

Reduction in Acetylcholine Release in the Hippocampus of Dopamine D5 Receptor-Deficient Mice

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Activation of the dopamine D₁-like receptor stimulates acetylcholine (ACh) release in the hippocampus, apparently through the molecularly defined d5 receptor. In the present study, we used a transgenic mouse completely deprived of functional d5 receptor (d5^{-/-}) to confirm the role and elucidate the possible function of the d5 receptor subtype on hippocampal cholinergic neurotransmission. ACh release was measured using *in vivo* microdialysis in the mouse dorsal hippocampus of 4 months old homozygous (d5^{-/-}), heterozygous (d5^{+/-}), and the wild-type (d5^{+/+}) littermates. Using the no net flux technique, a significant reduction in basal hippocampal ACh level was found in the d5^{-/-} compared to d5^{+/-} and d5^{+/+} mice. Moreover, the administration of SKF 38393, a D₁-like receptor agonist, systemically (2.0 and 10.0 mg/kg ip), or locally through the dialysis probe (10 and 50 μM), produced a dose-dependent enhancement of ACh release in the d5^{+/+}, a moderate stimulation in the d5^{+/-} but had no effect in the d5^{-/-} mice. Quantitative receptor autoradiography revealed significant increases in M₁-like but not in M₂-like muscarinic receptor binding sites in the hippocampal formation. These results confirm and extend the role of the d5 receptor in the modulation of hippocampal ACh release and provide evidence for long-term alteration of hippocampal cholinergic neurotransmission resulting from the absence of the d5 receptors including chronically reduced ACh release and change in M₁-like receptor levels.

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INTRODUCTION

Dopamine (DA) exerts a modulatory role on acetylcholine (ACh) release in the hippocampus (Day *et al*, 2001; Hersi *et al*, 1995a, b). Interactions between these two neurotransmitter systems play key roles in learning and memory (Levin *et al*, 1990) and these systems, particularly the cholinergic innervation, are severely affected in Alzheimer's Disease (Araujo *et al*, 1988; Auld *et al*, 2002; Davies and Maloney, 1976; Perry *et al*, 1992). Hippocampal cholinergic nerve terminals originate from the medial septum and the vertical limb of the diagonal band of Broca and project to the hippocampal formation via the fimbria fornix (Mesulam *et al*, 1983; Woolf, 1991). The hippocampus also receives dopaminergic projections arising mainly from the ventral tegmental area with little contribution of the substantia nigra, pars compacta (Gasbarri *et al*, 1994; Verney *et al*, 1985).

Numerous studies demonstrated that DA stimulates hippocampal ACh release through the activation of D₁-like receptors (Day and Fibiger, 1994; Imperato *et al*, 1993), most probably through receptors located on hippocampal cholinergic nerve terminals (Hersi *et al*, 1995a). Moreover, behavioral studies revealed that peripheral administration of a D₁-like agonist improved cognitive performance in rats (Steele *et al*, 1996, 1997), reduced age-related deficits in spatial memory in mice (Bach *et al*, 1999), and attenuated learning deficits in aged memory impaired rats possibly through the stimulation of hippocampal ACh release (Hersi *et al*, 1995b).

The D₁-like receptor subfamily includes the molecularly defined d1 and d5 subtypes (Deary *et al*, 1990; Sunahara *et al*, 1990, 1991; Zhou *et al*, 1990), which are both expressed in the hippocampus (Bergson *et al*, 1995; Tiberi *et al*, 1991). These two subtypes share approximately 50% sequence homology (80% in the transmembrane domains), which translates into a highly similar pharmacological profile (Seeman and Van Tol, 1993; Sunahara *et al*, 1991; Tiberi *et al*, 1991). No drugs are currently able to discriminate between d1 and d5 receptors. Recently, an antisense oligonucleotide approach revealed that the d5 (but not d1) receptor was the subtype likely involved in the modulation of hippocampal ACh release (Hersi *et al*, 2000).

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In the present study, we used an *in vivo* microdialysis technique in mice lacking functional d5 receptors (d5^{-/-}) to elucidate further the role of the receptor subtype in hippocampal ACh release. The results reported here confirm the tonic role of the d5 receptor in the dopaminergic modulation of hippocampal ACh release and demonstrated long-term alterations in M₁-like muscarinic receptors in the knockout animal model.

MATERIALS AND METHODS

Materials

The d5^{-/-} mouse with a genetic background of 129/SvJ1 and C57BL/6J was generated as described earlier (Hollon *et al*, 2002) and provided by one of the authors (D Sibley). Immunohistochemical detection of d5 receptors revealed that the frontal cortex in the d5^{-/-} mouse was, as expected, completely devoid of this receptor (Hollon *et al*, 2002). Animals were reproduced in our animal facilities by standard inbreeding. Females were isolated to give birth and pups were weaned at postnatal day 21. The genotype of each mouse was determined using a PCR technique as previously described (Hollon *et al*, 2002). Experiments were performed in homozygous d5^{-/-}, heterozygous (d5^{+/-}), and wild-type (d5^{+/+}) littermates. Homozygous d5^{-/-} mice are viable, develop normally and are fertile and capable of reproduction (Hollon *et al*, 2002). These animals were housed two to six per cage at room temperature and on a 12-h light/dark schedule. The animals were fed *ad libitum* with standard lab chow and tap water up to the time of the experiments, according to protocols and guidelines approved by the McGill University Animal Care Committee and the Canadian Council for Animal Care (CCAC).

