends cholinergic efferents to the dorsolateral frontal and parietal cortex and to the basolateral amygdala (Heckers and Mesulam 1994). PREG-S infusion into the nucleus basalis has been shown to increase ACh release in frontal cortex and amygdala, with a subsequent improvement of spatial and emotional memory (Gold 2003; Pallarés *et al.* 1998; Tinsley *et al.* 2004). In the present study, no significant difference was found between administration into the right and the left nostril on ACh release in the ipsilateral amygdala. This result is in line with the findings of Parthasarathy and Bhalla (2013), who found no difference in symmetry and laterality of information flow in the nostrils of rats, and observed no significant activity on the contralateral side after unilateral presentation of odors, indicating that ipsilateral odorant input has no effect on the response at the glomerular level of the contralateral olfactory bulb.

The higher ACh efflux in the amygdala compared to the other investigated regions

induced by the higher IN-PREG dose (11.2 mg/mL) might be related to the higher density of cholinergic terminals in the basolateral and lateral amygdala (Nagai *et al.* 1982; Woolf and Butcher 1982) Besides, the differences in ACh release might be due to tissue-specific differences in cholinergic regulation, which depends on the expression and density of specific cholinergic receptors subtypes such as the presynaptic muscarinic m2 and m4 receptor, the muscarinic m2 and m4 autoreceptor (Fitzgerald 2009), and the postsynaptic alpha-7 nicotinic receptor (Arroyo *et al.* 2014).

There is extensive proof that the amygdala is involved in emotional memory and that emotional arousal stimulates the amygdala resulting in the modulation of long-term memory storage in other brain regions (McGaugh *et al.* 1996). Experimental studies in animals and humans suggest that highly arousing experiences will be remembered more precisely, more easily and over a longer period of time compared to less arousing experiences (Cahill *et al.* 1996; McGaugh *et al.* 1996; Schafe *et al.* 2001). More specifically, amygdala and hippocampus act synergistically to form long-term memories of emotional events (Phelps 2004). It seems, therefore, reasonable to speculate that PREG, by increasing ACh release in the amygdala, can stimulate memory and learning. This is in line with previous findings, showing IN-PREG enhances memory and cognitive processes (Abdel-Hafiz *et al.* 2016). As a consequence, this neurosteroid may be relevant for the maintenance of cognitive function in conditions which are characterized by a deficiency of ACh, such as early dementia or manifest Alzheimer´s disease.

5. Conclusions

The present study yields first-time evidence that IN-PREG increases frontal and limbic ACh in a dose- and time-dependent fashion. Application into one nostril increased ACh levels in the ipsilateral amygdala, whereas no effect was observed in the amygdala of the contralateral hemisphere. This implies that IN-PREG reached the brain mainly via the direct olfactory epithelial pathway. Results demonstrate an effective modulation of the cholinergic system by IN-PREG, suggesting a therapeutic value in age-related neurodegenerative diseases linked to memory disturbances and depressed ACh activity, such as in Alzheimer's disease.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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LEGENDS

Figure 1. Timeline of protocol. **A** Pre-dialysis, **B** Microdialysis. Exp (Experiment), IN (IN), PREG (Pregnenolone). F (frontal cortex), H (hippocampus), Ar/Al (amygdala right, left).

Figure 2. Schematic representation of the placement of microdialysis cannulae. The bars represent the location of the dialysis membranes. In experiment I, each rat had implants in frontal cortex **(A)** and hippocampus **(C)** in the same hemisphere and in the amygdala in the opposite hemisphere **(B**). The implant location (left or right hemisphere) was randomized. In experiment II, each rat had implants in both right and left amygdala **(D)**. Numbers indicate the distance from bregma in mm (Paxinos & Watson, 2007)

Figure 3. Effects of intranasal (bilateral) administration of pregnenolone on extracellular acetylcholine (ACh) levels in frontal cortex **(A)**, hippocampus **(B)** and amygdala **(C)**, measured with in-vivo microdialysis in anesthetized rats. Samples were taken every 10 minutes. Values (mean + standard errors of the mean [SE]) are presented as % of baseline, with six baseline samples taken as 100%. Pregnenolone 5.6 mg/mL, 11.2 mg/mL, and vehicle were administrated intranasally into both nostrils, 5µL each, at T0. Sample size: **(A)** vehicle (n=9), 5.6 mg/mL (n=6), 11.2 mg/mL (n=8); **(B)** vehicle (n=9), 5.6 mg/mL (n=7), 11.2 mg/mL (n=12); **(C)** vehicle (n=11), 5.6 mg/mL (n=8), 11.2 mg/mL (n=6). * $p < 0.050$, compared to baseline; # $p < 0.050$ compared to vehicle, $ $p < 0.050$ comparison of PREG doses (5.6 mg/mL vs 11.2 mg/mL).

Figure 4. A. Lateralized effects of unilateral intranasal administration of 11.2 mg/mL PREG on ACh in the amygdala. The intranasal administration was performed at T0. Each rat received 5µL of 11.2 mg/mL PREG into one nostril and 5µL of vehicle into the other. #

p<0.050 comparison of ACh in the amygdala ipsilateral to the nostril, which had received PREG to ACh in contralateral amygdala (ipsilateral to nostril, which had received vehicle). * p<0.050, compared with baseline mean. Sample size n=10. **B.** Comparison of the effect on ACh in the ipsilateral amygdala after PREG administration in the right vs left nostril. The graph shows the level of ACh in the ipsilateral amygdalae (right and left) before and after administration. * p<0.050, compared with baseline mean. Sample size: right (n=7), left (n=3). Values are presented as % of baseline with six baseline samples taken as 100% (mean + SE).

Intra-nasal dopamine alleviates cognitive deficits in tgDISC1 rats which overexpress the human DISC1 gene

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ABSTRACT

The Disrupted-in-Schizophrenia 1 (DISC1) gene has been associated with mental illnesses such as major depression and schizophrenia. The transgenic DISC1 (tgDISC1) rat, which overexpresses the human DISC1 gene, is known to exhibit deficient dopamine (DA) homeostasis. To ascertain whether the DISC1 gene also impacts cognitive functions, 14–15 months old male tgDISC1 rats and wild-type controls were subjected to the novel object preference (NOP) test and the object-based attention test (OBAT) in order to assess short-term memory (1 h), long-term memory (24 h), and attention. Results:The tgDISC1 group exhibited intact short-term memory, but deficient long-term-memory in the NOP test and deficient attention-related behavior in the OBAT. In a different group of tgDISC1 rats, 3 mg/kg intranasally applied dopamine (IN-DA) or its vehicle was applied prior to the NOP or the OBAT test. IN-DA reversed cognitive deficits in both the NOP and OBAT tests. In a further cohort of tgDISC1 rats, post-mortem levels of DA, noradrenaline, serotonin and acetylcholine were determined in a variety of brain regions. The tgDISC1 group had less DA in the neostriatum, hippocampus and amygdala, less acetylcholine in neostriatum, nucleus accumbens, hippocampus, and amygdala, more serotonin in the nucleus accumbens, and less serotonin and noradrenaline in the amygdala. Conclusions:Our findings show that DISC1 overexpression and misassembly is associated with deficits in long-term memory and attention-related behavior. Since behavioral impairments in tgDISC1 rats were reversed by IN-DA, DA deficiency may be a major cause for the behavioral deficits expressed in this model.

1. Introduction

The Disrupted-in-Schizophrenia 1 (DISC1) gene was identified in a Scottish family with high linkage to various psychiatric disorders (Blackwood et al., 2001; Millar et al., 2000; St Clair et al., 1990). Several association studies have confirmed a role for DISC1 in schizophrenia, bipolar disorder, depression and autism (reviewed in Chubb, Bradshaw, Soares, Porteous, and Millar (2008), Thomson et al. (2016)). In these studies clinical diagnoses were not exclusively related to DISC1 genotypes, indicating that DISC1 should be conceptualized as a general vulnerability factor for mental illness (Brandon & Sawa, 2011; Korth, 2012).

The DISC1 protein has multiple roles in pre- and post-natal neuronal development, consistent with neurodevelopmental theories of mental illness, including regulation of the proliferation and migration of neuronal progenitor cells as well as synapse formation and maintenance (reviewed in Brandon and Sawa (2011)). It acts as a molecular hub that binds and interacts with many proteins, including components of dopamine(DA)ergic neurotransmission such as the DA D2 receptor (D2R) and DA transporter (DAT) (Su et al., 2014; Trossbach et al., 2016;

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Yerabham et al., 2017), thus linking it to the DA system, which is considered key in regulating multiple behavior systems (Dahoun, Trossbach, Brandon, Korth, & Howes, 2017).

The DISC1 gene has also been linked to cognitive processes, including deficits in object recognition (Cui et al., 2016; Ibi et al., 2010; Nagai et al., 2011). Deficits in the novel object- and novel place-preference tests were reversed by clozapine, a D1 receptor agonist, in the L100P mutant mouse model (Cui et al., 2016) and in human dominant-negative DISC1 transgenic mice (Ibi et al., 2010) and a 24 h novel object recognition deficit in a knockdown model of endogenous DISC1 (Niwa et al., 2010). Haloperidol reversed prepulse inhibition (PPI) deficits and blocked amphetamine-induced hyperactivity in DISC1-L100P mice (Lipina et al., 2010). Also, bupropion, a DA and norepinephrine reuptake inhibitor, was reported to ameliorate depressive-like behavior in the forced swim test and a PPI deficit in Q31L mice (Clapcote et al., 2007), underlining the interaction between DISC1 and DAergic systems.

Intranasal (IN) administration of substances is an alternative method for targeting the brain by bypassing the blood-brain-barrier (BBB). DA, which cannot cross the BBB due to its lipophobic properties, can be delivered directly into the brain via the nasal mucosa (Dahlin, Jansson, & Bjork, 2001). Previous studies have shown that IN-DA increased extracellular DA in the striatum (de Souza Silva, Topic, Huston, & Mattern, 2008), had antidepressant-like effects (Buddenberg et al., 2008) and reduced neostriatal DAT binding, suggesting an enhanced DA availability (de Souza Silva et al., 2016). IN-DA attenuated fear responses (Talbot, Mattern, de Souza Silva, & Brandao, 2017) and restored deficient object-place memory in aged rats (Trossbach, de Souza Silva, Huston, Korth, & Mattern, 2014). Furthermore, in a rat model of attention-deficit hyperactivity disorder (ADHD), it reduced hyperactivity and improved attention when administrated during the prepuberal period (Ruocco et al., 2014).

Here, we used the transgenic rat overexpressing the full-length non-mutant human DISC1 gene (tgDISC1) (Hamburg et al., 2016; Trossbach et al., 2016). In previous studies with this tgDISC1 rat, an 80% increase of high-affinity D2R was observed in the ventral striatum as well as an increased translocation of the DAT to the plasma membrane and a corresponding increase of DA reuptake into the presynaptic terminal (Trossbach et al., 2016). Rats also showed behavioral alterations, such as increased locomotion and rearing upon amphetamine challenge relative to controls (amphetamine supersensitivity) (Trossbach et al., 2016).

The DAergic systems play a key role in reward and learning processes (Guzman-Ramos et al., 2012; Schultz, Apicella, & Ljungberg, 1993; Wise, 2005). DA is a key neurotransmitter in the control of motivation, attention and memory known to degrade with age (Kaasinen et al., 2000; Trifilieff et al., 2013; Wang et al., 1998). Given its close relationship with the DISC1 gene (Dahoun et al., 2017; Su et al., 2014; Trossbach et al., 2016), we hypothesized that overexpression of non-mutant full-length human DISC1 gene would disrupt cognitive functions, specifically attention and memory processes. We further hypothesized that the administration of DA via the nose-brain pathway would alleviate behavioral deficits induced by the overexpression of the human DISC1 gene, which might be of future relevance for the pharmacological targeting of DISC1-dependent behavioral changes.

In the present study, we assessed cognitive function in tests of short-term (1h interval) and long-term (24 h interval) memory with the novel object preference (NOP) test (Ennaceur & Delacour, 1988), and of attention-related behavior with the object-based attention test (OBAT) (Alkam et al., 2011, 2013). We found both memory and attention-deficits in this study. Since we had already observed alterations of DA function in tgDISC1 rats (Trossbach et al., 2016) and IN-DA had repeatedly been found to ameliorate cognitive functions (Ruocco et al., 2014; Trossbach et al., 2014), we then assessed the effect of IN-DA on long-term memory (24h interval) in the NOP test and on attention-related behavior in the OBAT test. In a further group of tgDISC1 rats, post-mortem high-performance liquid chromatography (HPLC) analysis was conducted to assess possible changes of DA, noradrenaline (NA), serotonin (5-HT) and acetylcholine (ACh) levels in a variety of brain regions, which are relevant for learning and memory (neostriatum, nucleus accumbens, amygdala, hippocampus, frontal cortex, cerebellum).

2. Materials and methods

2.1. Animals

TgDISC1 Sprague Dawley rats and their littermate controls, so-called wild-type (WT), were bred at the local animal facility (ZETT, Heinrich-Heine University, Düsseldorf, Germany). For the behavioral studies a total of 33 male tgDISC1 and 15 male WT controls (aged 14–15 months) were housed in Makrolon cages (Type IV; 60 × 38 × 20 cm) in groups of 2–3 rats. Each pair of parents contributed one WT rat and one tgDISC1 rat. A further cohort of 24 animals (aged 4–5 months) underwent post-mortem HPLC analysis of neurotransmitter levels in various brain regions. The age of this cohort, relatively younger than the age used in the behavioral experiments, was chosen to compare the neurochemical changes in the tgDISC1 animals and the age-matched WT. This is a starting step to establish a basic understanding about the DISC1 gene on neurotransmitter systems in naïve adult animals.

Animals had free access to food and water and were placed in an environment with controlled humidity and temperature and reversed light-dark cycle rhythm (lights off from 7:00 to 19:00). After over 2 weeks of habituation to the environment, each rat was handled for 5 min/day for 10 consecutive days. From one week prior to the beginning of the experiments until the end of the study they were given restricted access to food (15 g/rat/day). This procedure was applied to increase the level of alertness in the animals for object exploration. The study was approved by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) NRW and carried out in accordance with the “Principles of laboratory animal care” (NIH publication No. 86-23, revised 1985) and the German Law on the Protection of Animals.

2.2. Apparatus and behavioral testing

A rectangular open field (60 × 60 × 40 cm) made of wood and painted black, was placed in a sound-attenuating chamber. A camera connected to a DVD recorder and computer with the tracking software was hung 2 m above the open field. Two figures (a white circle and a square made of black-white stripes) were attached to the black walls of the chamber as spatial cues. Four LED spotlights (~5 lx) were placed over the open field. Three different sets of objects made of glass or porcelain, with different shapes (cylinder, rectangular or octagonal columns), colors (white, green, transparent), sizes (25–32 cm height, 7–11 cm diameter) and textures (smooth, rough) were used as objects for the animals to explore. All objects weighed over 1.5 kg to prevent being moved by the rats. Acetic acid solution (0.1%) was used to clean the open field and the objects after each trial.

Physical contact with forepaws, snout or vibrissa toward an object was regarded as object explorative behavior. Experimenters who were blind to the experimental design registered the time of onset and duration of object exploration using Ethovision software 3.1 (Noldus, Wageningen, The Netherlands). Behavioral measures included locomotor activities (distance traveled, velocity in cm/s) and center time, as well as duration of grooming and rearing behavior. These were also analyzed automatically or manually with the Ethovision software. Behavioral studies were conducted between 10:00 and 17:00 h.

2.2.1. Novel object preference (NOP) test

Rats have a natural tendency to explore a novel object more than a familiar one. From this it can be inferred that they have a memory of the familiar object and, thus, show an explorative bias for the novel one

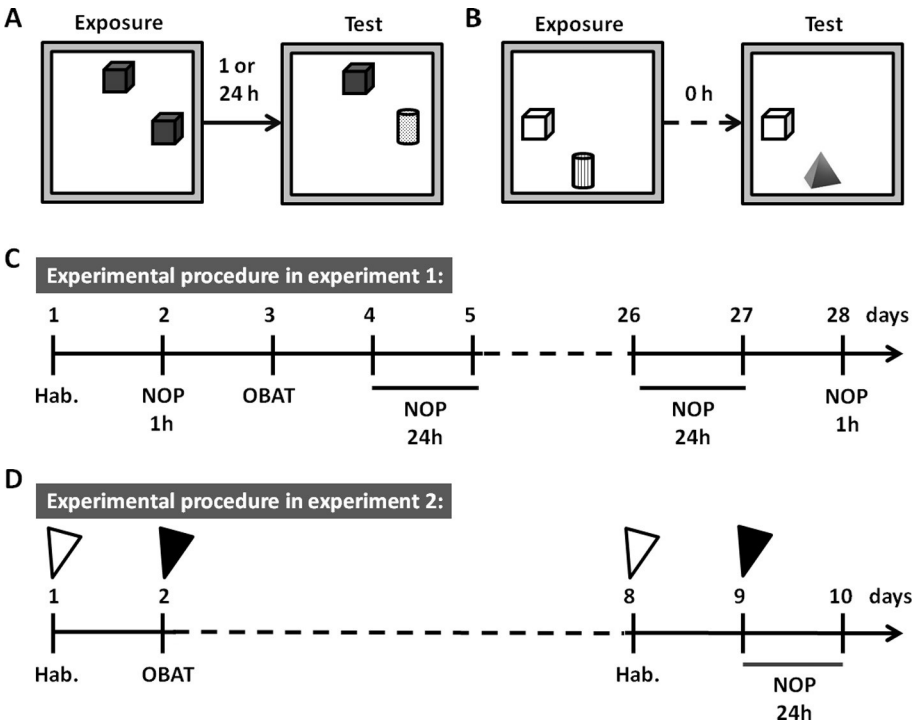

Fig. 1. Schematic representation of possible location of objects in the open field in tests for novel object preference (NOP) (A) and the object-based attention test (OBAT) (B). Different sets of objects are applied in these tests. Schedule of the behavioral tests in experiments 1 (C) and 2 (D). The dashed lines represent washout periods. The white triangles indicate intranasal administration of vehicle and the black triangles indicate intranasal administration of vehicle or dopamine before testing in the two experimental groups, interchangeably. Abbreviation: Hab., habituation; NOP1h, test with 1 h-interval; NOP24h, test with 24 h-interval.

