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Indirect modulation by α7 nicotinic acetylcholine receptors of noradrenaline release in rat hippocampal slices: interaction with glutamate and GABA systems and effect of nicotine withdrawal

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Running title: α7 nAChR modulate noradrenaline release

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Abbreviations: nAChR, nicotinic acetylcholine receptor; NA, noradrenaline; DA, dopamine; α-CTxMII, α-conotoxin-MII; α-CTxImI, α-Conotoxin-ImI; DHβE, dihydroβerythroidine; MLA, methyllycaconitine; αBgt, αbungarotoxin; α-CnTxAuIB, α-conotoxinAuIB; DNQX, 6,7-dinitroquinoxaline-2,3dione; AnTx, anatoxin-a; GluR, glutamate receptor.

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Abstract

Nicotinic acetylcholine receptors (nAChR) can modulate transmitter release. Striatal [^{3}H]dopamine ([^{3}H]DA) release is regulated by presynaptic nAChR on dopaminergic terminals and α7 nAChR on neighbouring glutamatergic afferents. Here, we explored the role of α7 nAChR in the modulation of [^{3}H]noradrenaline ([^{3}H]NA) release from rat hippocampal slices. The nicotinic agonist anatoxin-a (AnTx) evoked monophasic [^{3}H]NA release (EC_{50}=1.2 μM) that was unaffected by α-conotoxin-MII or dihydro-beta-erythroidine, antagonists of α3/α6β2* and β2* nAChR respectively. In contrast AnTx-evoked striatal [^{3}H]DA release was biphasic (EC_{50}=138.9 nM; 7.1 μM) and blocked by these antagonists. At a high AnTx concentration (25 μM), α7 nAChR antagonists (methyllycaconitine, α-conotoxin-ImI) and glutamate receptor (GluR) antagonists (kynurenic acid, DNQX) partially inhibited [^{3}H]NA release. The α7 nAChR-selective agonist choline evoked [^{3}H]NA release (E_{max}=33% of that of AnTx) that was blocked by GluR antagonists, supporting a model in which α7 nAChR trigger glutamate release that subsequently stimulates [^{3}H]NA release. A GABAergic component was also revealed: choline-evoked [^{3}H]NA release was partially blocked by the $GABA_A$ receptor antagonist bicuculline, and co-application of bicuculline and DNQX fully abolished this response. These findings support α7 nAChR on GABAergic neurones that can promote GABA release that, in turn, leads to [^{3}H]NA release, probably by disinhibition. To investigate the impact of chronic nicotine exposure on this model, rats were exposed for 14 days to nicotine (4 mg/kg/day) with or without 3 or 7 days withdrawal. α7 nAChR responses were selectively and transiently up-regulated after 3 days withdrawal. This functional up-regulation could contribute to the withdrawal effects of nicotine.

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Introduction

Neuronal nicotinic acetylcholine receptors (nAChR) have a pivotal role in modulating the release of various neurotransmitters from different brain areas including the hippocampus and the striatum (for review see Wonnacott, 1997, Vizi and Kiss, 1998). The hippocampus is a key structure implicated in memory formation where changes in long term synaptic potentiation are considered to be a cellular mechanism underlying aspects of learning and memory. Nicotinic agonists, through activation of neuronal nAChR, improve cognitive performance in both animals and humans (Levin and Simon, 1998; Newhouse et al., 2004), and hippocampal nAChR, including α7 nAChR influence synaptic plasticity through facilitation of presynaptic and postsynaptic mechanisms (Ji et al., 2001). Furthermore, Alzheimer's disease patients exhibit memory deficits characterized by a marked decline in cholinergic transmission and a decreased number of nAChR binding sites in the cortex and hippocampus (Gotti and Clementi, 2004), and nicotine can ameliorate the cognitive deficit in these patients (Newhouse et al., 2004). Therefore, the modulatory properties of nAChR contribute to both normal and pathological brain functions.

The noradrenergic input to the hippocampus largely arises from the ascending dorsal noradrenergic bundle that projects from the locus coeruleus (Ungerstedt, 1971). In vivo, intra-hippocampal administration of nicotine evoked noradrenaline (NA) release in a mecamylamine-sensitive manner (Mitchell, 1993). The presence of presynaptic nAChR on noradrenergic terminals in the hippocampus is consistent with nicotine-evoked [^{3}H]NA release from hippocampal synaptosomes (Clarke and Reuben, 1996; Luo et al., 1998). Pharmacological studies to characterise the nAChR mediating [^{3}H]NA release from hippocampal synaptosomes and slices provided evidence for nAChR heterogeneity, but the paucity of specific antagonists has limited the resolution of the subtypes of nAChR involved (Clarke and Reuben, 1996; Sershen et al., 1997; Anderson et al., 2000; see Vizi and Kiss, 1998). Luo et al. (1998) demonstrated that α-conotoxinAuIB (α-CnTxAuIB) blocked ~30% of

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the NA release elicited by nicotine from hippocampal synaptosomes, consistent with α3β4* nAChR on noradrenergic terminals being responsible for this proportion of the response.

It is likely that [^{3}H]NA release from slice preparations will reflect additional, indirect contributions of nAChR present on neighbouring neurons. Leslie et al. (2002) suggested an indirect GABAergic component in the modulation of NA release, in a hippocampal slice preparation, because [^{3}H]NA release evoked by nicotine (100μM) was partially blocked by the $GABA_A$ antagonist bicuculline. Functional glutamate heteroreceptors on hippocampal synaptosomes have been reported (Risso et al., 2004), and there is a large body of evidence from electrophysiological recordings from hippocampus that α7 nAChR regulate both glutamatergic and GABAergic neurotransmission (e.g. Gray et al., 1996; McQuiston and Madison, 1999; Alkondon and Albuquerque, 2000a; Buhler and Dunwiddie, 2001). Hence, α7 nAChR are a plausible candidate for an indirect modulation of NA release.

