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Caroline Bouvrais-Veret[1,2*], Stéphanie Weiss[1,2*], Annie Andrieux[3], Annie Schweitzer[3], J. Michael McIntosh[4], Didier Job[3], Bruno Giros[1,2] and Marie-Pascale Martres[1,2#]
[*]equal contribution

[1]Inserm, U513, Laboratoire de Neurobiologie et Psychiatrie, Créteil, F-94000 France; [2]Univ Paris 12, Créteil, F-94000 France. [3]Inserm, U366, Laboratoire du Cytosquelette, CEA, Grenoble, F-38000 France. [4]Department of Psychiatry, University of Utah, Salt Lake City, UT 84112

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Footnote: For nAChRs, * indicates the association with other subunits

#Address correspondence to Marie-Pascale Martres, Inserm U513, Laboratoire de Neurobiologie et Psychiatrie, Faculté de Médecine, 8 rue du Général Sarrail, Créteil, F-94010 France. Tel: (33) 149 813 658; Fax: (33) 149 813 685; E-mail: Marie-Pascale.Martres@creteil.inserm.fr.

ABSTRACT

Mice deficient in the microtubule stabilizing protein STOP (Stable Tubule Only Polypeptide) show synaptic plasticity anomalies in hippocampus, dopamine hyper-reactivity in the limbic system and severe behavioral deficits. Some of these disturbances are alleviated by long-term antipsychotic treatment. Therefore, this mouse line represents a pertinent model for some aspects of schizophrenia symptomatology. Numerous data support dysfunction of nicotinic neurotransmission in schizophrenia and epidemiological studies show increased tobacco use in schizophrenic patients, in whom nicotine has been reported to improve cognitive deficits and impairment in sensory gating.

In this study, we examined potential alterations in cholinergic (ACh) and nicotinic components and functions in STOP mutant mice. STOP KO mice displayed no variation of the density of ACh esterase and β2* nicotinic receptors (nAChRs), large reductions in the density of vesicular ACh transporter and α6* nAChRs and marked increases in the density of α7 nAChRs, in some brain areas. STOP KO mice were hypersensitive to the stimulating locomotor effect of nicotine and, interestingly, their impaired performance in learning the cued version of the water maze were improved by administration of the preferential α7 nAChR agonist choline.

Altogether, our data show that the deletion of the ubiquitous STOP protein elicited restricted alterations in ACh components. They also suggest that nicotinic neurotransmission can be deficient in STOP KO mice and that mutant mice can represent a meaningful model to study some nicotinic dysfunctions and therapeutic treatments.

INTRODUCTION

Neuronal microtubules play a structural key role in morphogenesis and protein transports in dendrites and axons. Recently, microtubules and their effectors, such as microtubule-associated proteins (MAP1B, STOP) and sequestering proteins, have been implicated in synaptic plasticity (Bosc et al., 1996; Guillaud et al., 1998; van Rossum and Hanisch, 1999). STOP knock-out (KO) mice exhibit synaptic abnormalities in the hippocampus, including depleted glutamatergic vesicle pools, decreased long-term potentiation and depression (Andrieux et al., 2002) and decreased mRNA transcripts of synaptic proteins (e.g. synaptophysin, GAP-43 and spinophilin; Eastwood et al., 2006). These mice show severe behavioral deficits, such as purposeless and disorganized activity, impaired social interactions and maternal behavior, deficits in prepulse inhibition (PPI) of the startle reflex and hypersensitivity to mild stress and to the stimulant locomotor effect of amphetamine (Andrieux et al., 2002; Brun et al., 2005; Fradley et al., 2005). STOP KO mice also display dopamine (DA) hyper-reactivity in the limbic system (Brun et al., 2005). Whereas basal extracellular DA levels are not modified in the striatum and the nucleus accumbens of mutant mice, electrically evoked DA release is selectively increased in the nucleus accumbens of STOP KO mice. Importantly, some of these dysfunctions can be alleviated by chronic antipsychotic drug treatment (Andrieux et al., 2002; Brun et al., 2005). Therefore, STOP KO mice may represent a useful experimental model for studying several features of schizophrenia, a notion consistent with recent models implicating synaptic disorders in the etiology of schizophrenia (Mirnics et al., 2001; Frankle et al., 2003). The pertinence of this experimental mouse line is strengthened by recent studies showing an association between schizophrenia and polymorphisms in the MAP6 gene, the human homolog of STOP gene (Shimizu et al., 2006).

Numerous data implicate nicotinic neurotransmission dysfunction in several psychiatric disorders, including schizophrenia. Indeed, post-mortem studies show decreased number of nicotinic receptors in various brain areas of schizophrenic patients (Freedman et al., 1995; Breese et al., 2000; Mihailescu and Drucker-Colin, 2000). Moreover, linkage studies strongly suggest that the P50 auditory sensory deficit in schizophrenia is linked to the α7 nicotinic receptor gene (Freedman et al., 1997; Leonard et al., 2002). In addition, epidemiological studies show a 2-3 fold increase in tobacco use in schizophrenic patients (Glassman, 1993; de Leon et al., 1995). It has been suggested that nicotine serves as a self-medication to improve a number of cognitive deficits associated with schizophrenia, to enhance the therapeutic effect of antipsychotics, to alleviate negative symptoms and/or to reduce the side-effects of antipsychotic drugs (Kumari and Postma, 2005). Thus, deficits in

sensorimotor gating, often encountered in schizophrenic patients and their first-degree relatives, is alleviated by nicotine, probably via its action through hippocampal α7 nicotinic receptors (Freedman et al., 1997; Adler et al., 1998; Leonard et al., 2002).

Thus, STOP KO mice, which serve as a pertinent experimental model for some schizophrenic related symptoms, present an opportunity to characterize key components of the cholinergic/nicotinic neurotransmission. We first measured the density of acetylcholine esterase (AChE), of vesicular ACh transporter (VAChT) and of β2*, α6* and α7 nicotinic receptors (nAChR) in various brain areas of wild-type (WT) and STOP KO mice. Then, in order to evaluate the potential consequences of the modified expression of cholinergic/nicotinic markers, we chose to characterize two behaviors that involved nicotinic neurotransmission, namely nicotine-induced locomotor activity and mouse learning in the Morris water maze.

MATERIALS AND METHODS

ANIMALS

Homozygous STOP KO mice and their WT littermates were obtained from the intercross (F2) of heterozygous 50:50 BALBc/129 SvPas-F1 mice (Andrieux et al., 2002). The genotype of the mice was determined from tail biopsies as previously described (Andrieux et al., 2002). Animals were housed four to six per cage by gender and litter. They were kept under standard conditions, i.e., laboratory chow and water available *ad libitum*, temperature at 23±2 °C, humidity at 55±10% and a light cycle of 12 h light:12 h dark (lights on at 7:30 a.m.). Experiments were carried out in accordance with the European Communities Council directive (86/809/EEC) and approved by the local ethical committee. Mice were allowed to habituate to the animal holding room for at least one week prior to use. All experiments were conducted on naive WT and KO mice from the same litters (about 60%/40% females/males), three to four months old.