(Ennaceur & Delacour, 1988). The NOP test has been widely employed to assess object recognition in rodents (Dere, Huston, & De Souza Silva, 2007). Exposure trial: two identical objects were placed in two of the corners of the apparatus (Fig. 1A). Test trial: after different delays, (1 h for the assessment of short-term and 24 h for the assessment of long-term memory), one of the objects was replaced by a novel object, which had shown the same level of preference for exploration in previous studies, while the other one remained in the open field. The delays of 1 h and 24 h were chosen on the basis of pilot studies, where aged wild-type Sprague Dawley rats had shown intact memory. For each trial, the animal was put into the apparatus, facing a wall that did not contain an object. It was removed from the testing chamber and returned to its home cage during the inter-trial interval. All trials lasted for 4 min. In order to examine the reliability of findings, the NOP1h and NOP24h tests were repeated 20 days later.

2.2.2. Object-based attention test (OBAT)

This test was modified from a paradigm described by Alkam et al. (2011, 2013), who named it “object-based attention test” and presented evidence that it may be uses as a test for attention-related processes. On the other hand, the task can also be considered a “ultra short-term working memory task” with practically no time lapse between exposure and test trial. The OBAT procedure was identical to the NOP procedure, except that two distinct objects were used in the exposure trial (Fig. 1B) and there was no inter-trial interval. Immediately after the exposure, both objects were removed from the open field. One object identical with the one that had been previously used was placed at the identical position, while the other object was replaced by a novel one. Animals remained in the apparatus during this transition and the test trial commenced at once. Both trials lasted for 4 min. Different sets of objects were employed for each object exploration test and were counterbalanced for each subject.

2.3. Experimental procedures

2.3.1. Experiment 1: Time-dependent test for novel object preference in the tgDISC1 rat

TgDISC1 and WT controls (n = 15 each) were transported from their colony to a room with dim light, which was next to the testing chamber, and were undisturbed for at least one hour. Then they were individually placed into the open field for 10 min in order to familiarize with the apparatus. One day after the habituation trial, tgDISC1 and WT groups were subjected to the behavioral tests for examining object memory and attention-related behavior: The NOP1h test, followed by the OBAT test on the next day, and the NOP24h test on the day after. The NOP1h and NOP24h tests were repeated 20 days later. The experimental procedure is summarized in Fig. 1C.

2.3.2. Experiment2: Effect of intranasal dopamine on deficits in object-based attention and long-term novel object preference in the tgDISC1 rat

TgDISC1 rats were randomly divided into two groups (A and B), receiving treatment with either IN-DA (n = 9) or vehicle (n = 9).

Habituation: One day before each test, all rats received an IN vehicle application in order to familiarize them with the nasal gel and the application procedure. Ten min later they were placed into the open field for 10 min. This procedure was also considered as a baseline measurement for locomotor, rearing, grooming and center-time behaviors.

The OBAT and the NOP24h tests were conducted consecutively, separated by a washout period of 6 days. Group A was administered vehicle on day 2 (OBAT) and DA on day 9 (NOP test), 10 min before the trials. Group B was treated with DA on day 2 (OBAT) and vehicle on day 9 (NOP test), 10 min before the trials. A schematic diagram is presented (Fig. 1D).

All behavioral protocols were identical to experiment 1.

2.3.3. Drug

Dopamine hydrochloride (Sigma-Aldrich, Taufkirchen, Germany) was suspended in gel of a viscous castor oil mixture (M & P Pharma, Emmetten, Switzerland). It was prepared immediately before usage and applied in a dose of 3 mg/kg in a volume of 10 µl of gel. It was kept on ice and protected from light throughout the experiment. Into each nostril, 5 µl of DA or its vehicle was applied to a depth of 2 mm using an applicator pipette for viscous liquids (Microman, Gilson, Villiers le Bel, France) and corresponding tips (CP10, Gilson, Villiers le Bel, France). The drug injection was performed over 8 s/nostril.

The dose of DA was chosen on the basis of previous studies, in which the IN application of 3 mg/kg had stimulated DA release (de Souza Silva et al., 2008), as decreased of DAT binding in the striatum (de Souza Silva et al., 2016) as well as exerted antidepressant-like effects (Buddenberg et al., 2008) and facilitated object place recognition (Trossbach et al., 2014). Behavioral testing was started 10 min post application. This ten-min duration was also chosen on the basis of the above-mentioned neurochemical and behavioral studies.

2.3.4. Experiment 3: Post-mortem analysis HPLC

TgDISC1 and WT controls (n = 12 each) were anaesthetized with CO_2 and decapitated. Each brain was dissected and then placed in cold Ringer's solution (B. Braun Melsungen AG, Germany). Neostriatum, nucleus accumbens, amygdala, hippocampus, frontal cortex and cerebellum were collected for each animal and the content of DA, NA, 5-HT and ACh was assessed via high-performance liquid chromatography with electrochemical detection (HPLC-EC). The separation of monoamines (DA, NA, 5-HT) was achieved by a 125 mm long analytical column filled with Nucleosil C-18 (reversed-phase with 5-µm particle size; Macherey & Nagel, Duren, Germany). The mobile phase was composed of 75 mM NaH_2PO_4, 4 mM KCl, 20 µM EDTA, 1.5 mM sodium dodecylsulfate, 100 µl/l diethylamine, 12% methanol and 12% acetonitrile and adjusted to pH 6.0 using phosphoric acid (de Souza Silva et al., 1997; Pum et al., 2009). The electrochemical detection was performed by the ISAAC reference electrode (Antec, Zoeterwoude, The Netherlands), which was set at 500 mV at 30 °C. The separation of ACh was achieved with a 75 mm long analytical column filled with ChromSpher 5C18 (Merck KGaA, Darmstadt, Germany) and loaded with sodiumdodecylsulfate (Sigma–Aldrich, Saint Louis, Missouri, US). For enzymatic cleavage, an enzyme reactor was connected to the column. The enzyme reactor was filled with LiChrosorb-NH2 (Merck), activated by glutaraldehyde (Merck, Darmstadt, Germany), and then loaded with acetylcholineesterase (Sigma–Aldrich, Saint Louis, Missouri, US). The enzymes were covalently bound to the stationary phase. The enzyme reactor converted ACh to hydrogen peroxide, which was electrochemically detected at a platinum electrode set at a potential of 350 mV. The reference electrode was a ISAAC (Antec, Fremont, California, US). The mobile phase was composed of 1 mM tetramethylammonium chloride and 0.18 M K_2HPO_4 and adjusted to pH 8.0 with KH_2PO_4 (Merck, Darmstadt, Germany) (de Souza Silva et al., 2013). It flowed at the rate of 0.3 µl/min, using a HPLC pump (Merck, Darmstadt). The neurotransmitter content was analyzed with the help of a Chrom Perfect Software (Justice Laboratory Software, Denville, NJ, USA).

2.4. Statistics

For all object exploration tests, mixed two-way ANOVAs with the within-subjects variable, "object", and the between-subjects factor, "group", were applied. Since the exploration times of objects were not independent of each other, two-tailed paired-sample t-tests were used for the comparisons. Independent-sample t tests were applied to analyze locomotor and emotional behaviors, as well as neurochemical data. Results were expressed as mean ± SEM and all tests were with the level of significance set at $P < .05$.

3. Results

3.1. Experiment 1: TgDISC1 rats are deficient in long-term novel object memory and object attention

3.1.1. Open field

Distance moved (cm), velocity (cm/s), time in the center area (s) and durations of grooming and rearing (s) were not significantly different between WT and tgDISC1 groups (Table 1). Both groups performed comparably on measures of locomotor activity and emotional behavior (time in the center as measure of anxiety-like behavior) (Prut & Belzung, 2003).

Table 1
Results of open field test in experiment 1. Values are presented as mean ± SEM. Number/group is 14. No significant differences between the experimental groups were detected in the presented behavioral parameters.

	WT	tgDISC1
Distance moved (cm)	2643.2 ± 124.7	2707.3 ± 93.7
Velocity (cm/s)	4.4 ± 0.2	4.5 ± 0.2
Center duration (s)	28.0 ± 5.4	21.6 ± 3.9
Grooming duration (s)	68.3 ± 10.9	44.8 ± 8.6
Rearing duration (s)	66.8 ± 10.5	53.3 ± 6.1

3.1.2. Novel object preference test

In the NOP1h test, a significant main effect of "object" ($F(1, 24) = 22.517$, $P = .0001$), but not of "group" ($F(1, 24) = 0.140$, $P = .711$) and "object × group" ($F(1, 24) = 0.454$, $P = .507$) was found. Paired-sample t-tests revealed that both groups spent more time exploring the novel object than the familiar one (WT group: d.f. = 12, $t = -2.878$, $P = .014$; tgDISC1 group: d.f. = 12, $t = -3.833$, $P = .002$; Fig. 2A). In the analysis of the repeated NOP1h test, a two-way ANOVA revealed a main effect of "object" ($F(1,24) = 19.388$, $P = .0001$), but not of "group" ($F(1, 24) = 0.268$, $P = .610$) or "interaction" ($F(1, 24) = 0.401$, $P = .532$). Paired-sample t-test showed that the WT group explored the novel object more than the familiar one (d.f. = 13, $t = -2.708$, $P = .018$; Fig. 2B). Accordingly, the tgDISC1 group spent more time exploring the novel object than the familiar one (d.f. = 11, $t = -3.537$, $P = .005$; Fig. 2B). The results indicate that both groups had intact short-term memory for novel objects after a delay of 1 h.

In the NOP24h test, a significant interaction between "object" and "group" ($F(1, 23) = 15.574$, $P = .001$) and a significant main effect of "object" ($F(1, 23) = 10.859$, $P = .003$), but not of "group" ($F(1, 23) = 2.450$, $P = .131$) was found. Paired-sample t-test showed a significant difference in exploration time (d.f. = 13, $t = -5.192$, $P = .0001$) between familiar object and novel object in the WT group. The tgDISC1 group explored both objects for an approximately equal time (exploration time: d.f. = 10, $t = 0.468$, $P = .650$; Fig. 2C), showing that they cannot distinguish the old from the novel object. Similar results were found in the repeated NOP24h test. There was an "object" effect ($F(1, 21) = 8.585$, $P = .008$), but no effect of "group" ($F(1, 21) = 0.004$, $P = .949$) or "interaction" ($F(1, 21) = 3.240$, $P = .086$). The WT group explored the novel object longer than the familiar one (time: d.f. = 11, $t = -3.087$, $P = .01$). In the tgDISC1 group, there was no difference in exploration time (d.f = 10, $t = -0.903$, $P = .388$) between the familiar and the novel object (Fig. 2D). In all of the test trials, the total time of exploration of objects was not significantly different between the groups ($ps > 0.05$, independent t-tests, data not shown). Thus, the tgDISC1 rats, unlike the WT controls, did not show intact long-term object memory when the inter-trial interval was extended to 24 h.

3.1.3. Object-based attention test

The ANOVA revealed a significant main effect of "object" ($F(1, 24) = 6.482$, $P = .018$), but no effect of either "group" ($F(1, 24) = 3.016$, $P = .095$) or "interaction" ($F(1, 24) = 2.744$, $P = .111$). The novel object was explored for a longer time (d.f. = 12, $t = -3.626$, $P = .003$) compared to the familiar one in the WT group. In contrast, the tgDISC1 group did not show a significant difference in exploration time between the novel and the familiar object (d.f. = 12, $t = -0.546$, $P = .595$) (Fig. 2E). No significant difference between groups was found in total time of object exploration in the test trial ($p > .05$, data not shown). In summary, these results show that the tgDISC1 animals are deficient in long-term object memory and in object-based attention,

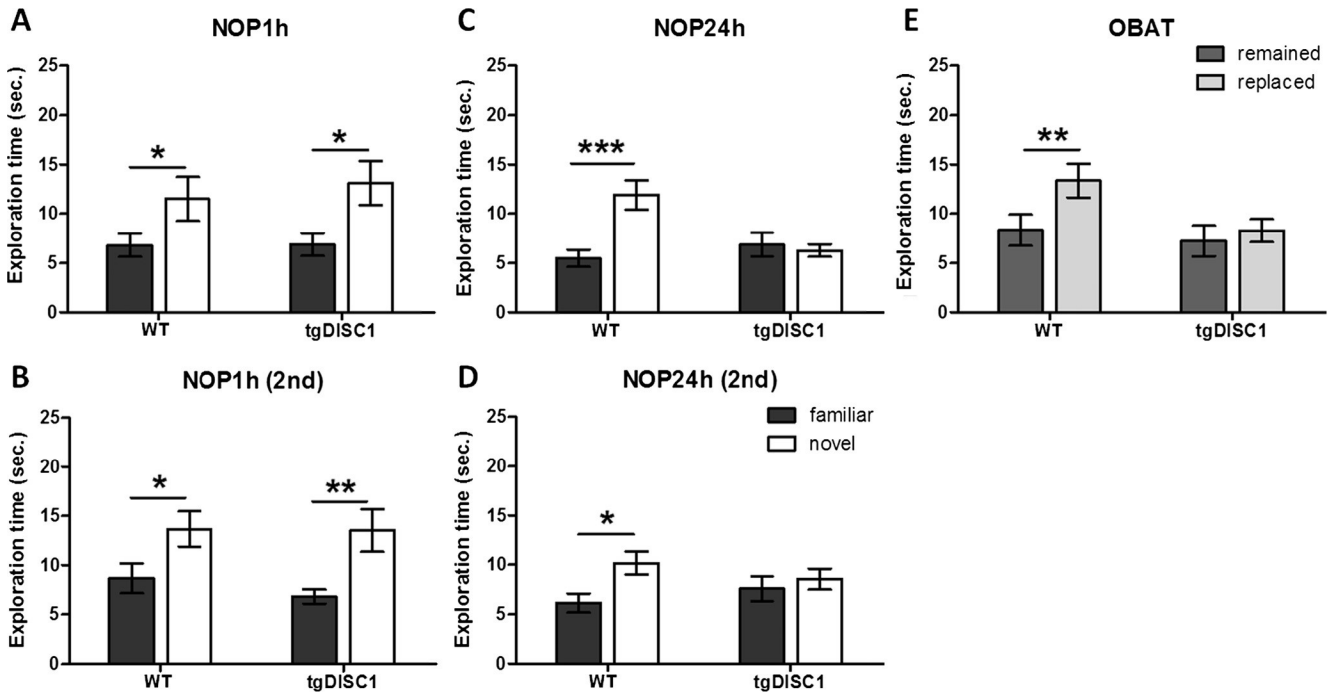

Fig. 2. Object exploration in experiment 1. The tgDISC1 group showed intact short-term memory in the novel object preference (NOP) test with 1 h-interval in the first (A) and second (B) test, but not with the 24 h-interval (C) in the first and in the second test (D), nor in the object-based attention test (OBAT) (E). The wild-type (WT) group showed intact short- and long-term memory and attention-related behavior. N = 12–14 in the WT group. N = 11–13 in the tgDISC1 group. Values represent mean ± SEM. *p < .05, **p < .01, ***p < .001 compared via paired-sample t-tests.

while their short-term object memory is intact.

3.2. Experiment 2: Intranasal dopamine application reverses deficits in long-term object memory and object attention in tgDISC1 rats

Since tgDISC1 rats failed to perform intact long-term object memory and object-based attention, we hypothesized that these behaviors were aberrant due to the known regulation of the DAergic system by DISC1 and that administration of DA to the brain would alleviate these deficiencies. Thus, IN-DA was administrated in the tgDISC1 animals and OBAT and NOP24h tests were subsequently repeated.

3.2.1. Object-based attention test

The ANOVA revealed a main effect of "object" ($F(1,15) = 5.205$, $P = .038$), but neither of "treatment" ($F(1,15) = 0.026$, $P = .873$) nor of the interaction between "object" and "treatment" ($F(1,15) = 2.404$, $P = .142$). The vehicle-treated tgDISC1 animals consistently failed to distinguish between the objects corroborating the results of experiment 1. They showed comparable exploration times of the novel and familiar objects (d.f. = 8, $t = -0.528$, $P = .612$). In contrast, the animals treated with IN-DA explored the novel object significantly longer than the familiar one (d.f. = 7, $t = -2.662$, $P = .031$; Fig. 3A). There was no significant group difference in the comparison of total time for object exploration between WT and tgDISC1 animals in the test trial ($p > .05$, data not shown). Thus, IN-DA treatment reversed the impairment in object-based attention of the tgDISC1 animals and restored a putative attention deficit.

3.2.2. Novel object preference test

For the NOP24h test, the two-way ANOVA revealed a main effect of "object" ($F(1,13) = 8.403$, $P = .012$) but not of "treatment" ($F(1,13) = 0.061$, $P = .809$) and the interaction between "object " and "treatment" ($F(1,13) = 1.518$, $P = .240$). When treated with vehicle, the animals showed comparable exploration times of the novel and familiar objects (exploration time: d.f. = 5, $t = -1.137$, $P = .307$; Fig. 3B). In contrast, the animals treated with IN-DA explored the novel object for a significantly longer time than the familiar one (d.f. = 8, $t = -3.164$, $P = .013$; Fig. 3B), and, thus, showed intact long-term object memory. The total time of exploration of objects was not significant between groups in all of the test trials ($ps > 0.05$, data not shown). Thus, IN-DA treatment also reversed the dysfunction of long-term object memory in the tgDISC1 rats.