In the present work, we have examined the role of α7 nAChR in the modulation of [^{3}H]noradrenaline ([^{3}H]NA) release from hippocampal slices, which preserve some of the existing neuroanatomical connections, enabling the investigation of neurotransmitter cross-talk. Pharmacological dissection of the response provides evidence for the indirect modulation of [^{3}H]NA release by α7 nAChR, via activation of both GABA and glutamate release. This is shown to differ from the nicotinic control of striatal [^{3}H]dopamine ([^{3}H]DA) release. We have also demonstrated that the indirect α7 nAChR modulation of [^{3}H]NA release was selectively up-regulated during nicotine withdrawal.

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Material and Methods

Drugs and reagents (±)Anatoxin-a fumarate (AnTx), (R)-baclofen, muscimol, CGP 54626 hydrochloride, kynurenic acid, GABA, (RS)-AMPA, kainic acid, (-)-bicuculline methochloride 1(S) 9(R) (bicuculline), 6,7-dinitroquinoxaline-2,3dione (DNQX), GBR 12909 dihydrochloride, nisoxetine hydrochloride and α-conotoxin MII (α-CnTxMII) were obtained from Tocris Cookson (Avonmouth, UK). Dihydro-beta-erythroidine hydrobromide (DHβE), (+)-MK801 maleate, pargyline, N-methyl-D-aspartic acid (NMDA), choline tartrate, ascorbic acid, L-glutamic acid, methyllycaconitine citrate (MLA), (-)-nicotine hydrogen tartrate and α-conotoxin ImI (α-CnTxImI) were purchased from Sigma Chemical Co. (Poole, Dorset, UK). [7,8-^{3}H]noradrenaline (35 Ci/mmol), [7,8-^{3}H]dopamine (40 Ci/mmol), [I^{125}]αbungarotoxin (256 Ci/mmol) and [^{3}H]epibatidine (54 Ci/mmol) were obtained from Amersham International (Buckinghamshire, UK).

Animals Male Sprague Dawleys rats (250-320g) were obtained from the University of Bath Animal House breeding colony. Food and water were provided *ad libitum*. The experimental procedures were in accordance with the guidelines of the Animals (Scientific Procedures) Act 1986.

[^{3}H]noradrenaline and [^{3}H]dopamine release Assessment of hippocampal [^{3}H]noradrenaline ([^{3}H]NA) or striatal [^{3}H]dopamine ([^{3}H]DA) release from slices was performed using a 96 well assay (Anderson et al., 2000; Jacobs et al., 2002). For each experiment, 2 rats were killed by cervical dislocation. Brains were rapidly removed and hippocampi and striata dissected and transferred to ice cold Krebs buffer (KB: NaCl 118 mM, KCl 2.4 mM, $CaCl_2$ 2.4 mM, KH_2PO_4 1.2 mM, $MgSO_4.7H_2O$ 1.2 mM, $NaHCO_3$ 25 mM, D-glucose 10 mM, ascorbic acid 1 mM, gassed with 95 % O_2 and 5 % CO_2 for at least 1 h at 37°C; pH was adjusted to 7.4); for experiments in Mg^{2+}-free KB, $MgCl_2$ was omitted from the buffer. Tissue was chopped 3 times (2 rotations at 60°) using a McIlwain tissue chopper to give prisms of 150 µm. Following 2 washes with warm KB, hippocampal and striatal slices

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were incubated for 30 min with 70 nM [^{3}H]NA and 50 nM [^{3}H]DA, respectively, in 5 ml KB supplemented with 10 µM pargyline (to prevent [^{3}H]catecholamine degradation) at 37°C. To remove excess of tritium, 5 washes were performed over 25 min in KB containing 10 µM pargyline and 0.5 µM nomifensine (to prevent [^{3}H]catecholamine reuptake). Slices were then loaded onto a 96 well filter plate (model MABVN1250, Millipore, Hertfordshire, UK) and incubated for 5 min with buffer in the presence or absence of antagonist or tetrodotoxin (this pre-incubation was extended to 10 min for α-CnTxImI). Following this, buffer was removed by filtration (basal values) and collected in a 96-well Packard Optiplate™ (Perkin Elmer, Belgium). Buffer (70 µl) containing agonists and/or antagonists was then added to each well (in each experiment, a buffer stimulation was included to determine the fractional release of [^{3}H]NA or [^{3}H]DA evoked by buffer alone). After a further 5 min at 37°C, buffer was collected by filtration in an Optiplate™ to determine the fraction of [^{3}H]catecholamine released. Microscint™ (170 µl per well) was added to optiplates and each well was counted for 1 min using a Microbeta liquid scintillation counter (Wallac 1450 Microbeta Trilux, Perkin Elmer, Finland), counting efficiency 30%. To determine the amount of tritium remaining in the slices, each filter of the 96-well filter plate was removed, transferred to scintillation vials containing 4ml of Optiphase™ and counted for 1 min using a Packard 1600 Tri-carb liquid scintillation counter (counting efficiency 45%). The amount of [^{3}H]catecholamine released was expressed as a percent of total radioactivity taken up in the slices prior to stimulation (i.e. amount of tritium released + tritium remaining in the tissue), giving a fractional release of [^{3}H]catecholamine. Each experiment was performed in 6 replicates and repeated at least 3 times.

