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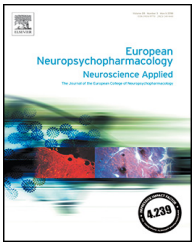

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Atypical but not typical antipsychotic drugs ameliorate phencyclidine-induced emotional memory impairments in mice

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Abstract
Schizophrenia is associated with cognitive impairments related to hypofunction in glutamatergic N-methyl-D-aspartate receptor (NMDAR) transmission. Phencyclidine (PCP), a non-competitive NMDAR antagonist, models schizophrenia-like behavioral symptoms including cognitive deficits in rodents. This study examined the effects of PCP on emotional memory function examined in the passive avoidance (PA) task in mice and the ability of typical and atypical antipsychotic drugs (APDs) to rectify the PCP-mediated impairment. Pre-training administration of PCP (0.5, 1, 2 or 3 mg/kg) dose-dependently interfered with memory consolidation in the PA task. In contrast, PCP was ineffective when administered after training, and immediately before the retention test indicating that NMDAR blockade interferes with memory encoding mechanisms. The typical APD haloperidol and the dopamine $D_{2/3}$ receptor antagonist raclopride failed to block the PCP-induced PA impairment suggesting a negligible role of D_2 receptors in the PCP impairment. In contrast, the memory impairment was blocked by the atypical APDs clozapine and olanzapine in a dose-dependent manner while risperidone was effective only at the highest dose tested (1 mg/kg). The PCP-induced impairment involves 5-HT_{1A} receptor mech-

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anisms since the antagonist NAD-299 blocked the memory impairment caused by PCP and the ability of clozapine to attenuate the impairment by PCP. These results indicate that atypical but not typical APDs can ameliorate NMDAR-mediated memory impairments and support the view that atypical APDs such as clozapine can modulate glutamatergic memory dysfunctions through 5-HT_{1A} receptor mechanisms. These findings suggest that atypical APDs may improve cognitive impairments related to glutamatergic dysfunction relevant for emotional memories in schizophrenia.

1. Introduction

Schizophrenia is a complex psychiatric disorder characterized by positive, e.g. delusions, and negative symptoms, e.g. social withdrawal, as well as cognitive dysfunctions (Goldman-Rakic, 1994; Weinberger and Gallhofer, 1997). The positive symptoms have been related to hyperactivity of cortical and limbic dopamine (DA) D_2 receptors (Creese et al., 1976; Seeman and Lee, 1975) while negative symptoms have been associated with hypoactivity in prefrontal D_1 receptor transmission (Goldman-Rakic, 1994; Toda and Abi-Dargam, 2007).

Schizophrenic patients suffer from dysfunctions in several cognitive domains (Weinberger and Gallhofer, 1997) considered to be related to abnormalities in glutamate transmission, particularly hypofunction of N-methyl-D-aspartate receptor (NMDAR) transmission (Coyle et al., 2003; Javitt and Zukin, 1991; Krystal, 2015; Krystal et al., 2003; Lewis and Lieberman, 2000; Olney and Farber, 1995). The strongest evidence for the hypoglutamatergic hypothesis of schizophrenia (Javitt and Zukin, 1991; Krystal, 2015; Olney and Farber, 1995) is based on the observations that administration of the non-competitive NMDAR antagonists such as phencyclidine (PCP) or ketamine can induce schizophrenia-like cognitive disturbances in healthy individuals (Malhotra et al., 1996) and exacerbate such symptoms in schizophrenic patients (Lahti et al., 1995; Malhotra et al., 1996).

The first generation of antipsychotic drugs (FGAs) such as haloperidol (Citrome, 2011; Leucht et al., 2009a,b) diminish the positive symptoms of schizophrenia through blockade of limbic D_2 receptors (Seeman et al., 1976). However, the FGAs cause movement disorders, e.g. extrapyramidal side effects (EPS), through striatal D_2 receptor blockade (Nord and Farde, 2011). Moreover, haloperidol does not improve the negative and cognitive deficits seen in schizophrenic patients (Toda and Abi-Dargam, 2007). There is, however, evidence (Kane et al., 1988; Keefe et al., 2007; Meltzer, 2013) that the second generation of APDs (SGAs) such as clozapine can improve cognitive functions in schizophrenic patients. Such improvements can also be observed when patients switched from the FGAs to the SGAs (Hagger et al., 1993; Woodward et al., 2005).

Both FGAs and SGAs ameliorate the positive symptoms in schizophrenia related to their affinities for D_2 receptors (Creese et al., 1976; Seeman and Lee, 1975). Clozapine, classified as the prototypical atypical APD, stands out as an overall more efficient APD than haloperidol in reducing positive symptoms in treatment-resistant schizophrenia (Kane et al., 1988) with a low propensity for causing EPS (Leucht et al., 2009a,b; Meltzer and Huang, 2008). In addition, clozapine may reduce affective symptoms and also improve negative and cognitive symptoms. In contrast, the clinical advantages including the low propensity of the SGAs e.g. clozapine for causing EPS compared with haloperidol (Leucht et al., 2009a,b; Meltzer and Huang, 2008) has been associated with their relatively higher affinity for multiple 5-HT receptor subtypes particularly the 5-HT_{2A} than for D_2 receptors (Meltzer and Huang, 2008; see Table 1). An alternative explanation for the low EPS propensity of the SGAs is their "loose D_2 binding" or "fast off kinetics" at the D_2 receptor related to their ~100-fold faster dissociation from dopamine D_2 receptors) compared with the FGAs (Seeman, 2014). However, this hypothesis is not supported by a recent study (Sahlholm et al., 2016).

It is notable that the receptor mechanism(s) by which the SGAs such as clozapine may improve human cognition are not well defined (Leucht et al., 2009a,b). Unlike haloperidol the SGAs share a moderate affinity for D_2 receptors with varying affinities for multiple serotonergic receptors, particularly the 5-HT_{2C}, 5-HT_{2A} and 5-$HT_{6/7}$ receptors (Meltzer, 2013; Meltzer and Huang, 2008; Nikiforuk, 2014; Oymada et al., 2015; Richtand et al., 2008; Woodward et al., 2005) which all have been implicated in rodent cognitive functions (Eriksson et al., 2008, 2012; Horisawa et al., 2013; Idris et al., 2010; Madjid et al., 2006; Ögren et al., 2008; Stiedl et al., 2015). The multimodal receptor profile of SGAs also include actions on histaminergic, cholinergic and adrenergic transmission (Table 1) which may contribute to their effects on cognitive performance (Bymaster et al., 2003; Meltzer and Huang, 2008).