Ketamine, xylazine, and acepromazine were obtained from Vetrepharm (Belleville, ON), Novopharm (Toronto, ON), and Wyert-Ayerst (Montréal, PQ), respectively. The dialysis membrane used was made from AN69 Hospal Industry (molecular weight cut off 60 000 Da, i.d. = 0.22 mm, o.d. = 0.31 mm; Hospal, Lyon, France). The membrane was covered with epoxy glue along its whole length except for 4 mm corresponding to the area of dialysis. 1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol (SKF 38393) and neostigmine bromide were obtained from Sigma RBI (Oakville, ON).

The microliter syringe pump was from Harvard Apparatus Inc. (South Natick, MA), ESA 580 HPLC dual piston pump from ESA (Chelmsford, MA), CMA 260 degasser from Cargenie Medicin (Stockholm, Sweden), Lichrosorb NH₂ (10 µm) was from Merck (Darmstadt, Germany), platinum and Ag/AgCl reference electrodes (Antec VT-03/Decade) from Decade (Leiden, the Netherlands), and column hardware and packing materials were from Chrompack (Raritan, MA). [³H]SCH 23390 (73.0 Ci/mmol), [³H]pirenzepine (79.3 Ci/mmol), and [³H]AFDX-384 (137.0 Ci/mmol) were from New England Nuclear (Boston MA) and tritium-sensitive films from Amersham (Oakville, ON).

All other reagents and chemical for the *in vivo* microdialysis, HPLC, and receptor autoradiography were obtained from Sigma Chemical Co (St Louis, MO).

Probe Implantation

The microdialysis probe was implanted in the dorsal hippocampus according to procedures described by our group for rats with minor modifications (Day *et al*, 2001; Hersi *et al*, 1995a; Kitaichi *et al*, 1999). Male mice, 4-month old, were anesthetized using a mix of ketamine (100 mg/kg), xylazine (20 mg/kg), and acepromazine (10 mg/kg) administered intramuscularly. Animals were placed in a stereotaxic apparatus. The skull was uncovered and lateral muscle displaced to expose parietal bone. One hole (about 2 mm diameter) was drilled on each side. The dialysis transverse probes were inserted through the holes at the level of the dorsal hippocampus, 2.2 mm posterior to bregma and 2.0 mm below the surface of the skull (Franklin and Paxinos, 1997). After insertion, the ends of the dialysis tube were connected to a stainless steel cannulae using epoxy glue. Steel cannulae were secured on the top of the skull using dental cement and fixed on the skull with a screw. *In vivo* microdialysis were performed 48 h after surgery.

In Vivo Microdialysis

At the beginning of each dialysis experiment, animals were placed in a lidless cage and the probes connected to a microliter syringe pump in a manner as to allow mice to move freely in the cage. The dialysis probe was perfused at a constant rate of 2.5 µl/min with a cerebrospinal fluid-like solution containing (in mM) NaCl 123, KCl 3, CaCl₂ 1.3, MgCl₂ 1 NaHCO₃ 23, and sodium phosphate buffer 1 pH 7.4. Neostigmine bromine (10 nM), an acetylcholinesterase inhibitor, was added to the solution. After a 1 h washout period, 20 min dialysate fractions were collected. SKF 38393 (2.0 and 10.0 mg/kg in sodium chloride 0.9% solution) was administered by intraperitoneal (i.p.) injection or locally (10 or 50 µM) through the dialysis probe. Each animal was dialysed once and tested for only one pharmacological treatment.

No Net Flux Method

The no net flux microdialysis technique is used to determine the *in vivo* extracellular concentration of a neurotransmitter (Day *et al*, 2001; Justice, 1993). Another group of animals was perfused with the same cerebrospinal fluid-like solution used in the former experiment without neostigmine bromine but containing a known concentration of ACh (2.5, 5.0, 7.5, or 10.0 nM) [ACh_{in}] perfused through the dialysis tube at a constant rate of 5 µl/min. When [ACh_{in}] is lower than that surrounding the probe, ACh will flow down its concentration gradient into the probe, increasing the resultant ACh concentration measured in the dialysate [ACh_{out}]. When [ACh_{in}] is higher than that surrounding the probe, ACh will flow out of the probe and [ACh_{out}] will be smaller than [ACh_{in}]. By plotting [ACh_{in-out}] as a function of [ACh_{in}], the point where the line cross the x-axis can be extrapolated (ie where [ACh_{in-out}] = 0, where there is no net flux) to give a measure of [ACh] surrounding the probe. For this experiment, dialysate samples were collected every 20 min and assayed for ACh content.