Release experiments following chronic nicotine administration (see below) were carried out with minor modifications of the protocol previously described. Release was assessed from tissue originating from each animal individually. The incubation with 70 nM [^{3}H]NA was completed in a volume of 2.5 ml, and the final resuspension of the tissue, before loading into one half of a 96-well filter plate, was into 5 ml. On each plate, slices obtained from a saline-

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and a nicotine-treated animal were assessed in parallel. Chronic nicotine treatment had no effect on basal release or responses to buffer stimulation.

[^{3}H]NA uptake Hippocampal slices, obtained from 1 rat (~200 mg of tissue), were re-suspended in 5ml KB (supplemented with 10 µM pargyline) and divided into 200 µl aliquots. Each tube received an equal volume of either buffer alone (control) or buffer containing nisoxetine (1 µM) or GBR 12909 (10 or 50 nM). [^{3}H]NA was added to give a final concentration of 70 nM and slices were maintained at 37°C for 30 min. The samples were then filtered through GF/C glass filters soaked in cold KB using a Millipore filtration manifold. Following 3 washes with 5 ml of cold KB, filters were added 4 ml of Optiphase and counted for radioactivity using a Packard 1600 Tri-carb liquid scintillation counter. Experiments, performed in triplicate, were repeated 4 times with tissue from 3 animals.

Chronic nicotine administration Rats were anaesthetized with Isofluorane and Alzet Osmotic minipumps (model 2002) were implanted subcutaneously; osmotic minipumps were filled with saline (control) or nicotine bitartrate, dissolved in saline and pH adjusted to 7.4, to deliver nicotine (4 mg/kg/day, free base) at a rate of 0.5 µl/h for 14 days. Nicotine administration was without effect on weight gain. To study the withdrawal period, osmotic minipumps were surgically removed after 14 days and animals were sacrificed by cervical dislocation and decapitation 3 or 7 days later. Each timepoint was determined for at least 8 rats, with saline- and nicotine-treated animals paired.

Nicotine and cotinine levels Following decapitation, trunk blood was collected in heparinised Eppendorf tubes. Blood was centrifuged at 2500 rpm for 15 min at 4°C, the supernatant was collected and re-centrifuged at 1500 rpm for 30 min at 4°C; the final supernatant was frozen in liquid nitrogen and stored until submitted for nicotine and cotinine analysis. Following 14 days of nicotine administration, nicotine and cotinine levels were 49.8 ± 3.0 ng/ml and 335.3 ± 18.2 ng/ml respectively (n=16).

Radioligand binding *Membrane preparation* Individual rat brains (minus cerebellum, hippocampus and striatum) were homogenized in ice cold 0.32 M sucrose containing 1 mM EDTA, 0.1 mM PMSF and 0.01 % NaN_3 (pH 7.4, 10% w/v). The homogenate was centrifuged

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at 15 000 g for 25 min. The pellet was resuspended in 50 mM phosphate buffer (40 mM K_2HPO_4, 10 mM KH_2PO_4, 1 mM EDTA, 1 mM PMSF and 0.01% NaN_3, pH 7.4) and centrifuged at 15 000 g for 25 min. The wash step was repeated and the final pellet was resuspended in 2.5 ml/mg original weight in ice cold 50 mM phosphate buffer and frozen until use. Protein concentration was estimated by using a colorimetric protein dye reagent.

[^{3}H]epibatidine binding assay [^{3}H]epibatidine binding was performed on 150 µg of brain membranes, in a final volume of 1 ml (NaCl 118 mM, KCl 4.8 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 2 mM, Hepes 20 mM, Tris 20 mM, PMSF 0.1 mM and 0.01% sodium azide, pH 7.4). The final concentration of [^{3}H]epibatidine was 500 pM. Non specific binding (~5-10% of total binding) was determined in the presence of 1 mM nicotine. Samples were incubated for 1.5 h at room temperature, followed by 30 min at 4^{o}C. Then samples were filtered through Gelman GFA filters, presoaked overnight in 0.3% polyethylene immine (PEI) using a Brandel cell harvester. Filters were washed 3 times with ice cold PBS, and counted for radioactivity using a Packard 1600 Tricarb scintillation counter (counting efficiency 45%). Each assay was conducted in triplicate.

[^{125}I]αbungarotoxin binding assay [^{125}I]αbungarotoxin ([^{125}I]αBgt) binding was performed on 250 µg of membranes in a final volume of 200 µl of phosphate buffer supplemented with 0.1 % BSA, (pH 7.4) and [^{125}I]αBgt to give a final concentration of 10 nM. Non specific binding (~15-20% of total binding) was determined in the presence of 1 mM nicotine. Samples were incubated for 3 h at 37°C and then 1 mL of buffer was added to each tube prior to 1 h incubation at 37°C. Samples were transferred at 4°C for 30 min before filtration through Gelman GFA filters, presoaked overnight in 0.3% PEI and 4% milk powder, using a Brandel cell harvester. Filters were then washed and counted as described above for [^{3}H]epibatidine binding assay. Counting efficiency for [^{125}I]αBgt was 60%.

Data analysis Data are presented as mean ± S.E.M from 3 to 10 experiments. Statistical significance was determined using Student's unpaired t-test, paired t-test and one way ANOVA with *post hoc* Tukey's or Bonferroni's test, as stated in the figure legends (Sigma

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Stat, Jandel scientific, Ekhrath, Germany). Values of at least $p<0.05$ were taken to be statistically significant.