The behavioral syndrome induced by administration of NMDA antagonists such as PCP or MK-801 has been suggested to be an animal model of schizophrenia (Javitt and Zukin, 1991; Krystal, 2015; Olney and Farber, 1995). Acute and subchronic PCP administration causes cognitive impairments in monkey and rodent tasks related to working, executive and spatial memory (Åhlander et al., 1999; Beraki et al., 2008; Didriksen et al., 2007; Jentsch and Roth, 1999; Podhorna and Didriksen, 2005). Studies in a variety of cognitive tasks and electrophysiological studies have provided evidence that SGAs but not haloperidol can block the behavioral effects of PCP through modulation of brain glutamate transmission (Beraki et al., 2008; Didriksen et al., 2007; Idris et al., 2010; Madjid et al., 2006; Ninan et al., 2003; Rujescu et al., 2006). For instance, the spatial memory impairment caused in Morris water maze performance by the 0.5 mg/kg dose of PCP was blocked by concomitant treatment with clozapine (0.5 mg/kg) but not with haloperidol (0.05 mg/kg; Beraki et al., 2008). Furthermore, the SGAs clozapine, risperidone and sertindole at low doses

Table 1 The receptor binding profile of antipsychotic drugs (APDs) classified as belonging to the first (FGA) and second generation of APDs (SGA) with affinity constants (K_i, nM) for monoamine receptor subtypes implicated in cognitive functions.

APD class	Compound	Receptor subtypes								
		5-HT_{1A}	5-HT_{2A}	5-HT_{2C}	5-HT_6	5-HT_7	α_1	D_2	H_1	M_1
FGA	Haloperidol	7930	78	3085	>5000	263	46	1	3630	1475
SGA	Clozapine	770	12	8	4	6.3	7	125	6	1.9
SGA	Risperidone	490	0.6	26	425	1.4	2	3	155	ND
SGA	Olanzapine	>1000	4	11	2.5	104	19	11	7	1.9

The classification the compounds used was adapted to the neuroscience-based nomenclature by Millan et al. (2015) and Zohar et al. (2015). The results of the receptor affinities are taken from Bymaster et al. (1996), Roth et al. (1994) and Schotte et al. (1996). The classification is based on the calculated affinity for different receptors as measured in vitro. For information of the experimental conditions for the radioligand studies, please see the above references. ND, not determined.

were found to reverse the PCP-induced learning and memory deficits in mice (Didriksen et al., 2007).

The first aim of this study was to investigate whether hypofunction of glutamatergic transmission caused by a single dose of the NMDAR antagonist PCP will alter emotional memory learning, a cognitive domain often disturbed in schizophrenia (Herbener, 2008). This design is motivated by clinical and animal studies showing that a single dose of PCP causes a transient state of schizophrenia-like behavioral effects (Javitt and Zukin, 1991; Krystal, 2015). With the use of pre- and post-training, and pretest administration of PCP, its effects were assessed on the temporal phases of learning, e.g. acquisition, consolidation or retrieval/expression of the memory. Second, the study examined whether the PCP effects can be modulated by pre-training administration of SGAs (clozapine, olanzapine and risperidone), the FGA haloperidol and the $D_{2/3}$ receptor antagonist raclopride. Finally, the study examined the potential role of 5-HT_{1A} and D_2 receptors in the memory modulating effect of PCP, and the role of 5-HT_{1A} receptors in the mechanism by which clozapine may modify the effects of PCP on emotional memory consolidation. The study was conducted in male C57BL/6J mice using the passive avoidance task (PA), a one-trial emotional memory task combining fear conditioning with an instrumental response (Ögren and Stiedl, 2015) which is dependent on hippocampal and amygdala function (LeDoux, 2000; Baarendse et al., 2008).

2. Experimental procedures

2.1. Animals

Experiments were performed in 433 adult male C57BL/6J mice (Taconic, Denmark) with body weights of 25–30 g. Mice were housed in groups of 4–6 individuals in Macrolon® type III cages (41 × 25 × 15 cm) in climate-controlled rooms (21±1 °C and 60±5% humidity; 12-h light/dark cycle, light on at 6.00 a.m.) with standard lab chow (Ewos R36, Ewos AB, Sweden) and tap water ad libitum. Experimental procedures and housing followed the provisions of the Swedish animal protection legislation and were approved by the local Animal Ethical Committees (N423/12) and (N138/16) in compliance with the European Council Directive (2010/63/EU) as well as the legislation at the Department of Pharmacology, College of Medicine, UAEU, Al Ain, United Arab Emirates.

Mice were habituated to the animal facility for five days and were handled daily for three consecutive days before any experiments started. The experiments were conducted in the 12-h light phase (light on at 7 a.m.) from 10:00 a.m. until 3:00 p.m. After transfer to the experimental room on the experimental day 1 mice were habituated for 30–60 min prior to the start of the experiment. All experiments were conducted in experimentally naïve mice.

2.2. Drugs and administration

2.2.1. Dose levels of phencyclidine

The doses of phencyclidine (PCP) to model schizophrenia-like behavior in rodents vary considerably in the published literature depending on the behavioral function studied. This complicates interpretation of the results obtained in learning tasks which are sensitive to confounding effects, e.g., on locomotor activity. PCP administered in the dose range of 5–10 mg/kg in rats and mice causes disorganized hyperactivity (Castañé et al., 2015; Corbett et al., 1995; Moghaddam and Jackson, 2003; Ögren and Goldstein, 1994) and negative-like symptoms such as reduced social interactions and disruption of pre-pulse inhibition (Cadinu et al., 2018; Javitt and Zukin, 1991). Thus, PCP given at this dose-range interferes with learning performance via non-cognitive effects. The use of low doses of PCP allows an analysis of its effect on learning by minimizing nonspecific effects on cognition caused by locomotor and sensory disturbances (Beraki et al., 2008; Didiksen et al., 2007). Previous results have shown that it is possible at low doses of PCP to dissociate the spatial learning impairment in the water maze from the adverse behavioral manifestations of NMDA receptor blockade (Beraki et al., 2008). The dosage of phencyclidine (PCP) used in mice here is based on earlier studies using low doses of PCP to minimize behavioral changes which can confound measures of learning and memory (Åhlander et al., 1999; Beraki et al., 2008). Notably, acute PCP treatment can produce a dose- (ED_{50} 4.2 mg/kg s.c.) and time-dependent neurotoxic insult on cortical and limbic neurons (Olney, 1989; Podhorna and Didriksen, 2005). Repeated PCP administration (5.45 mg/kg s.c.) can produce a neurotoxic effect in large areas of the rat brain including the entorhinal, cingulate and retrosplenial cortices as well as ventral hippocampus (Jentsch and Roth, 1999; Morris et al., 2005).

PCP (Sigma-Aldrich, USA) was dissolved in saline and used in doses ranging from 0.5 to 3 mg/kg.

2.2.2. Classification of antipsychotic drugs and doses

The study focused on commonly prescribed APDs which are referred to as typical or atypical APDs or belonging to the FGAs or SGAs (Zohar et al., 2015). Drugs characterized as SGAs such as clozapine, olanzapine and risperidone cause limited EPS and generally have fewer side-effects in humans than for instance the FGA haloperidol (Leucht et al., 2009a,b). This classification can be questioned since

each compound has its own clinical efficacy and side-effect profile (Leucht et al., 2009a,b). Moreover, this classification does not take into regard that "atypicality" can be a relative matter since moderate or higher doses of some compound can produce marked EPS in a substantial number of patients. Therefore, it seems reasonable to designate APDs as belonging to the FGAs or SGAs groups of APDs, since it avoids any implication of motor side effects, although also this classification is challenged (Leucht et al., 2013). In this study, the typical/atypical designation will be used when referring to drugs with a low EPS potential.