ACh Measurement

ACh content in dialysate fractions was determined using HPLC separation, postcolumn enzymatic reaction, and electrochemical detection (Day *et al*, 2001). ACh was separated from other molecules in the dialysate on a reverse phase column (75 × 2.1 mm) pretreated with sodium lauryl sulfate. From this column, the eluate then passes through an enzyme reactor (10 × 2.1 mm) containing acetylcholinesterase (AChE; EC 3.1.1.7; type VI-S) and choline oxidase (EC 1.1.3.17) covalently bound to gluteraldehyde-activated Lichrosorb NH₂. The separated ACh reacts with the enzymes to give stoichiometric yield of hydrogen peroxide, which is electrochemically detected with a platinum electrode at a potential of +500 mV vs an Ag/AgCl reference electrode. The mobile phase consist of a 0.2 M aqueous potassium phosphate buffer, pH 8.0, containing 1 mM tetramethylammonium hydroxide and is delivered at 0.35–0.45 ml/min and is degassed online. ACh elutes at ~4 min, and the best detection limit of the assay is ~10 fmol/injection. Sample concentrations were calculated by comparison with known standards.

Receptor Autoradiography

Animals were killed by decapitation. Brains were removed and frozen in 2-methylbutane at –40°C and stored at –80°C. Coronal brain sections (20 μm) were cut at –18°C on a cryostat, thaw-mounted on gelatin-coated slides and stored at –80°C until use.

D1-like DA receptors. D1-like receptor binding sites were visualized using [³H]SCH 23390 as described before (Flores *et al*, 1996). Brain sections were preincubated for 10 min at room temperature in buffer containing 50 mM Tris-HCl, pH 7.4, 154 mM NaCl, 1 mM EDTA, and 0.1% of bovine serum albumin. Sections were incubated at room temperature in the same buffer containing 2 nM [³H]SCH 23390 and 30 nM ketanserin to mask possible binding of the ligand to serotonergic 5-HT₂ sites. Consecutive sections were also incubated in presence of 1 μM (+)-butaclamol to ascertain the specificity of the binding. Incubations were terminated by dipping slides into ice-cold buffer followed by two consecutive 10 min washes in buffer and a rapid dip into ice-cold distilled water. Slices were air-dried and autoradiograms were generated by apposing sections alongside tritium standards to tritium-sensitive films for 15 days.

Muscarinic receptors. Muscarinic M₁-like and M₂-like receptor binding sites were visualized using [³H]pirenzepine and [³H]AFDX-384, respectively, as described elsewhere (Vaucher *et al*, 2002). Brain sections were preincubated for 10 min in Krebs buffer (NaCl 120 mM; KCl 4.7 mM; CaCl₂ 2.5 mM; KH₂PO₄ 1.2 mM; MgSO₄ 1.2 mM; glucose 5.6 mM; NaHCO₃ 25 mM, pH 7.4) at room temperature before a 60-min incubation into the same buffer containing either 10 nM [³H]pirenzepine or 2 nM [³H]AFDX-384. Consecutive sections were incubated in the presence of atropine (1 μM) to ascertain the specificity of the labelling. Sections were rinsed three times (4 min each) in ice-cold Tris-HCl buffer (50 mM, pH 7.4) followed by a rapid dip in ice-cold distilled water to removed buffer salts

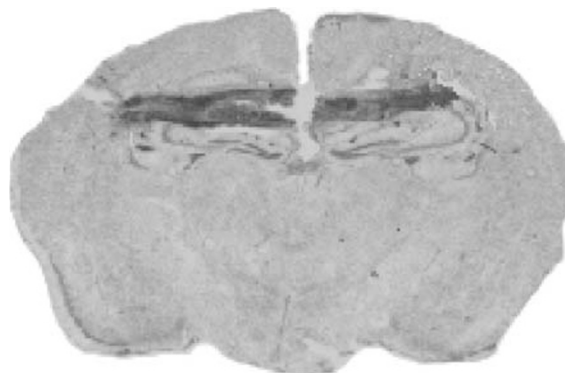


Figure 1 Histological representation of a coronal section from a 4-month old mouse brain stained with cresyl violet demonstrating the location of the microdialysis probe into the dorsal hippocampus.

and sections were air-dried. Autoradiograms were generated by apposing sections alongside tritium standards to tritium-sensitive films for 15 days for [³H]pirenzepine binding and 3 weeks for [³H]AFDX-384 binding.

Films were developed as described before (Quirion *et al*, 1981) and the specific labelling was quantified (fmol/mg tissues wet weight) using a computer-assisted microdensitometric image analysing system (MCID System, Imaging Research Inc., St Catherines, Ontario, Canada).