Dose response curves for agonists (except choline) were fitted to a single- or double-site model as previously described by Kaiser and Wonnacott (2000). The choline dose response curve was fitted to a bell-shaped model as described by:

$$Y = [Ymax/(1+(EC_{50}/C)^{n1})] \times [1/(1+(IC_{50}/C)^{n2})],$$

where, Y is the amount [^{3}H]NA elicited by a concentration C of choline; Ymax is the maximum amount of [^{3}H]NA release; EC_{50} and IC_{50} are values at the inflection points and n_1 and n_2 are the Hill coefficients (slope parameters).

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Results

Specificity of [^{3}H]NA uptake by hippocampal slices. The hippocampus receives modest dopaminergic projections from the midbrain (Jay et al., 2003) and nAChR activation has been shown to modulate DA release from hippocampal slices (Cao et al., 2004). To determine whether the uptake of [^{3}H]NA was specific to noradrenergic terminals, we carried out uptake experiments in the presence of specific inhibitors of the DA and NA transporters, GBR 12909 and nisoxetine respectively. In the absence of inhibitor, hippocampal slices incubated with 70 nM [^{3}H]NA accumulated 19423 ± 1576 cpm (n=4). However, following a 30 min pre-incubation with nisoxetine (1 µM), a concentration that blocked the uptake of [^{3}H]NA into rat frontal cortical synaptosomes (Cheetham et al., 1996), the amount of [^{3}H]NA taken up was substantially decreased, by 82.1 ± 1.7 % (n=4, $p<0.01$, one way ANOVA with *post hoc* Tukey's test). The potent and selective dopamine transporter inhibitor GBR 12909, at 10 and 50 nM (concentrations corresponding to 1.5 and 7.5 times the IC_{50} for inhibition of striatal dopamine reuptake, Ghorai et al., 2003), produced only weak inhibition of [^{3}H]NA uptake, by 16.3 ± 5.0 % and 21.6 ± 4.4 % respectively. Only the latter value was significantly different from control (n=4, $p<0.05$, one way ANOVA with *post hoc* Tukey's test). Therefore, under the conditions used for release experiments, 80 % of the accumulated [^{3}H]NA was transported into noradrenergic terminals.

Pharmacological characterization of nAChR-mediated [^{3}H]NA release from hippocampal slices. In agreement with previous reports (Sershen et al., 1997), the potent nicotinic agonist anatoxin-a (AnTx) concentration-dependently evoked [^{3}H]NA release that best fitted a single site model, with an EC_{50} of 1.2 µM (Fig. 1A). This is in contrast to AnTx-evoked striatal [^{3}H]DA release which was biphasic (EC_{50} values of 138.9 nM and 7.1 µM, Fig. 1B), in agreement with Kaiser and Wonnacott (2000). To distinguish the nAChR subtype(s) responsible for AnTx-evoked [^{3}H]NA release from hippocampal slices, we tested the effects of antagonists on responses to 1 µM (~EC_{50}) and 25 µM (maximal response) AnTx. The

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broad spectrum antagonist mecamylamine (20 μM) fully abolished release of [^{3}H]NA elicited by both concentrations of AnTx (Fig. 1A). [^{3}H]NA release evoked by AnTx (1 μM; 25 μM) was not decreased in the presence of DHβE (10μM), a β2* selective antagonist (Fig. 1C, D). Consistent with data from hippocampal synaptosomes (Luo et al., 1998), the α3/α6β2*-selective antagonist α-CnTxMII (200nM; Nicke et al., 2005) also failed to inhibit responses elicited by 1 μM AnTx (Fig. 1C). On the other hand, striatal [^{3}H]DA release elicited by 1 μM AnTx was partially antagonised by α-CnTxMII (60.0 ± 4.6 % of control) and virtually abolished by DHβE (6.0 ± 5.6 % of control) (Fig. 1B). This is consistent with the high affinity component of AnTx-evoked striatal [^{3}H]DA release comprising α6β2* and α4β2* nAChR (Champtiaux et al., 2003).

The lack of involvement of α3/α6β2* nAChR in hippocampal [^{3}H]NA release enabled us to use MLA as an α7-selective antagonist in this preparation (MLA also potently inhibits α6β2* nAChR, Mogg et al., 2002). Pre-incubation of hippocampal slices with 20 nM MLA had no effect on responses to 1 μM AnTx (Fig. 1C). However MLA and the structurally unrelated α7-selective antagonist, α-CnTxImI (1 μM; Nicke et al., 2005) inhibited responses elicited by a higher concentration of AnTx (25 μM) by 35.3 ± 9.2 % and 17.1 ± 5.2 % respectively (Fig. 1D). Thus, α7 nAChR are activated only by concentrations in the upper range of the AnTx concentration response curve, as is the case for AnTx-evoked striatal [^{3}H]DA release (Kaiser and Wonnacott, 2000). Whereas the wide difference in sensitivity to AnTx of the β2* and α7 nAChR components modulating [^{3}H]DA release result in a biphasic concentration response curve (Kaiser and Wonnacott, 2000; Fig. 1B), the lower sensitivity of the non-β2, non-α7 nAChR component of [^{3}H]NA release must overlap that of the α7 nAChR component sufficiently to result in a single concentration response curve best fitted to a simple site (Fig. 1A). This is compatible with the 8 fold lower affinity of AnTx for heterologously expressed α3β4 nAChR compared with forebrain membranes that represent predominantly α4β2 (Xiao et al., 1998).