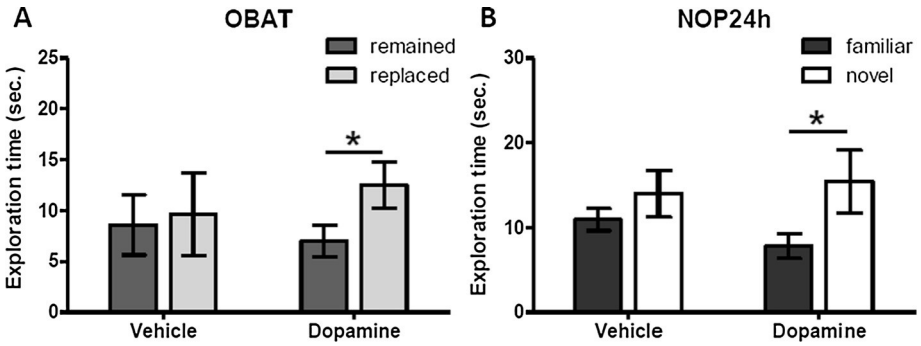

Fig. 3. Effects of intranasal dopamine administration on object explorative behavior in the object-based attention test (OBAT) (A) and novel object preference (NOP) test with a 24 h-interval (B) in tgDISC1 rats. The intranasal dopamine-treated group explored the novel object significantly more in both the OBAT and NOP24h tests; whereas the vehicle-treated group did not. Values represent mean ± SEM. *p < .05, compared via the pair-sample t-tests.

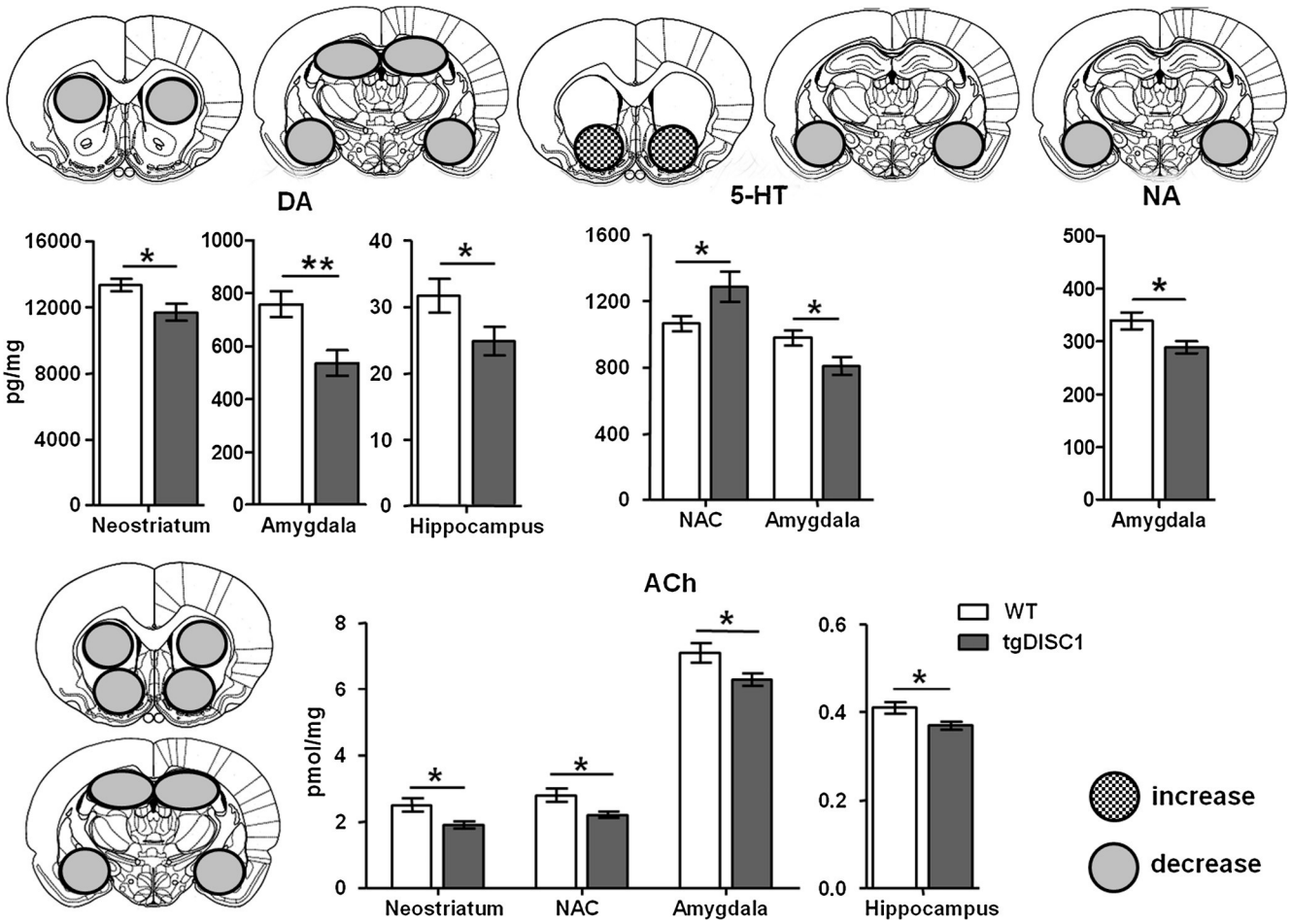

Fig. 4. Neurochemical quantification of dopamine (DA), noradrenaline (NA), serotonin (5-HT) and acetylcholine (ACh) in the WT and tgDISC1 groups. The mean contents of DA in the neostriatum, amygdala and hippocampus, 5-HT in the amygdala, and NA in the amygdala, was significantly lower in the tgDISC1 group. The mean content of 5-HT in the nucleus accumbens (NAC) was significantly higher in the tgDISC1 group. Lower levels of ACh were found in the neostriatum, NAC, amygdala and hippocampus in the tgDISC1 animals, when compared to WT groups. (The DA results have been published in a previous article.**** [9] N = 11–12 each group. Values represent mean ± SEM. *p ≤ .05, **p < .01, compared to the WT group via independent t-test.

3.3. Neurochemistry of tgDISC1 and WT brains

3.3.1. Monoamines

DA concentrations were significantly decreased in the neostriatum, amygdala and hippocampus of the tgDISC1 rats relative to WT controls (d.f. = 22, t = 2.646, P = .015; d.f. = 20, t = 3.254, P = .004; d.f. = 21, t = 2.060, P = .052, respectively; Fig. 4) (These results of DA levels have been part of a previous publication (Trossbach et al., 2016).

NA levels were significantly decreased in the amygdala of tgDISC1 rats relative to WT controls (d.f. = 22, t = 2.553, P = .018; Fig. 4). No significant differences between tgDISC1 rats and WT controls were observed in the other brain regions (P > .05; data not shown).

5-HT levels were increased significantly in the nucleus accumbens of tgDISC1 rats relative to WT controls (d.f. = 22, t = −2.126, P = .045; Fig. 4). In the amygdala, 5-HT concentrations were reduced in tgDISC1 compared with the WT controls (d.f. = 22, t = 2.442, P = .023; Fig. 4). No significant differences between tgDISC1 rats and WT controls were observed in other brain regions (P > .05; data not shown).

3.3.2. Acetylcholine

ACh levels were significantly lower in the neostriatum (d.f. = 22, t = 2.495, P = .021), nucleus accumbens (d.f. = 22, t = 2.619, P = .045), hippocampus (d.f. = 22, t = 2.440, P = .023) and amygdala (d.f. = 22, t = 2.131, P = .045) of tgDISC1 rats relative to WT controls (Fig. 4). No difference was found in the frontal cortex (P > .05; data not shown).

4. Discussion

The main findings of the present study on tgDISC1 rats that overexpress the full-length non-mutant human DISC1 gene are: (1) TgDISC1 rats were deficient in long-term (24 h) object memory, as well as in attention-like behavior, but not short-term (1 h) memory, (2) in tgDISC1 rats, administration of IN-DA reversed the deficits of long-term object memory and attention-like behavior, and (3) in the tgDISC1 rats post mortem baseline levels of DA were significantly lower in the neostriatum, hippocampus and amygdala. ACh levels were lower in the neostriatum, nucleus accumbens, hippocampus and amygdala. NA was lower in the amygdala, while 5-HT levels were reduced in the amygdala, but elevated in the nucleus accumbens.

In the present study, tgDISC1 rats were impaired in long-term memory for NOP and in attention-related behavior as tested with the OBAT. Thus, it appears that DISC1 overexpression impairs working memory during a very short delay between exposure and test trials, suggesting that the DISC1 gene may affect memory processes in rodents, when tasks require responses within a short time window. This is consistent with findings on humans with a haplotype of the DISC1 gene, who displayed both poor short-term visual memory and attention (Hennah et al., 2005). The dual effect of the DISC1 gene - deleterious performance in OBAT (ultra short-term) and long-term NOP test - implies that at least two distinct processes are influenced by the

overexpression of the DISC1 gene. One deficit involves long-term memory. Since short-term memory at the 1 h interval was intact, the question arises as how to interpret the deficit in the OBAT test with no interval between exposure to the objects and the test trial. This test can be considered either to assess ultra short-term memory and/or attention-like behavior (in accordance with the interpretation preferred by the inventors Alkam et al., 2011, 2013). Given that the total exploration time of objects was comparable in both groups in all the test trials, the level of motivation for object exploration should not be a confounding factor here.

Novel object exploration memory is considered to involve the hippocampus and its adjacent cortical areas, including the entorhinal, perirhinal, and parahippocampal cortices. The hippocampus receives input from the perirhinal cortex, including visual, olfactory, and somatosensory information, which is crucial for object recognition (Clarke, Cammarota, Gruart, Izquierdo, & Delgado-Garcia, 2010). The dorsal hippocampus plays an important role in NOP (Broadbent, Gaskin, Squire, & Clark, 2010; Cohen et al., 2013) and for the consolidation of NOP memory (Chao, Huston, Nikolaus, & de Souza Silva, 2016; Oliveira, Hawk, Abel, & Havekes, 2010; Reger, Hovda, & Giza, 2009). The prefrontal cortex is relevant for visual working memory and attention and interacts with the hippocampus for the processing of long-term memory (Preston & Eichenbaum, 2013; Simons & Spiers, 2003).

There is ample evidence that the DISC1 gene is associated with prefrontal function. Transgenic mice, expressing a putative dominant-negative DISC1 gene, were impaired in reversal learning and reinforcement devaluation performance, along with increased oxidative stress in the prefrontal cortex (Johnson et al., 2013). Deficient dendrites and a decreased spine density were also found in the frontal cortex of DISC1 mutant mice (Lee et al., 2011). Furthermore, in the tgDISC1 rat interneurons were shifted to deeper layers in the sensorimotor cortex (Hamburg et al., 2016). Thus, it is likely that prefrontal cortex functions are disrupted in the tgDISC1 rat. Disrupted prefrontal cortex function may account for both the deficit in long-term NOP memory and the poor performance in the OBAT test. Further investigations are required in order to clarify the contribution of the hippocampus and of the interaction between these regions to the cognitive deficits observed in the present study.

Previous findings on tgDISC1 rats showed elevated DAT function and an increase of D2R density in the ventral striatum (Trossbach et al., 2016). Moreover, in DISC1-L100P mutant mice, the D2R formed a protein complex with DISC1, facilitating D2R-mediated glycogen synthase kinase-3 signaling and inhibiting agonist-induced D2R internalization (Su et al., 2014). Given the DAergic connections between the dorsal/ventral striatum and prefrontal cortical areas (Haber, 2014), which are relevant for both motor and cognitive functions, alterations of DA functions are likely to be involved in the behavioral impairments observed in tgDISC1 rats. This is underlined by the ameliorative effects of IN-DA. It may be argued that the increased availability of D2 heteroreceptors in the ventral striatum of tgDISC1 rats, firstly, leads to an increased inhibition of substantia nigra/ventral tegmental area, and, thus, to a disinhibition of the prefrontal cortex. Secondly, the increased availability of D2 autoreceptors increases feedback inhibition, which – together with the observed increase of DAT function in tgDISC1 rats – is likely to result in a shortage of extracellular DA. It may be that this deficit is compensated by the administration of IN-DA. After IN-DA delivery, DA was detected in the CSF and brain (Dahlin et al., 2001) and increased DA levels were found in the neostriatum and nucleus accumbens (de Souza Silva et al., 2008). IN-DA administration led to reduced DAT binding in the dorsal striatum, which suggests an elevation of synaptic DA activity by this treatment (de Souza Silva et al., 2016). IN-DA in rats also increased locomotor activity (de Souza Silva et al., 2008) and had anti-depressant-like effects (Buddenberg et al., 2008). An anxiolytic effect and higher locomotion were found in mice with IN-DA treatment (Kholodar, Amikishieva, & Anisimov, 2013). In aged rats, IN-DA compensated memory deficits tested in object-place preference test (Trossbach et al., 2014). Treatment with IN-DA attenuated deficient spatial attention and working memory in an animal model of ADHD (Ruocco et al., 2014). Given that the DISC1 gene influences the DAergic systems (for review, see Dahoun et al. (2017)), treatment with DA may “re-balance” the system and, thus, compensate for DA-related deficits. A working hypothesis is that DISC1 interacts with striatal DAergic systems and, thereby, influences the prefrontal cortex to impede short-term/working memory, attention and long-term memory deficits.

Post-mortem analysis revealed lower total DA levels in neostriatum, hippocampus and amygdala of tgDISC1 rats. Neurochemical differences, however, were not confined to DA. TgDISC1 rats also had lower levels of ACh in the neostriatum, nucleus accumbens, hippocampus and amygdala, which are major projection areas of the basal forebrain cholinergic neurons (Dani & Bertrand, 2007). The cholinergic system in the hippocampus is acknowledged to regulate memory processes (Everitt & Robbins, 1997; Hasselmo, 2006). The lower levels of hippocampal ACh observed in the present study may be related to the impaired long-term memory in the NOP test. Striatal cholinergic interneurons are targeted by the nigrostriatal dopaminergic system (Pickel & Chan, 1990). DA agonists regulate the inhibition of ACh release in the striatum via D2R on cholinergic neurons (Ikarashi, Takahashi, Ishimaru, Arai, & Maruyama, 1997; Stoof, Drukarch, de Boer, Westerink, & Groenewegen, 1992). Since we also found a significant decrease of hippocampal DA levels, disruption of the DAergic system might have impaired cholinergic functions by a variety of mechanisms.

The higher 5-HT and lower ACh concentrations observed in the nucleus accumbens might indicate an interaction between these two neurotransmitter systems. 5-HT can have direct effects on cholinergic neurons, resulting in their inhibition (Van Bockstaele, Chan, & Pickel, 1996). Along with DA, 5-HT and NA levels were reduced in the amygdala, which controls motivated behaviors along with prefrontal cortex and hippocampus (Gruber & McDonald, 2012; Robbins & Everitt, 1996). The neurotransmitter changes induced by the DISC1 gene in nucleus accumbens and amygdala may be related to reward-based learning, since previous studies on transgenic mice expressing putative dominant-negative DISC1 revealed a deficiency in reinforcement devaluation (Johnson et al., 2013). Taken together, both the monoaminergic and cholinergic systems are dysregulated in the tgDISC1 rats, which may be related to the observed behavioral deficits. Post-mortem levels of neurotransmitters, as assessed here,indicate changes in the systems involved, but must be interpreted with caution as they do not necessarily reflect extracellular activity.

Further studies are necessary to establish the developmental course of the memory deficits in the tgDISC1 rat as well as to determine whether early intranasal DA treatment can prevent the neurochemical and cognitive deficits.

In conclusion, the overexpression of the human DISC1 gene impaired object long-term memory and ultra-short memory or attention, which was alleviated by IN-DA administration. In the tgDISC1 rats, DA, 5-HT, NA and ACh levels were affected in neostriatum, nucleus accumbens, amygdala and hippocampus, suggesting an interaction between the DISC1 gene and these systems, possibly in relation to the observed behavioral deficiencies.

Rare mutations of the DISC1 gene have been closely linked to various psychiatric disorders, including susceptibility to schizophrenia (Blackwood et al., 2001; Chubb et al., 2008). A functional interaction between DISC1 and the DA system would seem to be compatible with DISC1 functions in schizophrenia. On the other hand, deficits in attention and working memory correspond to symptoms of ADHD. In fact, IN-DA was shown to alleviate the behavioral ADHD markers, attention and working memory deficit, in an animal model of ADHD (Ruocco et al., 2014). Therefore, rather than to assign DISC1 dysfunction, be it genetic or on the posttranslational level, to a clinical diagnosis, we

consider it a vulnerability factor based on deficient DA homeostasis.

Several antipsychotic drugs, which affect the DAergic system were also found to alleviate DISC1-related cognitive impairments. The DAT inhibitor bupropion improved the antidepressant-like behavior in the forced swim test (Clapcote et al., 2007) and ameliorated social deficits (Lipina, Fletcher, Lee, Wong, & Roder, 2013), while the D1R agonistic clozapine reversed the impairment of short-term object memory (Nagai et al., 2011) and long-term spatial memory (Cui et al., 2016). Likewise, the finding that administration of DA via the nose-brain pathway can restore cognitive functions in the tgDISC1 rat indicates that DA deficiency may be a major cause for the behavioral deficits of the tgDISC1 rat, and that cognitive deficits in DISC1-related disorders, may be ameliorated by administration of IN-DA.