DRUGS

Nicotine bitartrate and choline hydrochloride were purchased from Sigma-Aldrich (St Louis, MO). Drugs were dissolved in physiological saline as free bases and were administered sub-cutaneously in a volume of 100 µl per 20 g weight. [^{125}I]-Epibatidine (74 TBq/mmol) and (3-[^{125}I]-iodotyrosyl) α−bungarotoxin (74 TBq/mmol) were from GE Healthcare. [^{125}I]-α-Conotoxin-MII was synthesized as previously described (Whiteaker et al., 2000). Before use, rabbit [^{125}I]-Ig F (ab')2 fragment antibodies (28-111 TBq/mmol, GE Healthcare, Orsay, France) were purified by gel filtration onto a PD10 column (Sephadex G25 M, Pharmacia) in PBS (50 mM NaH_2PO_4/Na_2HPO_4, 154 mM NaCl) containing 0.1% gelatin. The first 0.25 ml eluate was discarded and the following 3.5 ml eluate was put into 400 ml PBS supplemented with 3% bovine serum albumin (BSA), 1% goat serum, 1 mM NaI and 0.02% sodium azide. This solution was kept at 4°C, for four to six weeks.

QUANTITATIVE AUTORADIOGRAPHY

Mice were killed by cervical dislocation (between 10:00 a.m. and 4:00 p.m.) and their brains rapidly removed and frozen in isopentane at –30°C. Serial 10 µm coronal sections were cut at –20°C, thaw-mounted on Superfrost Plus® slides and stored at –80°C until use. Coronal levels used are IA=4.90-5.22 mm for the nucleus accumbens and the striatum, IA=1.86-2.22 mm for the dorsal hippocampus and IA=0.52-0.72 mm for the substantia nigra and the ventral tegmental area.

Immunoautoradiography of acetylcholine esterase (AChE) and vesicular acetylcholine transporter (VAChT)

Brain sections were fixed with 4% paraformaldehyde for 15 min at room temperature, extensively washed with PBS and then incubated for 1 h at room temperature in PBS containing 3% BSA, 1% goat serum and 1 mM NaI. Sections were incubated for 2 h at room temperature (or overnight at 4°C) in the presence of AChE antiserum (1:1,000 dilution, polyclonal AChE antibody characterized by Marsh et al., 1984) or VAChT antiserum (1:8,000 dilution; polyclonal antibody directed against mouse VAChT Ser^{478}-Ser^{530} peptide). After washes, slides were incubated for 2 h at room temperature with purified anti-rabbit [^{125}I]-IgG (370-740 MBq/100 ml). Sections were extensively washed with PBS, dried and exposed to Biomax MR films for 1-4 days.

Autoradiographic determination of β2*, α6* and α7 nicotinic receptor densities

To rule out possible binding of endogenous acetylcholine to nicotinic receptors, sections were pre-incubated in buffer before addition of radioactive ligands.

Labelling of β2* subunits was performed according to Perry and Kellar (1995). Slides were pre-incubated three times for 5 min at room temperature in 50 mM Tris-HCl buffer, pH 7.4, containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (Tris-ions buffer). Sections were incubated for 40 min at room temperature in the same fresh buffer, in the presence of 0.4 nM [^{125}I]-epibatidine with or without 300 μM nicotine to determine non-specific binding. Slides were then washed twice for 5 min in Tris-ions buffer at 4 °C, dipped in ice-cold water, dried and exposed to Biomax MR films for 2-3 days.

Labelling of α6* subunits was performed according to Whiteaker et al. (2000). Slides were pre-incubated for 15 min at room temperature in 20 mM HEPES buffer, pH 7.5, containing 144 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$ (standard binding buffer), supplemented with 0.1% BSA and 1 mM phenylmethylsulfonyl fluoride. They were incubated in the presence of 0.8 nM [^{125}I]-α-conotoxin-MII for 2 h at room temperature in standard binding buffer supplemented with 0.1% BSA, 5 mM EDTA, 5 mM EGTA and 1 mg/100 ml of aprotinin, leupeptin and pepstatin A (protease inhibitors). Non-specific binding was determined in the presence of 1 μM epibatidine. Slides were washed for 30 sec at room temperature and then at 4°C in standard binding buffer plus 0.1 % BSA. Afterwards, sections were washed twice for 5 sec at 4°C in standard binding buffer with 0.01% BSA and, finally, twice for 5 sec at 4°C in 5 mM HEPES buffer pH 7.5. After dipping in ice-cold water and drying, they were exposed to Biomax MR films for 24-72 h.

Labelling of α7 nicotinic receptors was performed according to Spurden et al. (1997). The slides were pre-incubated for 30 min at room temperature in 50 mM Tris–HCl buffer, pH 7.4, containing 0.1% BSA. Sections were next incubated for 2 h at room temperature in the same buffer in the presence of 0.5 nM [^{125}I]-α-bungarotoxin, with or without 1 mM nicotine to determine non-specific binding. Slides were then washed four times for 10 min in ice-cold Tris-HCl buffer, dipped in ice-cold water, dried and exposed to Biomax MR films for 1 week.

Quantification of autoradiographic labelling

Standard radioactive-microscales (GE Healthcare) were exposed on each autoradiographic film to ensure that labelling densities were in the linear range. The autoradiograms were scanned and densitometry was performed with MCID^{TM} analysis software. Values were expressed in nCi/mg and, after subtraction of non-specific labelling, specific densities of four sections per area were averaged for each mouse.

BEHAVIORAL STUDIES

Locomotor activity

The horizontal (locomotion) and vertical (rearing) activities were individually assessed in transparent activity cages (20 x 15 x 25 cm), with automatic monitoring of photocell beam breaks, located at 1.5 cm (horizontal activity) and 6.5 cm (vertical activity) above the floor (Imetronic, Bordeaux, France). For the dose-response analysis of nicotine and choline, locomotor activities of mice were recorded for 60 min immediately after sub-cutaneous drug administration, between 9:00 a.m. and 2:00 p.m.

