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RESEARCH PAPER

Catecholamine outflow from mouse and rat brain slice preparations evoked by nicotinic acetylcholine receptor activation and electrical field stimulation

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Background and purpose: Mice with targeted deletions of neuronal nicotinic acetylcholine receptor (nAChR) subunit genes are valuable models to study nAChR function such as catecholamine outflow by presynaptic receptor activation. Contrary to the rat, our present knowledge on presynaptic nAChRs in mice primarily relies on observations made with synaptosomes. We have now used brain slices to investigate nicotine-induced catecholamine outflow in wild type (WT) and nAChR (β_2 and α_5) knockout mice for a comparison with rat brain slice preparations.
Experimental approach: Brain slices from rat and mouse hippocampus, parieto-occipital neocortex, and corpus striatum were loaded with either [^{3}H]-noradrenaline or [^{3}H]-dopamine. We provoked catecholamine outflow by electrical field stimulation and nicotinic agonists.
Key results: When set in relation to electrical field stimulation, nicotine-evoked catecholamine release was sizeable in the striatum but low in the neocortex of both rats and mice. [^{3}H]-noradrenaline outflow was, on the other hand, substantial in the rat but low in the mouse hippocampus. About 10% (or less) of nicotine-induced catecholamine release persisted in the presence of tetrodotoxin in all our preparations.
Conclusions and implications: Targeted deletion of the β_2 subunit gene essentially abolished the effect of nicotine, indicating that this subunit is an essential constituent of nAChRs that indirectly (*via* action potentials) induce catecholamine release from hippocampal and striatal slices in mice. The impact of nAChRs in catecholaminergic projection areas differs between species and has thus to be considered when extrapolating results from animal models to human conditions.
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Keywords: catecholamine outflow; brain slice; hippocampus; neocortex; corpus striatum; electrical field; nicotinic ACh receptors; null mutation; α_5 subunit; β_2 subunit

Abbreviations: α-CtxMII, α-Contotoxin MII; DMPP, 1,1,-dimethyl-4-phenylpiperazinium; KO, knockout; nAChR, nicotinic acetylcholine receptor; R_{max}, maximally induced release; TTX, tetrodotoxin; WT, wild type

Introduction

Nicotinic acetylcholine receptors (nAChRs) modulating the release of neurotransmitters from presynaptic terminals have a definite role in the central nervous system (reviewed by Role and Berg, 1996; Wonnacott, 1997). Our knowledge of these receptors rests to a large extent on the neurochemical analysis of transmitter release from either synaptosomes or brain slices. In contrast to synaptosomes, brain slices retain local anatomical integrity which may account for significantly differing results between the two preparations (Kaiser *et al*., 1998; Wonnacott *et al*., 2000; Leslie *et al*., 2002). [^{3}H]dopamine release is, for instance, evoked by anatoxin-a with two affinities (0.24 and 5.1 μM) in rat striatal slices, whereas a single EC_{50} (0.13 μM) was determined in striatal synaptosomes (Kaiser and Wonnacott, 2000; Wonnacott *et al*., 2000). Likewise, the sensitivity to tetrodotoxin (TTX) of nicotine-induced catecholamine release differs significantly between the two preparations (reviewed in Wonnacott, 1997). To appreciate functions of nAChRs in terminal fields it is thus essential to supplement results on nAChR activation in synaptosomes with observations from brain slice preparations.

Although previous work allows such comparison for the rat, neurochemical studies of presynaptic nAChRs in mice have primarily taken advantage of synaptosomal

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preparations (Marks *et al*., 1993, 1995, 2000; Grady *et al*., 2002; Champtiaux *et al*., 2003; Salminen *et al*., 2004; Azam and McIntosh, 2006). We have now set out to analyse catecholamine outflow from hippocampal, neocortical and striatal brain slices of wild-type (WT) C57BL/6J mice. The impact of the β_2 subunit on nicotine-evoked catecholamine outflow was investigated in the hippocampus and the striatum taken from animals with a targeted deletion of the nAChR β_2-subunit gene (Picciotto *et al*., 1995). We used, in addition, α_5-knockout (KO) mice (Salas *et al*., 2003) to explore the function of this subunit in striatal slice preparations. Our results confirm previous observations on the crucial role the β_2 subunit plays in nicotine-induced catecholamine release from the hippocampus as well as the striatum. However, and unlike the peripheral nervous system where a null mutation of the α_5-subunit gene dramatically enhances nicotine-evoked [^{3}H]noradrenaline outflow (Fischer *et al*., 2005), this KO had no significant effect on [^{3}H]dopamine release from striatal slices.

The catecholamine outflow evoked by nAChR activation in mouse brain slice preparations is mainly caused by an indirect (TTX-sensitive) component. In hippocampal and striatal slices, this component critically depends on the presence of the nAChR β_2 subunit. Our comparative studies with rat brain slice preparations indicate major species and regional differences of catecholamine release in response to nAChR activation.

Methods

Animals

About 3–4-week-old male Sprague–Dawley rats were obtained from the Institute of Biomedical Research, Medical University of Vienna (Himberg, Austria). WT-C57BL/6J mice and mice deficient in the α_5 nAChR subunit gene were from Avi Orr–Urtreger (Salas *et al*., 2003). The β_2-KO strain was from J-P Changeux (Picciotto *et al*., 1995). The two KO strains have been backcrossed onto a C57BL/6J background for seven and 12 generations, respectively. We used 6–8-week-old male mice bred from homozygous parent animals at our in-house animal facilities. All animals were kept in thermostable rooms (21°C) at a light–dark schedule of 22:14 h in-group cages, with food and water freely accessible.