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Quantitative Proteomics of Synaptosomal Fractions in a Rat Overexpressing Human DISC1 Gene Indicates Profound Synaptic Dysregulation in the Dorsal Striatum

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Contribution: sacrificed and collected the brain samples

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Quantitative Proteomics of Synaptosomal Fractions in a Rat Overexpressing Human DISC1 Gene Indicates Profound Synaptic Dysregulation in the Dorsal Striatum

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Disrupted-in-schizophrenia 1 (DISC1) is a key protein involved in behavioral processes and various mental disorders, including schizophrenia and major depression. A transgenic rat overexpressing non-mutant human DISC1, modeling aberrant proteostasis of the DISC1 protein, displays behavioral, biochemical and anatomical deficits consistent with aspects of mental disorders, including changes in the dorsal striatum, an anatomical region critical in the development of behavioral disorders. Herein, dorsal striatum of 10 transgenic DISC1 (tgDISC1) and 10 wild type (WT) littermate control rats was used for synaptosomal preparations and for performing liquid chromatography-tandem mass spectrometry (LC-MS)-based quantitative proteomics, using isobaric labeling (TMT10plex). Functional enrichment analysis was generated from proteins with level changes. The increase in DISC1 expression leads to changes in proteins and synaptic-associated processes including membrane trafficking, ion transport, synaptic organization and neurodevelopment. Canonical pathway analysis assigned proteins with level changes to actin cytoskeleton, Gαq, Rho family GTPase and Rho GDI, axonal guidance, ephrin receptor and dopamine-DARPP32 feedback in cAMP signaling. DISC1-regulated proteins proposed in the current study are also highly associated with neurodevelopmental and mental disorders. Bioinformatics analyses from the current study predicted that the following biological processes may be activated by overexpression of DISC1, i.e., regulation of cell quantities, neuronal and axonal extension and long term potentiation. Our findings demonstrate that the effects of overexpression of non-mutant DISC1 or its misassembly has profound consequences on protein networks essential for behavioral control. These results are also relevant for the interpretation of previous as well as for the design of future studies on DISC1.

Keywords: DISC1, proteomics, synapses, animal model, dopaminergic system, axon guidance, striatum

INTRODUCTION

Disrupted-in-schizophrenia 1 (DISC1) is a gene originally identified as a translocation mutation in an extended Scottish pedigree where carriers suffered from diverse mental disorders comprising schizophrenia and affective disorders (Millar et al., 2000). Similarly, the DISC1 haplotype was associated with schizophrenia in a Finnish cohort (Hennah et al., 2003). A second family was later identified with a missense mutation and associated diverse clinical phenotypes (Sachs et al., 2005), and genetic association studies have supported association of DISC1 with mental disorders (Chubb et al., 2008). A role of the DISC1 gene for adaptive behavior was also suggested by various animal studies (Brandon and Sawa, 2011; Dahoun et al., 2017).

The DISC1 protein has features of a scaffold protein (Yerabham et al., 2013) and several subdomains have an intrinsic tendency to form high molecular multimers (Yerabham et al., 2017). Insoluble DISC1 protein has been identified in human post mortem brains with mental disorders (Leliveld et al., 2008), indicating that the DISC1 protein can be subject to aberrant proteostasis *in vivo*. For modeling the effects of aberrant proteostasis *in vivo*, a transgenic rat model overexpressing (approximately 11-fold) the full length, non-mutant human DISC1 gene (transgenic DISC1, tgDISC1 rat) was generated that exhibited perinuclear aggregates throughout the brain, accentuated in dopamine-rich regions such as in the striatum (Trossbach et al., 2016). The tgDISC1 rat exhibited phenotypes such as amphetamine supersensitivity, an increase in D2R high receptors, and dopamine transporter mislocalization and dysfunction consistent with phenotypes observed in schizophrenia (Trossbach et al., 2016). Also, at the neuroanatomical level fewer dopaminergic neurons and projections into the dorsal striatum, as well as aberrant interneuron positioning was observed indicating subtle neurodevelopmental disturbance (Hamburg et al., 2016).

These findings, induced by aberrant proteostasis of the DISC1 protein, leading to its misassembly and perinuclear deposition, suggest an important role of the DISC1 protein and its correct assembly for protein networks involved in adaptive behavior. Such protein networks have been described both at the protein and the genetic level. At the genetic level, Teng et al. (2017) carried out targeted sequencing of 59 DISC1 interactome genes and 154 regulome genes in psychiatric patients, identifying altered regulation of schizophrenia candidate genes by DISC1. In an attempt to dissect DISC1 function through protein-protein interactions based upon a yeast two-hybrid system along with bioinformatic methods, a comprehensive network around DISC1 was generated (Camargo et al., 2007). Using this iterative yeast two-hybrid system, a framework was provided to explore the function of DISC1, and interrogation of the proposed interactome has shown DISC1 to have protein-protein interactions consistent with that of an essential synaptic protein (Camargo et al., 2007). Current evidence suggests that DISC1 functions as a neuronal intracellular trafficking regulator that includes transport of neurotransmitter receptors, vesicles, mitochondria and mRNA, rendering synaptic regulation vulnerable to DISC1 dysfunction (Devine et al., 2016).

The objective of this study was to identify the proteomic signatures of the tgDISC1 rat model vs. its littermate wild type (WT) control to gain insights onto the DISC1-regulated proteins and downstream synaptic processes and to identify molecular circuitry regulated by relatively modest changes in expression level leading to DISC1 misassembly. Identification of changes in protein networks relevant for behavioral processes would raise the possibility for the DISC1 protein to represent a non-genetic interface with exogenous influences for mental disorders.

There is mounting evidence for a focal role of the DISC1 protein in striatal functions, and particularly on dopamine homeostasis in relation to behavioral changes (Trossbach et al., 2016; Wang et al., 2017). Therefore we chose to select proteins from the synapse-enriched membrane fractions (synaptosomes) from the dorsal striatum for this study. Differential proteomics by isobaric labeling (TMT 10plex) enable multiplexed protein identification and quantitative analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS). This allows the unbiased analyses of approximately 6000 proteins and targets synaptic proteins including receptors, transporters and channels that have been implicated in psychiatric disorders. Combining proteomics and bioinformatics approaches enabled a comprehensive view on the *in vivo* protein changes and the biological functions of DISC1.

MATERIALS AND METHODS

Animals

Previously described tgDISC1 Sprague-Dawley rats and WTs were used in this study (Trossbach et al., 2016). Briefly, full-length, non-mutant human DISC1 as transgene with the polymorphisms F607 and C704 were integrated into the pronuclei of Sprague Dawley rats. Ten male tgDISC1 rats and 10 male WT littermate control rats, aged 14–15 months (ZETT, Heinrich Heine University, Düsseldorf, Germany) were used. One WT rat and one tgDISC1 rat were derived from each pair of parents. The study was carried out in accordance with the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985), and the German Law on the Protection of Animals. It was approved by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) NRW.

Preparation of Synaptosomal Fractions

Dorsal striata from fresh brains were dissected and stored at −80°C. Synaptosomal fractions from bilateral regions were prepared for individual animals (for tgDISC1 and WT; n = 10 each), using a microscale discontinuous sucrose gradient modified from previous protocols (Hahn et al., 2009; Sialana et al., 2016). Collected synaptosomes from 1.25/1.0 M sucrose interface were diluted with 10 mM HEPES, divided into two and pelleted at 15,000 $\times g$ for 30 min. Pelleted synaptosomal samples were reconstituted in urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 50 mM TEAB supplemented with protease inhibitors) for LCMS analyses and SDS buffer

(1.5% SDS,100 mM NaCl, 20 mM Tris supplemented with protease inhibitors) for WB analyses and were sonicated for 1 min. Protein amounts were estimated using the Pierce 660 protein assay or BCA protein assay (ThermoFisher Scientific).

Proteolytic Digestion and Isobaric Labeling

Fifty micrograms of samples were digested with a Trypsin-LysC enzyme mixture (1:100 w/w, Promega) using the filter-aided sample preparation (FASP), as previously described, with minor modifications (Wisniewski et al., 2009). The resulting peptide samples were purified with reversed-phase C18 and labeled with TMT 10-plex according to the instructions supplied by the manufacturer. Two TMT-10plex experiments were performed, with each experiment consisting of five tgDISC1 and five WT animals (n = 10 biological replicates per group). For each TMT experiment, ten isobarically labeled peptide samples were pooled, the peptides separated by high pH reversed-phase LC into 100 time-based fractions and pooled into 25 samples (Gilar et al., 2005). The peptides were vacuum concentrated and reconstituted in 5% formic acid. Details of the procedure are essentially as described previously (Sialana et al., 2016) and in the Supplementary Figure S1.

Liquid Chromatography and Tandem Mass Spectrometry

Samples were injected onto a Dionex Ultimate 3000 system (ThermoFisher Scientific) coupled to a Q-Exactive Plus mass spectrometer (ThermoFisher Scientific, Schwerte, Germany). Software versions used for the data acquisition and operation of the Q-Exactive were Tune 2.8.1.2806 and Xcalibur 4. HPLC solvents were as follows: solvent A consisted of 0.1% formic acid in water and solvent B consisted of 0.1% formic acid in 80% acetonitrile. From a thermostated autosampler, 10 µL that correspond to 1 µg of the peptide mixture were automatically loaded onto a trap column (PM100-C18 3 µm, 75 µm × 20 mm, ThermoFisher Scientific, Austria) with a binary pump at a flow rate of 5 µL/min using 2% acetonitrile in 0.1% TFA for loading and washing the pre-column. After washing, the peptides were eluted by forward-flushing onto a 50 cm analytical column with an inner diameter of 75 µm packed with 2 µm-C18 reversed phase material (PepMap-C18 2 µm, 75 µm × 500 mm, ThermoFisher Scientific, Austria). For label free quantification (LFQ), the LCMS analyses was performed using a single-shot LCMS approach with 4-h gradient with LCMS parameters as described previously (Stojanovic et al., 2017).

The fractionated TMT10plex labeled peptides were eluted from the analytical column with a 120 min gradient ranging from 5% to 37.5% solvent B, followed by a 10 min gradient from 37.5% to 50% solvent B and finally to 90% solvent B for 5 min before re-equilibration to 5% solvent B at a constant flow rate of 300 nL/min. The LTQ Velos ESI positive ion calibration solution (Pierce, IL, USA) was used to externally calibrate the instrument prior to sample analysis and an internal calibration was performed on the polysiloxane ion signal at m/z 445.120024 from ambient air. MS^1 scans were performed from m/z 375–1400 at a resolution of 70,000. Using a data-dependent acquisition mode, the 15 most intense precursor ions of all precursor ions with +2 to +7 charge were isolated within a 1.2 m/z window and fragmented to obtain the corresponding MS/MS spectra. The fragment ions were generated in a higher-energy collisional dissociation (HCD) cell at 32% normalized collision energy with a fixed first mass at 100 m/z and detected in an Orbitrap mass analyzer at a resolution of 35,000. The dynamic exclusion for the selected ions was 30 s. Maximal ion accumulation time allowed in MS and MS^2 mode was 50 and 100 ms, respectively. Automatic gain control was used to prevent overfilling of the ion trap and was set to 3×10^6 ions and 1×10^5 ions for a full Fourier transform MS and MS^2 scan, respectively.

Protein Identification and Quantification

All MS-MS^2 spectra were searched against UniProtKB/Swiss-Prot rat protein database version v 2016.04.14 (27,815 sequences, including isoforms). In addition, sequences of the human DISC1 protein and 11 isoforms produced by alternative splicing with the polymorphisms F607 and C704 were appended to the rat database. All spectra files were processed in Proteome Discoverer 2.1 (Thermo Scientific, Germany) platform with Mascot using mass tolerances of ± 10 ppm and ±0.02 Da for precursor and fragment ions. One missed tryptic cleavage site was allowed. Oxidation of methionine was set as variable modification, whilst carbamidomethylation of cysteine residues, TMT 10-plex labeling of peptide N-termini and lysine residues were set as fixed modification. Thresholds were determined via the target-decoy approach using a reversed protein database as the decoy by imposing 1% false discovery rate (FDR). Label-free quantitation was implemented using the Minora feature of Proteome Discoverer 2.2. The following parameters are used: maximum retention time alignment of 10 min with minimum of S/N of 5 for feature linking mapping. Abundance were based precursor/peptide area intensities. Normalization was performed such that the total sum of the abundance is the same for all sample channels. Imputation was performed by replacing the missing values with random values from the lower 5% of the detected values. For TMT 10-plex labeled samples, relative abundances of proteins were determined from the TMT reporter ions without imputation. Protein abundance ratios were calculated based on unique and razor peptides. Relative protein levels were determined from the sum of the reporter ion intensities per quantitative channel that correspond to each biological animal replicate.

The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2014) partner repository with the dataset identifier PXD008123.

Bioinformatics

Quantitative data were analyzed using Perseus statistical package (version 1.5.1.6, Tyanova et al., 2016). Statistical significance of differences in protein levels between the groups were evaluated using a two-sided T-test with $P < 0.05$ (either Student's or Welch's as required). Enrichment of GO annotations were

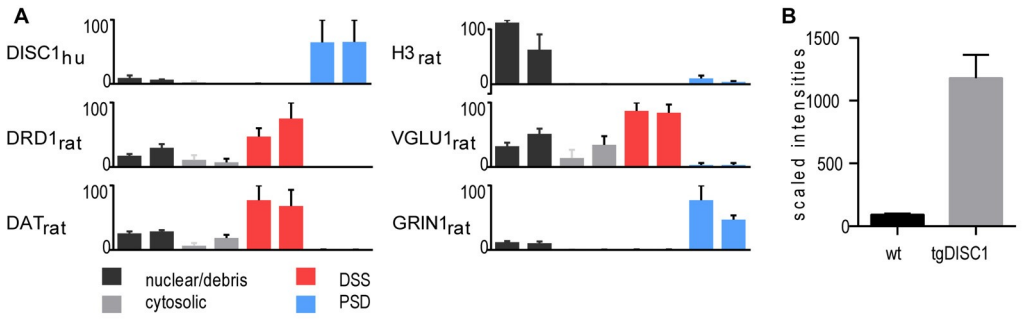

FIGURE 1 | Proteomic profile of the transgenic DISC1 (tgDISC1) fractions. (A) Protein levels of representative proteins for the following biochemical fractions of the dorsal striatum: nuclear/debris, cytosolic, detergent-soluble synaptosomal preparation (DSS), postsynaptic density (PSD) preparation. Protein levels of representative synaptic markers were estimated from label-free LCMS analyses. Protein levels of the nuclear (H3), presynaptic (VGLU1) and postsynaptic (GRIN1) protein markers are enriched in nuclear/debris, DSS and PSD preparations, respectively. The majority of the human Disrupted-in-schizophrenia 1 (DISC1) protein was enriched in the PSD preparations. The level of overexpression is approximately 10-fold higher than the endogenous DISC1 protein in the whole synaptosomes (B).

performed on the significant proteins using GOA database (v30.08.2017) using the ClueGO via the Cytoscape platform (Bindea et al., 2009; Huntley et al., 2015). To reduce redundancy of GO terms the fusion option was selected. Enriched GO terms (Benjamini–Hochberg P-value < 0.05) are functionally grouped into networks linked by their kappa score level (≥ 0.40). Functionally related groups partially overlap and only the most significant terms per group are labeled. Pathway analyses on the significant proteins were performed through the use of IPA (Ingenuity[r] Systems[1]). The differentially expressed genes were categorized to related canonical pathways. Only those experimentally observed or highly predicted molecules and/or relationships from tissues and cells from the nervous system were considered. The top enriched categories of canonical pathways with a P-value $< 10^{-3}$ as well as representative differentially expressed proteins in each canonical pathway is reported. Curated gene-disease annotations were obtained from Comparative Toxigenomics database (Davis et al., 2015). The IPA regulation z-score algorithm was used to predict biological functions that are expected to be activated (z-score ≥ 2; $P \leq 0.05$). The z-scores take into account the directional effect of one protein on a process and the direction of change of molecules in the dataset.

Immunoblotting

The following antibodies were used according to the instructions supplied by the manufacturer: mouse anti-PSD95 (124011, Synaptic Systems), mouse anti-SYP (sc-55507, Santa Cruz Biotechnology), rabbit anti-NMDAR1 (ab32915, Abcam), mouse anti-VGLUT1 (135311, Synaptic Systems), rabbit anti-GAPDH (ab9485, Abcam), rabbit anti-DAT1 (ab111468, Abcam) and mouse anti-huDISC1 (3D4, Korth lab; Ottis et al., 2011). Immunoblot data were normalized to corresponding whole-lane densitometric volumes of protein-stained membranes (Welinder and Ekblad, 2011). Immunoblotting conditions were as previously described (Sialana et al., 2016) and antibody dilutions are provided in the Supplementary Table S1.