Cued version of the water maze

The water maze consists of a circular stainless steel pool (150 cm diameter, 29 cm height) filled to a depth of 16 cm with water at 20-22 °C and made opaque using a white aqueous emulsion (Acusol® OP 301 opacifier, Rohm Ihaas, France). The pool was located in a sound-attenuated and brightly illuminated room. A video tracking system, including an overhead camera connected to an image analyzer and a computer (View Point, France), was used to monitor activity. Mice were trained to find and escape onto a rough stainless steel platform (9 cm diameter), submerged one cm below the water surface and made visible by a small contrasted ball (4.5 cm diameter) fixed 11 cm above the platform. The pool was surrounded by curtains to hide other spatial cues. Both the platform location and the animal starting position were pseudo-randomly changed for each trial. Trials ended when mice climbed onto the platform, with a maximum searching time of 90 s. Mice that did not find the platform were

gently guided and placed on it for 20 s. Animals were trained with two trials per day, for five consecutive days. The first trials were conducted from 10:00 a.m., mice were left undisturbed in their home cage before the second trials at 2:00 p.m. Saline or choline (0.30 mg/kg, free base, s.c.) was injected and mice were tested 15 min later. The mean latency (sec), the mean distance traveled (m), the swimming speed (m/min) and the number of successful trials were calculated for each mouse.

STATISTICAL ANALYSIS

Data from autoradiographic experiments were subjected to factorial one- and two-way analysis of variance (ANOVA), with genotype and area as between-group factors. Significant main effects were further analyzed by post hoc comparison of means using Fisher's exact test. Behavioral data were subjected to factorial two-, three- or four-way ANOVA, with genotype, sex and treatment as between-group factors and time as within-group factor. Significant main effects were further analyzed by comparison of means using Fisher's test. The numbers of successful trials in the water maze tests were compared by the Kolmogorov-Smirnov non-parametric test.

RESULTS

Quantification of the acetylcholine/nicotinic components

Statistical analysis of the density of some cholinergic/nicotinic components showed no significant effect of genotype on ACh esterase (AChE; Fig. 1, Table 1), but a significant effect of genotype on the vesicular ACh transporter (VAChT; genotype: $F_{1,64}$=4.92, p<0.05; genotype x area: $F_{6,64}$=2.17, p=0.05). The density of VAChT was decreased by 33% in the CA1 field of the hippocampus in STOP KO compared with WT mice (p<0.01), with no significant modification in the CA3 field nor in the entire dorsal hippocampus. The density of VAChT was not modified in the other areas tested.

In all areas tested in this study, [^{125}I]-epibatidine at low concentration is a specific ligand of β2* subunits (Marks et al., 2006): its autoradiographic labelling is entirely suppressed in mice lacking the β2 subunit gene (Zoli et al., 1998) and recovered by its re-expression in the ventral tegmental area (Maskos et al., 2005). No variation in β2* nAChR subunit density was found between WT and STOP KO mice (Fig. 1, Table 2). Analysis of the density of α6* subunits showed a significant effect of genotype (genotype: $F_{1,44}$=15.87, p<0.01; genotype x area: $F_{4,44}$=2.85, p<0.05). The density of α6* subunits was significantly decreased in STOP KO mice by 19% in the striatum (p=0.01) and by 33% in the shell part of the nucleus accumbens (p<0.01). It was not modified in the substantia nigra, the ventral tegmental area and the core part of the nucleus accumbens. Analysis of the density of α7 nAChRs showed a significant effect of genotype (genotype: $F_{1,78}$=8.32, p<0.01; genotype x area: $F_{9,78}$=3.02, p<0.01). The density of α7 nAChRs in STOP KO mice was significantly increased by 24% in the dorso-lateral striatum (p=0.05) and in the entire dorsal hippocampus (p<0.05). This latter effect was accounted for by the 51% increase in the CA1 field (p<0.01) and the 67% increase in the CA3 field (p<0.01). Density of α7 nAChRs was not modified in the substantia nigra, the ventral tegmental area, the nucleus accumbens, the medial septum and the cingulate cortex.

These data suggest that the deletion of the protein STOP elicited alterations in the density of ACh markers, restricted to some proteins and to some areas.

Effects of nicotine on the locomotor activity

In order to evaluate the potential consequences of the altered VAChT and nAChR densities, we first studied the locomotor effects of nicotine (Fig. 2). Since male and female mice of each genotype did not show any significant difference in locomotor activity (not shown), their data were pooled. Statistical analysis showed a significant effect of genotype, treatment and time on the horizontal locomotor activity of mice (genotype: $F_{1,1078}$=22.68, p<0.01; treatment:

$F_{6,1078}$=10.17, p<0.01; genotype x treatment: $F_{6,1078}$=2.34, p<0.05; genotype x treatment x time: $F_{66,1078}$=1.29, p=0.05).

Nicotine at 0.5 mg/kg had no significant effect on the horizontal locomotion of WT mice, but significant increased by 130% the locomotor activity of STOP KO mice (genotype: $F_{1,132}$=10.26, p<0.01; Figs. 2A, B, C). At doses greater than 0.5 mg/kg, nicotine elicited a dose-dependent hypolocomotor effect in both WT and STOP KO mice (genotype: $F_{1,396}$=14.89, p<0.01; treatment: $F_{2,396}$=55.70, p<0.01; genotype x treatment: $F_{2,396}$=5.10, p<0.05), with a significant effect of genotype at 1.0 mg/kg (genotype: $F_{1,132}$=15.77, p<0.01). Analysis of the dose-effect of nicotine gave an ID50%=1.6 mg/kg and a maximal inhibition (Imax)=100% for mice of both genotypes (Fig. 2C).

Statistical analysis of the nicotine effects on the vertical activity showed a significant effect of genotype and treatment (genotype: $F_{1,1078}$=8.40, p<0.01; treatment: $F_{6,1078}$=8.03, p<0.01; Fig. 2D). Effect of nicotine at 0.1 mg/kg was significantly different in WT and STOP KO mice (genotype: $F_{1,132}$=14.87, p<0.01). At doses greater than 0.5 mg/kg, nicotine elicited a dose-dependent hypolocomotor effect in WT and STOP KO mice (treatment: $F_{2,396}$=11.84, p<0.01), with an ID50%=1.0 mg/kg for WT mice and 1.1 mg/kg for mutant mice and an Imax=100% in both mouse lines. Finally, the absence of effect of nicotine at 0.25 mg/kg on the vertical locomotor activity of STOP KO mice demonstrated that the increased horizontal locomotion induced by nicotine at this dose was not due to an inhibition of vertical activity.

These results suggest that STOP KO mice were hypersensitive to the stimulant locomotor effect of nicotine, but not to its depressant effect.

Effects of choline on the locomotor activity

Statistical analysis showed a significant effect of genotype and choline treatment on the horizontal locomotor activity of mice (genotype: $F_{1,572}$=19.65, p<0.01; treatment: $F_{3,572}$=2.54, p=0.06; genotype x treatment: $F_{3,572}$=2.81, p<0.05; Fig. 3). Choline 0.3 mg/kg had no significant effect on the horizontal locomotion of WT mice and STOP KO mice. At higher doses, choline had no effect on the locomotion of WT mice, but significantly increased the locomotor activity of STOP KO mice, by 116% at 3.0 mg/kg (genotype: $F_{1,143}$=7.55, p<0.05) and by 166% at 30 mg/kg (genotype: $F_{1,154}$=15.51, p<0.01).