Clozapine (Sigma) was dissolved in saline with few drops of glacial acetic acid and neutralized to pH 6.5–7.0 with 5 N NaOH and used at the doses 0.05 and 0.3 mg/kg. Olanzapine (Sigma), S(-)-raclopride, (3,5-dichloro-N-)1-ethylpyrrolidine-2-ylmethyl)-2-hydroxi-6-methoxybenzamide (+)-tartrate salt, Astra Zeneca R&D, Sweden) (Ögren et al., 1986) and risperidone (Sigma) were dissolved in saline and used in the dose range 0.1–1 mg/kg. Haloperidol (Sigma) was dissolved in 40% propyleneglycol (PG) solution (v/v) in water and tested at the doses 0.1 and 0.3 mg/kg.

2.2.3. Other drugs, general administration route and timing

The 5-HT_{1A} antagonist NAD-299, (Robalzotan; R(-3-N,N-dicyclobutylamino-8-fluoro-3,4-dihydro-3H-1benzopyran-5-carboxamidehydrogen(2R,3R)-tartrate monohydrate, Astra Zeneca R&D (Johansson et al., 1997) was dissolved in saline.

The drugs were injected subcutaneously (s.c.) into the scruff of the neck at a volume of 4 ml/kg. The s.c. route of administration was used to minimize the importance of the first-pass metabolism. For pre-training administration, the APDs were always given 30 min prior to PA training and 15 min before PCP administration on day 1. Post-training injections were given immediately after training and pre-test injections occurred 15 min prior to the retention test on day 2 of the experiment. Saline control groups were always run concurrently. However, in the haloperidol experiments the control group received 40% PG solution.

2.3. Passive avoidance

PA is an associative emotional learning task based on Pavlovian fear-conditioning and instrumental conditioning (Baarendse et al., 2008; Ögren and Stiedl, 2015) and conducted as described earlier (Madjid et al., 2006). Briefly, a computer-controlled system was used for automatic recording of step-through latencies (Model 256000, TSE-System, Bad Homburg, Germany), within two equal sized compartments with a floor of stainless steel bars. The compartments were separated by a 10×10-cm sliding door. The conditioning (dark) compartment was black and only illuminated via indirect light with a light intensity of 3 lx. The light compartment was illuminated with a light intensity of 330 lx.

PA training was conducted in a single session on day 1. The animals were treated with the test compounds as described above and after the defined time interval placed in the light compartment with the sliding door closed (i.e., no access to the dark compartment for 60 s). After this delay the sliding door was automatically opened allowing the mouse access to the dark compartment. The latency to enter the dark compartment (training latency) with all four paws was recorded in all animals. Upon entering the dark compartment, the sliding door was automatically closed and a weak electrical current delivered via the grid floor (scrambled current: 2-s duration, 0.35 mA). The reactions to the electric current were scored for each animal to assess the responsiveness to unconditioned stimulus (UCS) according to the following scale; (1) a flinch (a sudden, brief vertical movement of one of the legs) (2) vocalization and (3) jumping. The scoring system means that mice could display a total of 3 scores in its response to the UCS. After USC exposure the mouse remained for 30 s in the dark compartment before being transferred to its holding cage to increase the associative strength between the UCS and the training context. Retention latencies were determined 24 h after training on day 2. The mouse was placed in the light compartment, with access to the dark compartment within 15 s. The latency on day 2 (step-through latency or retention latency) to enter the dark compartment with all four paws was automatically measured with a cut-off time of 300 s.

3. Statistical analysis

The study was designed as a between subjects (independent groups) experiment (i.e. each animal was used only once). The data were analyzed for normality by assessing the sample distribution or skewness (-1.5 to $+1.5$ considered normally distributed). After passing the tests for normality data were analyzed using one-way analysis of variance (ANOVA followed by the post-hoc test Fisher's Least Significant Difference (LSD) (Graph Pad Prism, San Diego, CA). The Cochran's Q test, a non-parametric statistical test was used to analyze the behavioral response to the UCS, the electrical current. A significance level of $p < 0.05$ was accepted as statistically significant. The results are presented as means $\pm$SEM.

4. Results

4.1. Behavioral observations and training latencies

Behavioral observations aimed to determine potential unspecific drug affects through altered locomotion and pain perception. The training latencies, the intervals between opening of the sliding door and entrance to the dark compartment during day 1, ranged from 25 to 55 s and were not significantly affected by treatment with PCP, the APDs or the APDs and PCP combinations (Table 2). This indicates that PCP and APD treatments with the used dosages did not interfere with motor performance to an extent that significantly affected training latencies. The scoring of behavioral responsiveness during training in the dark compartment did not indicate that the pharmacological treatments caused any significant change in the 3 variables scored unrelated to the response to the UCS (Table 3). This finding suggests that the PCP and the APDs did not interfere with sensory perception and processing which could have led to significant alteration of UCS responsivity. Thus, based on these findings unspecific drug effects on information processing for memory formation are highly unlikely.

4.2. Pre-training administration of phencyclidine impairs emotional memory

These experiments aimed to characterize the effective dose range for PA impairment by pretraining PCP. Mice treated with saline (control mice) before training displayed retention latencies of approximately 200 s, indicating that they had acquired the PA task. The training latencies of the PCP-treated groups were also in the range of 25–30 s and they did not differ significantly between the doses of PCP

Table 2 PA training latencies after various treatments with the APDs and PCP.

Control	Treatment 1	Treatment 2	Treatment 3	Treatment 4	*F*-/*p*-values
Saline	PCP 0.5 mg/kg	PCP 1 mg/kg	PCP 2 mg/kg	PCP 3 mg/kg	$F_{(4,25)}$=0.38 p>0.80
26.0 ± 4	**28.8 ± 7**	**24.8 ± 6**	**31.3 ± 3**	**32.7 ± 5**	
Saline	Clozapine 0.05 mg/kg	Clozapine 0.1 mg/kg	Clozapine 0.3 mg/kg	-	$F_{(3,22)}$=0.15 p>0.90
32.8 ± 10	**38.8 ± 12**	**39.9 ± 13**	**42.0 ± 17**	-	
Saline	Haloperidol 0.1 mg/kg	Haloperidol 0.3 mg/kg	-	-	$F_{(2,21)}$=1.50 p>0.20
31.1 ± 7	**32.5 ± 7**	**45.4 ± 8**	-	-	
Saline	Olanzapine 0.1 mg/kg	Olanzapine 0.3 mg/kg	Olanzapine 1 mg/kg	-	$F_{(3,20)}$=0.02 p>0.90
25.3 ± 4	**26.8 ± 5**	**26.5 ± 7**	**27.2 ± 5**	-	
Saline	Risperidone 0.1 mg/kg	Risperidone 0.3 mg/kg	Risperidone 1 mg/kg	-	$F_{(3,28)}$=1.78, p>0.15
32.9 ± 6	**44.1 ± 4**	**40.8 ± 4**	**49.1 ± 6**	-	
Saline	Raclopride 0.1 mg/kg	Raclopride 0.3 mg/kg	Raclopride 1 mg/kg	-	$F_{(3,28)}$=0.90, p>0.40
38.1 ± 10	**51.0 ± 5**	**52.4 ± 8**	**55.5 ± 9**	-	

Training latency of all experiments after treatment with PCP, clozapine, haloperidol and combined treatment of PCP and clozapine including controls. Results with the combined treatment with PCP and the APDs are not shown since none of the PCP and drug combinations differed significantly from the control values. The bold numbers represent the average training latency (s) ±S.E.M.