To confirm the involvement of α7 nAChR, we used the selective agonist choline (Alkondon et al., 1997). Choline evoked both striatal [^{3}H]DA release (Fig. 1B) and hippocampal [^{3}H]NA

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release (Fig. 1A) with a bell-shaped concentration response curve. The maximum response in hippocampal slices was reached by 5 mM choline and corresponds to 33.3 ± 1.4 % of that achieved by AnTx. In both tissues, choline-evoked responses were nAChR-mediated as they were fully abolished by mecamylamine (20 µM) (Fig. 1A, B). Choline-evoked [^{3}H]NA release was also fully blocked by α-CnTxImI (Fig. 1D). These results support the modulation of [^{3}H]NA by multiple subtypes of nAChR, including α7 nAChR.

Ionotropic glutamate receptors stimulate the release of [^{3}H]NA from hippocampal slices. Glutamate evoked [^{3}H]NA release with a low potency (EC_{50}= 177.0 µM, Fig. 2A). The selective agonists kainate and AMPA evoked [^{3}H]NA release with respective EC_{50} values of 31.6 µM and 28.4 µM, similar to values reported by Pittaluga and Raiteri (1992) (Fig. 2B). Responses elicited by glutamate (100 and 500 µM), AMPA (100 and 500 µM) and kainate (100 and 500 µM) were largely abolished by the selective AMPA/Kainate antagonist DNQX (200 µM, Fig. 2A and B). To circumvent the block by Mg^{2+} of NMDA receptors, in order to investigate their ability to elicit the release of [^{3}H]NA, we used Mg^{2+}-free Krebs buffer (see Methods). Under these conditions, NMDA induced [^{3}H]NA release with an apparent EC_{50} value of 33.0 µM, consistent with previous studies (Pittaluga and Raiteri, 1992, Risso et al., 2004; Fig. 2A). The specific NMDA antagonist MK801 (5 µM) fully blocked the response to 500 µM NMDA (Fig. 2A). Antagonists had no effect on basal release in the absence of agonists.

To explore possible crosstalk between glutamate receptors (GluR) and nAChR, we determined the AnTx concentration response curve for [^{3}H]NA release in the presence or absence of the non-selective GluR antagonist kynurenic acid (800 µM). Significant inhibition of 23.8 ± 10.8 % was seen only at the highest agonist concentration examined (25µM, Fig. 2C). A similar block was obtained in the presence of DNQX (200 µM, 25.0 ± 5.9 % of control, Fig. 2C). As seen in Fig. 1D, [^{3}H]NA release evoked by 25 µM AnTx is also partially blocked α7 nAChR antagonists. Therefore, we investigated the effect of the same GluR antagonists on responses evoked by the selective α7 nAChR agonist choline. Both DNQX and kynurenic

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acid partially inhibited [^{3}H]NA release evoked by 1 mM choline (41.2 ± 5.1 % and 51.1 ± 9.4 % respectively, $p<0.05$, one way ANOVA with Bonferroni's test, Fig. 2D). In Mg^{2+}-free Krebs buffer, 1 mM choline elicited greater [^{3}H]NA release, although this increase was not significantly different from control choline responses in the presence of $MgCl_2$ (Fig. 2D). Choline-evoked [^{3}H]NA release in Mg^{2+}-free conditions was blocked by 88.4 ± 19.9 % in the presence of 5 µM MK-801 ($p<0.05$, Student's t-test, Fig. 2D).

Effect of GABA agonists and antagonists on [^{3}H]NA release from rat hippocampal slices. GABA can evoke [^{3}H]NA release from hippocampal synaptosomes (Fassio et al., 1999). In the hippocampal slice preparation, GABA (100 µM) induced a fractional release of [^{3}H]NA of 6.4 ± 0.8 %, similar to the value reported by Fassio et al. (1999, Fig. 3A). CGP 54626 (1 µM), a potent $GABA_B$ receptor antagonist, had no significant effect on GABA stimulation, whereas bicuculline (100 µM), a $GABA_A$ receptor selective antagonist, abolished the GABA-mediated [^{3}H]NA release (Fig. 3A). Pre-incubation for 5 min with bicuculline did not alter basal release. To confirm the predominant role of $GABA_A$ receptors in GABA-evoked [^{3}H]NA release, we stimulated [^{3}H]NA release with selective agonists for each subtype. Muscimol (100 µM), a $GABA_A$ receptor agonist, mimicked the response to GABA and this release was also completely blocked by bicuculline (Fig. 3A). On the other hand, the $GABA_B$-selective agonist, baclofen (100 µM), was ineffective (Fig. 3A).

Subsequent experiments examined the possibility of crosstalk between the α7 nAChR and $GABA_A$ receptor-mediated responses. Co-application of choline and GABA evoked [^{3}H]NA in a non-additive manner (8.1 ± 1.1 %, compared to choline and GABA alone, 7.7 ± 1.4 % and 7.0 ± 0.7 % respectively, n=3, $p>0.05$, one way ANOVA with Bonferroni's test). Stimulation of [^{3}H]NA release by choline (1 mM) was decreased by 80.3 ± 4.6 % in the presence of bicuculline (Fig. 3B); this inhibition was additive with the blockade by DNQX, as co-application of both antagonists fully abolished the release induced by choline (4.7 ± 3.5 %, significantly different from bicuculline or DNQX alone, $p<0.05$ one way ANOVA with

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Bonferroni's test, Fig. 3B). This suggests that α7 nAChR promote both glutamate- and GABA-evoked [^{3}H]NA release. In contrast, in striatal slices choline-evoked fractional release of [^{3}H]DA release was similar in the presence or absence of bicuculline (6.8 ± 0.9 % and 7.2 ± 0.9 % respectively, data not shown, n=6), consistent with the failure of GABA to evoke [^{3}H]DA release (0.3 ± 2.4 %, n=3).