These results suggest that choline at relatively high doses had stimulant locomotor effect in mutant mice.

Effect of choline on performance of WT and STOP KO mice in the cued version of the water maze

Animals of both genotypes received saline or 0.30 mg/kg choline, 15 min before each trial (Fig. 4). At this time and dose, choline had no effect on the swimming

speed of either WT or STOP KO mice (not shown). Analysis of performance of mice showed a significant effect of genotype and treatment between day 2 and day 5 on the latency (genotype: $F_{1,105}=5.03$, $p<0.05$; treatment: $F_{1,105}=11.88$, $p<0.01$; genotype x treatment: $F_{1,105}=4.01$, $p=0.05$; genotype x treatment x training: $F_{3,105}=2.89$, $p<0.05$) and the distance (genotype: $F_{1,105}=9.30$, $p<0.01$; treatment: $F_{1,105}=15.09$, $p<0.01$; genotype x treatment: $F_{1,105}=3.73$, $p=0.06$; genotype x treatment x training: $F_{3,105}=7.85$, $p<0.01$).

The performance of STOP KO mice were significantly lower, compared with that of WT mice. The latency time of mutant mice was longer by 27% between day 2 and day 5 (genotype: $F_{1,51}=4.59$, $p<0.05$). Mutant mice traveled more distance to reach the platform, by 14% between day 2 and day 5 (genotype x training: $F_{3,51}=3.92$, $p<0.05$) and by 26% between day 3 and day 5 (genotype: $F_{1,34}=5.56$, $p<0.05$). Finally, the proportion of successful trials of STOP KO mice was significantly lower than WT mice ($Chi2=3.20$, $p<0.05$).

Choline treatment had no effect on the latency and distance traveled by WT mice, but significantly increased their proportion of successful trials ($Chi2=4.35$, $p<0.05$). In contrast, choline treatment improved the performance of STOP KO mice, assessed by the three parameters. Compared with saline-treatment between days 2 and 5, choline decreased the latency time by 27% (treatment: $F_{1,51}=8.51$, $p<0.01$) and the distance swum by 17% (treatment: $F_{1,51}=15.51$, $p<0.01$) and increased the proportion of successful trials of STOP KO mice ($Chi2=3.76$, $p<0.05$). Finally, performance of choline-treated STOP KO mice and saline-treated WT mice were no longer different (genotype: latency $F_{1,48}=1.55$, distance $F_{1,48}=0.21$, number of successful trials $Chi2=0.20$).

Our data show that choline at low doses improved the impaired performance of STOP KO mice in the cued Morris test to a higher extent than WT mice.

DISCUSSION

The constitutive inactivation of the ubiquitous protein STOP elicited modified VAChT and nicotinic receptor densities, in an area- and sub-type dependent-manner. The altered densities of ACh markers have functional consequences, since STOP KO mice were hypersensitive to nicotine- or choline-induced locomotor hyperactivity. Interestingly, the impaired performance of mutant mice in the cued version of the Morris water maze were greatly improved by choline at low dose, to a higher extent than WT mice.

In STOP KO mice, while the density of the ACh inactivating enzyme AChE was not modified in any brain areas, the density of the vesicular ACh transporter VAChT was selectively decreased in the granular layer of the hippocampal CA1 field, but not in the CA3 field and in the median septum containing the somas of these cholinergic neurons. These results suggest that modulation of VAChT expression depends on a specific regional/neuronal environment. Importantly, a two-fold decrease in glutamatergic synaptic vesicles and in alterations of the long term potentiation and depression have been found in CA1 field of mutant mice, but not in CA3 field, also suggesting area-specific modifications (Andrieux et al., 2002). The decreased density of VAChT could result from a decreased number of cholinergic terminals and/or vesicular transporters per vesicle and/or vesicles per terminal. In any event, reduction in VAChT density might result in decreased ACh release. Interestingly, modified VAChT density was found in terminals of septo-hippocampal cholinergic neurons thought to be involved in learning and memory (Leanza et al., 1995).

The density of β2* nAChRs was not modified in any area tested. In contrast, α6 nAChR subunit density was largely decreased in the striatum and the shell part of the nucleus accumbens and α7 nAChR density was greatly increased in the dorso-lateral striatum and in some layers of the dorsal hippocampus, but not in the median septum. Such opposite effects on α6* and α7 nAChR density were also found in DA transporter (DAT) KO mice, a constitutively hyperdopaminergic mouse line pertinent to schizophrenia (Giros et al., 1996; Gainetdinov et al., 2001; Weiss et al., 2007b). Altogether our results, as well as previous data (Andrieux et al., 2002; Brun et al., 2005), indicate that the deletion of the ubiquitously expressed protein STOP elicits selective alterations in various neurotransmitter systems. Indeed, these alterations depend on both proteins and brain areas. Further studies should be necessary to know if these restricted alterations are related to differential functions of STOP protein and/or other STOP-like proteins during mouse cerebral ontogenesis.

In order to evaluate the potential consequences of the modified expression of cholinergic/nicotinic markers, we characterized two behaviors involving nicotinic neurotransmission. We chose nicotine-induced locomotor activity in relation with nicotinic alterations in the striatum and the nucleus accumbens and learning performance in the Morris water maze in relation with nicotinic alterations in the hippocampus and the striatum.

Mutant mice were hypersensitive to the stimulating locomotor effect of nicotine, in agreement with previous results with amphetamine in STOP KO mice (Brun et al., 2005) and with nicotine in DAT KO mice (Weiss et al., 2007a,b). In rodents, nicotine can increase locomotion in a familiar environment probably via activation of DAergic pathways through distinct nAChRs (Menzaghi et al., 1997; Picciotto et al., 2001). In our case, habituation of STOP KO mice to actimeter cages was not different from their WT counterparts (compare the time course of basal activities after saline administration, Fig. 1A and B). The hypersensitivity of STOP KO mice to the stimulating locomotor effect of nicotine could be due to an enhanced DA release effect, since nicotine induces DA release in the nucleus accumbens, as all drugs of abuse (Pontieri et al., 1996). The pertinence of this hypothesis is reinforced by recent studies showing that the DA efflux evoked by electrical stimulation is highly increased in the nucleus accumbens of STOP KO mice (Brun et al., 2005). Studies of various nAChR subunit KO mice indicate that nicotine exerts its DA releasing effect mainly via β2* nAChRs (Picciotto et al., 1998; Maskos et al., 2005). The density of nicotinic β2 subunits was not modified in STOP KO mice but their sensitization state was not determined. Increased striatal α7 nAChRs, if they were functional, could also participate in nicotine-induced DA release, taking into account the localization of α7 nAChRs on glutamatergic terminals in DAergic areas (Kaiser and Wonnacott, 2000). Finally, other non-cholinergic receptors could mediate the hypersensitivity of STOP KO mice to the stimulating effect of nicotine.