Histology

After each *in vivo* microdialysis experiment, animals were killed by cervical dislocation and brains were removed and frozen in 2-methylbutane at –40°C and stored at –80°C. Then, 20 μm coronal sections were mounted onto gelatin-coated slides and stained with cresyl violet to confirm probe location into both dorsal hippocampi (Figure 1). Animals whose probe was not implanted in the appropriate area were excluded from the study.

Statistical Analysis

For *in vivo* microdialysis experiments, data were analyzed by a repeated measure three-way analysis of variance (ANOVA), for the main effect of genotype, main effect of pharmacological treatments, main effect of time as repeated measures, and interaction between these three factors. *Post hoc* Bonferonni analyses were conducted when appropriate. For the no net flux experiments, basal ACh release and for receptor autoradiography, significant differences between experimental groups were determined using one-way ANOVA with *post hoc* Dunnett's multiple comparisons with the d5 +/+ genotype, *p* < 0.05 being considered significant.

RESULTS

Determination of Basal Hippocampal ACh Level

Using the no net flux technique, we established the extracellular concentration of ACh into the dorsal hippocampus to 4.4 ± 0.2 nM for the d5 +/+ (*n* = 11), 4.0 ± 0.2 nM for d5 +/- (*n* = 7), and 2.8 ± 0.1 nM for the d5 -/- (*n* = 11) mice (Figure 2). A significant reduction of

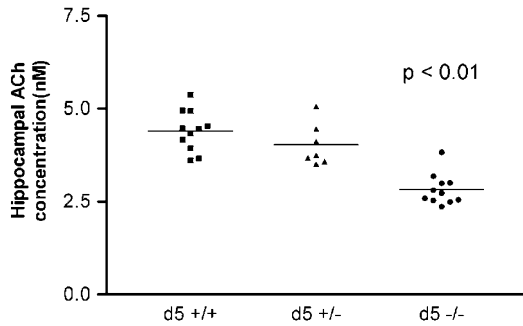


Figure 2 Extracellular levels of ACh as determined using the no net flux method in dopamine d5 knockout ($d5^{-/-}$) heterozygous ($d5^{+/-}$) and wild-type ($d5^{+/+}$) mice. Each point represents individual animals. *Post hoc* analysis revealed significant differences between $d5^{+/+}$ and $d5^{-/-}$ ($p < 0.01$).

basal hippocampal ACh level was hence observed in $d5^{-/-}$ mice compared to $d5^{+/+}$ controls ($F_{(2,26)} = 28.43$, $p < 0.01$), whereas $d5^{+/-}$ had no significant reduction compared to the wild-type mice.

Before pharmacological treatments, the average basal efflux of ACh was 274 ± 28 fmol/50 μ l dialysate in $d5^{+/+}$ ($n = 31$), 239 ± 19 fmol/50 μ l dialysate in $d5^{+/-}$ ($n = 29$), and 180 ± 18 fmol/50 μ l dialysate in $d5^{-/-}$ ($n = 30$). In accordance with data obtained using the no net flux method, a significant reduction of basal hippocampal ACh release was observed in $d5^{-/-}$ mice compared to $d5^{+/+}$ controls ($F_{(2,89)} = 4.44$, $p = 0.015$).

Effect of SKF 38393, a D₁-like Agonist

Three-way ANOVA revealed significant interaction between the main effects of genotype, treatment, and time ($F_{(44,539)} = 2.70$, $p < 0.0001$). Saline administration slightly increased ACh release likely as a consequence of the animal handling. However, no significant differences in saline effects were observed between the three animal genotypes (Figure 3a). The systemic administration of SKF 38393 (2.0 and 10.0 mg/kg ip) differentially affected ACh release in the hippocampus of the animal subgroups studied here. The D₁-like agonist increased hippocampal ACh release to a greater extent at 20, 40, and 60 min, postinjection in $d5^{+/+}$ compared to $d5^{+/-}$ and $d5^{-/-}$ mice at both 2.0 mg/kg (Figure 3b) ($p < 0.0001$) and 10.0 mg/kg (Figure 3c) ($p < 0.0001$).

Moreover, systemic administration of SKF 38393 at both doses significantly increased hippocampal ACh release in $d5^{+/+}$ mice compared to saline injection ($p = 0.0005$, 2.0 mg/kg; $p < 0.0001$, 10.0 mg/kg). In $d5^{-/-}$, the administration of SKF 38393 did not increase hippocampal ACh release compared to saline. In $d5^{+/-}$, a significant increase in ACh release was observed only following the administration of SKF 38393 at the higher dose used here ($p < 0.0001$) (Figure 3c).