RESULTS

In the current study, a high-throughput proteomic approach was employed to generate a comprehensive view of the *in vivo* protein changes in striatal synaptosomes of the tgDISC1 rat model (experimental workflow, Supplementary Figure S1). Methodologically, tissue fractionation was initially performed on the dorsal striatum of tgDISC1 rats to determine the subcellular expression of tgDISC1 and which enrichment steps would be employed in this study (Phillips et al., 2001; Sialana et al., 2016). Dorsal striata of tgDISC1 rats were fractionated into nuclear/debris, cytosolic, detergent soluble synaptosome (DSS) and postsynaptic density (PSD) preparations. LCMS-based proteomic analyses of the biochemical fractions resulted in the identification and LFQ of 5002 protein groups (Supplementary Data 1). Distribution of the nuclear (H3), presynaptic (VGLU1) and postsynaptic (GRIN1) protein markers enriched in nuclear/debris, DSS and PSD preparations is given in Figure 1A. Although DISC1 was observed in all preparations, the majority of the human DISC1 protein was enriched in the Triton-X100-resistant PSD fractions. This is in agreement with previous immunoblotting studies of DISC1 in adult rats (Hayashi-Takagi et al., 2010). We have previously shown that dopaminergic pathways are modulated in the striatum of the tgDISC1 rat (Trossbach et al., 2016). Taking into account that dopamine receptor 1 and the dopamine transporter were highly enriched in the DSS preparations (Figure 1A), it was decided to study the *whole synaptosome* for quantitative proteomics experiments. Immunoblots of postsynaptic (GRIN1 and PSD95) and presynaptic (VGLU1 and SYP) proteins show enrichment of synaptosomal proteins on the biochemical fraction (Supplementary Figure S2). The level of overexpression is approximately 10-fold higher than

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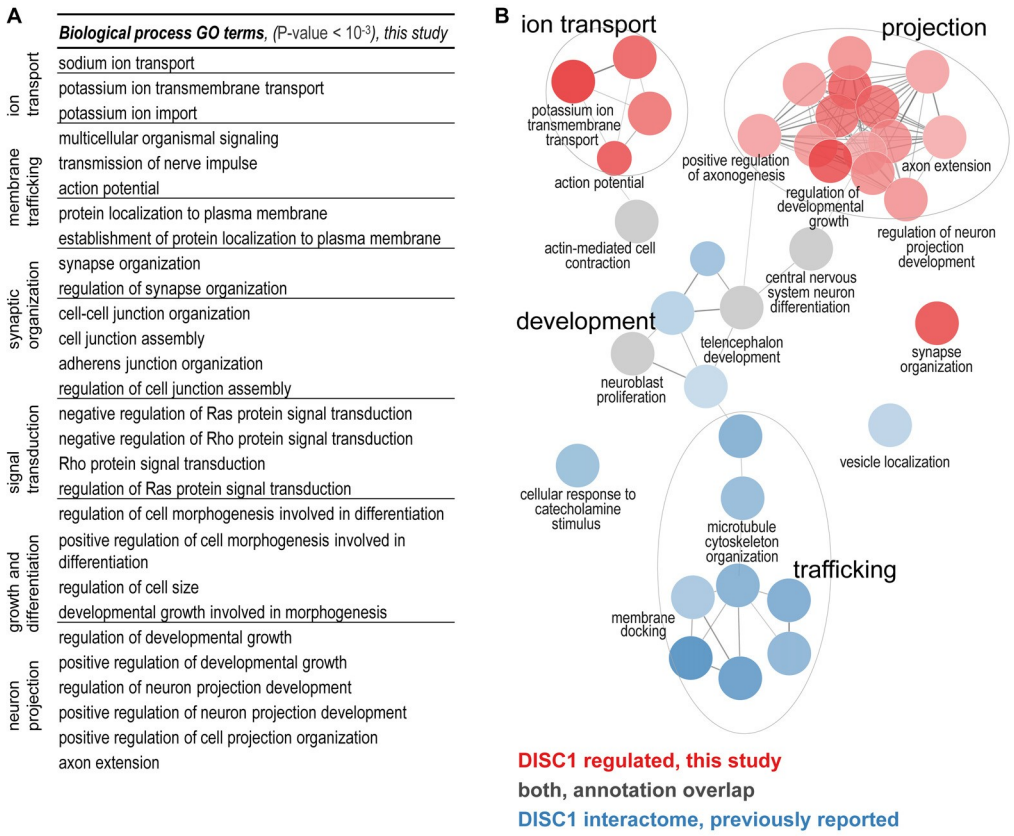

FIGURE 2 | (A) Biological processes regulated by DISC1. Enriched GO terms (P-value < 10^{-3}) are functionally grouped. The synaptic components terms as well as membrane trafficking, ion transport, synaptic organization and neurodevelopment processes are well represented. (B) Comparison of the DISC1-regulated proteins and the previously reported interacting proteins. In the current study ion transport, projections and synaptic organization were novel findings. Developmental processes from previous studies were confirmed (gray GO enrichment analyses was performed using ClueGO. Enriched GO terms (Benjamini–Hochberg P-value < 10^{-3}) are functionally grouped into networks linked by their kappa score level (≥0.40). Functionally related groups partially overlap and only the most significant terms per group are labeled.

the endogenous DISC1 protein in the whole synaptosomes (Figure 1B).

DISC1 Regulated Proteins—Proteomic Profiling of Striatal Synaptosomes

An expression proteomics experiment was performed to identify the proteins potentially regulated by DISC1. Synaptosomal fractions of bilateral dorsal striata of 10 wt and 10 tgDISC1 rats using TMT10plex were analyzed in two separate 10-plex experiments (5 tgDISC1 and 5 WT). In total, 7227 protein groups were identified (Supplementary Data 2) including 252 receptors and 672 transporters/channels. Out of the 6153 quantifiable protein groups, 213 proteins were statistically different between the tgDISC1 and WT rats (Supplementary Table S2, Supplementary Data 3). Protein levels were considered statistically different between groups when $P \leq 0.05$ using a two-sided T-test (either Student's or Welch's as required). Given the large number of comparisons made and the possibility of Type 1 error, the p values given cannot be interpreted in terms of "significance", but rather as "measures of effect".

As we used a good number of biological replicates for TMT-based proteomics (10 animals per group), we opted to use T-test that performs "individual proteins-based" hypotheses test (T-test) rather than a background "all-proteins-based" hypothesis test (FDR). TMT-based proteomics experiments are sensitive and precise but quantification is known to undergo ratio compression (Ow et al., 2011). The values from FDR corrections depend on effect size; smaller differences yield higher P-corrected (q-values); thus only two proteins passed the corrected thresholds. An additional filter is applied when enrichment analyses (GO annotation, IPA) is employed. Slight differences in the levels of multiple proteins should cluster relevant processes and the proteins from the top enriched processes/pathways are of higher emphasis (Pascoviciet al., 2016).

Immunoblotting analyses of DAT1, GRIN1 and DISC1 of WT and tgDISC1 indicated that the direction of fold differences measured by TMT-proteomics and western blotting (Supplementary Figure S3) was consistent.

A

Canonical Pathways	P-value	Proteins
Signaling by Rho Family GTPases	4.57E-06	MYL6, ARHGEF4, RAF1, ROCK1, GNAZ, FGFR3, DES, PIK3CA, ARPC1B, CDH20, GNAS, ITGA3
Axonal Guidance Signaling	1.07E-04	MYL6, LINGO1, RAF1, ABLIM2, EPHA6, ROBO2, ROCK1, GNAZ, FGFR3, SEMA7A, PIK3CA, ARPC1B, GNAS, ITGA3
RhoGDI Signaling	2.57E-04	MYL6, ARHGEF4, ROCK1, GNAZ, ARPC1B, CDH20, GNAS, ITGA3
Ephrin Receptor Signaling	2.69E-04	RAF1, EPHA6, ROCK1, GNAZ, ARPC1B, GNAS, ITGA3, GRIN1
Actin Cytoskeleton Signaling	3.47E-04	MYL6, ARHGEF4, RAF1, ROCK1, CSK, FGFR3, PIK3CA, ARPC1B, ITGA3
Gαq Signaling	8.71E-04	RAF1, ADRA1A, ROCK1, CSK, FGFR3, PIK3CA, GNAS
Dopamine-DARPP32 Feedback in cAMP Signaling	9.33E-04	CACNA1A, ADCY3, KCNJ4, KCNJ11, GNAS, KCNJ12, GRIN1

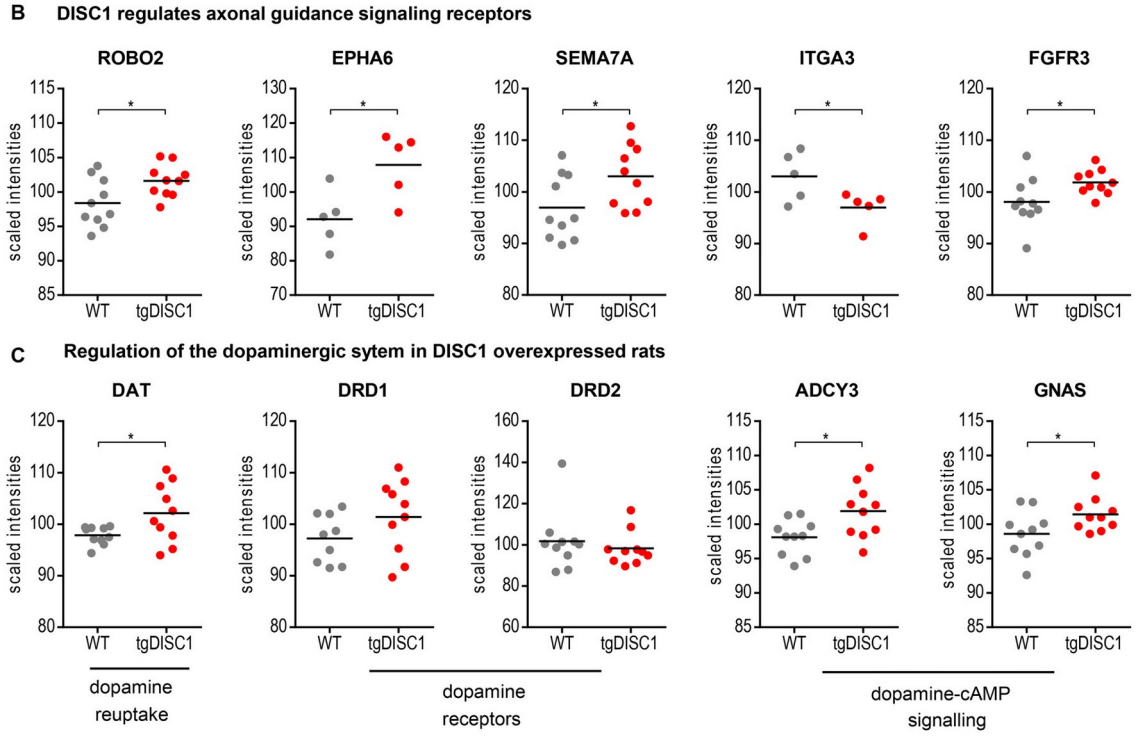

FIGURE 3 | Pathways regulated by DISC1. Significantly enriched canonical pathways (Fishers' exact test, $P < 10^{-3}$, IPA) of the proteins altered in by tgDISC1 rats in the dorsal striatum (A). Representative proteins from the dopaminergic (B) and axonal guidance signaling pathway (C) are shown. Values represent $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared using two-sided *T*-tests.

Functional Classification of Proteins Modulated in tgDISC Rats

The biological functions of the 213 proteins with highly different protein level changes between wildtype and tgDISC1 rats were explored using GO enrichment analyses. Enrichment of synaptic components such as axons, dendritic spines, membrane rafts, neuron projection membrane, and the ion channel complex were revealed (Supplementary Table S3, Supplementary Figure S4). The voltage gated ion channels were the major protein classes represented (Supplementary Table S4). The results suggest that the modest overexpression of the full-length human DISC1 alters proteins linked to synaptic processes including membrane trafficking, ion transport, synaptic organization and neurodevelopment (Figure 2A).

Functional Comparison of the DISC1 Regulated Proteins to Known Interacting Proteins

To determine the biological functions unique to DISC1 regulated proteins, we performed enrichment analyses for the

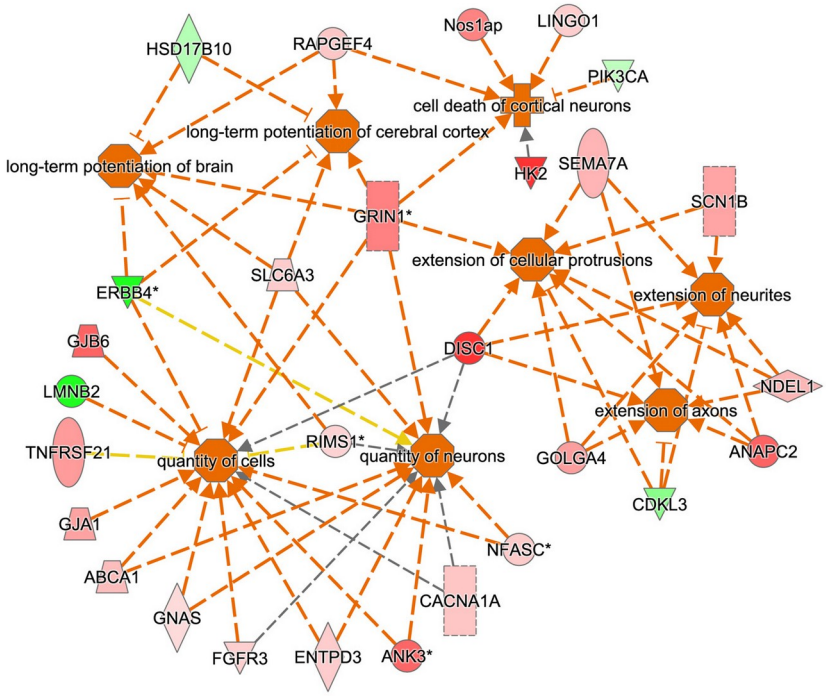

FIGURE 4 | Predicted biological functions of the tgDISC1 regulated proteins as evaluated by IPA. The IPA regulation *z*-score algorithm was used to predict the activation of biological functions in tgDISC1 rats relative to wild type (WT) according to our proteomics data. The network displays functional interactions between proteins (z-score $\geq$ 2 and p-value $\leq$ 0.05). Dashed lines indicate direct or indirect interactions. Proteins up-regulated in tgDISC1 rats are colored in shades of red; proteins down/regulated are colored in green.

DISC1 regulated proteins in comparison to previously reported interacting proteins (Camargo et al., 2007; Boxall et al., 2011; Bradshaw and Porteous, 2012; Thomson et al., 2013) as compiled by a recent study (Teng et al., 2017). Using ClueGO, 36 biological processes with strong enrichment ($P < 10^{-6}$) were revealed (Figure 2B; Supplementary Figure S5). The clusters of biological processes exclusive to the proteins regulated by DISC1 include: "regulation of neuron projection development", "positive regulation of axonogenesis", "action potential/potassium ion transport and synapse organization". Terms associated with microtubule development and neuronal transport were highly represented in the DISC1-interacting proteins. Biological processes such as "CNS differentiation" and "telencephalon development" were enriched in both, DISC1 regulated and interacting protein data sets.

Prediction of Canonical Pathways and Biological Function

To investigate the molecular mechanisms modulated by DISC1, data were analyzed through the use of Ingenuity Pathway analysis (IPA; Ingenuity Systems[2]). The differentially expressed proteins were categorized to related canonical pathways. Canonical pathway analysis assigned proteins with level changes to actin cytoskeleton, Gαq, Rho family GTPase and Rho GDI-, axonal guidance, ephrin receptor and dopamine-DARPP32 feedback in cAMP signaling (Fisher's exact test, $P < 10^{-3}$, Figure 3A, Supplementary Figure S6). Only robustly predicted or experimentally observed molecules and/or relationships from tissues and cells from the nervous system were considered. Receptors from the axonal guidance signaling and the dopamine-DARPP32 feedback from the cAMP signaling canonical pathway are illustrated in Figures 3B,C).

The IPA regulation *z*-score algorithm was used to predict biological functions that are expected to be activated in tgDISC1 rats rather than in wildtype (positive *z*-score) according to own proteomics data (z-score $\geq$ 2; $P \leq 0.05$). The *z*-scores take into account the directional effect of one protein on a process and the direction of change of molecules in the dataset. From the expression data of the regulated proteins, the following processes are predicted to be activated: "activation regulation of cell quantities", "neuronal and axonal extension", "long term potentiation" and "apoptosis" (Figure 4, Supplementary Table S5).

Annotation of the DISC1 altered protein levels revealed that 54 proteins are associated with mental disorders and/or nervous system diseases as implemented by the Comparative Toxicogenomics Database (CTD; Davis et al., 2015). Disease-gene associations were based on genomic, transcriptomic and proteomic studies on the sequence variation

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and expression changes associated with brain diseases and disorders. Over-represented disease-protein associations (Fishers' exact test, $P < 0.05$) include: neurodevelopmental disorders, autistic disorders, schizophrenia spectrum, anxiety disorders, substance-related disorders (e.g., cocaine) and intellectual disability (Table 1). In particular, the schizophrenia-associated proteins including dopamine transporter 1 (SLC6A3), receptor tyrosine-protein kinase erbB-4 (ERBB4), glutamate ionotropic receptor NMDA type subunit 1 (GRIN1), membrane-associated guanylate kinase WW and PDZ domain containing 2 (MAGI2) and regulator of G-protein signaling 12 (RGS12) were also regulated by DISC1 (Mateos et al., 2006; Silberberg et al., 2006; Xu et al., 2011; Koide et al., 2012; Guipponi et al., 2014; Jaros et al., 2015; Zhang et al., 2015; Li et al., 2017).

DISCUSSION

By the use of quantitative proteomics of synapse-enriched membrane (synaptosomal) fractions of the dorsal striatum of the tgDISC1 rat, we have identified novel protein networks and signaling pathways regulated by an increase of non-mutant DISC1 expression or DISC1 misassembly. These results suggest that the DISC1 protein and its disturbed proteostasis can have an effect on mental disorder-relevant protein networks independent of genetic mutations. Likely, multiple exogenous or endogenous factors other than overexpression could lead to a failure of DISC1 proteostasis, such as exposure to high dosages of dopamine or other oxidants, making DISC1 protein an oxidation "sensor" (Atkin et al., 2012; Trossbach et al., 2016).

In the tgDISC1 rat, an about 11-fold overexpression, leading to DISC1 misassembly, changed proteins and synaptic-associated processes including membrane trafficking, ion transport, synaptic organization and neurodevelopment is observed. Furthermore, dysregulation of DISC1 potentially modulates pathways including actin cytoskeleton, Gαq, Rho family GTPase and Rho GDI-, axonal guidance, ephrin receptor and dopamine-DARPP32 feedback in cAMP signaling associated with the synaptic pathologies. DISC1-regulated proteins are also highly associated with neurodevelopmental disorders, autistic disorder, schizophrenia spectrum, anxiety disorders, substance-related disorders and intellectual disability (Figure 5).