Table 3 The behavioral responsivity to the unconditioned stimulus (UCS) after treatment with phencyclidine (PCP), clozapine and haloperidol.

Treatment/Behavior	Flinch	Vocalization	Jumps	Total *n*
Saline	1	3	2	6
PCP 0.5 mg/kg	2	2	2	6
PCP 1 mg/kg	1	2	3	6
PCP 2 mg/kg	1	2	3	6
PCP 3 mg/kg	1	2	3	6
Saline	3	4	3	10
Clozapine 0.05 mg/kg	4	4	2	10
Clozapine 0.1 mg/kg	4	3	2	9
Clozapine 0.3 mg/kg	2	3	1	6
Saline	3	4	1	8
Haloperidol 0.1 mg/kg	3	3	2	8
Haloperidol 0.3 mg/kg	2	4	2	8

Each column presents the number of mice responding with the specific behavior (flinch, vocalization or jump) to the unconditioned stimulus (UCS) of the total number (*n*) of mice tested. The behavior of the mice was scored by the observer in the dark compartment during training after treatment with PCP, clozapine and haloperidol and the combined treatment of PCP and clozapine. Each given behavior e.g. flinch was statistically analyzed separately. There was no significant difference between the control or the drug-treated groups according to Cochrane *Q* test.

($F_{(4,25)}$=0.38, p>0.80) and the saline-treated group. Compared to the saline-treated mice, the PCP-treated mice (0.5, 1, 2 and 3 mg/kg) caused a dose-dependent decrease in their step-through latency (one-way ANOVA, treatment effect: $F_{(4,25)}$=10.70, p<0.001), with a significant impairment by the dose of 2 mg/kg (p<0.01, LSD) indicating a highly impaired PA memory retention (Fig. 1(A)). Based on these results the 3 mg/kg dose of PCP was used for subsequent experiments with APDs.

4.3. Post-training and pre-test administration of phencyclidine fails to impair emotional memory

Next, we examined the temporal memory phase (consolidation or expression/retrieval phase) possibly underlying the memory deficit induced by pre-training administration of PCP using two injection protocols. In contrast to the results from pre-training, immediate post-training (Fig. 1(B)) or pretest (Fig. 1(C)) administration of PCP (3 mg/kg) did not significantly impair PA retention. Thus, no significant difference was observed between saline- and PCP-treated mice ($F_{(2,11)}$=0.39, p=0.68 and $F_{(2,11)}$=0.31, p=0.74 respectively, NS). These results indicate that the impairing effect of PCP is related to the acquisition or encoding phase of the memory trace rather than the early consolidation or the retrieval/ memory expression phase.

4.4. Effects of atypical APDs on PA retention in drug-naïve and in phencyclidine-treated mice

Based on the results from pre-training we examined if the atypical APDs could modify the PCP-induced impairment of

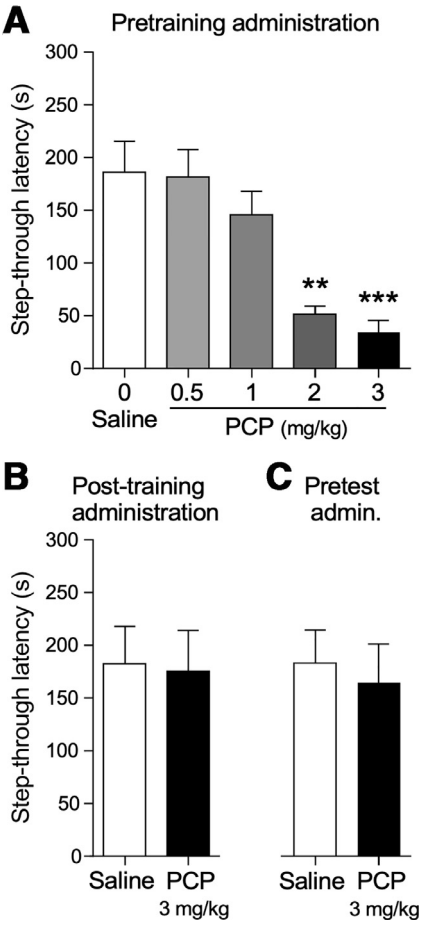

Fig. 1 The dose-effects of phencyclidine (PCP) on passive avoidance retention, using three different protocols. The graphs represent the retention latency (s) ±S.E.M. $^{***}p<0.001$ vs. saline control group (Fisher's post-hoc comparison). (A) Dose-response effect of pretraining administration of PCP (0.5, 1, 2 and 3 mg/kg, $n=6$/group). (B) Effect of post-training administration of PCP (3 mg/kg, $n=7$/group). (C) Effect of pre-test administration of PCP (3 mg/kg, $n=7$/group).

encoding (acquisition) prior to memory formation. As described earlier, the atypical APDs were administered 30 min prior to training either alone or in combination with PCP (3 mg/kg) which was injected 15 min before training. When given alone clozapine (0.05, 0.1 and 0.3 mg/kg) at very low doses caused a dose-dependent inverted "U" shape effect on retention latency (Fig. 2(A); one-way ANOVA, treatment effect: $F_{(3,31)}=4.24$, $p<0.01$). In fact, of the three doses used (0.05, 0.1 and 0.3 mg/kg), only 0.1 mg/kg enhanced memory retention whereas the other two doses were ineffective compared to saline-injected controls (Fisher's post-hoc comparison). Importantly, Clozapine (3 mg/kg) blocked the PCP-induced impairment of PA retention (Fig. 2(B); one-way ANOVA, treatment effect: $F_{(3,22)=}17.64$, $p<0.0001$) with a significant effect at both 0.1 and 0.3 mg/kg. It is notable that the training latencies of the clozapine-treated groups were not significantly changed ($F_{(3,31)}=0.47$, $p>0.70$). Again, the training latencies of the clozapine/PCP-treated groups did not differ significantly from the saline- and individual drug-treated groups ($F_{(3,22)}=0.15$, $p>0.90$).

It is crucial to compare clozapine with olanzapine since they are chemical analogues with a similar in vitro receptor binding profile (Table 1). Olanzapine administered alone (0.1, 0.3 and 1 mg/kg) did not by itself affect the PA retention latency (Fig. 2(C)). However, like clozapine, olanzapine dose-dependently blocked the PCP-induced impairment of PA retention (Fig. 2(D); one-way ANOVA, treatment effect: $F_{(3,22)}=23.30$, $p<0.0001$) with a significant blockade at the 0.1 and 0.3 mg/kg.

Unlike haloperidol, risperidone has a much higher affinity for the 5-HT_{2A} than for the D_2 receptor (Table 1) considered to be essential for its atypical profile (Meltzer and Huang, 2008). Risperidone by itself significantly facilitated PA retention latency compared to saline at the highest dose (1 mg/kg) tested (Fig. 2(E); one way ANOVA, treatment effect: $F_{(3.28)}=3.70$, $p<0.05$). Risperidone also significantly ($p<0.05$) blocked the PCP-induced impairment of PA retention (Fig. 2(F); ANOVA, treatment effect: $F_{(3,28)}=35.82$, $p<0.001$), only at the 1 mg/kg dose. These experiments indicated that all atypical APDs could ameliorate the PCP-induced impairment of PA memory.