Superfusion experiments

All animals were killed by CO_2 anaesthesia and decapitation according to the Guidelines of the Animal Care Committee of the Medical University of Vienna. The brains were rapidly removed and placed in ice-cold superfusion buffer containing (mM): NaCl 118; KCl 4.8; $CaCl_2$ 2.5; $MgSO_4$ 1.2; $NaHCO_3$ 25; KH_2PO_4 1.2; Na_2-ethylenediaminetetraacetic acid 0.03; glucose 11; ascorbic acid 0.57; fumaric acid 0.5; sodium pyruvate 5.0; saturated with 95% O_2/5% CO_2. Hippocampus, striatum and (parieto–occipital) neocortex were cut into 300 μm slices by means of a McIllwain tissue chopper. Loading of radioactivity was achieved by adding either 0.03 μM [^{3}H]noradrenaline or 0.2 μM [^{3}H]dopamine and 1 mM ascorbic acid to buffer, followed by incubations for 60 min at 36.5°C. The brain slices were then placed in small chambers between two platinum wire electrodes and superfused for 1 h with buffer (kept at 26°C and continuously bubbled with 95% O_2/5% CO_2) in the presence of 0.5 μM clorgyline. We collected 4 min fractions at a flow rate of 1 ml min^{-1}.

Stimulated [^{3}H] outflow was achieved by either adding nAChR agonists to the superfusion buffer for 30 s or by electrical field stimulation (100 pulses, 10 Hz, 0.5 ms, 50 V cm^{-1}, 40 mA). Each slice was tested with a single application of a nicotinic agonist (see Figure 1a and d). Mecamylamine and TTX were added to the superfusion buffer 12 min before and during the stimulation with nicotine. By taking advantage of the slow off-kinetics, the effects of α-conotoxin MII (α-CtxMII) were tested by pre-exposing cultures for 12 min before applying a nicotine stimulus (Salminen *et al*., 2004). The radioactivity retained by slices at the end of an experiment was recovered by extraction with 1% sodium dodecyl sulphate and sonication.

Calculations

Fractional rates of [^{3}H] outflow were calculated by dividing the radioactivity of a 4 min fraction by the total radioactivity of slices at the beginning of the corresponding 4 min collection period. The total radioactivity at a given time was obtained by adding up the residual radioactivity retained by slices with the radioactivity of all fractions collected from this point. Stimulation-evoked outflow was calculated as the difference between the total [^{3}H] outflow during and after stimulation on one hand, and the estimated basal outflow on the other hand, assuming that basal release follows a linear decline. Both fractional rates and stimulated outflow were multiplied by 100 and thus expressed as % of the total radioactive contents of a slice. Radioactivity in extracts and collected fractions was determined by liquid scintillation counting.

Data analysis

Data are shown as means ± s.e.m. The significance of differences was evaluated with the unpaired Student's *t*-test, unless indicated otherwise. Concentration–response curves for agonists were fitted by unweighted non-linear regression to the logistic equation with GraphPad Prism software. The programme uses *F* statistics as the 'extra sum of squares' introduced when testing for independent (i.e. significantly different at $P<0.05$) versus shared (i.e. not significantly different) parameters of curves (EC_{50}, slope, maximal release R_{max}).

Materials

Materials and reagents were from the following sources: (−)-[ring-2,5,6 ^{3}H]noradrenaline (60–75 Ci mmol^{-1}, Perkin-Elmer, Waltham, MA, USA); [^{3}H]dopamine (9.9 Ci mmol^{-1}, Amersham-Biosciences, GE-Healthcare, Chalfont St Giles, GB); α-CtxMII was synthesized as previously described (Cartier *et al*., 1996); TTX HCl (TTX: Latoxan, 26000 Valence – France). 1,1,-Dimethyl-4-phenylpiperazinium