To further explore transmitter crosstalk in the hippocampus, we tested the effects of bicuculline and DNQX on glutamate (500 µM)- and GABA (100 µM)-evoked [^{3}H]NA release respectively. Interestingly, DNQX inhibited GABA-evoked responses by 65.3 ± 7.3 %, whereas bicuculline failed to inhibit glutamate-evoked responses (95.0 ± 5.7 % compared to control 100.0 ± 9.2 %, $p>0.05$ Student's t-test, Fig. 3C). These results suggest that GABA acts upstream of glutamate stimulation of [^{3}H]NA release.

Tetrodotoxin (TTX) sensitivity of [^{3}H]NA release from rat hippocampal slices. To assess the requirement for voltage-gated Na^+ channels for the release of [^{3}H]NA from hippocampal slices, experiments were conducted in the presence or absence of tetrodotoxin (TTX, 1 µM). The voltage-gated Na^+ channel activator veratridine (50 µM) evoked [^{3}H]NA release that was entirely blocked by TTX (Table 1). Consistent with previous reports (Sershen et al., 1997; Leslie et al., 2002) we found a substantial block by TTX of nAChR-mediated [^{3}H]NA release evoked by AnTx (1µM) or choline (1 mM) (Table 1). As the response to choline was blocked by bicuculline and DNQX (Fig. 3B), we compared the effect of TTX on GABA- and glutamate-evoked [^{3}H]NA release. Responses to both transmitters were virtually abolished in the presence of TTX (Table 1), consistent with Na^+ channel activation in the mediation of these responses.

Effect of chronic nicotine treatment on nAChR-mediated [^{3}H]NA release. Rats were treated with nicotine for 14 days by osmotic minipump (4 mg/kg/day). Assays were conducted on day 14, or following 3 or 7days withdrawal. Brain [^{3}H]epibatidine binding sites were up-regulated by 37.0 ± 15.7 % at 14 days nicotine treatment and remained significantly

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elevated by 42.0 ± 10.3 % and 46.9 ± 11.3 % at 3 and 7 days withdrawal respectively. In contrast, numbers of α7 nAChR labelled with [^{125}I]αBgt were unchanged (Table 2).

Hippocampal [^{3}H]NA release elicited by 1 μM AnTx was similar in saline- and nicotine-treated animals following 14 days' treatment and during the withdrawal period (Fig. 4A). α7 nAChR-mediated [^{3}H]NA release evoked by 1 mM choline was unchanged following 14 days' treatment with nicotine, but was significantly up-regulated by 61.6 ± 19.4 % at 3 days withdrawal (Fig. 4B). This functional up-regulation returned to control levels at 7 days withdrawal.

Inhibition of choline-evoked [^{3}H]NA release by DNQX or bicuculline was similar at the 3 time points (Table 3), indicating a lack of effect of the nicotine treatment at non-NMDA GluR and $GABA_A$ receptors. This is consistent with the lack of change in AMPA- and GABA-evoked [^{3}H]NA release following chronic nicotine exposure and its withdrawal (Table 4).

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Discussion

In this study, we have characterised the modulation of [^{3}H]NA release from rat hippocampal slices by nAChR. The broad spectrum nAChR agonist AnTx provided evidence for the participation of multiple nAChR subtypes in the regulation of [^{3}H]NA release. A high concentration of AnTx (25 µM) elicited [^{3}H]NA release that was partially blocked by α-CnTxImI and MLA, two α7 nAChR antagonists. The involvement of α7 nAChR was supported by the ability of the α7-selective agonist choline to evoke [^{3}H]NA release. [^{3}H]NA release was positively modulated by ionotropic glutamate receptor and $GABA_A$ receptor agonists, and α7 nAChR-mediated responses evoked by choline were fully abolished by co-application of DNQX and bicuculline. These results are consistent with the hypothesis that α7 nAChR present on both glutamatergic and GABAergic neurons indirectly modulate [^{3}H]NA release by promoting the release of glutamate and GABA, probably involving a mechanism of disinhibition of the glutamatergic/GABAergic network (see model, Fig. 5). After chronic nicotine treatment, the α7 nAChR-mediated responses were selectively and transiently up-regulated at 3 days withdrawal.

Subtypes of nAChR mediating [^{3}H]NA release from hippocampal slices. Pharmacological identification of nAChR subtypes mediating NA release has been restricted by the limited availability of drugs to discriminate different combinations of nAChR subunits. The involvement of β2* nAChR in the nicotinic modulation of [^{3}H]NA release is controversial: some studies reported sensitivity to DHβE (Clarke and Reuben, 1996; Anderson et al., 2000), whereas others failed to show any effect of this antagonist (Sershen et al., 1997; Leslie et al., 2002; and the present work). In contrast, the presence of the β2* nAChR on striatal dopaminergic terminals is consistent with pharmacological data (Kaiser and Wonnacott, 2000) and functional comparisons of tissue from wildtype and β2 null mutant mice (Champtiaux et al., 2003). Consistent with the lack of β2* nAChR on hippocampal noradrenergic terminals, AnTx was much less potent in evoking hippocampal [^{3}H]NA release (EC_{50} 1.2 µM, Fig. 1A) compared with striatal [^{3}H]DA release (EC_{50} 138.9 nM, inset Fig.1A;

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see Kaiser and Wonnacott, 2000). The ineffectiveness of α-CnTxMII with respect to AnTx- (Fig. 1B) or nicotine-evoked [^{3}H]NA release from hippocampal preparations (Luo et al., 1998) rules out α6(α3)β2* nAChR, whereas partial inhibition by α-CnTxAuIB (Luo et al., 1998) supports the presence of α3β4* nAChR. However these results do not exclude the possibility of an α6(α3)β4* combination (see Léna et al., 1999).