Local perfusion of SKF 38393 (10 and 50 μ M) directly through the dialysis probe significantly increased ACh release to 200 and 290% over basal values in the wild-type mice (Figure 4a, b), but only over 140 and 150% in $d5^{+/-}$ mice (Figure 4a, b) while having no effect $d5^{-/-}$ mice (Figure 4a, b). Three-way ANOVA revealed a significant

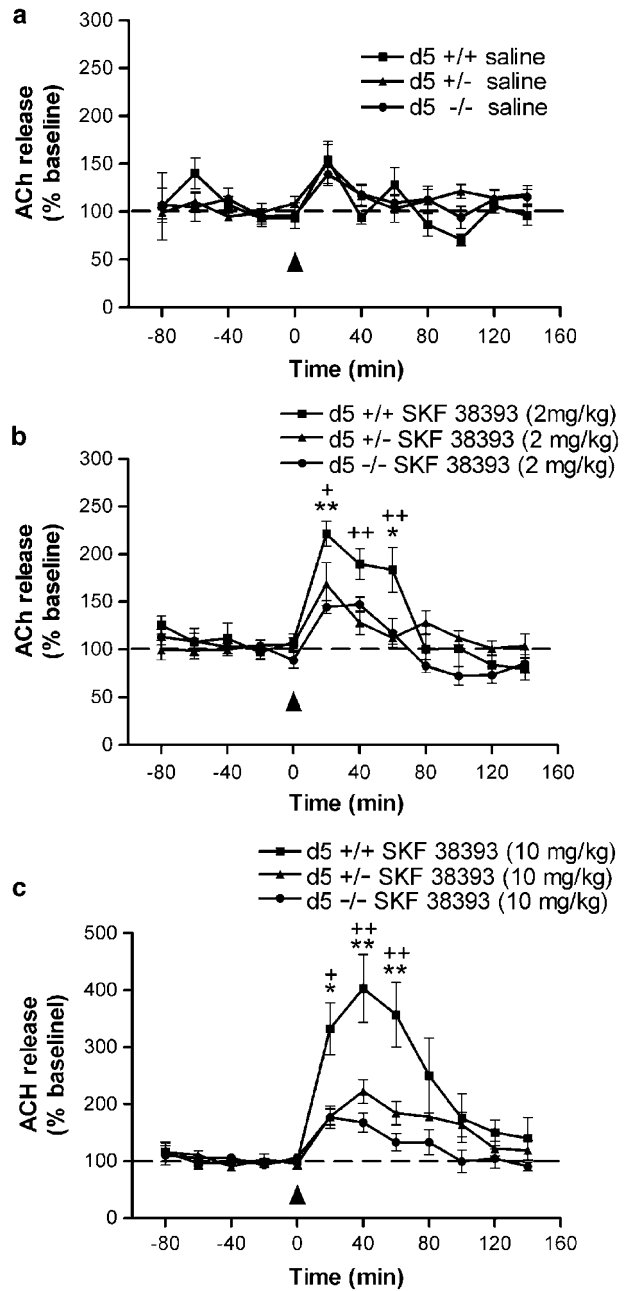


Figure 3 ACh release in the dorsal hippocampus in mice ($d5^{-/-}$, $d5^{+/-}$ and $d5^{+/+}$) following intraperitoneal administration of (a) saline solution, (b) SKF 38393 at 2.0 mg/kg, and (c) SKF 38393 at 10.0 mg/kg. Data represent mean \pm SEM ($n = 6-7$ in each group). Dialysate ACh content is expressed as percent of baseline. Baseline was calculated from the average of three samples preceding drug injection (arrow). For both doses, *post hoc* analysis revealed significant differences of drug effect between $d5^{+/+}$ and $d5^{-/-}$: ** $p < 0.001$; * $p < 0.01$ and between $d5^{+/+}$ and $d5^{+/-}$: + $p < 0.05$; ++ $p < 0.01$.

interaction between the main effects of genotype, treatment, and time ($F_{(22,264)} = 2.26$, $p = 0.0001$). A significant difference in hippocampal ACh release following stimulation with SKF 38393 is observed between $d5^{+/+}$ and $d5^{-/-}$ at both concentrations tested in this study ($p < 0.0001$ for both concentrations) and a significant difference is observed between $d5^{+/-}$ and $d5^{-/-}$ only at 50 μ M ($p = 0.002$).