Numerous studies in humans and rodents demonstrate the pro-cognitive effect of nicotine, via α7 nAChR stimulation (Adler et al., 1998; Levin, 2002; Levin et al., 2002; Young et al., 2004; Buccafusco et al., 2005). The highly increased α7 nAChR density in the hippocampus and the dorso-lateral striatum of STOP KO mice prompted us to test the cognitive performance of mice in the Morris water maze. Since the higher increase in density of α7 nAChRs was found in the hippocampus of STOP KO mice, we first assessed spatial learning. In this paradigm, WT mice did not learn to find the hidden platform even after eight days of training (not shown). The poor performance could result from the genetic background of the mice (50:50 BALBc/129SvPas), since both parental strains are poor performers (van Dam et al., 2006). Accordingly, we employed the cued platform version of the water maze (place task), in which mice were

trained to swim to a platform mounted with a visible cue and placed in a different location on each trial. Such learning is thought to be mediated by the basal ganglia, in particular the dorsal striatum (Packard and Knowlton, 2002), where α7 nAChR density was also increased in STOP KO mice. In cued version, STOP KO mice showed impaired performance compared with WT littermates. Contrary to WT mice whose performance improved during the successive trials, mutant mice did not learn to find the platform during the five-day training period. Consequently, we tested the effect of choline, a preferential α7 nAChR agonist at low doses (Papke et al., 1996; Matsuyama and Matsumoto, 2003). We first assessed its effect on locomotor activity. Whereas choline had no effect on the locomotion of WT mice, it increased the activity of STOP KO mice. However, this effect was elicited by choline at relatively high doses, for which it can serve as substrate for ACh synthesis and can stimulate non-α7 nicotinic and muscarinic AChRs. Thus, we chose the lowest ineffective dose of choline (0.3 mg/kg) to test its potential effect on learning in WT and STOP KO mice. At this dose, choline had no effect on the swimming speed of mice.

Administered 15 minutes before trials, choline improved performance of both WT and STOP KO mice, but to a higher extent in mutant mice. Indeed, choline increased the proportion of successful trials of WT mice, but had no effect on their latency and distance traveled. In contrast, choline significantly decreased the latency and distance swam by STOP KO mice and increased their number of successful trials. Altogether, the performance of choline-treated STOP KO mice were no longer different from saline-treated WT mice. The pro-cognitive effect of choline is thought to be a central one, since choline did not affect the swimming speed of WT and STOP KO mice and since its effect was rather selective for mutant mice. The preferential pro-cognitive effect of choline in STOP KO mice can be related to the pro-cognitive effect of nicotine in DAT KO mice, both in the spatial and cued versions of the Morris water maze (Weiss et al., 2007a).

A recent study demonstrates that stimulation of both α4β2 and α7 nAChRs, by epibatidine and choline respectively, are essential for full-development of long-term potentiation in the mouse dentate gyrus (Matsuyama and Matsumoto, 2003). Reports also indicate that working memory in rats is improved by stimulation or co-stimulation of α4β2 and α7 nAChRs in the hippocampus and/or amygdala (Levin, 2002; Levin et al., 2002). Various studies also implicate cholinergic neurotransmission in the dorsal striatum in learning tasks that associate a stimulus with a response (Packard and Knowlton, 2002). The failure of STOP KO mice to learn the cued version of the water maze can hardly be explained by the increased α7 nAChR density in the dorso-lateral striatum. This suggests that the up-regulated α7 nAChRs are non-functional, being desensitized or under-stimulated. The latter hypothesis seems more likely:

first, in hippocampus, another area implicated in cognitive behavior, up-regulated α7 nAChRs were associated with decreased density of VAChT, suggesting reduced ACh release in that region. Second, choline improved the impaired performance of STOP KO mice. The pro-cognitive effect of choline at low dose on STOP KO mice, probably via α7 nAChR stimulation, was reinforced by recent data of our lab showing that impaired performance of DAT KO mice in the cued version of the water maze was improved by DMXB-A, another α7 nAChR agonist (El Khoury et al., in preparation). Another non-exclusive hypothesis to explain the impaired performance of STOP KO mice would be an imbalance between β2* (not modified) and α7 (increased) nAChR densities in some brain areas, in agreement with previous reports (Levin et al., 2002).

Finally, the beneficial effect of choline on performance of WT mice and its higher effect in STOP KO mice can be due to a hypersensitive response of mutant mice to the pro-cognitive effect of choline. They are also reminiscent of studies on cognitive performance in humans. Although the pro-cognitive effect of nicotine in healthy humans is debated, there is clearer evidence for nicotinic modulation of cognitive dysfunction in several neuropsychiatric disorders, including Alzheimer's and Parkinson's diseases, ADHD and schizophrenia (Sacco et al., 2004).

In conclusion, our present study shows that the deletion of the microtubule-regulating protein STOP can induce significant adaptation of the cholinergic/nicotinic neurotransmission. Paradoxically, the lack of STOP, an ubiquitously expressed protein, induced alterations of cholinergic/nicotinic transmission in a protein- and area-dependent manner. Altogether, our data suggest that nicotinic neurotransmission is probably deficient in STOP mutant mice and that these mice may represent a useful experimental model for studying cognitive deficits and testing the potential benefits of therapeutic treatments by nicotine or nicotinic agonists.

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Table 1 Density of cholinergic markers in WT and STOP KO mice

Marker	Area	WT	KO	% KO/WT
AChE	SNC	(4) 235±7	(4) 263±14	+12% ns
	SNR	(5) 167±9	(5) 177±10	+6% ns
	VTA	(3) 269±13	(6) 290±11	+8% ns
	Striatum	(6) 335±12	(5) 331±12	-1% ns
	Acc Shell	(4) 352±23	(4) 356±16	+1% ns
	Acc Core	(3) 295±15	(4) 294±22	0%
	Dorsal Hipp	(6) 57±1	(5) 60±3	+5% ns
	CA1	(5) 90±3	(5) 88±4	-2% ns
	CA3	(6) 71±4	(6) 73±5	+3% ns
VAChT	SN	ND	ND	
	VTA	ND	ND	
	Striatum	(6) 182±10	(6) 181±7	-1% ns
	Acc Shell	(6) 224±18	(6) 233±20	+4% ns
	Acc Core	(6) 156±9	(6) 166±8	+6% ns
	Dorsal Hipp	(5) 39.5±1.6	(5) 32.8±1.6	-17% ns
	CA1	(5) 30.6±1.3	(5) 20.6±1.4	-33% **
	CA3	(5) 46.8±2.9	(5) 48.7±5.1	+4% ns
	Septum	(6) 180±13	(6) 154±15	-14% ns

Densities are the means ± SEM of immunolabeling in nCi/mg. The number of mice is indicated in parentheses. ND, not detectable; Acc, nucleus accumbens; AChE, acetylcholine esterase; CA1, CA3, CA1 and CA3 fields of hippocampus; Dorsal Hipp, dorsal hippocampus; SN, substantia nigra; SNC, substantia nigra, pars compacta; SNR substantia nigra, pars reticulata; VAChT, vesicular acetylcholine transporter; VTA, ventral tegmental area.
Fisher's test: **$p<0.01$.