The ability of choline to elicit [^{3}H]NA release, and inhibition of responses elicited by high concentrations of AnTx (25 μM) by both α-CnTxImI and MLA, implicate α7 nAChR in the modulation of [^{3}H]NA release. Clarke and Reuben (1996) did not find any blockade by 10 nM MLA of nicotine-evoked [^{3}H]NA release from hippocampal synaptosomes, implying that α7 nAChR are not present on noradrenergic terminals. Together these data suggest that at least 2 distinct nAChR populations modulate [^{3}H]NA release from hippocampal slices: α7 nAChR activated by choline and high AnTx concentrations, that can indirectly modulate [^{3}H]NA release, and a non-β2* nAChR population, likely to be β4-containing nAChR, that resides on the noradrenergic terminals to directly influence [^{3}H]NA release.

α7 nAChR on glutamatergic and GABAergic neurons: model of disinhibition. In adult rat hippocampus, α7 nAChR have been immunolocalised on both glutamatergic and GABAergic neurons (Fabian-Fine et al., 2001). In the CA1 region, several electrophysiological studies (Alkondon and Albuquerque, 2000a; Buhler and Dunwiddie, 2001) have recorded fast synaptic desensitising inward currents that are sensitive to MLA, characteristic of α7 nAChR. In whole-cell patch-clamped hippocampal neurons, Gray et al. (1996) provided evidence for presynaptic nAChR sensitive to αBgt that could elicit glutamate release in response to nicotine. In the present study, responses to choline and AnTx (25 μM) were partially blocked by antagonists of both NMDA and non-NMDA GlutR (Fig. 2), consistent with α7 nAChR on glutamatergic afferents promoting glutamate exocytosis and subsequent glutamate-evoked [^{3}H]NA release (Fig. 5). The presence of glutamate heteroreceptors on noradrenergic afferents is supported by the ability of selective GluR agonists to stimulate [^{3}H]NA release (Fig. 2; Pittaluga and Raiteri, 1992).

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In addition to glutamatergic influences elicited by α7 nAChR activation, we also show that α7 nAChR can modulate a GABAergic component of [^{3}H]NA release. GABAergic interneurons represent 10-15% of the total neuronal population in the hippocampus, predominantly located in the stratum oriens and stratum radiatum (Freund and Buzsáki, 1996). GABAergic interneurons are heterogeneous with respect to their nAChR complement: (i) >50% express exclusively α7 nAChR, (ii) ~30% express a combination of both α7 and non-α7 nAChR and (iii) the remainder give no response to nicotine (Alkondon and Albuquerque, 2000a; McQuiston and Madison, 1999; Buhler and Dunwiddie, 2001). Activation of nAChR on interneurons induces the release of GABA (Kofalvi et al., 2000) that, depending on the GABAergic innervation, can strongly inhibit pyramidal cells (Freund and Buzsáki, 1996) or inhibit another interneuron, resulting in disinhibition of pyramidal cells (McQuiston and Madison, 1999; Ji and Dani, 2000; Bulher and Dunwiddie, 2001). This process of disinhibition has also been described for cerebral cortical interneurons (Alkondon et al., 2000b).

The abolition of α7 nAChR-mediated [^{3}H]NA release by the additive blockade of both GluR and $GABA_A$ receptor antagonists (Fig. 3B) implies that α7 nAChR are present on both glutamatergic and GABAergic neurons. The association of α7 nAChR with the latter is further supported by the non-additivity of co-applied choline and GABA, suggesting that α7 nAChR and GABA act in series to modulate [^{3}H]NA release. This is consistent with the selective block by bicuculline of choline-evoked [^{3}H]NA release and is compatible with the study of Leslie et al. (2002). One explanation is that activation of α7 nAChR on GABAergic cells evokes the exocytosis of GABA that in turn inhibits a second interneuron, generally considered to be GABAergic, to disinhibit pyramidal cells, via repression of a tonic inhibition (see Fig. 5). This hypothesis is in agreement with the findings of Ji and Dani (2000), from electrophysiological recordings in the CA1 region. If a tonic GABAergic inhibition occurs in the in vitro slice preparation, bicuculline should enhance basal release. This was not observed (see Methods), suggesting that under the experimental conditions there was no tonic GABAergic inhibition, but other inhibitory systems cannot be excluded. The complete block of choline-mediated [^{3}H]NA release by the Na^+ channel blocker TTX (Table 1) suggests

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an action potential-dependent mechanism, consistent with a somatic or preterminal localisation of α7 nAChR on GABAergic interneurons, in agreement with the observations of Alkondon et al. (2000a).

An alternative explanation of GABA-evoked [^{3}H]NA release is that GABA has a direct, excitatory effect on noradrenergic varicosities. Although this is well established in development, an excitatory effect of GABA in adult rodent brain has also been reported (Vizi and Kiss, 1998; Fassio et al., 1999; for review see Stein and Nicoll 2003) and we cannot rule out a depolarising effect of GABA on noradrenergic afferents in the hippocampus as an explanation of the positive modulation of [^{3}H]NA release observed in the present study.

The involvement of the GABAergic system in mediating the nicotinic modulation of hippocampal NA release is in contrast to the mechanisms modulating striatal DA release. Although a common feature of the 2 regions lies in the ability of α7 nAChR to indirectly stimulate striatal dopamine and hippocampal noradrenaline release via the release of glutamate (Kaiser and Wonnacott, 2000 and the present work, respectively), an additional GABAergic component is present in the hippocampal preparation, conferring a more complex picture of neurotransmitter crosstalk (Fig. 5).