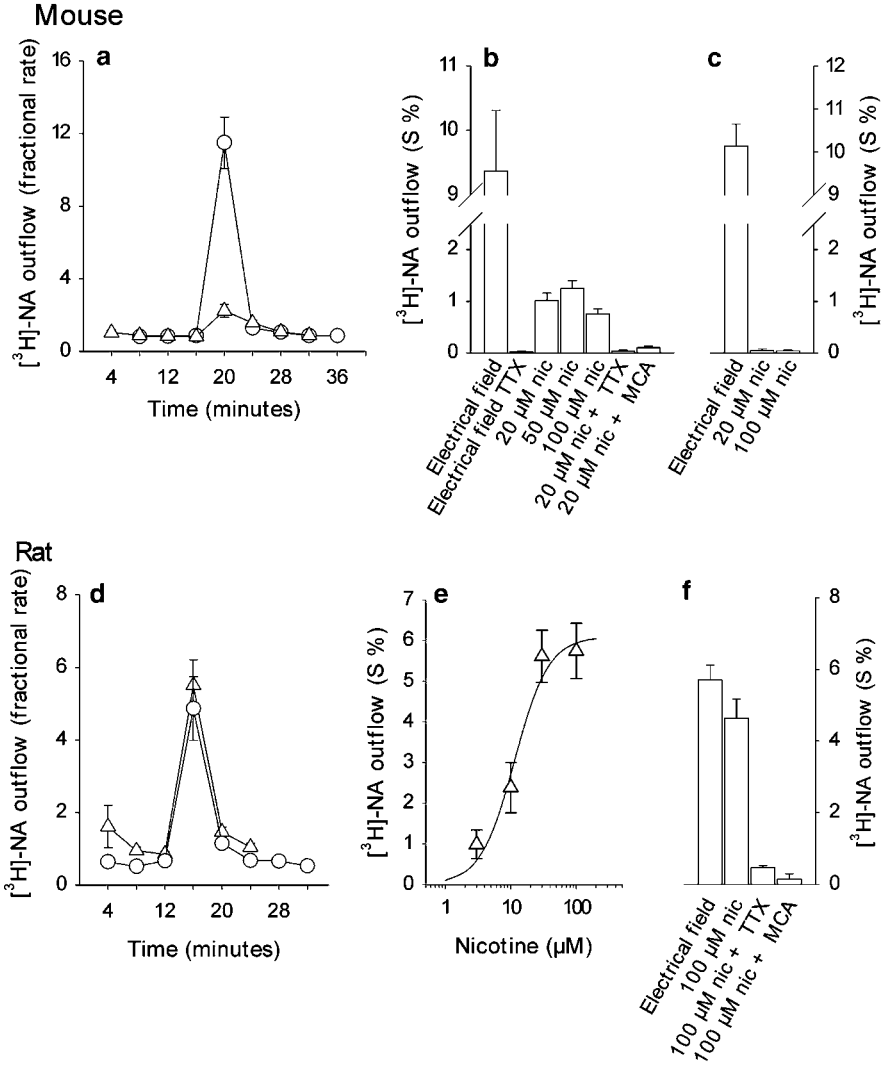

Figure 1 [^{3}H]noradrenaline outflow from mouse (**a–c**) and rat (**d–f**) hippocampal slices. (**a**) Fractional rates of outflow in response to electrical field stimulation (circles) or to the application of 100 μM nicotine (triangles). Data are means $\pm$ s.e.m. ($n=3$). (**b**) [^{3}H]noradrenaline outflow in response to indicated stimuli (shown as a percentage of total radioactivity in slices) in WT mice ($n=6$–27). (**c**) [^{3}H]noradrenaline outflow in response to indicated stimuli in β_2-KO mice ($n=6$–9). Compared to WT mice (**b**), the outflow induced by 20 μM nicotine was reduced by 95.2% in the KO. (**d**) Fractional rates of [^{3}H]noradrenaline outflow in response to electrical field stimulation (circles) or to the application of 100 μM nicotine (triangles; $n=3$). (**e**) [^{3}H]noradrenaline outflow in response to indicated concentrations of nicotine (calculated EC_{50}: 19.6 μM; $n=3$). (**f**) [^{3}H]noradrenaline outflow in response to indicated stimuli ($n=3$–12). nic, nicotine; TTX, 0.5 μM TTX; MCA, 5 μM mecamylamine.

iodide (DMPP, D5891), (−)-nicotine (N3876), (−)-cytisine (C2899), mecamylamine hydrochloride (M9020), and clorgyline hydrochloride (M3778) were from Sigma-Aldrich (St Louis, MO, USA). Other chemicals were from Merck (Darmstadt, Germany), analytical grade.

Results

Hippocampal brain slice preparations from mice and rats

Electrical field stimulation caused a large outflow of [^{3}H]noradrenaline from mouse hippocampal brain slices (Figure 1a–c) that was entirely abolished in the presence of 0.5 μM TTX (Figure 1b).

The nicotine-induced release was small by comparison (Figure 1a and b). Calculating a ratio of [^{3}H]noradrenaline outflow in response to a maximally effective nicotine concentration (Figure 1b) and electrical field stimulation (Figure 1b) yielded a 'standardized nicotine index' of 0.13 (Table 1). The nicotine-induced [^{3}H]noradrenaline outflow was largely prevented not only by adding the nicotinic antagonist mecamylamine (5 μM, Figure 1b) but also by the sodium channel blocker (TTX, 0.5 μM). In the presence of TTX, the [^{3}H]noradrenaline outflow evoked by 20 μM nicotine amounted to just $0.034\pm0.022\%$ ($n=8$) of the radioactive contents of the slices (Figure 1b).

Targeted deletion of the β_2 nAChR subunit gene essentially abolished all nicotine-evoked [^{3}H]noradrenaline release in these mice, whereas the [^{3}H]noradrenaline outflow in response to electrical field stimulation was unaffected (Figure 1c).

The impact of nAChR activation was quite different in rat hippocampal brain slices (Figure 1d–f) with a 'standardized nicotine index' of 0.81 (Table 1). Hence, 100 μM nicotine

Table 1 Standardized nicotine indices

	C57Bl/6J WT mouse	*Rat*
Hippocampus	0.13 (1.25, 9.37%)	0.81 (4.63, 5.70%)
Neocortex	0.08 (0.77, 9.42%)	0.21 (1.11, 5.17%)
Striatum	0.93 (1.73, 1.86%)	1.45 (0.90, 0.62%)

Abbreviation: Wt, wild type.
Standardized nicotine indices were obtained by dividing nicotine-induced catecholamine outflow (first number in parenthesis) by the outflow in response to electrical field stimulation of brain slices (second number in parenthesis). Data for this table were taken from experiments shown in Figures 1–3 with a nicotine concentration yielding maximal outflow.