Table 2 Density of nicotinic receptors in WT and STOP KO mice

Receptor	Area	WT	KO	% KO/WT
β2*	SN	(5) 126±5	(4) 119±11	-6% ns
	VTA	(5) 333±16	(4) 313±13	-6% ns
	Striatum	(6) 242±12	(6) 215±18	-11% ns
	Acc Shell	(6) 193±11	(6) 211±2	+9% ns
	Acc Core	(5) 166±9	(5) 180±11	+8% ns
	Dorsal Hipp	(6) 133±10	(6) 126±6	-5% ns
	Cing Cx	(5) 225±5	(4) 216±10	-4% ns
α6*	SNC	(5) 155±9	(4) 156±9	0%
	VTA	(4) 225±27	(5) 210±21	-7% ns
	Striatum	(6) 179±6	(6) 145±4	-19% **
	Acc Shell	(6) 225±7	(6) 151±8	-33% ***
	Acc Core	(6) 192±5	(6) 173±10	-10% ns
α7	SN	(5) 50.0±4.9	(5) 48.0±8.3	-4% ns
	VTA	(6) 143±11	(4) 132±20	-8% ns
	Str DL	(5) 65.0±4.7	(4) 80.4±2.4	+24% *
	Acc Shell	(5) 15.3±1.4	(5) 14.1±2.4	-8% ns
	Acc Core	(4) 16.1±1.4	(4) 17.0±2.3	+6% ns
	Dorsal Hipp	(6) 74.2±3.3	(5) 92.2±10.3	+24% *
	CA1	(6) 44.1±4.2	(4) 66.8±4.2	+51% **
	CA3	(6) 58.3±3.6	(5) 97.5±14.8	+67% ***
	Septum	(5) 40.1±3.7	(5) 38.6±2.6	-4% ns
	Cing Cx	(5) 35.2±2.4	(4) 32.5±2.8	-8% ns

Densities are the means ± SEM of specific radioligand binding in nCi/mg. The number of mice is indicated in parentheses. Acc, nucleus accumbens; CA1, CA3, CA1 and CA3 fields of hippocampus; Cing Cx, cingulate cortex; Dorsal Hipp, dorsal hippocampus; SN, substantia nigra; SNC, substantia nigra, pars compacta; Str DL, dorso-lateral striatum; VTA, ventral tegmental area.
Fisher's test: *$p<0.05$; **$p<0.01$; ***$p<0.001$.

LEGENDS TO FIGURES

Fig. 1: Autoradiographic representation of acetylcholine esterase (AChE), vesicular acetylcholine transporter (VAChT), β2*, α6* and α7 nicotinic receptors in WT and STOP KO mice.

Fig. 2: Hypersensitivity of STOP KO mice to the stimulant locomotor effect of nicotine.

Time course of the horizontal activity of WT (A) and STOP KO (B) mice after s.c. administration of saline (Sal) or nicotine (Nic) at increasing doses. Means ± SEM of photocell counts over a 5 min period. Dose-response effects of nicotine administration on the horizontal (C) and vertical (D) locomotor activities of WT and STOP KO mice. Data represent means ± SEM of photocell counts over a 60 min period, expressed as percentages of respective basal activities (65.5±9.0 and 63.3±7.0 counts/60 min for horizontal activity of WT and STOP KO, respectively; 299±60 and 246±55 counts/60 min for vertical activity of WT and STOP KO mice, respectively). Number of mice in parentheses: Sal (15 WT, 13 KO), Nic (7 WT or KO per dose).
Fisher's test: ** $p<0.01$, comparison between genotypes.

Fig. 3: Stimulant locomotor effect of choline in STOP KO mice.

A: time course of the horizontal activity of WT and STOP KO mice after s.c. choline administration at various doses. Means ± SEM of photocell counts over a 5 min period for 7-8 mice per dose. B: dose-response effects of choline administration on the horizontal locomotor activity of WT and STOP KO mice. Data represent means ± SEM of photocell counts over a 60 min period, expressed as percentages of respective basal activity (61.6±6.0 and 35.7±8.0 counts/60 min for horizontal activity of WT and STOP KO, respectively; 101±36 and 151±51 counts/60 min for vertical activity of WT and STOP KO mice, respectively).
Fisher's test: ** $p<0.01$, comparison between genotypes.

Fig. 4: Choline improved the performance of STOP KO mice in the cued version of water maze.

15 Min before each trial, mice received s.c. administration of saline (Sal) or choline 0.3 mg/kg (Chol). Performance of mice are expressed as latency (seconds, s) and mean distance traveled (meters, m) and proportion of successful trials. Values are means ± SEM for 9 (WT Sal), 10 (KO Sal and KO Chol) and 11 (WT Chol) mice.