4.5. The typical APD haloperidol and the $D_{2/3}$ receptor antagonist raclopride failed to block the PCP-induced PA impairment

Next, we examined if the typical APD haloperidol and the $D_{2/3}$ receptor antagonist raclopride could ameliorate or block the PCP-induced PA impairment. Haloperidol is regarded as the prototype for typical APDs which improves positive symptoms, through its D_2 receptor blocking action. The training latencies of the haloperidol-treated groups were not significantly altered ($F_{(2,21)}=1.50$, $p>0.20$). Moreover, in contrast to clozapine, haloperidol (0.3 mg/kg) significantly impaired PA retention compared to saline (Fig. 3(A); one-way ANOVA, treatment effect: $F_{(2.21)}=12.96$, $p<0.01$). Importantly, unlike clozapine, haloperidol failed to attenuate the PCP-induced PA impairment. In fact, haloperidol (0.3 mg/kg) significantly enhanced the PCP-induced impairment (Fig. 3(B); one-way ANOVA, treatment effect: $F_{(3,20)}=50.51$, $p<0.01$).

Haloperidol was compared to the highly selective $D_{2/3}$ receptor antagonist raclopride, an experimental drug with probable antipsychotic effects. Like haloperidol, raclopride significantly impaired PA retention at the highest dose tested (1 mg/kg) when compared to saline controls (Fig. 3(C); one way ANOVA, treatment effect: $F_{(3.28)}=8.59$, $p<0.05$). Raclopride also failed to block the PCP-induced impairment of PA retention (Fig. 3(D)) but unlike haloperidol it did not further enhance the impairment caused by PCP.

4.6. Effects of the 5-HT_{1A} receptor antagonist NAD-299 alone or in combination with clozapine and PCP on PA retention

To determine if the blocking effect of clozapine on the PCP-induced impairment of PA retention could involve serotoner-

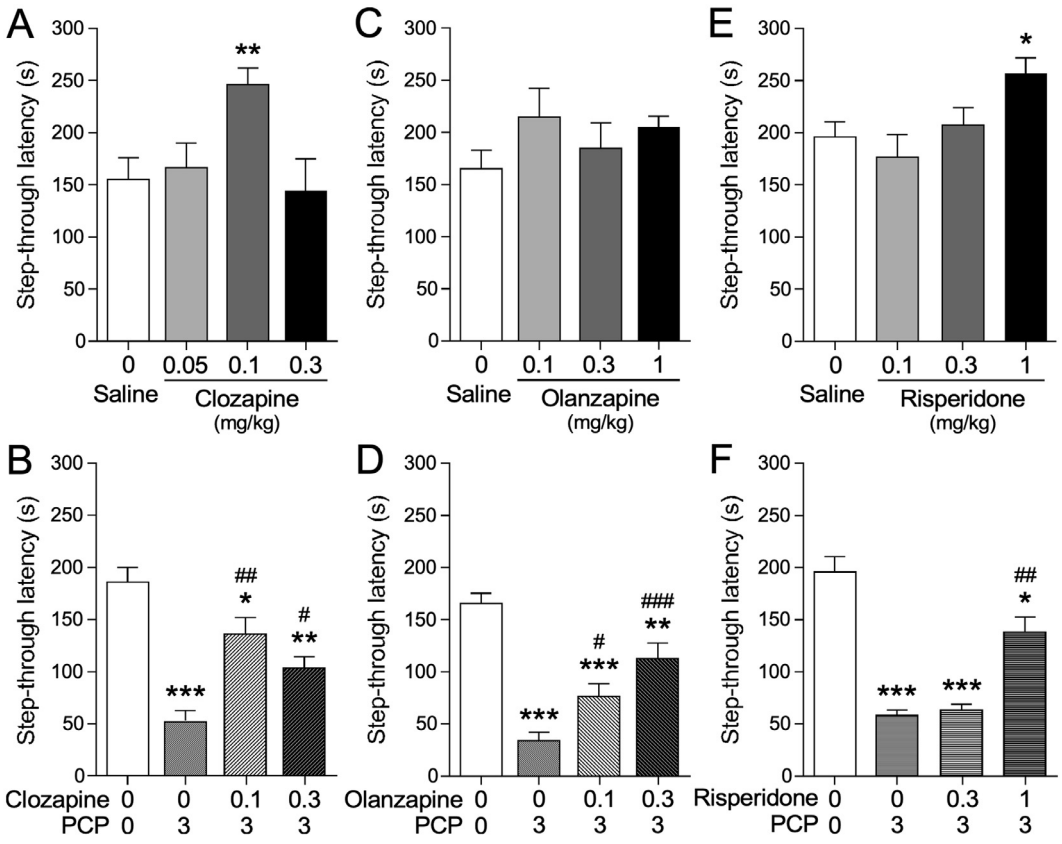

Fig. 2 The effects of the atypical antipsychotics clozapine, olanzapine and risperidone alone or in combination with PCP (3 mg/kg) on passive avoidance retention. The graphs represent the retention latency (s) ±S.E.M. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, vs. saline control group; $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ vs. PCP-treated group (Fisher's post-hoc comparison). (A) Dose-response effect of clozapine (0.05, 0.1 and 0.3 mg/kg; $n \geq 6$/group). (B) Effect of clozapine (0.1 and 0.3 mg/kg; $n \geq 6$/group) in combination with PCP. (C) Dose-response effect of olanzapine (0.1, 0.3 and 1 mg/kg; $n=6$/group). (D) Effect of olanzapine (0.1 and 0.3 mg/kg; $n=6$/group) in combination with PCP. (E) The dose-response effect of risperidone (0.1, 0.3 and 1 mg/kg; $n=8$/group). (F) Effect of risperidone (0.3 and 1 mg/kg; $n=8$/group) in combination with PCP.

gic receptors, we studied the effect of the selective 5-HT_{1A} receptor antagonist NAD-299 alone or in combination with clozapine and PCP on PA retention.

NAD-299 produced a dose-dependent increase in PA retention (Fig. 4(A); $F_{(3,28)}=3.76$, $p<0.05$) with a significant increase at the 1 mg/kg dose. NAD-299 also blocked the PCP-induced memory impairment significantly at the 0.1 mg/kg dose which by itself did not improve cognition (Fig. 4(B); $F_{(3,28)}=15.15$, $p<0.001$). NAD-299 also significantly blocked the improving effect of clozapine on PCP-induced impairment of PA retention (Fig. 4(C); $F_{(3,22)}=11.28$, $p<0.001$). This finding indicates that the effect of clozapine on the PCP-induced impairment involves 5-HT_{1A} receptor activation.