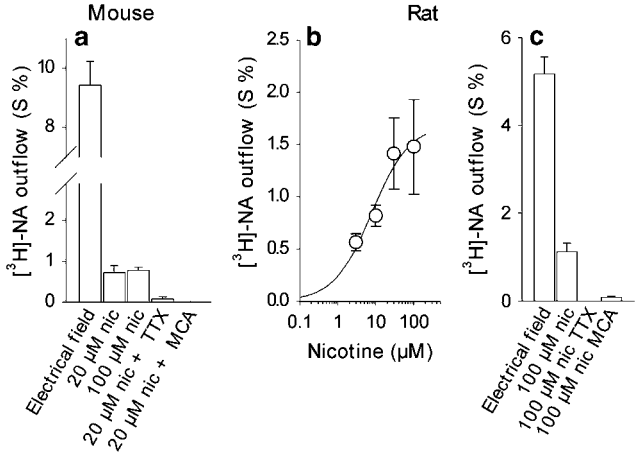

Figure 2 [^{3}H]noradrenaline outflow from mouse (**a**) and rat (**b, c**) parieto–occipital neocortical slices. (**a**) [^{3}H]noradrenaline outflow in response to indicated stimuli (shown as a percentage of total radioactivity). Data are means ± s.e.m. ($n = 3$–6). (**b**) [^{3}H]noradrenaline outflow in response to indicated concentrations of nicotine (EC_{50}: 7.8 μM; $n = 3$). (**c**) [^{3}H]noradrenaline outflow in response to indicated stimuli ($n = 5$–15). nic, nicotine; TTX, nicotine in the presence of 0.5 μM TTX; MCA, nicotine in the presence of 5 μM mecamylamine.

caused almost as much [^{3}H]noradrenaline outflow as did electrical field stimulation (Figure 1f). We found nicotine EC_{50} values (19.6 μM; log EC_{50}: −4.707 ± 0.126) and maximal effects (6.9 ± 0.92%; Figure 1e) in good agreement with a previous study (Leslie *et al*., 2002). As in mouse hippocampal brain slices, the nicotine-induced outflow was largely prevented in the presence of 0.5 μM TTX or 5 μM mecamylamine (Figure 1f).

As we used rats of younger age (3–4 weeks) than mice (6–8 weeks), and as age-dependent effects of nicotine on the outflow of [^{3}H]noradrenaline from both hippocampal synaptosomes and slice preparations have been reported in either species (Leslie *et al*., 2002; Azam and McIntosh, 2006) we checked whether mice of similar age showed larger effects in response to nicotine. However, the outflow of [^{3}H]noradrenaline induced by 100 μM nicotine did not differ significantly between 20, 48 and 70-days-old mice (20 days: 0.95 ± 0.12%, $n = 9$; 48 days: 1.17 ± 0.13%; $n = 6$; 70 days: 0.95 ± 0.19%, $n = 9$; $P > 0.05$, one-way analysis of variance).

Neocortical brain slice preparations from mice and rats

The [^{3}H]noradrenaline outflow from mouse (parieto–occipital) neocortical slices was similar to the mouse hippocampus: high in response to electrical field stimulation, but low upon nAChR activation (Figure 2a), yielding a 'standardized nicotine index' of just 0.08 (Table 1).

In rat neocortical slices, nicotine was about as potent (EC_{50}, 7.8 μM; log EC_{50}, −5.107 ± 0.585) as in hippocampal slices, though clearly less efficient in inducing [^{3}H]noradrenaline outflow (Figure 2b). Owing to a sizeable outflow in response to electrical field stimulation, the small effect of a maximally effective nicotine concentration resulted in a 'standardized nicotine index' of 0.21 (Figure 2c; Table 1). TTX and mecamylamine effectively inhibited nicotine-induced [^{3}H]noradrenaline outflow in both mice (Figure 2a) and rats (Figure 2c).

Striatal brain slice preparations from mice and rats

[^{3}H]dopamine outflow from mouse striatal slice preparations in response to electrical field stimulation (Figure 3b) was significantly smaller than [^{3}H]noradrenaline outflow from either the hippocampus (Figure 1b) or the neocortex (Figure 2a). Likewise, electrical field stimulation of rat brain slices caused a significantly smaller outflow of [^{3}H]dopamine from the striatum (Figure 3e) than [^{3}H]noradrenaline outflow from the hippocampus (Figure 1f) or the neocortex (Figure 2c).

The lower [^{3}H]dopamine outflow in response to electrical field stimulation was not paralleled by a reduced effect of nicotine in the striatum, resulting in a 'standardized nicotine index' of 0.93 (mouse) and 1.45 (rat) (Figure 3b and e; Table 1). In mice, 50 μM nicotine-evoked [^{3}H]dopamine outflow from striatal slices (Figure 3b) was comparable to the nicotine-induced [^{3}H]noradrenaline release from the hippocampus (Figure 1b) and significantly larger than in the neocortex (Figure 2a). In line with previous reports (Sacaan *et al*., 1995; Wonnacott *et al*., 2000), nicotine-induced [^{3}H]noradrenaline outflow from rat striatal slices with high potency (EC_{50}, 0.84 μM; log EC_{50}, −6.073 ± 0.332) but low efficacy (maximal effect: 1.3 ± 0.8%; Figure 3d).