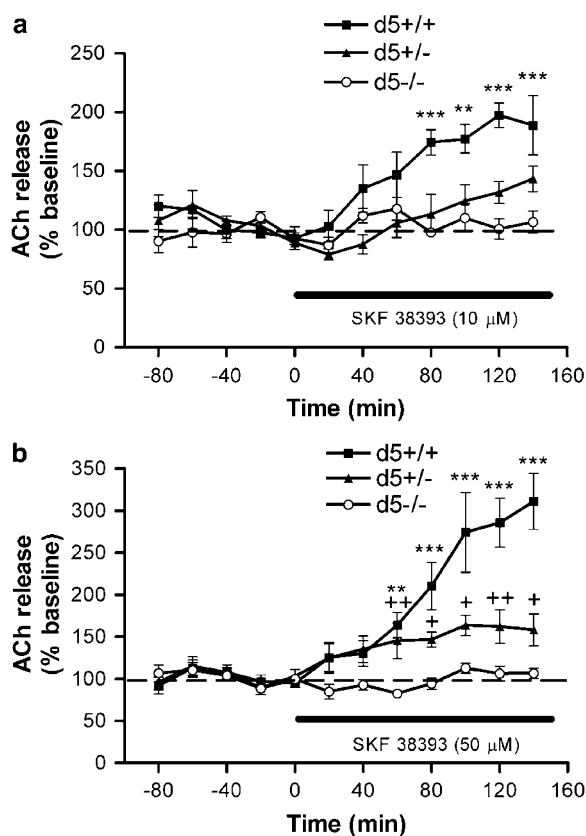


Figure 4 ACh release in the dorsal hippocampus in mice ($d5^{-/-}$, $d5 + / -$ and $d5 + / +$) following local perfusion of (a) $10 \mu\text{M}$ and (b) $50 \mu\text{M}$ SKF 38393. Data represent mean \pm SEM ($n = 5-6$ in each group). Dialysate ACh content is expressed as percent of baseline. Baseline was calculated from the average of three samples preceding drug perfusion (line). Post hoc analysis revealed significant differences of the drug effect between $d5 + / +$ and $d5 - / -$: $**p < 0.01$; $***p < 0.001$ and between $d5 + / -$ and $d5 - / -$: $+p < 0.05$; $++p < 0.01$.

Quantitative Receptor Autoradiography

Deletion of d5 receptors in $d5^{-/-}$ mice resulted in significant reductions in specific [^3H]SCH 23390 binding in all areas of the hippocampal formation (50% in the dentate gyrus and 73% in other regions) compared to wild-type animals (Figure 5a, b) ($p < 0.01$). $d5 + / -$ mice exhibited intermediate declines in specific [^3H]SCH 23390 binding (from 25 to 40% reduction; $p < 0.05$ for CA3 subfield and $p < 0.01$ for other regions) compared to wild-type animals.

Hippocampal [^3H]pirenzepine M_1 -like and [^3H]AFDX-384 M_2 -like muscarinic receptor binding sites are expressed in all subareas of the hippocampal formation (Figure 6a, c). Specific [^3H]pirenzepine binding is significantly increased in all subregions of the hippocampal formation in the $d5$ knockout mouse (Figure 6b). Heterozygote mice displayed moderate increases in specific [^3H]pirenzepine binding in the CA2 and CA3 subfields compared to $d5 + / +$ (Figure 6b). Specific [^3H]AFDX-384 M_2 -like binding sites was not significantly changed in the three groups studied here (Figure 6d).

DISCUSSION

The main findings of the present study are that extracellular levels of hippocampal ACh are chronically decreased,

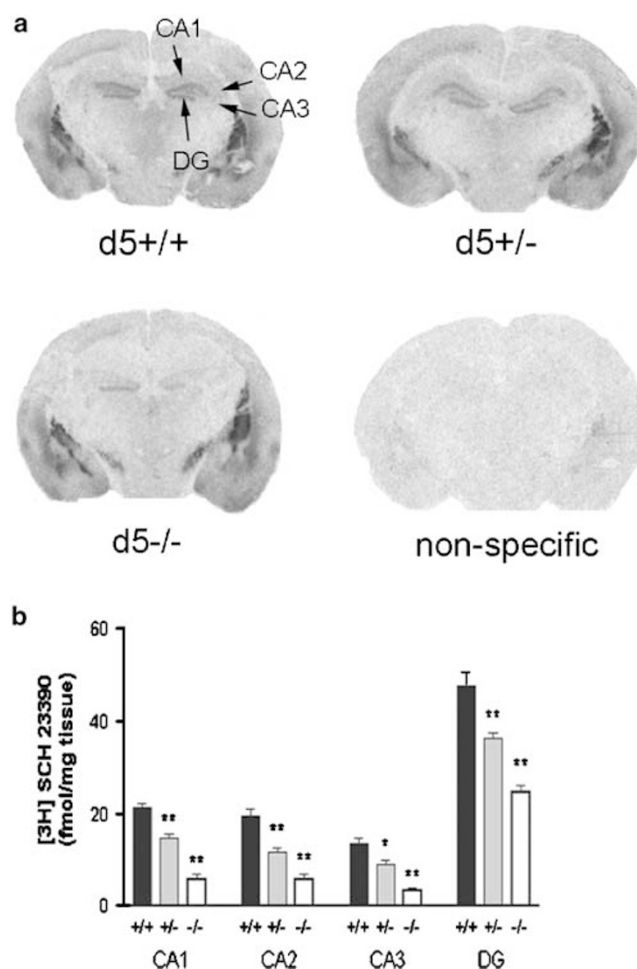


Figure 5 Quantitative autoradiography of D_1 -like DA receptor binding sites at the level of the hippocampal formation. (a) D_1 -like of dopaminergic receptors are known to be expressed in all subareas of the hippocampus. [^3H]SCH 23390 exhibited 80% of specific binding in the normal mice. (b) D_1 -like receptors were quantified in all genotypes. Data represent mean \pm SEM ($n = 6-8$ in each group). ANOVA with Dunnett's multiple comparisons revealed significant difference between $d5 + / +$ and $d5 + / -$ and between $d5 + / +$ and $d5 - / -$: $*p < 0.05$; $**p < 0.01$. Abbreviations: CA1, CA2, CA3, subfields of the Ammon's horn of the hippocampus. DG, dentate gyrus.