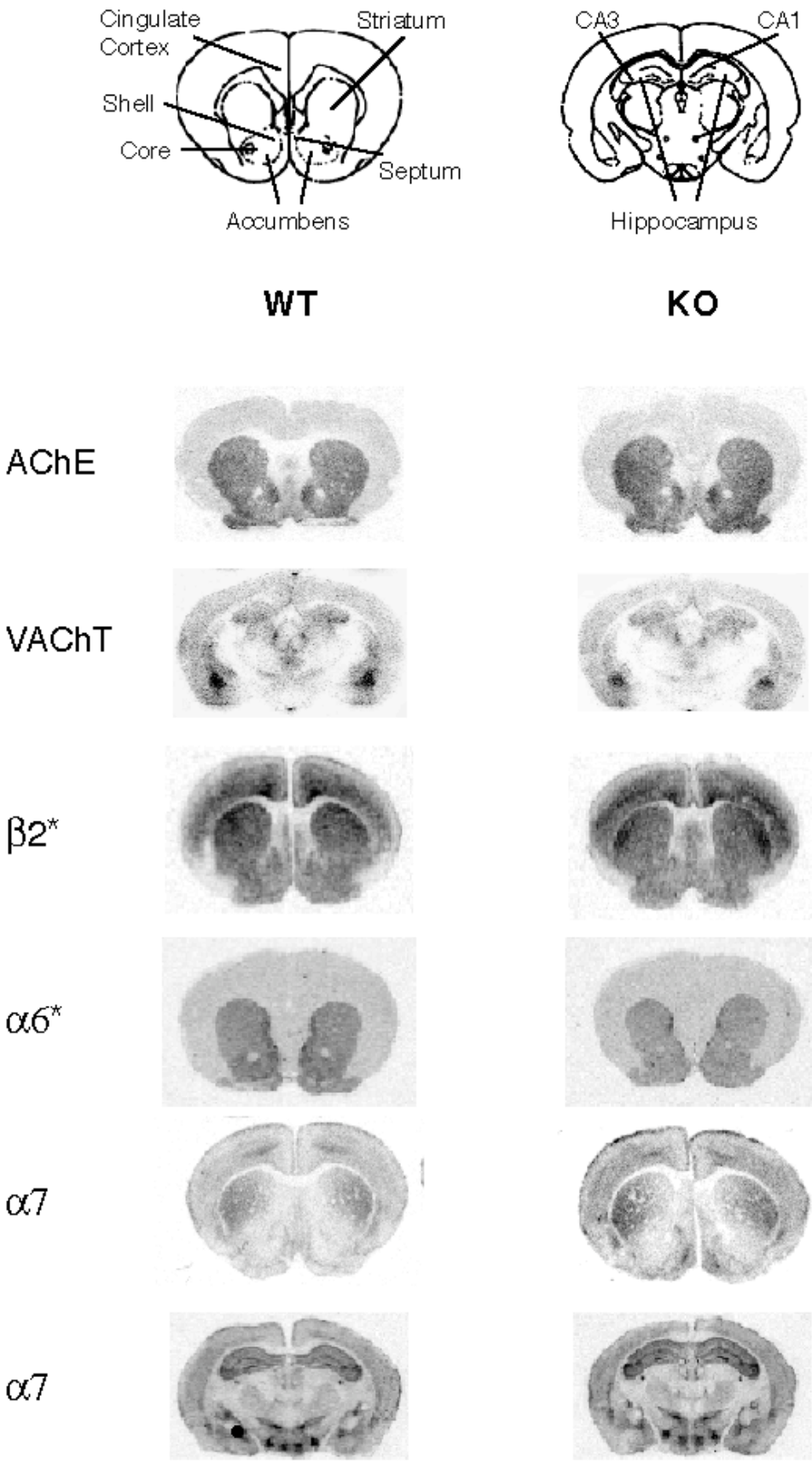
Cingulate Cortex
Striatum
Shell
Core
Septum
Accumbens
CA3
CA1
Hippocampus
WT
KO
AChE
VAChT
β2*
α6*
α7
α7

Figure 1

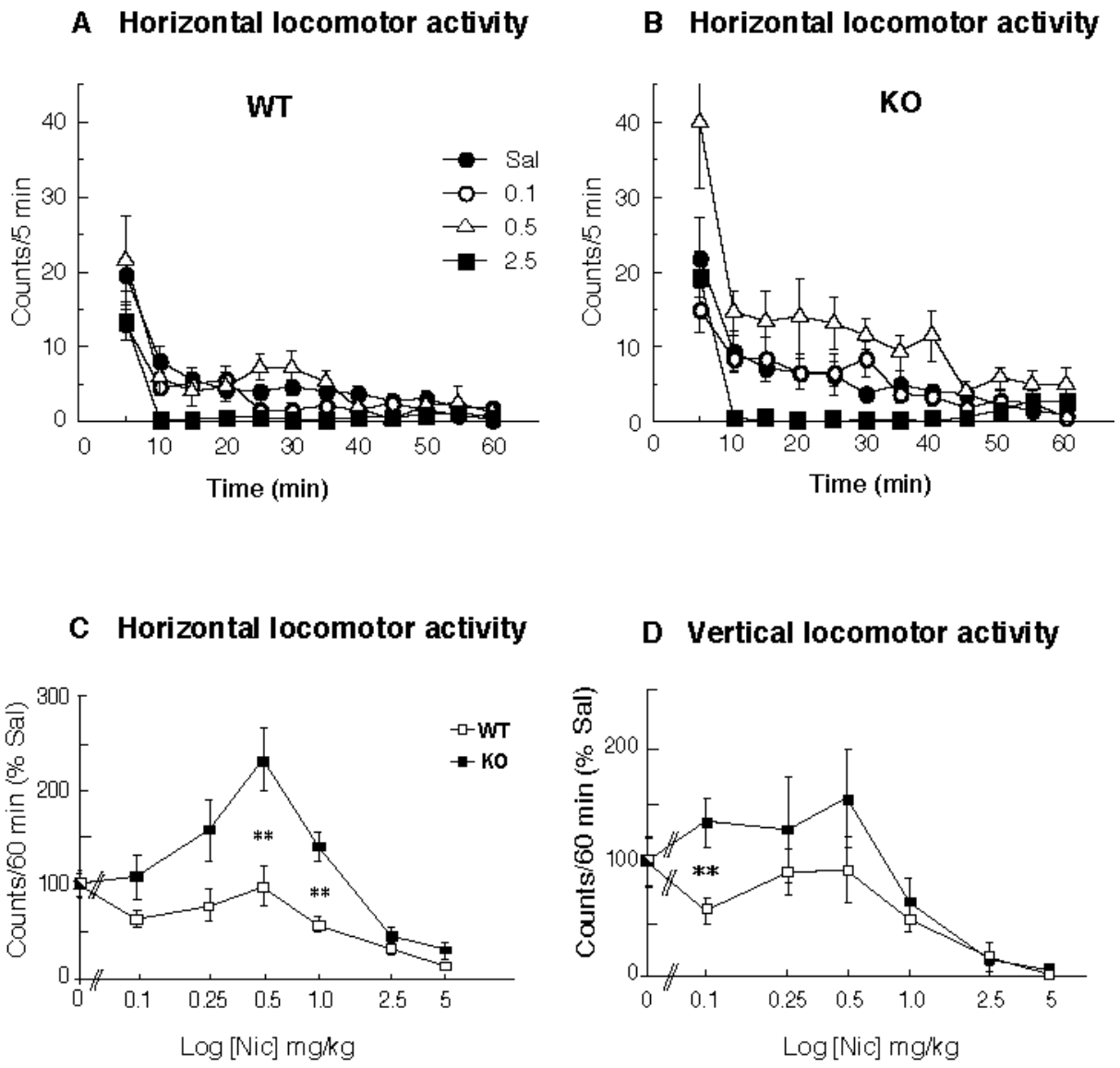
A Horizontal locomotor activity
WT
Sal
0.1
0.5
2.5
Counts/5 min
Time (min)
B Horizontal locomotor activity
KO
Counts/5 min
Time (min)
C Horizontal locomotor activity
WT
KO
Counts/60 min (% Sal)
**
**
Log [Nic] mg/kg
D Vertical locomotor activity
Counts/60 min (% Sal)
**
Log [Nic] mg/kg