The rather large 'standardized nicotine index' in mice suggested the possibility that [^{3}H]dopamine outflow in response to electrical field stimulation was caused, in part, by ACh released from cholinergic neurons. We tested this hypothesis by adding 5 μM mecamylamine between two electrical stimuli set 20 min apart to our superfusion buffer. In the absence of MCA, the second stimulus was 80.6 ± 12.8% (means ± s.e.m., $n = 10$) of the first stimulus. In the presence of 5 μM mecamylamine (added 12 min before and during stimulation), the second stimulus was 80.4 ± 9.4% ($n = 12$). Hence, activation of nAChRs by endogenous ACh did not significantly contribute to [^{3}H]dopamine outflow in our paradigm.

The nicotine-induced [^{3}H]dopamine outflow from mouse striatal slices was examined in more detail. It was entirely dependent on the presence of calcium in the superfusion buffer (Figure 3a), largely reduced in the presence of 0.5 μM TTX (Figure 3a), blocked by mecamylamine in a concentration-dependent manner (Figure 3a), and partially inhibited

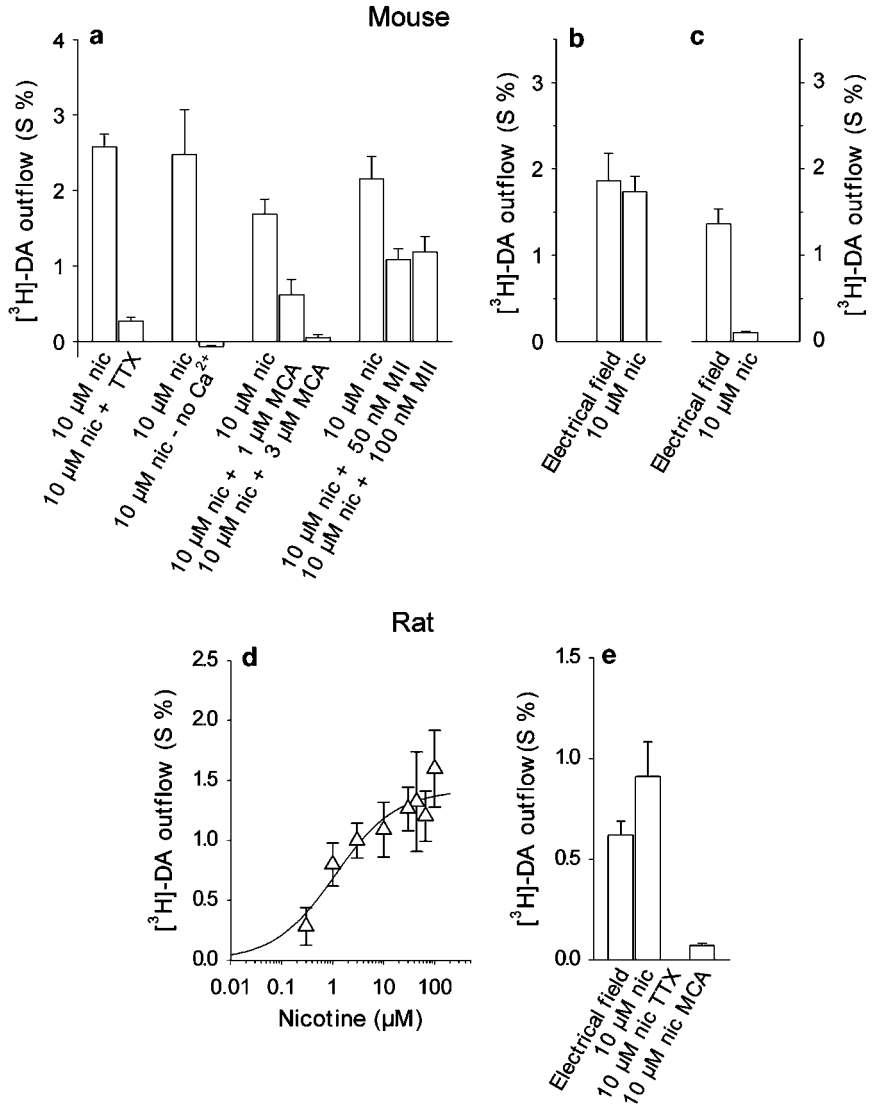

Figure 3 [^{3}H]dopamine outflow from mouse (**a–c**) and rat (**d–e**) corpus striatum slices. (**a**) [^{3}H]dopamine outflow (shown as a percentage of total radioactivity) in response to 10 μM nicotine and indicated experimental conditions. Each approach was run with its own set of [^{3}H]dopamine outflow evoked by 10 μM nicotine at control conditions. Data are means$\pm$s.e.m. ($n=3$–15). (**b**) [^{3}H]dopamine outflow in response to electrical field stimulation ($n=16$) or 10 μM nicotine ($n=17$) in WT mice. (**c**) [^{3}H]dopamine outflow in response to electrical field stimulation ($n=11$) or 10 μM nicotine ($n=6$) in β_2-KO mice. (**d**) [^{3}H]dopamine outflow in response to indicated concentrations of nicotine (EC_{50}, 0.84 μM; $n=3$–6). (**e**) [^{3}H]dopamine outflow in response to indicated stimuli ($n=3$–9). nic, nicotine; TTX, 0.5 μM TTX; no Ca^{2+}, Ca^{2+} omitted before and during nicotine application; MCA, 1 and 3 μM mecamylamine; MII, 50 and 100 nM α-CtxMII.

by α-CtxMII (to 50.2 and 54.8% in the presence of 50 and 100 nM α-CtxMII, respectively, Figure 3a). Null mutation of the β_2 nAChR subunit gene almost abolished [^{3}H]dopamine outflow in response to nicotine (Figure 3c). Electrically induced outflow was, on the other hand, not significantly affected by the KO (Figure 3b and c).