5. Discussion

The multiple cognitive impairments in schizophrenia has been postulated to be mediated by glutamatergic dysfunction attributed mainly to reduced NMDAR transmission (Javitt and Zukin, 1991; Olney and Farber, 1995; Tamminga,1998). This study provides in vivo evidence that NMDAR synaptic mechanisms play an important role in emotional memory mechanisms. Acute pre-training administration of the NMDR antagonist PCP in a low dose-range (0.5–3 mg/kg) caused a dose-dependent impairment of memory performance in C57BL/6J mice in the PA task, an associative and Pavlovian learning paradigm (Ögren and Stiedl, 2015). Moreover, the PCP-induced impairment appears to be related to mechanisms of encoding rather than mechanisms involving early memory consolidation or retrieval. The impairment seen in spatial learning tasks after pretraining administration of PCP implicates hippocampal encoding mechanisms in the acquisition of the Morris water maze (Åhlander et al., 1999; Beraki et al., 2008; Didriksen et al., 2007; Podhorna and Didriksen, 2005). Thus, the role of NMDAR in emotional memory is probably partly mediated via hippocampal glutamatergic mechanisms involving the acquisition or the encoding phase of learning (Ögren et al., 2008) consistent with fear conditioning studies in mice (Stiedl et al., 2000). Taken together, these results confirm the usefulness of NMDAR antagonist as animal models for analysis of the pathophysiology of cognitive deficits in schizophrenia (Cadinu et al., 2018).

The main finding of this study is that drugs characterized as SGAs blocked the memory impairment induced by

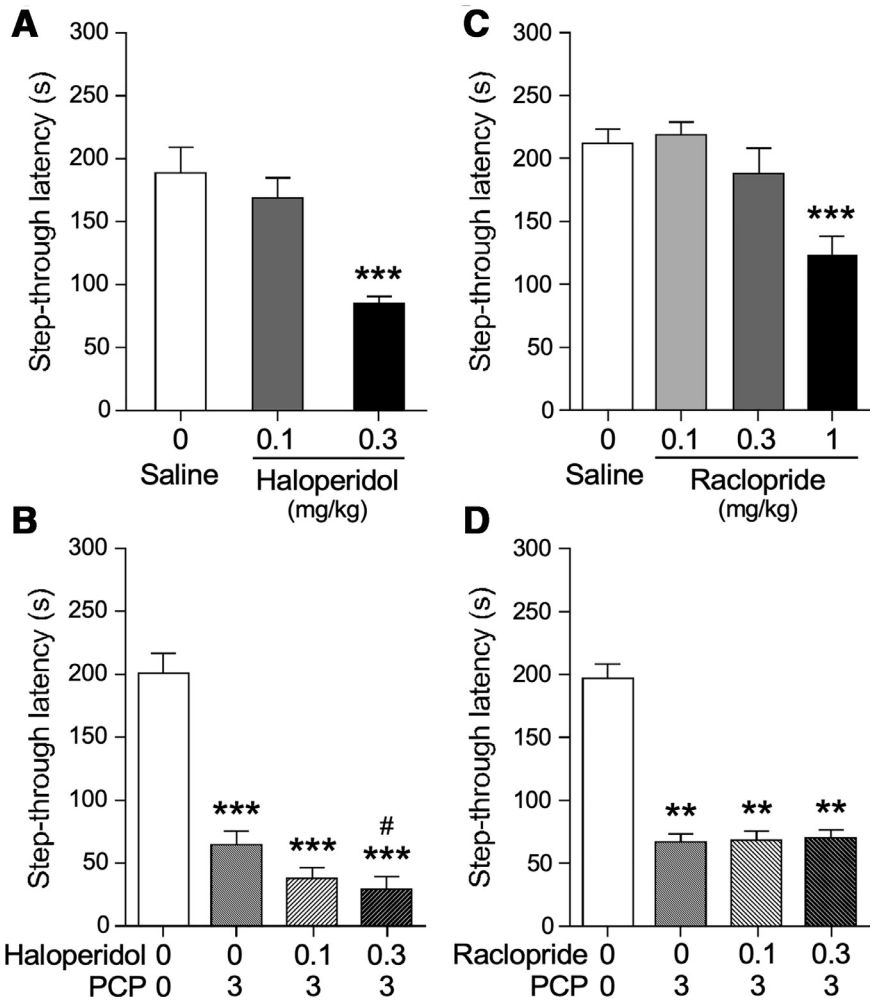

Fig. 3 The effects of the typical antipsychotic haloperidol and of raclopride alone or in combination with PCP (3 mg/kg) on passive avoidance retention. The graphs represent the retention latency (s) ±S.E.M. $^{***}p<0.001$, vs. saline control group; $^{\#}p<0.05$, vs. PCP-treated group (Fisher's post-hoc comparison). (A) The dose-response effect of haloperidol (0.1 and 0.3 mg/kg; $n=8$/group). (B) Effect of haloperidol (0.1 and 0.3 mg/kg; $n=6$/group) in combination with PCP. (C) The dose-response effects of raclopride (0.1, 0.3 and 1 mg/kg; $n=8$/group). (D) Effect of raclopride (0.3 and 1 mg/kg; $n=8$/group) in combination with PCP.

the NMDAR antagonist PCP. In contrast, the FGA haloperidol was found to be ineffective. In fact, haloperidol enhanced the memory impairment caused by PCP consistent with earlier findings in a spatial learning task in mice using both acute and subchronic PCP administration combined with haloperidol (Beraki et al., 2008; Didriksen et al., 2007). In contrast to the atypical APDs haloperidol by itself impaired PA retention at the 0.3 mg/kg dose but not at the 0.1 mg/kg dose while it enhanced the PCP effect at the 0.3 mg/kg dose. Since haloperidol did not significantly affect training latency, it seems likely that the result with the haloperidol/PCP combination is probably not due to interference with D_2 motor mechanisms. Interestingly, the $D_{2/3}$ receptor antagonist raclopride which unlike haloperidol has an atypical behavioral profile (Ögren et al., 1986) failed to modulate the PCP-effect without any evidence for motor disturbances. It is possible that raclopride with its combined affinity for $D_{2/3}$ receptors may attenuate the effect of D_2 blockade. Thus, blockade of D_3 receptors has been shown to attenuate EPS induction, e.g. catalepsy induced by haloperidol in rats (Millan et al., 1997). Taken together, these results indicate that D_2 receptor blockade cannot normalize emotional memory impairments related to glutamatergic NMDAR dysfunction.