Figure 2

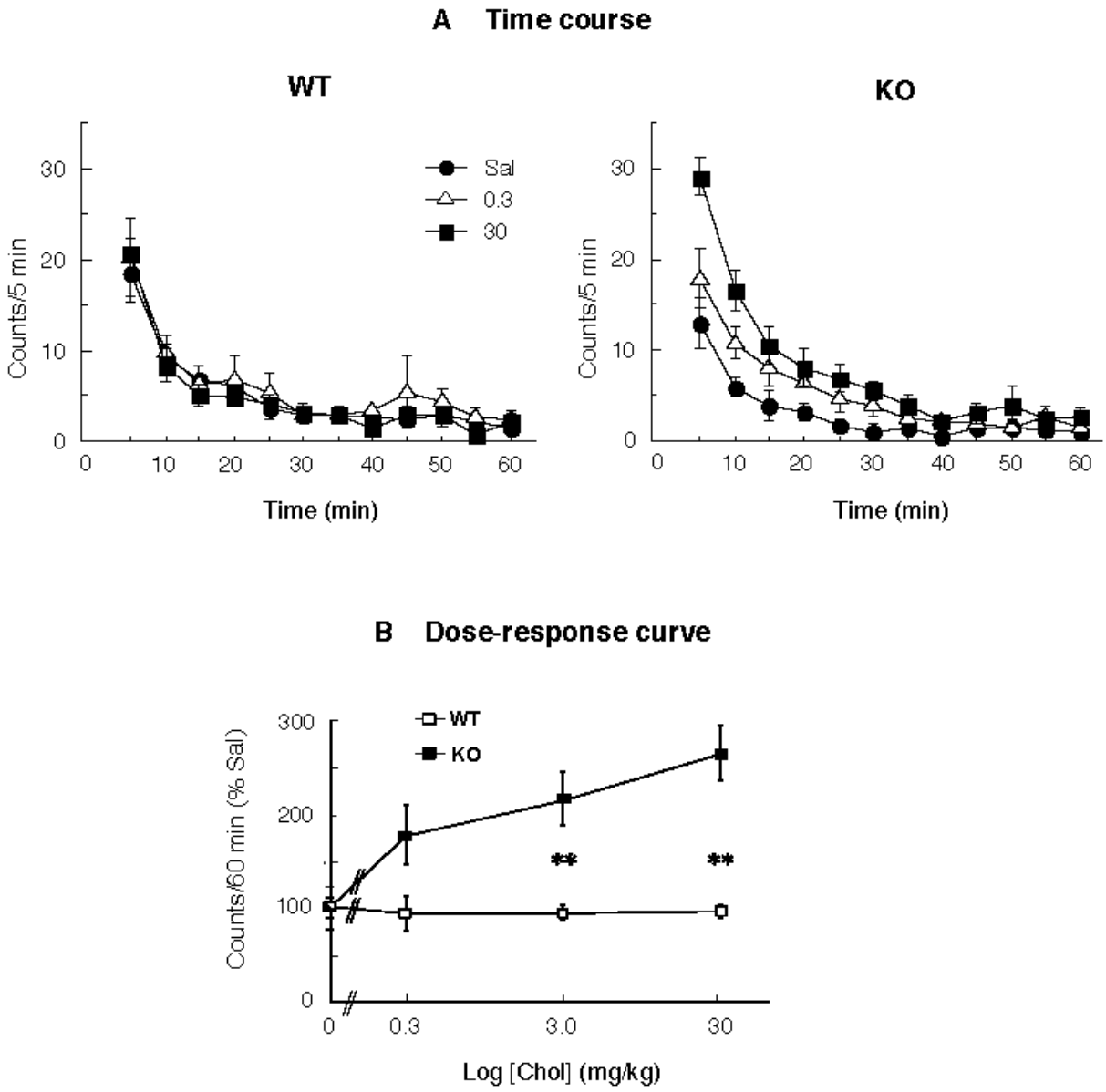
A Time course
WT
KO
Sal
0.3
30
Counts/5 min
Time (min)
B Dose-response curve
WT
KO
Counts/60 min (% Sal)
Log [Chol] (mg/kg)
**
**

Figure 3

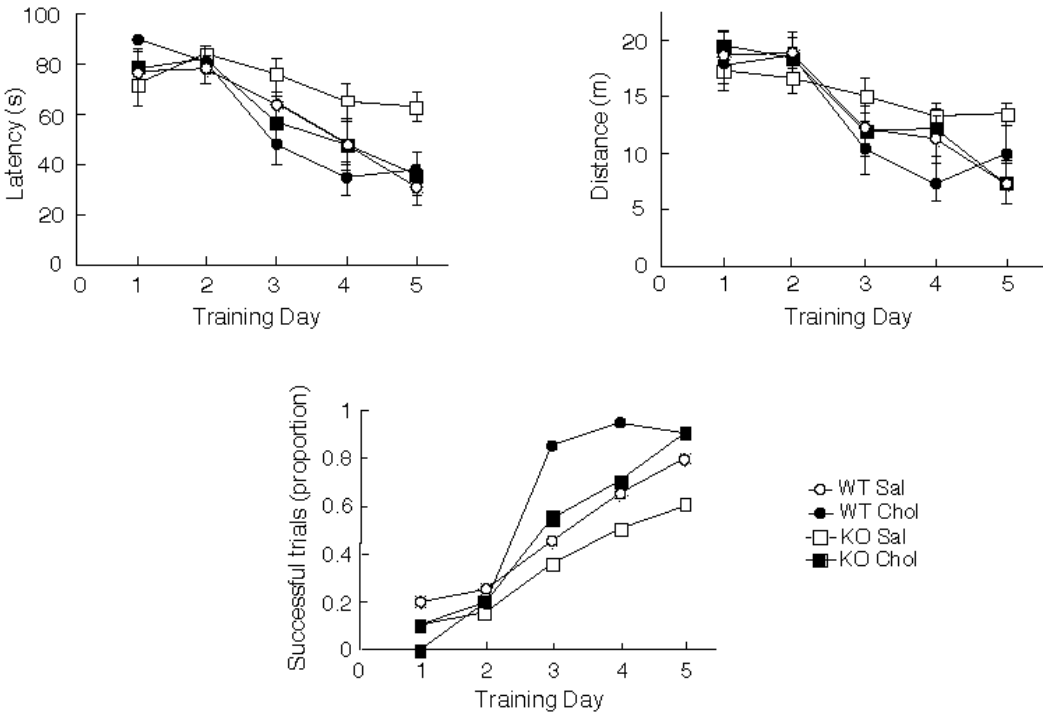
Latency (s)
Training Day
Distance (m)
Training Day
Successful trials (proportion)
Training Day
WT Sal
WT Chol
KO Sal
KO Chol

Figure 4