Null mutation of the α_5 nAChR subunit gene has been reported to affect ACh-induced [^{3}H]dopamine outflow from mouse striatal synaptosomes (Salminen *et al*., 2004). We thus compared the [^{3}H]dopamine outflow in response to nicotine, cytisine and 1,1,-dimethyl-4-phenylpiperazinium iodide (DMPP) from striatal slices of WT and α_5-KO animals. However, our experiments revealed no significant difference of either potencies or efficacies between the two genotypes (Figure 4; Table 2).

Discussion

The object of this study was to characterize properties of nAChRs triggering catecholamine release in brain slice preparations of *mice*. We furthermore compared the two species, *rats* and *mice*, and the outflow following two types of stimulus, nicotine and electrical field stimulation. The principal findings of our study confirm and significantly extend previous observations made on *mouse* synaptosomes and indicate major species and regional differences of catecholamine outflow: (1) nicotine-induced [^{3}H]noradrenaline outflow from hippocampal slices was significantly larger in *rats* than in *mice*. (2) Irrespective of species, electrical field stimulation of slices produced significantly more [^{3}H]noradrenaline release from both the hippocampus and the

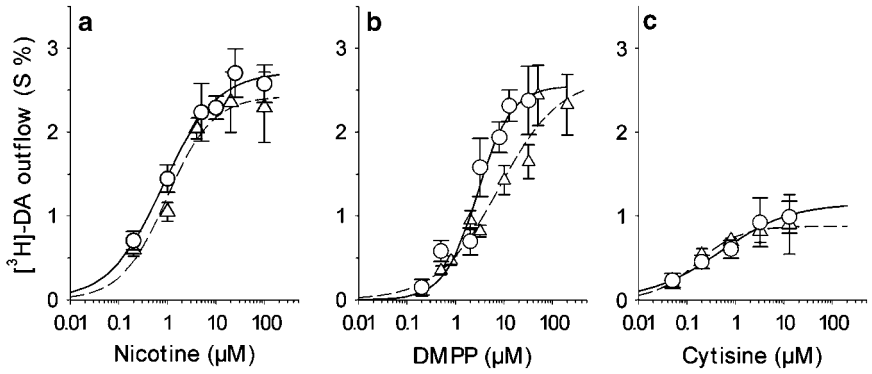

Figure 4 [^{3}H]dopamine outflow in response to nicotine (**a**), DMPP (**b**) and cytisine (**c**) from striatal slices taken from WT (circles, solid lines) or α_5-KO mice (triangles, dashed lines). Data points are means$\pm$s.e.m. ($n=3$–15). Curves were fitted to data points as described in Methods. See Table 2 for EC_{50} and R_{max} values.

Table 2 Agonist-induced [^{3}H]DA outflow from striatal slices of WT and α_5-KO mice

	EC_{50} (log EC_{50})	R_{max} *(S %)*
Nicotine, WT	0.80 μM (-6.09 ± 0.270)	2.85 ± 0.41
Nicotine, KO	1.00 μM (-5.99 ± 0.197)	2.26 ± 0.27
DMPP, WT	2.57 μM (-5.592 ± 0.175)	2.58 ± 0.40
DMPP, KO	7.59 μM (-5.120 ± 0.360)	2.78 ± 0.56
Cytisine, WT	0.46 μM (-6.336 ± 0.652)	1.15 ± 0.45
Cytisine, KO	0.14 μM (-6.827 ± 0.230)	0.86 ± 0.12

Abbreviations: DMPP, 1,1,-dimethyl-4-phenylpiperazinium; KO, knockout; WT, wild type.
EC_{50} (log $EC_{50}\pm$s.e.m.) and maximal release R_{max} are ($\pm$s.e.m.) obtained by fitting concentration–response curves to data points shown in Figure 4. Fit parameters (EC_{50} and R_{max}) did not differ significantly between WT and KO animals for any of the three agonists ($P>0.05$, see Methods for statistics applied).

neocortex than [^{3}H]dopamine outflow from the striatum. (3) The nicotine-evoked catecholamine release was largely prevented in all slice preparations by the presence of TTX. (4) Nicotine-induced [^{3}H]dopamine outflow from the striatum and [^{3}H]noradrenaline release from the hippocampus of *mice* required the presence of the β_2nAChR subunit. (5) Targeted deletion of the α_5 subunit gene had no significant effect on [^{3}H]dopamine outflow from the *mouse* striatum in response to nAChR activation.

The modulation [^{3}H]noradrenaline outflow from hippocampal slices by nAChR activation was species dependent

The robust outflow of [^{3}H]noradrenaline in response to nAChR activation in *rat* hippocampal slices is well documented (Sacaan *et al*., 1995, 1996; Sershen *et al*., 1997; Lena *et al*., 1999; Leslie *et al*., 2002; Amtage *et al*., 2004). By comparison with our standard electrical field protocol, we found about equal quantities of [^{3}H]noradrenaline release in response to stimulation with maximally effective nicotine concentrations ('standardized nicotine index': 0.81, Table 1).