and that a DA D_1 -like agonist failed to modulate this release in the hippocampus of $d5$ receptor knockout mice. A blunted, but still significant effect was observed in heterozygous mice ($d5 + / -$) while SKF 38393, the D_1 -like agonist, potentially facilitated ACh release in the hippocampus of wild-type mice. These results confirm and extend previous data mostly obtained in the rat suggesting that the $d5$ receptor subtype was the D_1 -like receptor involved in the regulation of hippocampal ACh release (Hersi et al, 1995a, 2000). The use of molecular approaches such as the one reported here based on knockout animals or oligonucleotide antisenses (Hersi et al, 2000) is required as currently available pharmacological tools cannot distinguish between $d1$ and $d5$ receptors, the two subtypes composing the D_1 -like family of DA receptors (Seeman and Van Tol, 1993; Sunahara et al, 1991).

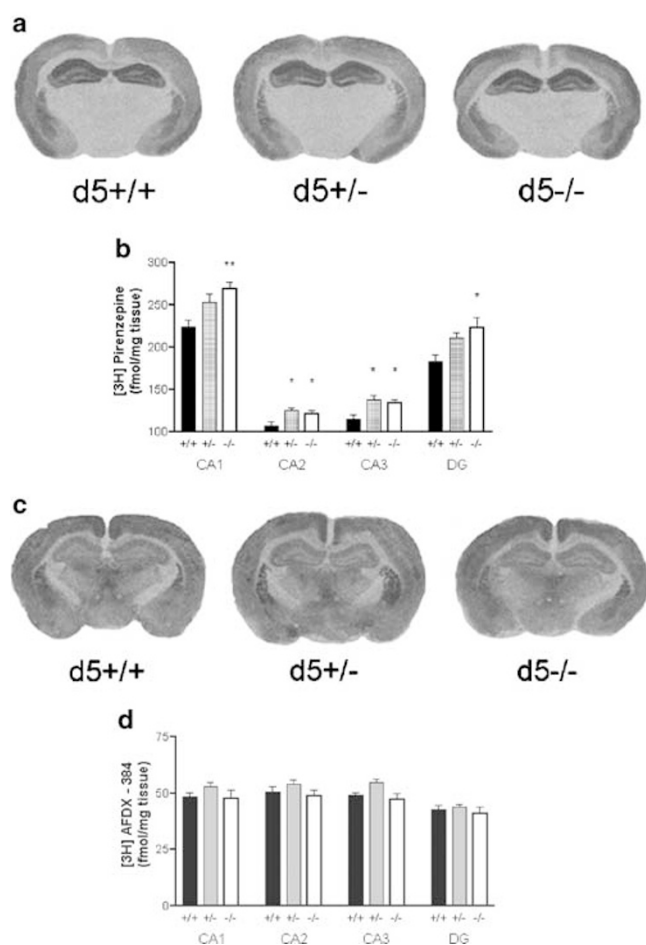


Figure 6 Quantitative autoradiography of muscarinic receptor binding sites in the hippocampal formation. Both M_1 and M_2 -like muscarinic receptor classes are expressed in all subareas of the hippocampus. [3H]pirenzepine and [3H]AFDX 384 exhibited almost 100% of specific binding. (a, b) M_1 -like and (c, d) M_2 -like muscarinic receptor binding sites were quantified in all genotypes. Data represent mean \pm SEM ($n = 7$ in each group). ANOVA with Dunnett's multiple comparisons revealed significant difference between $d5 +/+$ and $d5 +/-$ and between $d5 +/+$ and $d5 -/-$: * $p < 0.05$; ** $p < 0.01$. Abbreviations: Ca1, Ca2, Ca3, subfields of the Ammon's horn of the hippocampus. DG, dentate gyrus.