Previously known DISC1-protein interactors have been reported to modulate synaptic processes. The current study revealed that DISC1 regulates an array of synaptic proteins and processes that complements previous protein interaction results (Supplementary Figure S7). Proteins that were previously reported to interact with DISC1 (Millar et al., 2003; Camargo et al., 2007) were also modified in the current study in the tgDISC1 rat. These include microtubule protein spericentrin (PCNT), GRIP1 associated protein 1 (GRIPAP1), microtubule associated protein 1A (MAP1A), nudE neurodevelopment protein 1 (NDEL1) and microtubule-actin crosslinking factor 1 (MACF1) that are involved in neuronal cytoskeleton organization and membrane transport processes.

Dysregulation of DISC1 was reported to modulate glutamatergic and dopaminergic systems as previously reviewed (Hayashi-Takagi et al., 2010; Ramsey et al., 2011; Dahoun et al., 2017). Own results herein show that NMDAR1 is increased in the striatum of the tgDISC1 rat. A relationship between NMDAR1 and DISC1 has been shown, as knockdown and antagonists of NMDAR1 reduced numbers of synapses and synaptic DISC1 mainly in the striatum (Ramsey et al., 2011). Further, the DISC1 interactor GRIPAP1 is increased in the tgDISC1 rat. GRIPAP1 controls the AMPA receptors/GRIP-complex transport to the synapse by NMDA receptor activation (Ye et al., 2000).

As shown by MS, dopamine transporter levels were highly increased in the tgDISC1 rats, consistent with own previous studies by immunoblotting (Trossbach et al., 2016). Whereas levels of dopamine receptors 1 and 2 were not significantly altered, pathway enrichment analyses (Figure 3C) suggest that proteins (e.g., ADCY3, GNAS) from the dopamine-DARPP32 feedback of the cAMP signaling canonical pathway, may be involved in modulation of the known dopaminergic deficits in tgDISC1. Adenylate cyclase ADCY3 as a downstream effector of dopaminergic pathways catalyzes the formation of cAMP in response to G-protein signaling.

TABLE 1 | Disease-protein association of the DISC1 regulated proteins.

Disease name	*P*-value	Proteins
Neurodevelopmental disorders	1.02E-07	ANK3, ASIC2, CADM1, CTTNBP2, DISC1, GJA1, GNAS, GRIN1, KCNA2, KCNMA1, RIMS1, ROBO2, SCN2A, SLC4A4, SLC6A3, STAMBP, TCN2
Mental disorders	5.11E-07	ANK3, ASIC2, CADM1, CTTNBP2, DISC1, GC, GJA1, GNAS, GRIN1, KCNA2, KCNMA1, KLHL5, LINGO2, MAGI2, RGS12, RIMS1, ROBO2, SCN2A, SLC4A4, SLC6A3, STAMBP, TCN2
Autistic disorder	3.40E-05	ASIC2, CADM1, DISC1, GJA1, KCNMA1, RIMS1, ROBO2, TCN2
Schizophrenia spectrum and other psychotic disorders	4.10E-04	DISC1, GC, GRIN1, MAGI2, RGS12, SLC6A3
Anxiety disorders	2.53E-02	MAGI2, SLC6A3
Cocaine-related disorders	1.74E-02	GRIN1, KLHL5, SLC6A3
Intellectual disability	2.52E-02	ANK3, DISC1, GNAS, GRIN1, KCNA2, SLC4A4
Psychotic disorders	1.13E-02	GRIN1, SLC6A3
Schizophrenia	1.62E-03	DISC1, GC, MAGI2, RGS12, SLC6A3
Substance-related disorders	3.98E-02	GNAS, GRIN1, KLHL5, LINGO2, SLC6A3

Gene-disease associations on the DISC1 regulated proteins were implemented in the Comparative Toxicogenomics Database, CTD. Fifty-four DISC1-regulated proteins are associated with mental disorders and/or nervous system disease disorders. Over-represented disease-protein associations (Fishers' exact test, $P < 0.05$) are illustrated.

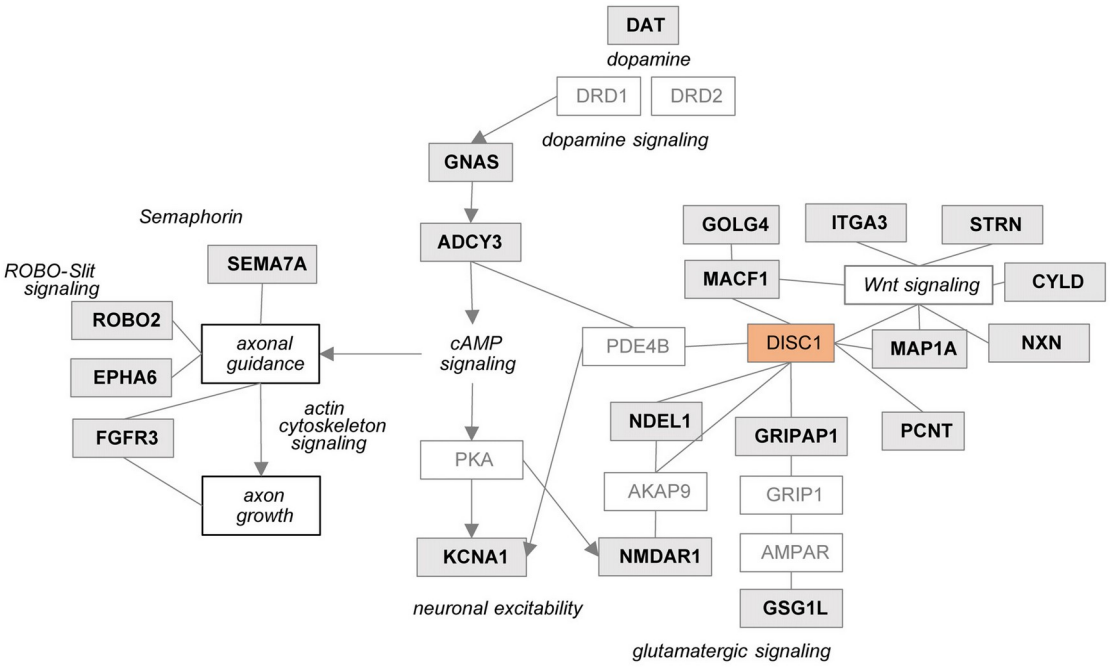

FIGURE 5 | Potential relationship between the DISC1 regulated proteins and synaptic processes. Proteins regulated in tgDISC1 rats from this study are marked in gray boxes.

The protein level changes of this enzyme along with the corresponding G-protein GNAS observed herein supports previous studies proposing dysregulation of cAMP signaling by DISC1 (Millar et al., 2005; Kvajo et al., 2011; Crabtree et al., 2017).

In a mouse Disc1 mutant model, functional reduction of Kv1.1/KCNA1 was proposed to contribute to alterations in neuronal excitability and short-term plasticity. Reduction of this channel was accompanied by reduced phosphodiesterase 4 activity and elevated cAMP levels in the PFC of *Disc1* mutant mice (Crabtree et al., 2017). Interestingly, in our DISC1 overexpressing transgenic model, we found an increase of this and several proteins in the voltage-gated potassium channel complex suggesting potential dysregulation of electrophysiological synaptic functions (Supplementary Figure S8).

Current data also revealed that proteins associated with axonal guidance pathways were altered by DISC1 overexpression: the axonal guidance receptors semaphorin 7A (SEMA7A), EPH receptor A6 (EPHA6), roundabout receptor 2 (ROBO2), fibroblast growth factor receptor 3 (FGFR3) and integrin subunit alpha 3/very late activation protein 3 receptor alpha-3 subunit (ITGA3) were shown to be modulated by DISC1 (Figure 3B). The leading edge of the axons contains receptors that sense guidance cues and aid in the navigation and migration of axons. The attraction or repulsion of cues promotes or decreases active actin polymerization, resulting in axonal extension or retraction by triggering the actin cytoskeleton signaling and Rho-GTPase pathways, as also proposed in the current pathway enrichment analysis (reviewed in Dent et al., 2011; Spillane and Gallo, 2014; Van Battum et al., 2015). The receptor SEMA7A stimulates axonal growth through integrins and MAPK signaling (Pasterkamp et al., 2003). The roundabout receptor 2, ROBO2 is the main receptor from the Slit-Robo pathway, that is involved in axon guidance and which is also associated with DISC1-interacting proteins SRGAP2 and 3 (Camargo et al., 2007). The Ephrin receptor signaling pathway, predicted to be regulated by DISC1, is critical for embryonic development and known as a mediator of axon guidance (Kvajo et al., 2011).

In perspective, alterations of these developmental pathways and processes could explain the subtle neurodevelopmental phenotypes in the tgDISC1, where the substantia nigra (SN) contains fewer dopaminergic neurons (DA), fewer projections into dorsal striatum, and a shift in the parvalbumin-positive interneurons (Hamburg et al., 2016). DA homeostasis deficiency and the proposed disturbed dopaminergic signaling could explain the observed decrease of DA neurons in the SN. The disturbed axonal guidance signaling could lead to the reduction of the projections into the dorsal striatum and the shift of the parvalbumin-positive interneurons. As protein profiles were obtained from adult tgDISC1 rats, it would be interesting to follow up by studying the profiles in the developing brain to reveal the etiopathology effects of DISC1 which exceeds the scope of this study.

Bioinformatics analyses from the current study predicted that the following biological processes were activated by overexpression of DISC1, i.e., regulation of cell quantities, neuronal and axonal extension and long term potentiation

(Figure 4). These results may be relevant for interpretation of previous as well as for the design of future studies on DISC1.

CONCLUSION

Our results suggest that overexpression and/or aberrant DISC1 proteostasis can lead to profound changes in protein networks relevant for mental disorders or endophenotypes and may signify a role for the DISC1 protein alone—in the absence of mutations—in behavioral and neural processes and disorders. DISC1 expression levels likely have to be controlled in a narrow expression window in order to execute adaptive behavior. These findings make the DISC1 protein and its post-translational modifications a molecular convergence point or sensor for environmental interactions such as oxidative stress. The findings also strongly support the earlier literature indicating involvement of the dopaminergic systems, particularly in the dorsal striatum in functional properties of the DISC1 protein.

AUTHOR CONTRIBUTIONS

FJS, SVT, CK, JPH, MASS and GL conceived and designed the experiments. FJS, A-LW, BF and MK performed experiments. FJS and RS collected data and processed the data. FJS, CK, JPH, MASS and GL interpreted the results. FJS, CK, JPH, MASS and GL wrote the article. CK, JPH, MASS and GL revised the intellectual content.

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SUPPLEMENTARY MATERIAL

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Aβ dimers induce behavioral and neurochemical deficits of relevance to early Alzheimer's disease

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Ab dimers induce behavioral and neurochemical deficits of relevance to early Alzheimer's disease

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abstract

We examined behaviors and neurotransmitter levels in the tgDimer mouse, a model for early Alzheimer's disease, that expresses exclusively soluble amyloid beta (A b) dimers and is devoid of Ab plaques, astrogliosis, and neuroinflammation. Seven-month-old mice were subjected to tests of motor activity, attention, anxiety, habituation learning, working memory, and depression-related behaviors. They were impaired in nonselective attention and motor learning and showed anxiety- and despair-related behaviors. In 7- and 12-month-old mice, levels of acetylcholine, dopamine, and serotonin were measured in neostriatum, ventral striatum, prefrontal cortex, hippocampus, amygdala, and entorhinal cortex by high-performance liquid chromatography. The tgDimer mice had lower serotonin turnover rates in hippocampus, ventral striatum, and amygdala relative to wild type controls. The aged tgDimer mice had less hippocampal acetylcholine than adult tgDimers. Stress-test results, based on corticosterone levels, indicated an intact hypothalamus-pituitary-adrenal axis in 12-month-old mice. Since neither A b plaques nor astrogliosis or neuroinflammation was responsible for these phenotypes, we conclude that A b dimers contribute to neurotransmitter dysfunction and behavioral impairments, characteristic for the early stages of Alzheimer's disease.

1. Introduction

An early diagnosis of Alzheimer's disease (AD) is pivotal because it is believed that pharmacotherapies are more efficient when initiated before the massive structural damage due to amyloid- b (Ab) plaque formation. The early stages of AD are not well explored. It is known, however, that about 30% of individuals with mild cognitive impairment convert to early stages of AD (Langa and Levine, 2014). Cognitive deficiency correlates higher with synaptic density than with neuronal loss. This is supported by the findings that A b promotes axonal pruning (Roselli et al., 2005; Shankar et al., 2007) and disrupts cognitive functions in the absence of neuronal loss (Cleary et al., 2005; Maurice et al., 1996; McDonald et al., 1994; Stéphan et al., 2001). As a consequence, soluble Ab oligomers rather than A b plaques are increasingly believed to be responsible for the progressive neuronal degeneration in AD (Cleary et al., 2005; Dickson et al., 1995; Haass and Selkoe, 2007; Krafft and Klein, 2010; Terry et al., 1991). Especially Ab dimers, a form of single A b oligomers, have been shown to be synaptotoxic and prevalent in the brain tissue of AD patients (Klyubin et al., 2008; Mc Donald et al., 2010; Shankar et al., 2008). Ab deposition has been linked to the massive loss of cholinergic neuron in the basal forebrain and other brain areas (Blusztajn and Berse, 2000). Accordingly, cholinergic deficits are considered to be the major pathological characteristic of AD and also represent the main target for current available pharmacological treatment (Geula et al., 2008; Schliebs and Arendt, 2006).

Soluble Ab oligomers may be responsible for both early cognitive (mild cognitive impairment) and affective symptoms (depression, anxiety) (Hefti et al., 2013). Until recently, it has not been possible to investigate in vivo the roles of the A b dimer through the life span of the host, due to the existing kinetic equilibrium of different

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multimers and the simultaneous occurrence of different forms of amyloid deposits. Intraventricular (i.c.v.) infusion of A b molecules in rodents was shown to decrease DA (Wang et al., 2007), 5-HT (Colaianna et al., 2010), and nerve growth factors including nerve growth factor and brain-derived neurotrophic factor (Colaianna et al., 2010). There is also evidence of behavioral action after i.c.v. infusion of Ab oligomers, such as disruption of sleep pattern (Kincheski et al., 2017), impairment of long-term object recognition (Balducci et al., 2010), forced swimming (Ledo et al., 2013, 2016), and sucrose preference (Ledo et al., 2016). However, there is no study on transgenic animal models, which has been successful in specifically assessing the impact of the A b dimer on neurochemical, cognitive, and emotional behavioral parameters.

The development of the transgenic A b-S8C mouse (tgDimer mouse) enabled us to investigate the role of biological effects of single Ab oligomers in early AD (Müller-Schiffmann et al., 2016). To stabilize A b dimers, 2 A b monomers were linked by a disulfide bridge that was incorporated into the amyloid precursor protein (APP) by the introduction of a cysteine amino acid at position 8 of the A b domain (corresponding to APP751-S679C). This led to the secretion of a neurotoxic A b dimer, which did not influence the processing of the APP by cellular secretases (Müller-Schiffmann et al., 2011). The Ab-S8C mutation in the tgDimer mouse resulted in a high concentration of A b dimers but not of monomers. Moreover, no insoluble amyloid species or plaques were generated over its life span. This allowed to distinguish between A b-specific effects and other neuropathological alterations such as amyloid plaques, cerebral amyloid angiopathy, and neurofibrillary tangles, as associated with other transgenic models. So far, the tgDimer mouse has been found to exhibit deficits in Morris water maze (MWM) learning and memory as well as in hippocampal long-term potentiation, comparable to findings in classical AD mice (Müller-Schiffmann et al., 2016). These results suggest that soluble Ab dimers, per se, can induce neurotoxicity and aberrant synaptic signaling and impair cognitive functions in the absence of plaque pathology or elicited effects, such as neuroinflammation and astrogliosis.

The rationale of the present study was the further behavioral and neurochemical characterization of the A b-S8C mutation in adult and aged (7 and 12 months old) tgDimer mice in comparison to age-matched wild type (WT) C57BL/6N controls. The 7-month-old mice underwent tests of motor activity, attention, emotionality, and habituation learning in the open field and radial arm maze (RAM), anxiety- and fear-related behaviors in the elevated plus-maze (EPM), depression-related behaviors in the forced swimming test (FST) and motor coordination and learning on the rotarod. Furthermore, in 12-month-old animals, blood corticosterone levels were determined after subjection to restraint stress to assess hypothalamus-pituitary-adrenal (HPA) axis functionality. In further batches of adult and aged tgDimer and WT mice, levels of acetylcholine (ACh), dopamine (DA), and serotonin (5-HT) were measured in neostriatum, ventral striatum, prefrontal cortex, hippocampus, amygdala, and entorhinal cortex via high-performance liquid chromatography with electrochemical detection.

2. Material and methods

2.1. Subjects

Twelve homozygous male tgDimer mice were tested behaviorally at 7 and 12 months of age. Nine WT C57BL/6N controls were tested at 7 months of age, and 8 were retested at 12 months of age. At the age of 7 months, they were tested in the MWM, followed by tests in the open field, EPM, RAM, FST, and on the rotarod. At the age of 12 months, they were again tested in the MWM, followed by assessment of corticosterone levels in the blood after restraint stress. (The MWM data were published as part of an initial report on the tgDimer mouse (Müller-Schiffmann et al., 2016)). For post-mortem analysis of neurotransmitter levels, 19 further male tgDimer (7 months old: n ¼ 10; 12 months old: n ¼ 9) and 21 WT C57BL/6N (7 months old: n ¼ 12; 12 months old: n ¼ 9) were employed. All animals were obtained from the Central Animal Laboratory of the University Hospital Essen, Germany and housed individually at 20 C 2 C, under a reversed 12:12 hours light-dark cycle (light off at 07:00 a.m.) and humidity (60% 2 C) with food and water ad libitum. Experiments were carried out in accordance with the German Animal Protection Law and approved by the local authority (Landesamt für Natur-, Umwelt-und Verbraucherschutz, North Rhine-Westphalia).