In contrast, this ratio diminished to 0.13 in *mouse* hippocampal slices, indicating that nAChR activation triggers NA release less efficiently in the *mouse* than in the *rat* hippocampus. This observation is in line with a recent report showing that nicotine-evoked [^{3}H]noradrenaline outflow from hippocampal synaptosomes is larger in *rats* than in *mice* (Azam and McIntosh, 2006). Such species difference has also been reported for the neocortex, where [^{3}H]noradrenaline outflow induced by nicotine was significantly larger in *human* than in *rat* brain slice preparations (Amtage *et al*., 2004). Whether properties of these nAChRs in the *human* hippocampus compare better with the *mouse* than with the *rat* is not known. Species differences in nAChRs become critical though when applying observations from animal models to neuropathological mechanisms in men (Kaiser and Wonnacott, 2000; Picciotto *et al*., 2001; Amtage *et al*., 2004).

The impact of nAChR activation on catecholamine outflow differs between brain regions

Transmitter outflow in response to electrical field stimulation is the sum of several parameters, such as the number of presynaptic specializations, reuptake and degradation mechanisms and feedback inhibition by presynaptic autoreceptors (Valenta *et al*., 1988; Boehm and Huck, 1995). Any of these parameters might contribute to the larger [^{3}H]noradrenaline release from neocortex and hippocampus compared to a smaller [^{3}H]dopamine outflow from the striatum. The smaller effect of electrical field stimulation on [^{3}H]dopamine outflow in the striatum did not go together with a reduction of nicotine effects. Hence, indices for nicotine-induced [^{3}H]dopamine outflow set in relation to electrical field stimulation in *mice* yielded 0.93 (striatum), opposed to 0.13 (hippocampus, Table 1). These data, and the indices calculated for *rat* neocortical, hippocampal and striatal slices (0.21, 0.81 and 1.45, respectively, Table 1) suggest that the impact of nAChR activation on catecholamine release distinctly differs in these structures.

Indirect mechanisms of nAChR-modulated catecholamine release markedly outweigh direct mechanisms

Our finding that TTX inhibited nicotine-evoked catecholamine outflow in all our preparations by about 90% are in line

with previous observations made on *human* and *rat* brain slice preparations (Sacaan *et al*., 1995; Marshall *et al*., 1996; Wonnacott, 1997; Lena *et al*., 1999; Leslie *et al*., 2002; Amtage *et al*., 2004; Barik and Wonnacott, 2006). Quite the opposite, nicotinic agonist-induced catecholamine release from *rat* synaptosomes is clearly less sensitive to an inhibition by TTX (Soliakov *et al*., 1995; Clarke and Reuben, 1996; Marshall *et al*., 1996; Leslie *et al*., 2002). To our knowledge, effects of TTX on nicotine-induced catecholamine release have not been studied in *mouse* synaptosomal preparations, though [$^{86}Rb^+$] efflux from *mouse* thalamic synaptosomes in response to nicotine was partly inhibited (by 42%) in the presence of TTX (Marks *et al*., 1995).

A sizeable effect of TTX indicates that a major part of nAChRs, though still on the catecholaminergic axon, is too far distant from active zones to trigger exocytosis directly by calcium influx through the nAChR channel pore (Wonnacott, 1997; Kristufek *et al*., 1999). As even synaptosomal preparations may show some TTX sensitivity (Soliakov *et al*., 1995; Marshall *et al*., 1996), nAChRs that trigger catecholamine release by a direct (TTX-resistant) and an indirect (TTX-sensitive) mechanism may coexist in relative close proximity (i.e., on isolated synaptosomes).

nAChRs modulating catecholamine outflow indirectly may in addition reside on glutamatergic axons, as shown in *rat* striatal slice preparations (Wonnacott *et al*., 2000; Kaiser and Wonnacott, 2000). Indirect effects of nAChR activation are more complex in the hippocampus, where both GABA-ergic and glutamatergic mechanisms are involved (Leslie *et al*., 2002; Barik and Wonnacott, 2006). In the rat, these indirect effects appear to be mediated by α_7 nAChRs (Kaiser and Wonnacott, 2000; Wonnacott *et al*., 2000; Barik and Wonnacott, 2006).

Our experiments revealed that nicotine-evoked [^{3}H]dopamine outflow from *mouse* striatal slices was reduced to 6% (compared to controls) in animals lacking the β_2nAChR subunit. These observations are in line with data obtained in striatal synaptosomal preparations, where β_2 null mutations were found to eliminate nicotine-induced [^{3}H]dopamine release (Whiteaker *et al*., 2000; Grady *et al*., 2002; Champtiaux *et al*., 2003; Salminen *et al*., 2004). However, our results imply that not only direct (TTX-resistant) but also indirect mechanisms depend on nAChRs containing the β_2 subunit, as most of the [^{3}H]dopamine outflow from *mouse* striatal slices in response to nicotine was owing to an indirect (TTX-sensitive) effect. nAChRs containing the β_2 subunit also mediate the indirect (action potential-dependent) dopamine release caused by endogenous ACh in striatal slice preparations (Zhou *et al*., 2001).

We have shown that β_2 null mutations greatly reduced catecholamine outflow not only from striatal but also from hippocampal slices. These data are in line with a recent study showing that [^{3}H]noradrenaline outflow in response to nicotine is abolished in hippocampal synaptosomes prepared from β_2-KO animals (Azam and McIntosh, 2006). By the combined use of subunit-specific conotoxins (α-MII, α-BuIA, α-PIA and α-AuIB) and KO animals (β_2, β_3, β_4 and α_4) the authors identified two types of nAChRs modulating [^{3}H]noradrenaline outflow from synaptosomes: $\alpha_6(\alpha_4)\beta_2\beta_3\beta_4$ and $\alpha_6(\alpha_4)\beta_2\beta_3$.