In contrast, both FGAs and SGAs are able to block the increase in locomotor activity, stereotypies and ataxia induced by PCP and MK-801 in rodents most likely partly related to their dopamine blocking action (Castañé et al., 2015; Gleason and Shannon 1997; Jackson et al., 1994; Kitaichi et al., 1994; Ögren and Goldstein, 1994). The difference between the effects on memory and locomotion is surprising since the PCP-induced hyperlocomotion has been suggested to be a surrogate marker for positive symptoms in schizophrenia (Mohn et al., 1999). The high potency of the SGAs to block the PCP-induced hyperlocomotion in mice (Gleason and Shannon, 1997) is surprising in view of their relatively low affinity for D_2 receptors (Table 1; Meltzer and Huang, 2008; Richtand et al., 2008; Schotte et al., 1996) but probably reflects their potent blocking action at 5-HT_{2A} receptor. Notably, 5-HT_{1A} receptor antagonists only weakly

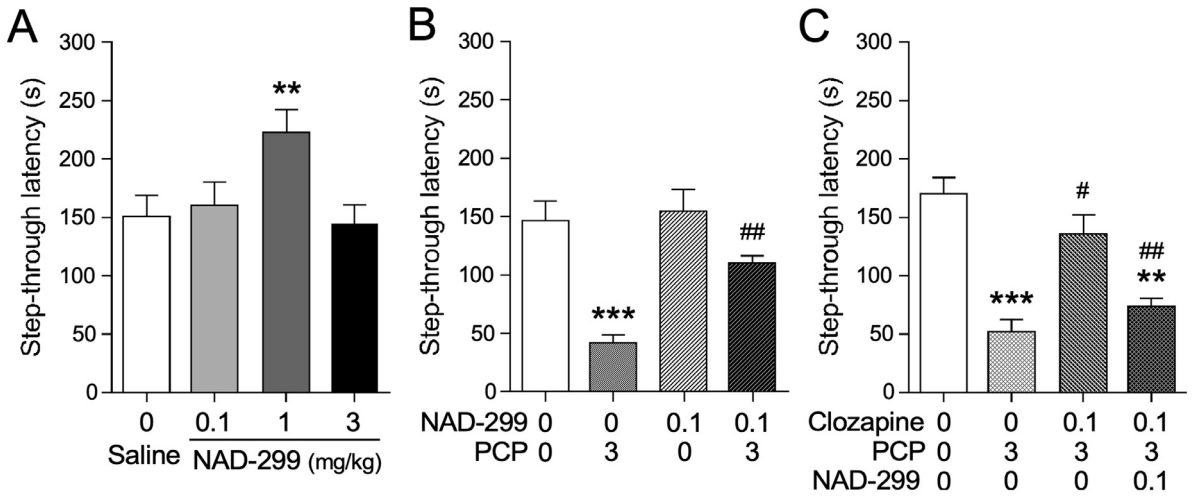

Fig. 4 The effects of NAD-299 alone or in combination with clozapine and PCP (3 mg/kg) on passive avoidance retention. The graphs represent the retention latency (s) ±S.E.M. $^{***}p<0.001$, vs. saline control mice; $^{\#\#\#}p<0.001$ vs. PCP-treated mice (Fisher's post-hoc comparison). (A) The dose-response effect of NAD-299 (0.3, 1 and 3 mg/kg, $n \geq 8$/group). (B) Effect of NAD-299 (0.3 mg/kg; $n = 8$/group) in combination with PCP. (C) Effect of NAD-299 (0.3 mg/kg; $n \geq 6$/group) in combination with clozapine (0.1 mg/kg) and PCP.

reduced PCP-induced locomotor activity (Gleason and Shannon, 1997).

Recent data suggest that PCP-induced motor activation is specifically mediated by activation of dopamine- and cAMP-regulated phosphoprotein (DARPP-32) in distinct populations of striatal medium spiny neurons in the direct motor pathway involving mainly D_1 receptors (Bonito-Oliva et al., 2016). In contrast, the memory deficit induced by PCP in the PA task was not linked to the expression of DARPP-32 in both the direct and the indirect striatal motor pathway. These results suggest that typical and atypical APDs block the PCP-induced hyperactivity via differential effects on the direct and indirect striatal motor pathways.

Since the PCP treatment was administered prior to training, it is critical to reduce its nonspecific or confounding effects on cognitive performance. An important aspect of this work was to select low doses of PCP and to include behavioral observations of the responses to drug treatments to monitor changes in performance independently of an effect on memory processes. Notably, there was a 24-h interval between drug injection and test of retention which made it unlikely that the drug treatments could directly affect retention performance at the time of testing. Alternatively, the PCP treatment could affect the relationship between the UCS and the training context by affecting the instrumental or motor response (Ögren and Stiedl, 2015) or the perception of pain. However, this study failed to show any apparent motor deficits caused by PCP or the drugs used in this study. Thus, PCP produced marked PA retention impairments without any major effects on the non-cognitive measures indicated by a non-significant alteration in training latency or responsivity to the electric current (UCS) (Tables 2 and 3).

The analysis of the possible mode of action of the SGAs underlying their anti-PCP effect will focus on their multiple actions on serotonin receptors implicated in cognition (Table 1). The SGAs but not haloperidol share high affinities for various combinations of 5-HT_{2A}, 5-HT_{2C}, 5-HT_6 and 5-HT_7 receptors but with low affinity for the 5-HT_{1A} receptors (Table 1; see below). It could be argued that the combination of this receptor binding profile is the mechanism behind the PCP blockade. However, the behavioral results indicate that although the SGAs share an anti-PCP effect, they differ clearly in their effect on emotional memory. For instance, the receptor binding profile of clozapine and olanzapine for serotonin receptors are rather comparable while they differ markedly from that of risperidone (Table 1). Clozapine, unlike its chemical analogue olanzapine, displayed also a pro-cognitive effect at a restricted dose-interval in control mice but caused a dose dependent blockade of the PCP-induced impairment. The difference between clozapine and olanzapine is intriguing in view of their similar receptor binding profile (Table 1) which combines high affinities for 5-HT_{2A} and 5-HT_{2C} receptors and with moderate affinity for the 5-HT_{1A} and D_2 receptors in case of clozapine. In contrast, the affinity of olanzapine for the D_2 receptors is in the same range as its affinities for the 5-HT_{2A}, 5-HT_{2C} and 5-HT_6 receptors. Unlike, clozapine, olanzapine has a 40-fold lower affinity for the 5-HT_7 compared to the 5-HT_6 receptor (Table 1) which may contribute to its different behavioral profile compared with clozapine. Based on the in vitro receptor binding profile it seems unlikely that clozapine or olanzapine can act via a direct action on 5-HT_{1A} receptors but rather via an indirect modulatory effect on serotonin transmission. However, since these compounds are partial agonists and not full antagonists at the 5-HT_{1A} receptors, the possible role of 5-HT partial agonist cannot be excluded. Thus, presynaptic doses of 5-HT_{1A} receptor agonists have been shown to enhance memory retention in mice in the PA task (Madjid et al., 2006; Ögren et al., 2008). Taken together, olanzapine and clozapine block the PCP-induced impairment in a low and narrow dose-interval which is partly

overlapping with their effects on memory retention in naïve mice.

Risperidone differed from clozapine and olanzapine by blocking the PCP-induced deficit only at a dose which significantly enhanced memory retention (Fig. 2(E) and (F)). The compound also differed from the other SGAs with its combined high affinity for both D_2, 5-HT_{2A} and 5-HT_7 receptors (Meltzer and Huang, 2008; Millan et al., 2015; Schotte et al., 1996). The enhancement of PA memory by risperidone is intriguing. It is possible that the difference between risperidone and haloperidol is related to its combined and potent affinity for 5-HT_{2A} and 5-HT_7 receptors which may mitigate its high blocking action at D_2 receptors which could result into a significant increase in EPS (Meltzer and Huang, 2008; Millan et al., 2015; Schotte et al., 1996).