2.2. Neurochemistry

From both hemispheres, the neostriatum, ventral striatum, prefrontal cortex, hippocampus, amygdala, and entorhinal cortex were dissected, and levels of ACh, DA, 5-HT, the DA metabolite dihydroxyphenylacetic acid (DOPAC), and the 5-HT metabolite 5-hydroxyindole acetic acid (5-HIAA) were determined using high-performance liquid chromatography with electrochemical detection.

Monoamines were analyzed, using a 125-mm long analytical column filled with Nucleosil C-18 (reversed-phase with 5- mm particle size; Macherey & Nagel, Düren, Germany). The mobile phase was composed of 0.86 mM sodium octylsulfate, 0.15 M chloroacetic acid, 0.12 M NaOH, 0.67 mM, 1.8% tetrahydrofuran, 3.5% acetonitrile, and EDTA and adjusted to pH 3.0 with phosphoric acid. The electrochemical detector (Intro, Antec, Leyden, Netherlands) was set at a potential of 530 mV versus an Ag/AgCl reference electrode at 30 C.

For ACh measurement, the analytical column was packed with ChromSpher 5C18 (Merck) and loaded with sodiumdodecylsulfate (SigmaeAldrich). The enzyme reactor was filled with LiChrosorb-NH2 (Merck), activated by glutaraldehyde (Merck), and then loaded with acetylcholineesterase (SigmaeAldrich). The enzyme reactor converted ACh to hydrogen peroxide, which was electrochemically detected, using a platinum electrode set at a potential of 350 mV. The mobile phase was composed of 1 mM tetramethylammonium chloride (Merck) and 0.18 M K_2HPO_4 (Merck) and adjusted to pH 8.0 with KH_2PO_4 (Merck). The flow rate was 0.3 mL/min.

Neurotransmitter and metabolite levels were analyzed with Chrom Perfect Software (Justice Laboratory Software, Denville, NJ, USA). For more details see (de Souza Silva et al., 2013).

2.3. Behavioral testing

In all experiments, behaviors were recorded via camera and DVD recorder and subjected to post hoc analysis, using tracking software (Ethovision X 8, Noldus, Netherlands). The tgDimer mice and WT controls (7 months of age) were tested for acquisition and retention in the MWM (results published in (Müller-Schiffmann et al., 2016)). The following behavioral tests were administered after the water maze test, beginning 17 days after its completion.

2.3.1. Seven-month-old mice

2.3.1.1. Open field. The open field consisted of a 45 45 40 cm wooden box, dimly lit by white LED lights, providing a light density of about 13 lx in the center and 9.5e12 lx in the corners. The animals were placed in the center of the open field and allowed to explore for 5 minutes. The behavior was analyzed in ten 30-second time-bins. The following parameters were determined: (a) distance

moved within the open field, (b) duration of immobility, (c) duration of rearing, and (d) frequency of rearing. Measures of emotionality (Prut and Belzung, 2003) were assessed by (e) the time spent in the center (dimension of the virtual center: 40 40 cm) and (f) time spent in the margin (thigmotaxis). The animals were tested once per day for 2 consecutive days to assess habituation learning (Schildein et al., 2002).

2.3.1.2. Elevated plus maze. The EPM is used to measure anxiety- and fear-related behaviors (Graeff et al., 1993). It was elevated 50 cm above the floor and consisted of 2 open and 2 closed arms (length: 30 cm; width: 5 cm; wall height: 15.25 cm) arranged around a central platform (5 5 cm). Illumination was provided by white LED lights placed around the maze, providing 105e112 lx in the open arms and 24e26 lx in the middle of the closed arms. A camera connected to a DVD recorder was mounted centrally above the maze.

The animals were placed onto the central platform, facing one of the open arms and were allowed to explore for 5 minutes. The behaviors assessed to evaluate anxiety-related behavior were: (a) time spent on the open and closed arms and the central platform, (b) "risk assessment" indexed by stretched-attend posture (head and forepaws exit the closed arm, with body remaining in the arm), (c) head dips on the open arms (bowing the head beneath the edge of the arm) (d) time spent on the distal region (length 15 cm) versus the proximal region (length 15 cm) of the open arm extending from the center platform. Also (e) distance moved as a measure of motor performance.

2.3.1.3. Eight-arm radial maze. The eight-arm radial maze (RAM) is employed to assess working memory and attention-related behaviors (Ruocco et al., 2014). The maze (length: 35 cm; width: 5.3 cm; height: 8 cm) with gray walls and open roof, was arranged radially from an octagonal center platform (diameter: 25 cm). It was surrounded by posters and objects, serving as extramaze cues. Diffuse illumination by LED lights provided about 105 lx in the center platform and 80 lx in the 8 arms.

A procedure involving nonbaited arms was used to assess "non-reinforced exploration". The animals were placed on the center platform of the maze and allowed 5 minutes of exploration. The behavioral parameters assessed: (a) velocity, (b) distance moved, (c) duration of rearing (as a measure of nonselective attention [Aspide et al., 1998]), and (d) the number of arms visited before the first repetition (first error) was used to measure working memory (Ruocco et al., 2014).

2.3.1.4. Rotarod. Motor coordination and learning were evaluated with the "rotarod", consisting of a rotating rod with a black rubber surface (diameter: 2.5 cm) (TSE GmbH, Germany). Performance was assessed on 2 consecutive days with 6 trials (intertrial interval: 90 seconds) per session. On day 1, mice were subjected to 6 trials and placed on the drum that rotated at the constant speed of 24 rpm. The cutoff time was 5 minutes. On day 2, 6 further trials were conducted over a period of 5 minutes with the speed accelerating up to 450 rpm. The time until the mouse fell off was recorded.

2.3.1.5. Forced swimming test. The FST has been employed to assess depression-related behavior ("learned despair'') as indicated by the duration of immobility and the number of attempts to escape from the water (Petit-Demouliere et al., 2005), although its value as a test for depression has been challenged (de Kloet and Molendijk, 2016). The apparatus consisted of a glass cylinder (height: 12.5 cm; diameter: 9 cm) filled with water (20 2 C) up to a height of 15 cm. In the first trial (pretest trial), the animal was placed into the cylinder for 15 minutes. Then, it was dried under red-light heating lamp before being returned to the home cage. Twenty-four hours later, it was placed again into the cylinder for 5 minutes (24 hours test trial). Behaviors analyzed were (a) duration of swimming, (b) latency for first-time floating, (c) duration of immobility (lack of motion except for movements to keep the head above water - possibly an index of "behavioural despair"), and (d) climbing (attempts to escape by movement of forepaws on the wall).

2.3.2. Twelve-month-old mice

Seven days after the second MWM test (Müller-Schiffmann et al., 2016), the 12-month-old tgDimer mice and WT controls were subjected to a restraint stress procedure to test for responsiveness of the HPA axis (Kaur et al., 2015). The animals were placed under red-light heating lamps, and 0.2 mL Ringer's solution was injected subcutaneously for 20 minutes to avoid fluid depletion. Blood samples were taken by a 1.0e1.5 mm long incision at the distal part of the tail. After a baseline blood sample (S1) was drawn, the animal was placed into a restrainer (Harvard Apparatus) for 10 minutes, and a second sample (S2) was taken. Subsequently, it was transferred to a single cage with water ad libitum. Additional samples were taken after 60 (S3), 120 (S4), and 180 minutes (S5). Blood was collected in 300 mL EDTA-coated vials (Microvette CB 300 Sarstedt). After centrifugation (4000 rpm, 10 minutes, 4 C), 50 mL plasma was stored at 80 C. Corticosterone plasma concentrations were analyzed with ELISA (IBL International, Hamburg, Germany).

2.4. Statistical analysis

For neurochemical analyses, 2-way analysis of variances (ANOVAs with the 2 between-group factors "genotype" and "age" were calculated. Repeated 2-way ANOVAs with the between-group factor "genotype" and the within-group factor "time" or "trial" were conducted for the analyses of behaviors. One-way ANOVAs were conducted when appropriate and Bonferroni adjustments were applied. Paired t-tests were applied post hoc, when main within-group effects had been found. All tests were 2-tailed with $p < 0.05$. Analyses were performed using IBM SPSS Statistics 22.0.

3. Results

3.1. Neurochemistry

3.1.1. Acetylcholine

In the hippocampus, 2-way ANOVA revealed a main effect of "age" ($F1, 34 = 6.017$, $p = 0.019$,) was found on ACh levels, but there were no effects of "genotype" and "genotype age" (ps >0.05). Hippocampal ACh was lower in the aged relative to the adult animals ($F1, 34 = 6.017$, $p = 0.019$). Analysis within each tested group showed no significant difference between ages ($p > 0.05$) in WT animals, whereas the aged tgDimer mice displayed lower ACh concentrations relative to the adult ones ($F1, 17 = 9.62$, $p = 0.006$, 1-way ANOVA; Fig. 1A). In neostriatum, ventral striatum, prefrontal cortex, amygdala, and entorhinal cortex, no significant effects of "genotype", "age", and their interaction were found (p >0.05; 2-way ANOVAs,data not shown).

3.1.2. Serotonin and 5-HIAA

There were no significant effects of "genotype", "age", and "genotype age" for 5-HT and 5-HIAA levels in the hippocampus (ps >0.05, 2-way ANOVAs). However, concerning the 5-HT turnover rate, 2-way ANOVAs revealed a main effect of "genotype", but no effects of "age" and "genotype age" were obtained ($F1,33 = 10.49$, $p = 0.003$). Thereby, tgDimer mice showed lower 5-HT turnover relative to the WT controls in hippocampal tissue ($F1, 33 = 10.49$, $p = 0.003$, 1-way ANOVAs; Fig. 1B).

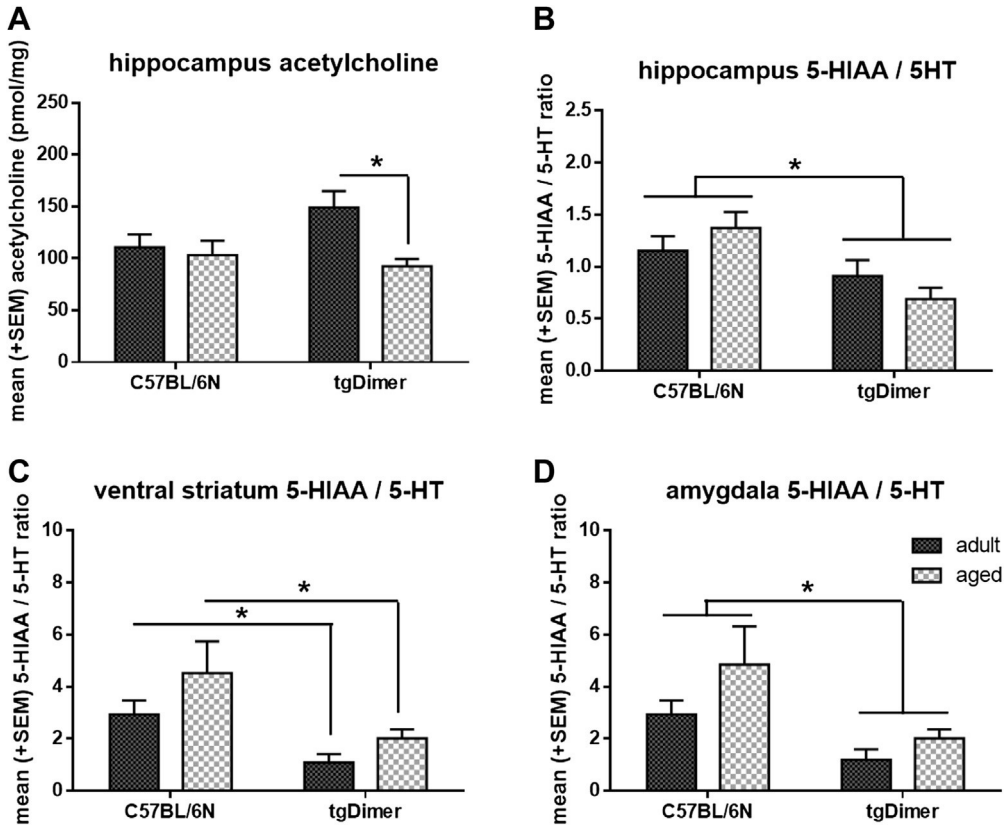

Fig. 1. Neurochemical evaluation. (A) A significant decrease in ACh levels in the hippocampus of the aged tgDimer mice compared to adult ones but not in C57BL/6N. (B) Hippocampus: 5-HIAA/5-HT turnover was significantly lower in tgDimer mice than in WT. (C) Ventral striatum: The 5-HIAA/5-HT ratio was significantly lower in adult and aged tgDimer mice relative to controls, respectively. (D) Amygdala: a significantly lower 5-HIAA/5-HT ratio in tgDimer mice compared to controls. (*p < 0.05). Abbreviations: ACh, acetylcholine; HIAA, hydroxyindole acetic acid; WT, wild type.

In the ventral striatum, no significant effects of "genotype", "age", and "genotype age" were obtained for both 5-HT and 5-HIAA levels (ps > 0.05, 2-way ANOVAs). The analysis of 5-HT turnover rate, however, yielded significant effects of "genotype" (F1,14 ¼ 15.05, p ¼ 0.002) and "age" (F1,14 ¼ 4.47, p ¼ 0.043), but no effect of "genotype age" (p > 0.05) when 2-way ANOVAs were applied. Significant effects of "genotype" were found for both the adult (F1, 8 ¼ 8.889, p ¼ 0.018, 1-way ANOVA) and the aged condition (F1, 6 ¼ 6.305, p ¼ 0.046, 1-way ANOVA). Thereby, tgDimer mice displayed a lower 5-HT turnover rate at both ages relative to WT controls (adult: F1, 8 ¼ 8.889, p ¼ 0.018; aged: F1, 6 ¼ 6.305, p ¼ 0.046, 1-way ANOVA; Fig.1C).

In the amygdala, no significant effects of "genotype", "age", and "genotype age " effects were found for either 5-HT or 5-HIAA levels (p > 0.05, 2-way ANOVAs). The analysis of 5-HT turnover rate, however, yielded a significant effect of "genotype" (F1,14 ¼ 9.209, p ¼ 0.009, 2-way ANOVAs) but not effects of "age" and no interaction (ps > 0.05, 2-way ANOVAs), with tgDimer mice showing a lower turnover rate relative to WT controls (F1,14 ¼ 9.209, p ¼ 0.009, 1-way ANOVA; Fig. 1D).

There were no significant effects of "genotype", "age", and "genotype age" on 5-HT levels, 5-HIAA levels, and turnover rate in neostriatum, prefrontal cortex, and entorhinal cortex (ps > 0.05, 2-way ANOVAs; data not shown).

3.1.3. DA and DOPAC

In the hippocampus, 2-way ANOVAs revealed a main effect of "age" (F1,26 ¼ 6.675, p ¼ 0.016) on DA levels was obtained but no effects of "genotype" and "genotype age" (p >0.05). Thereby, the adult animals had lower DA levels than the aged ones (F1,26 ¼ 6.675, p ¼ 0.016). When comparing adult and aged animals separately in each group, no significant differences were found in either group (p > 0.05, 1-way ANOVA). Analyses of DOPAC levels and DA turnover rate yielded no significant effects of "genotype ", "age", and "genotype age" (ps > 0.05, 2-way ANOVAs; data not shown).

There were no significant effects of "genotype", "age", and "genotype age" on DA levels, DOPAC levels, and turnover rate in the other examined brain regions (ps > 0.05, 2-way ANOVAs; data not shown).

3.2. Open field

Two-way ANOVA revealed significant effects of "genotype" (F1,19 ¼ 10.911, p ¼ 0.004),"time" (F9,171 ¼ 6.089, p < 0.001), and "genotype time" (F9,171 ¼ 2.962, p ¼ 0.003) on rearing behavior on day 2 but not on day 1 (ps >0.05). In the tgDimer animals, the duration of rearing was shorter relative to WT controls (F1,19 ¼ 10.911,p ¼ 0.003; Fig. 2), possibly indicating a deficit of nonselective attention (Ruocco et al., 2009). One-way ANOVAs, however, revealed significant "genotype" effects in the time bins 0e90, 90e120,120e180, 210e240, and 270e300 seconds (F1,19 ¼ 5.02, p ¼ 0.037; F1,19 ¼ 5.64, p ¼ 0.028; F1,19 ¼ 5.25, p ¼ 0.034; F1,19 ¼ 7.89, p ¼ 0.011; F1,19 ¼ 22.57,p < 0.001, respectively).

No significant effects of "genotype", "time", and "genotype time" were found on horizontal locomotion, frequency of rearing, duration of immobility, and time spent in the center and margins of the open field (ps > 0.05, 2-way ANOVAs; data not shown).