Pharmacological evidence suggests that receptors modulating [^{3}H]noradrenaline outflow in *rat* hippocampal synaptosomes are distinct from those in *mice* and contain the subunits α_3 and β_4 (Clarke and Reuben, 1996; Luo *et al*., 1998; Azam and McIntosh, 2006). Hence, α-Ctx MII fully inhibits [^{3}H]noradrenaline outflow in mice but is ineffective in rat synaptosomes. Likewise, α-Ctx PIA which reduces [^{3}H]noradrenaline outflow by about 80% in *mice* potentiates nicotine effects in *rat* hippocampal synaptosomes (Azam and McIntosh, 2006)). The significantly larger outflow of [^{3}H]noradrenaline in response to nicotine seen in *rat* compared to *mouse* hippocampal synaptosomes (Azam and McIntosh, 2006) may thus be caused by more efficient receptors, a higher number of receptors, or both.

Synaptosomes taken from adult *mice* do not display significant [^{3}H]noradrenaline outflow above baseline (Azam and McIntosh, 2006). As most of our experiments were performed on hippocampal slices of 6–8 weeks old *mice*, nAChRs modulating [^{3}H]noradrenaline release by indirect mechanisms not residing on noradrenergic projections may play a dominant role. The absence of nicotine-evoked [^{3}H]noradrenaline outflow from hippocampal slices taken from β_2-KO animals indicates that in *mice*, these indirect mechanisms crucially depend on the presence of the β_2 subunit.

Targeted deletion of the nAChR α_5 subunit gene

Three main types of hetero-oligomeric nAChRs have been identified in *mouse* dopamine terminal fields: $\alpha_4\beta_2$*, $\alpha_6\beta_2$* and $\alpha_4\alpha_6\beta_2$*. Whereas α6-containing receptors are sensitive to α-CtxMII, those made of $\alpha_4\beta_2$* are not (Champtiaux *et al*., 2003). α_5 was present in 9% of purified α_4* nAChRs but in only 1% of α_6* receptors (Champtiaux *et al*., 2003). Nonetheless, targeted deletion of the α_5 subunit gene affected not only α-CtxMII-insensitive (presumably mediated by $\alpha_4\beta_2$* receptors) but also α-CtxMII-sensitive (presumably mediated by α_6* receptors) ACh-induced outflow of [^{3}H]DA, albeit in an opposite manner: Whereas maximally induced release (R_{max}) in the presence of 100 nM α-CtxMII was reduced in the α_5 KO, the R_{max} of the α-CtxMII-sensitive component was enhanced (Salminen *et al*., 2004). The report makes no mention whether the two opposite effects of the α_5-KO level off when looking at overall outflow in the absence of α-CtxMII.

As in synaptosomal preparations (Kulak *et al*., 1997; Kaiser *et al*., 1998; Kaiser and Wonnacott, 2000; Grady *et al*., 2002; Champtiaux *et al*., 2003; Salminen *et al*., 2004) we found α-CtxMII to inhibit [^{3}H]dopamine release from *mouse* striatal slices by about 50% in response to nicotine. Our assay is, however, not sufficiently robust to distinguish possible effects of a deletion of the α_5 subunit on the α-CtxMII-sensitive and the α-CtxMII-insensitive component of [^{3}H]dopamine release, as carried out in *mouse* striatal synaptosomes (Salminen *et al*., 2004). The overall release (R_{max} and EC_{50} values in the absence of α-CtxMII) induced by three nicotinic agonists nicotine, cytisine and DMPP was not significantly affected in *mice* lacking the α_5 subunit. These results are in contrast to observations we made in cultured sympathetic neurons, where targeted deletion of the α_5 subunit gene

caused a large enhancement of nicotine-evoked [^{3}H]noradrenaline outflow (Fischer *et al*., 2005).

Impact of nAChRs located in the projection areas of catecholaminergic neurons

Excitation of dopaminergic neurons in the midbrain and of noradrenergic neurons in the locus coeruleus play a major, if not dominant, role in the release of catecholamines upon systemic application of nicotine (Mitchell, 1993; Fu *et al*., 1998; Mansvelder and McGehee, 2000; Picciotto and Corrigall, 2002; Mansvelder *et al*., 2003; David *et al*., 2006). The collective activation of projecting axons by electrical field stimulation in brain slices provides some indirect evidence on the overall release capacity of these systems. When set in relation to the electrically induced outflow, nicotine by acting on receptors in the terminal fields is about equieffective in stimulating [^{3}H]dopamine release from the striatum, but significantly less so in evoking [^{3}H]noradrenaline outflow from the neocortex of both *rats* and *mice*. The modulation of [^{3}H]noradrenaline outflow in the hippocampus by nicotine is species-dependent: sizeable in *rat*, but small in the *mouse* hippocampal slice preparation.

Our comparative observations made in striatal, hippocampal and neocortical slice preparations from *rats* and *mice* showed regional and species dependent differences of nAChR properties in the terminal fields of catecholaminergic projections. In *mice*, nicotine-evoked catecholamine outflow is primarily mediated by action potentials and dependent on nAChRs containing the β_2 subunit.

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Conflict of interest

The authors state no conflict of interest.

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