In the $d5 -/-$ mice, the lack of stimulatory effects of the D_1 -like agonist SKF 38393 on hippocampal ACh release was observed following systemic injections or intraprobe infusions of the agonist. In the heterozygous mice, significant effects of SKF 38393 were seen only at the higher concentration tested in this study, while the D_1 -like agonist potentially facilitated *in vivo* hippocampal ACh release at both concentrations tested. These results are likely due to the loss of D_1 -like receptors in the hippocampal formation of $d5 +/-$ and $d5 -/-$ mice. Indeed, we observed that specific [3H]SCH 23390 D_1 -like receptor binding levels were significantly decreased in $d5$ knockout and heterozygous mice. The residual labelling seen in these animals is likely reflecting the presence of low but significant levels of $d1$ receptors in the hippocampus (Bergson *et al*, 1995; Tiberi *et al*, 1991). However, this class of receptors does not appear to be involved in the direct regulation of hippocampal ACh release as we failed to observe any effects of SKF 38393 in

$d5 -/-$ animals. A previous study reported no reduction in specific [3H]SCH 23390 binding in the striatum; this is in accordance with the fact that this brain region is mostly enriched with $d1$ receptors (Hollon *et al*, 2002).

Anatomical evidence have shown that a subpopulation of hippocampal $d5$ -like receptors are located on axons in the hippocampus (Bergson *et al*, 1995). Moreover, fimbriectomy resulted in significant losses in D_1 -like receptors in the dentate gyrus of the hippocampus (Hersi *et al*, 1995a). These data may be taken as evidences that $d5$ receptors are present on septo-hippocampal cholinergic nerve terminals where they can directly modulate ACh release.

Using the technique of 'no net flux' *in vivo* microdialysis (Justice, 1993), the calculated level of extracellular ACh was in the range of 4.4 nM in the wild-type mouse dorsal hippocampus. This is comparable to the level reported in the rat dorsal hippocampus (Day *et al*, 2001). Interestingly, the basal level of ACh in the $d5$ knockout mouse (2.8 nM) was significantly lower than in heterozygous (4.0 nM) and wild-type (4.4 nM) mice. This result suggests that DA, acting through the $d5$ receptor, has a tonic role on the maintenance of normal ACh release in the hippocampus. Interestingly, the $d5$, but not $d1$, receptor subtype has been shown to be constitutively active even in the absence or in the presence of very low concentrations of DA (Tiberi and Caron, 1994; Demchyshyn *et al*, 2000). Such a constitutive activity could play a role in the maintenance of extracellular levels of ACh in the hippocampus.

What could be the functional relevance of hippocampal $d5$ receptors on the maintenance of normal release of ACh leading to enhanced extracellular levels? While it is well established that hippocampal cholinergic innervation plays a major role in attention and learning behaviors (Perry *et al*, 1999; Everitt and Robbins, 1997), the role of DA and dopaminergic receptors is not as clear, even if few studies have proposed that the D_1 -like receptors can facilitate cognitive behaviors (Packard and White, 1991; Arnsten *et al*, 1994). In a recent study, Holmes *et al* (2001) reported normal spatial memory in $d5 -/-$ mice in the Morris water maze, except for a minor deficit in hidden platform acquisition. Similar small deficits were seen in fear conditioning (Holmes *et al*, 2001). However, studies using pharmacological approaches have reported that D_1 -like agonists can facilitate the late phase of long-term potentiation (LTP) and restore memory impairments in aged mice (Bach *et al*, 1999), and aged memory-impaired rats (Hersi *et al*, 1995b). Behavioral tests performed in $d5 -/-$ mice involved learning to avoid aversive stimuli (Holmes *et al*, 2001). It would thus be appropriate to investigate further the abilities of $d5 -/-$ mice in attention and learning models that are more discriminating for various aspects of the cognitive process, such as reward-related memory mechanisms (Schultz *et al*, 1993). Also, a particular focus could be on attention as multiple studies have suggested a role for DA in attention behaviors over memory processes *per se* (Clark *et al*, 1987a, b; Nieoullon, 2002).

In addition to changes in extracellular ACh levels, increases in muscarinic M_1 -like, but not M_2 -like, receptors were found in the hippocampus of $d5 -/-$ mice. M_1 -like receptors are mostly postsynaptically located in the hippocampus and are believed to be involved in LTP and in the maintenance of normal cognitive functions

(Anagnostaras *et al*, 2003; Quirion *et al*, 1989). The increase in M₁-like receptors may be a compensatory mechanism associated to the decrease in extracellular ACh levels found in this brain region in d5 knockout mice. This compensatory mechanism could also possibly explain the apparent lack of learning deficits of d5^{-/-} mice in the Morris water maze task (Holmes *et al*, 2001). On other hand, M₂-like receptors are mostly presynaptic auto receptors exerting inhibitory effect on ACh release (Lapchak *et al*, 1989; Quirion *et al*, 1989).

In summary, deletion of the d5 receptor in a knockout mouse model results in a significant reduction in extracellular ACh levels in the hippocampal formation, suggesting a tonic role for this receptor in ACh release. Moreover, a D₁-like agonist fails to stimulate *in vivo* ACh release in this model demonstrating further the unique role of the d5 receptor in the regulation of hippocampal ACh release. Further studies are now in progress to establish more precisely the behavioral relevance of these findings.

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