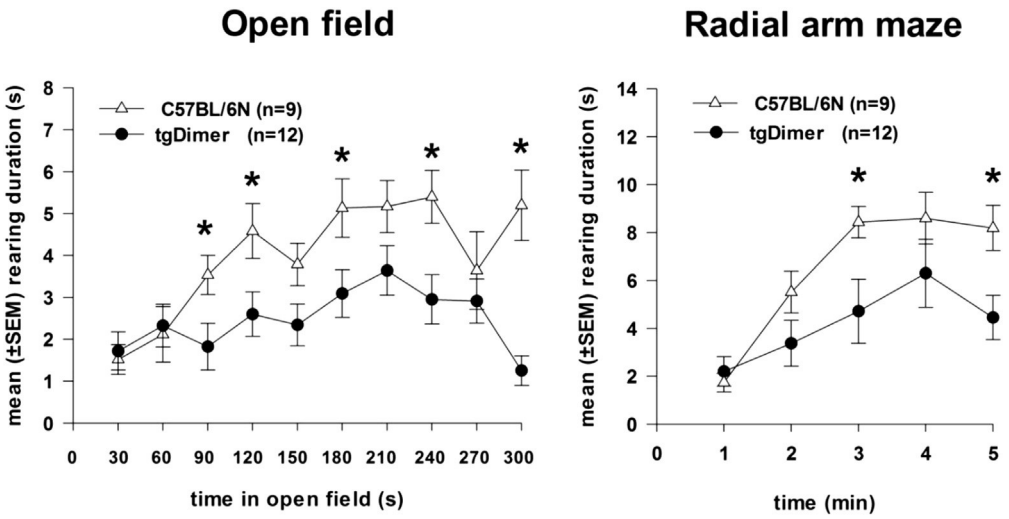

Fig. 2. Duration of rearing in the open field (left) and radial arm maze (RAM) (right) in tgDimer mice and WT controls. (*p < 0.05). Abbreviation: WT, wild type.

3.3. Elevated plus maze

The analysis of time spent in the open arms, closed arms, and center in the EPM failed to reveal significant differences between groups (Table 1). However, the tgDimer, but not WT mice, spent more time in the proximal compared to the distal region of the open arm (p ¼ 0.025, paired sample t-test) Moreover, the tgDimer animals exhibited more stretch-attend postures than the WT controls (F1,19 ¼ 12.272, p ¼ 0.002), indicative of elevated "risk-assessment", both findings indicate more anxiety-related behavior for the tgDimer mice.

The other parameters of measurement were not significant between the groups (ps > 0.05, 2-way ANOVAs; Table 1).

3.4. Radial arm maze

For the duration of rearing, the 2-way ANOVA revealed main effects of "time" (F4,72 ¼ 14.468, p < 0.001) and "genotype" (F1,18 ¼ 5.443, p ¼ 0.031) but no interaction (p > 0.05). The tgDimer mice spent less time rearing compared to WT controls in the total testing time (F1,18 ¼ 5.443, p ¼ 0.031). One-way ANOVAs, however, revealed significant "genotype" effects in the third and fifth minute (F1,18 ¼ 5.428, p ¼ 0.032; F1,18 ¼ 7.943, p ¼ 0.011, respectively), whereby the tgDimer mice spent less time in rearing relative to the WT controls, possibly indicating a deficit of nonselective attention (Aspide et al., 1998). There were no significant differences between groups in horizontal locomotor activity (velocity, distance moved) and the number of arms visited before the first repetition (as an index of working memory) (p > 0.05; Table 2).

3.5. Rotarod

In the analysis of time spent on the rotarod, when rotating with constant speed, a significant effect of "trial" (F5,95 ¼ 24.407, p < 0.001) was obtained, but no effects of "genotype" and "genotype trial" were obtained (p > 0.05). The subsequent 1-way ANOVAs for each trial showed no significant "genotype" effects (ps > 0.05). Thus, there was no between-group difference in motor coordination and fatigue resistance, when the speed remained constant (Fig. 3).

In the analysis of time spent on the rotarod, when rotating with accelerating speed, there was a significant effect of "genotype" (F1,19 ¼ 6.037, p ¼ 0.024), but no effects of "trial" and "genotype trial" (ps > 0.05). Thereby, tgDimer mice spent less time on the rotarod than the WT animals (F1, 19 ¼ 6.093, p ¼ 0.023; Fig. 3), suggesting deficits in motor learning.

3.6. Forced swimming test

There were no significant effects of "genotype" in the analysis of the pretrial parameters (duration of swimming, immobility, climbing, and latency to first-time floating) (ps > 0.05 1-way ANOVA; Table 3). In the 24 hours test trial, significant effects of "genotype" on duration of swimming (F1,17 ¼ 5.687, p ¼ 0.029, 1-way ANOVA) and floating latency (F1,17 ¼ 13.655, p ¼ 0.002, 1-way ANOVA) were obtained. Thereby, the tgDimer animals spent less time swimming and showed a shorter latency of first-time floating relative to the WT controls (F1,17 ¼ 5.687, p ¼ 0.029; F1,17 ¼ 13.655, p ¼ 0.002, respectively; 1-way ANOVAs), indicating

Table 1
Elevated plus-maze behavior, in 7-month-old tgDimer mice and WT controls

	Duration (s)					
	Open arm	Closed arm	Center region	Proximal closed arm	Proximal opened arm	Distal opened arm
WT	66.42 10.43	181.89 7.51	44.33 6.14	50.67 4.82	40.48 5.70	34.44 8.48
tgDimer	68.44 10.48	175.67 5.52	47.25 5.80	47.96 4.10	37.79 4.50	29.62 5.82

Duration (sec)		Distance (cm)	Frequency (count)		
Stretch/attend posture	Head dips	Distance moved	Open arm	Closed arm	Center region
2.170.50	24.20 6.12	1059.20 55.12	11.89 1.60	16.78 1.68	16.11 1.52
8.501.66 [a]	26.31 3.46	1229.17 73.40	12.75 1.30	20.83 2.46	19.00 1.45

Key: WT, wild type.
[a] p < 0.05, tgDimer versus WT mice.

Table 2
Radial arm maze. Performance of tgDimer and wild type mice in the radial arm maze

Genotype	Locomotion		First error (number)
	Distance moved (cm)	Velocity (cm/s)	
WT	7282.6 1157.6	24.6 4.0	4.0 1.8
tgDimer	7621.0 2820.0	25.7 9.6	3.41 1.2

Key: WT, wild type.

an increase in despair-related behavior. The effect of "genotype" on immobility marginally failed to reach statistical significance ($F_{1,17} = 13.551$, $p = 0.075$, 1-way ANOVA), whereas there were no effects on climbing duration (ps > 0.05; Table 3).

3.7. Restraint stress and corticosterone analysis

There was a significant effect of "trial" ($F_{4,64} = 16.478$, $p < 0.001$) but no effects of "genotype" and "genotype trial" on corticosterone levels (ps > 0.05; 2-way ANOVA). One-way ANOVAs, however, yielded no significant "genotype" effects for any of the samples taken on corticosterone levels (ps > 0.05; Fig. 4), indicating an intact HPA axis in tgDimer mice relative to WT controls.

4. Discussion

4.1. Neurochemistry

The present study showed an age-related reduction of hippocampal ACh levels in tgDimer mice, whereas hippocampal 5-HT and DA levels were unaltered in both tgDimer mice and WT controls. A 5-HT turnover, however, was decreased in ventral striatum, hippocampus, and amygdala of tgDimer mice compared to that of WT controls.

A significant age-related decline of hippocampal ACh was found, with aged tgDimer mice showing less hippocampal ACh than adult tgDimer mice. This was not the case in WT mice, suggesting that the tgDimer mouse is more vulnerable to age with respect to hippocampal ACh levels. The hippocampus and the structures highly interconnected with it are important for memory formation (Squire and Zola-Morgan, 1991; Young et al., 1997). They are also the earliest regions prone to AD pathology (Duffy et al., 2015; Serrano-Pozo et al., 2011; Van Hoesen et al., 1991) and A b toxicity (Pooler et al., 2015). Given that ACh deficiency in the hippocampus and its connected regions is one of the features of early AD (Hernandez et al., 2010; Riekkinen et al., 1987), the present finding support the hypothesis that A b dimers underlie the impairment of cholinergic function in the hippocampus and, thus, contribute to early AD-associated cognitive deficits (see below).

Although the absolute values of 5-HT and 5-HIAA levels did not differ between tgDimer mice and WT controls, a significant reduction of the 5-HIAA/5-HT ratio was observed in ventral striatum, hippocampus, and amygdala of tgDimer mice, indicating limbic 5-HT dysfunction (Fig. 1). This is supported by previous findings obtained after intraventricular injection of the toxic peptide A b 25e35, which reduced the 5-HT turnover rate in rats (Litvinova et al., 2015). Moreover, intraventricular injection of A b oligomers also reduced 5-HT levels in prefrontal cortex and hippocampus and disrupted forced swimming and sucrose preference in mice (Ledo et al., 2016). Whether the A b-S8C influences the neurotransmission systems similar to the direct application of A b oligomers requires further study.

4.2. Behavioral phenotyping

The present study on tgDimer mice showed a reduction of rearing duration in both the RAM tests and in the open field, indicating a deficit of nonselective attention. Moreover, adult tgDimer mice showed increased risk assessment in the EPM and a preference of the proximal over the distal part of the open arm, reflecting an elevation of anxiety-relating behavior. In addition, in the FST, duration of swimming was decreased with a shorter latency for

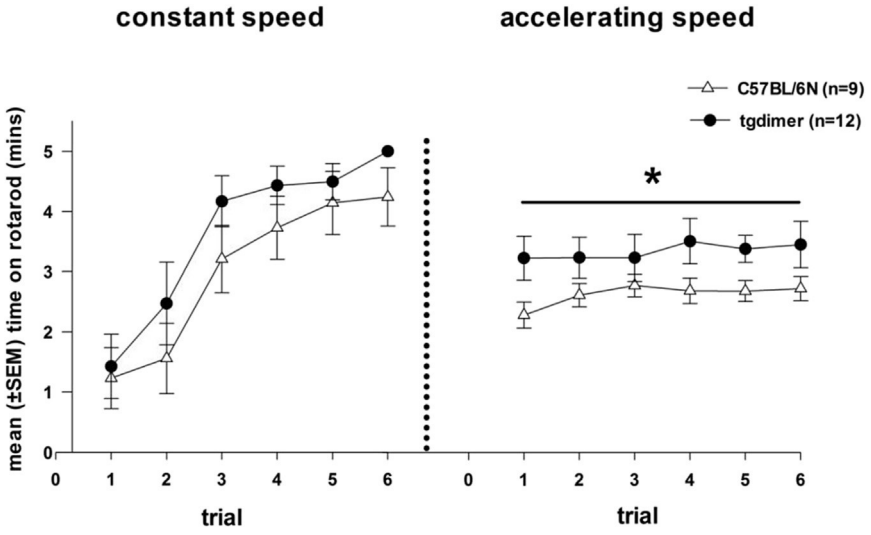

Fig. 3. Rotarod performance of 7-month-old tgDimer mice and WT controls at constant and accelerating speed. No group difference was found at constant speed, while the tgDimer mice showed less time spent on the rotarod compared to WT when speed was accelerating. Mean time (SEM; min) the mice remained on the rotating rod, with a cutoff of 5 minutes per trial (*p < 0.05). Abbreviation: WT, wild type.

Table 3
Forced-swimming test. Duration of immobility, swimming and climbing behaviors, and latency of first-floating behavior of 7-month-old tgDimer and WT mice

	Pretrial (sec)				24-h test trial (s)			
	Climbing	Immobility	Swimming	Latency of first floating	Climbing	Immobility	Swimming	Latency of first floating
WT	10,75 1,52	512,90 37,5	369.04 36.62	94.46 11.15	8.45 0.86	128.01 27.30	174.12 28.02 [a]	81.15 16.15 [a]
tgDimer	8.41 0.65	577.20 38.03	317.61 38.40	88.52 9.07	12.30 3.27	189.57 19.31	94.72 019.85	23.48 6.45

Key: WT, wild type.
[a] $p < 0.05$, tgDimer versus WT mice.

first-time floating, suggestive of an increase in depression-related behavior. The adult tgDimer were also deficient in motor learning in the rotarod test.

4.2.1. Cognition

The tgDimer mice exhibited intact working memory, as indicated by the comparable performance (first error score) of the tgDimer and WT mice in the RAM. In addition, in our previous study using the MWM, the tgDimer mice at both ages were impaired in both acquisition and retention (Müller-Schiffmann et al., 2016).

We found no significant differences between the tgDimer and WT mice in corticosterone levels in response to restraint stress over the course of 180 minutes of testing. This suggests an intact HPA axis response of the tgDimer mice and, therefore, permits to exclude HPA axis dysregulation as a cause for disturbed memory processing and dysregulated emotional behaviors (Lathe, 2001). Cholinergic integrity is essential for performance in the MWM, and the deterioration of water maze acquisition and retention caused by anticholinergic agents (e.g., scopolamine) is well established (Klinkenberg and Blokland, 2010; Saucier et al., 1996). It is also known that cholinergic neurons in hippocampal slices are sensitive to Ab toxicity. Therefore, the observed deficiencies in learning and memory may be due to the diminished hippocampal ACh levels observed in the present study.

Interestingly, the adult tgDimer mice exhibited less rearing behavior in the open field and in the RAM, which can be interpreted as deficient nonselective attention (Aspide et al., 2000). Attention deficits are one of the main elements of cognitive decline in AD (Perry and Hodges, 1999). In particular, impairments of orienting "nonselective'' and/or visiospatial "selective'' attention are common in dementia patients (Parasuraman et al., 1992) and can be ameliorated by cholinergic treatment (Warburton and Rusted, 1993). Notably, the tgDimer mice showed intact working-memory in the reward-free version of the RAM, suggesting that the cognitive deficits in this mouse are limited to reward-governed learning/memory and to attention-related processes.

4.2.2. Motor behavior

In the present study, motor performance of tgDimer mice was comparable to WT controls on the rotarod under constant speed and in open field horizontal activity. This is consistent with previous findings on adult rodents with APP mutations, which showed rotarod performance comparable to WT controls (Bellucci et al., 2006; Lalonde et al., 2004). Under accelerating speed, however, tgDimer mice were able to remain on the rotarod for a significantly shorter time. Before plaque deposition, Ab was found to affect the functional integrity of motor neurons. In a study on transgenic mice with intraneuronal A b accumulation, no plaques were detected, but there was axonal degeneration of motor neurons and deficient motor abilities (Wirths et al., 2007). Therefore, it is possible that the observed motor deficiency may be related to A b action on motor neurons. On the other hand, given the comparable performance of tgDimer mice and WT controls on the rotarod under constant speed, horizontal activity of the open field and RAM in the present study and in swimming speed in the MWM task (Müller-Schiffmann et al., 2016), it may be concluded that the tgDimer mice were deficient in motor learning, rather than in general motor coordination and in resistance to fatigue.

4.2.3. Depression- and anxiety-related behaviors

In the present study, and in line with previous findings of soluble Ab (Colaianna et al., 2010; Knight et al., 2016), emotionality parameters were not affected in the tgDimer mice in the EPM task when the conventional scoring parameters were employed. However, the tg Dimer mice spent more time in the "safe" proximal part of the open arm, and the measure of "risk assessment" was found to be augmented, indicating an increase in anxiety-related behavior. In the FST, the tgDimer mice exhibited less swimming and had a shorter latency of first-time floating than the WT controls. Increased immobility after i.c.v. injection of soluble A b oligomers (Colaianna et al., 2010; Ledo et al., 2016) as well as in the APPswe/PS1 mouse model of AD (Filali et al., 2009) were also found.

In summary, the present findings demonstrate a neurochemical and behavioral syndrome caused by an abundance of A b dimers in the absence of A b plaques, astrogliosis or neuroinflammation, and reminiscent of early stages of AD. This syndrome consists of deficits in MWM learning, retrieval, nonselective attention, motor learning, as well as depression- and anxiety-related behaviors. Because no differences in corticosterone levels were observed between tgDimer and WT mice, an action of Ab dimers on the HPA axis function may be excluded, and behavioral deficits cannot be accounted for by HPA axis dysregulation. However, the behavioral

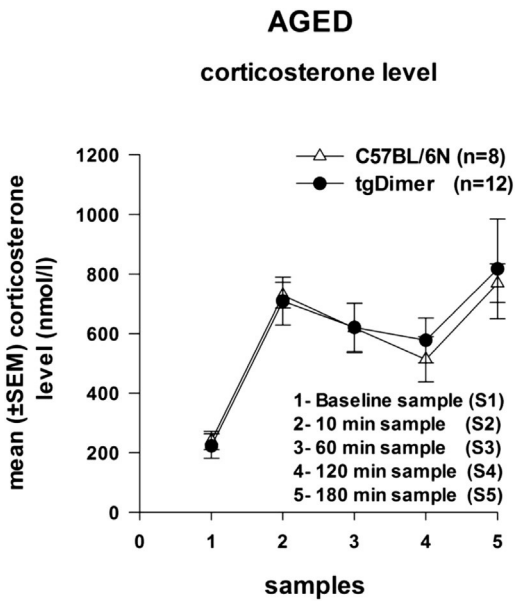

Fig. 4. Corticosterone levels in the blood of aged (12 months old) tgDimer mice and WT controls in baseline and 10, 60, 120, and 180 minutes after restraint stress.

phenotypes may be explained in part by effects of the A b dimer on cholinergic and 5-HTergic neurons. In particular, the results imply a physiological role of A b dimers in modulating ACh and 5-HT function, including neurotransmitter homeostasis. As the tgDimer mouse exhibits neither Ab plaques nor astrogliosis or neuroinflammation, it is likely that the A b dimers are responsible for the impairments found in behavioral and neurotransmitter functions. In so far as these findings have relevance for understanding the onset of behavioral deficits in early AD, they also argue for an attempt at therapeutic targeting of the A b dimer, per se.

Disclosure statement

The authors report no biomedical financial interests or potential conflicts of interest.

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10. Declaration

Die hier vorgelegte Dissertation habe ich selbständig und nur unter Verwendung der angegebenen Literaturquellen angefertigt. Diese Arbeit wurde in der vorgelegten oder ähnlichen Form bei keiner anderen Institution eingereicht. Zudem erkläre Ich, dass bisher keine erfolglosen Promotionsversuche unternommen habe.

Düsseldorf, den