Consistent with earlier findings the present results show that systemic administration of the 5-HT_{1A} receptor antagonist NAD-299 can facilitate PA memory performance related to its enhancement of hippocampal/cortical glutamatergic and cholinergic neurotransmissions (Madjid et al., 2006). This result also indicates an important role for brain serotonin in the action of PCP, since NAD-299 blocked the PCP-induced impairment at the dose of 0.1 mg/kg which did not improve PA memory performance in control mice. This may be attributed to blockade of the impairing effect of postsynaptic 5-HT_{1A} receptor activation in combination with enhanced 5-HT_7 receptor activation as indicated by our previous results (Eriksson et al., 2012; Stiedl et al., 2015). However, the contribution of the role of pre- versus postsynaptic 5-HT_{1A} receptor and their interaction with 5-HT_7 receptor activation needs to be analyzed in the action of the SGAs. The facilitatory role of a low dose (0.01–0.03 mg/kg) of 8-OH-DPAT in mice (Madjid et al., 2006) and rats (Lüttgen et al., 2005) has been attributed to presynaptic 5-HT_{1A} receptor activation thereby lowering postsynaptic 5-HT release which is expected to enhance, through reduced postsynaptic 5-HT_{1A} receptor signaling, glutamate release in cortical and hippocampal sites.

Surprisingly, NAD-299 also blocked the improving effect of clozapine on PCP-induced impairment of PA retention. These results suggest that the PCP-induced impairment involves activation of 5-HT_{1A} receptors and that the mechanisms underlying the effect of NAD-299 may differ in naïve mice vs. PCP-treated mice. Moreover, since NAD-299 blocked the ability of clozapine to attenuate the PCP impairment, it lends support to the involvement of 5-HT_{1A} receptors in mediating the ability of clozapine to ameliorate the impairing effect of PCP. This finding may seem inconsistent in view of the evidence that clozapine and olanzapine may attenuate the PCP impairment through an indirect 5-HT_{1A} receptor stimulation. There is, however, an important distinction between the experimental conditions when NAD-299 is given alone or in the combination with clozapine/PCP. Administration of PCP has been shown to activate a large number of cortical and subcortical brain regions and brain circuits, an effect normalized by clozapine (Santana et al., 2004). It seems possible that the different experimental conditions result into differential degree of brain activation which may explain these seemingly contradictory results. However, these findings are in agreement with the observation that the ability of clozapine to reverse the PCP-induced disorganization of prefrontal cortex (PFC) activity was (1) absent in 5-HT_{1A} receptor knockout mice and (2) blocked by the 5-HT_{1A} receptor antagonist WAY-100635 (Kargieman et al., 2012).

Since PA is a hippocampal-dependent task it is notable that electrophysiological data have shown that hippocampal CA1 pyramidal neurons are tonically inhibited by endogenous 5-HT through postsynaptic 5-HT_{1A} receptors during conditioned fear learning (Tada et al., 2004) partly via a direct regulation of NMDAR channels (Yuen et al., 2005) or through an indirect modulation of glutamatergic neurotransmission via activation of 5-HT_{1A} receptors. By blocking the hyperpolarizing action of endogenous 5-HT mediated by 5-HT_{1A} receptors, 5-HT_{1A} receptor antagonists could compensate for the reduction of NMDA receptor-mediated excitatory drive on pyramidal cells in the hippocampus/cortex. A similar mechanism may explain the finding that the PA impairment caused by the NMDAR antagonist MK-801 was blocked by NAD-299 (Madjid et al., 2006). Electrophysiological studies also suggest that SGAs may enhance glutamatergic neurotransmission in pyramidal cells of the medial PFC (Ninan et al., 2003). Clozapine and other SGAs but not haloperidol facilitated responses evoked by electrical stimulation of the forceps minor and by NMDA, but not by (±)-alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), of the medial PFC possibly related to the putative beneficial effect of atypical APDs on cognitive functions (Ninan et al., 2003).

The impairment of PA learning caused by PCP most likely involves hippocampal NMDARs. However, in view of the wide neuronal activation by PCP (Santana et al., 2004), the anti-PCP effect by the SGAs might also depend on modulation of non-hippocampal brain systems such as the PFC. Unlike haloperidol, SGAs such as clozapine, olanzapine and risperidone have been shown to activate the mesocortical dopamine pathway and thereby increase dopamine release in the PFC (Bortolozzi et al., 2010; Díaz-Mataix et al., 2005). This effect appears to be mediated by the direct or indirect activation of 5-HT_{1A} but not 5-HT_{2A} receptors (Díaz-Mataix et al., 2005). Dopaminergic transmission in the PFC has an important role in working memory function (Surmeier, 2007) and is in a position to modulate hippocampal cognition via reciprocal interactions with the hippocampus. Thus, PFC receives hippocampal excitatory afferents (Sesack et al., 1989) and in turn sends feedback projections into the hippocampus (Fuster, 2015).

The impairment in memory may appear paradoxically since blockade of NMDARs results in enhanced release of cortical glutamate (Moghaddam and Jackson, 2003) and probably also hippocampal glutamate. The enhanced excitatory transmission after NMDAR blockade could be related to the blocking action of PCP on NMDAR located on GABAergic interneurons mediating local inhibition of hippocampal glutamatergic pyramidal neurons via recurrent axon collaterals (Rujescu et al., 2006). Importantly, NMDA receptors located on hippocampal GABAergic interneurons are tenfold more sensitive to NMDA receptor blockade than glutamatergic pyramidal neurons (Grunze et al., 1996). Administration of PCP would, therefore, lead to inhibition of GABAergic interneurons and thereby a diminished inhibitory tone on glutamatergic neurons (Rujescu et al., 2006). This would theoretically result in an enhanced (overactivated), and thus, dysfunctional (increased because disinhibited) gluta-

mate receptor transmission. Importantly, this synaptic disorganization appears to normalized by 5-HT_{1A} receptor antagonism highlighting the importance of an optimal level of glutamate receptor transmission (signaling) in health versus disease (Zhou and Danbolt, 2014).

In conclusion, there exists a large body of evidence based on various rodent paradigms which indicate that SGAs may possess memory improving potential in schizophrenic patients. However, the translational value and clinical utility of these findings are still not demonstrated for the SGAs with the exception of clozapine. There are several explanations for this situation. First, most of the rodent data is based on spatial learning and not on emotional memory paradigms. Secondly, the present results indicate that the SGAs have anti-PCP effects at an optimal and low dose-range which underline the importance of cross-species drug kinetics. Finally, the results highlight the unique profile of clozapine compared to other SGAs and the potential of 5-HT_{1A} receptor mechanisms in the beneficial cognitive effects of clozapine. The results suggest that the ability of the SGAs to attenuate the cognitive deficits by PCP appears to involve multiple 5-HT receptors putatively modulating glutamatergic NMDA and GABAergic cortical and hippocampal functions. The present study provides a useful experimental basis to further investigate schizophrenic-like cognitive abnormalities in mice and to analyze in vivo the mechanisms of drugs with a potential for treating cognitive impairments in schizophrenia.

Contributors

NM and ABO performed the research; SOÖ and AA designed the research, analyzed the data and provided funds; SH analyzed the data and provided figures for the manuscript, OS made important comments on the scientific aspects of the manuscript, GF and ÅKG contributed to the discussion. All authors contributed to and approved the final manuscript.

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Conflicts of interest

The authors declare no conflict of interest.

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