Physiological implications of α7 nAChR modulation of NA release

Physiologically, the endogenous activation of α7 nAChR arises from the substantial cholinergic input to the hippocampus from the medial septum diagonal band complex of the basal forebrain that targets mainly GABAergic interneurons (Frotscher and Leranth, 1985). Because the cholinergic varicosities exhibit sparse synaptic contacts (~10-20%), interneuronal communication is likely to reflect volume transmission. Depending on the frequency of activation of the cholinergic fibres, the concentration of ACh (or its degradation product choline) might be sufficient to activate α7 nAChR, or to desensitise them. The GABAergic interneurons participate in the maintenance of rhythmic activities that include a nicotinic component in their modulation (Cobb et al., 1999), therefore imbalance in the regulation of excitation/inhibition could alter hippocampal functions. For example, we showed

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that chronic nicotine perturbs the nAChR modulation of the noradrenergic system by producing a transient enhancement of α7 nAChR-mediated [^{3}H]NA release (Fig. 4). This effect was seen only at 3 days withdrawal, and was not accompanied by any changes in responsiveness to GABA or AMPA, or any increase in α7 nAChR density. A selective enhancement of α7 nAChR function could reflect the insertion of more pre-existing receptors in the plasma membrane, as recently demonstrated for hippocampal interneurons (Cho et al., 2005). Interestingly, chronic infusion for 10 days with higher doses of nicotine caused an increase in nicotine- (Grilli et al., 2005), AMPA- and NMDA-evoked [^{3}H]NA release (Risso et al., 2004). In contrast, Jacobs et al. (2002) reported that repeated nicotine injections led to a decrease in nicotine-evoked [^{3}H]NA release. Therefore it appears likely that the mode of delivery of nicotine (intermittent versus sustained) and the dose administered can differentially influence the noradrenergic system.

The constant delivery of nicotine via osmotic minipumps reproduces the sustained plasma concentration of nicotine achieved by smokers. While the nicotinic modulation of hippocampal [^{3}H]NA release was unchanged by nicotine administration (day 14), the elevation during withdrawal is reminiscent of increased NA release in the hippocampus during opiate withdrawal (Done et al., 1992; Grasing et al., 1997). Moreover, a sustained elevation of NA, but not DA, in the brains of mice during withdrawal from nicotine delivered in the drinking water has been reported (Gaddnas et al., 2000). Thus the interplay of transmitter systems shown in the present study to mediate the nicotinic modulation of NA release in the hippocampus could contribute to altered noradrenergic function during nicotine withdrawal.

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Footnotes

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Figures legends

Fig. 1. nAChR modulation of [^{3}H]NA release from slices.

Rat hippocampal slices (main figure) or striatal slices (inset) were loaded with [^{3}H]NA and [^{3}H]DA respectively and released transmitter in response to drug treatment was determined by filtration, as described in the Methods. **A**. Hippocampal slices were incubated for 5 min with increasing concentrations of AnTx (n=4, closed circles) or choline (n=4, closed squares). The concentration response curve for AnTx-evoked [^{3}H]NA release was fitted to a one-site Hill equation (EC_{50} of 1.2 µM). The choline concentration response curve was fitted to a bell-shaped profile as described in the Methods. Mecamylamine (mec, 20 µM) was applied 5 min prior to and remained throughout the stimulation with AnTx (1 and 25 µM, n=3, open circles) or choline (1 and 5 mM, n=3, open squares). **B.** Striatal slices were incubated for 5 min with increasing concentrations of AnTx (n=4, closed circles) or choline (n=4, closed square). α-CnTxMII (200 nM, filled triangles, n=6) and DHβE (10 µM, open triangles, n=4) were applied 5 min prior to and and remained throughout the stimulation with 1 µM AnTx. AnTx-evoked striatal [^{3}H]DA was fitted to a two-site Hill equation (EC_{50} = 138.9 nM and 7.1 µM) and the choline concentration response curve was fitted to a bell-shaped profile as described in the Methods. **C**. [^{3}H]NA release from hippocampal slices stimulated with 1 µM AnTx alone (black bar) or in the presence (grey bars) of α-CnTxMII (200nM, n=6), DHβE (10 µM, n=9) or MLA (20 nM, n=4). **D**. [^{3}H]NA release from hippocampal slices stimulated with either 25 µM AnTx alone (black bars) or choline alone (black bars) or in the presence (grey bars) of DHβE (10 µM, n=4) or αCnTxImI (1 µM, n=3) or MLA (20 nM, n=4). Transmitter release in (A) and (B) is presented as fractional release, whereas in (C) and (D) release is expressed as a percent of the response to 1 µM or 25 µM AnTx alone, which gave a fractional release of 10.8 ± 1.4 % and 28.8 ± 0.9 % respectively. * $P<0.05$ and ** $P<0.01$, significantly different from corresponding control, Student's t-test.

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Fig. 2. Ionotropic glutamate receptors modulation of [^{3}H]NA release from rat hippocampal slices

Rat hippocampal slices were loaded with [^{3}H]NA and released transmitter in response to drug treatment was determined by filtration, as described in the Methods. **A**. Slices were challenged for 5 min by various concentrations of glutamate (closed diamonds) in normal Krebs buffer or NMDA (closed square) in Mg^{2+} free conditions. DNQX (200 µM, n=3) and MK801 (5 µM, n=3) were applied 5 min prior to stimulation with glutamate (100 and 500 µM, open diamonds) and NMDA (500 µM, open square) respectively. Glutamate and NMDA concentration response curves were fitted to a single site Hill equation with respective apparent EC_{50} values of 177.0 µM and 33.0 µM. **B** Hippocampal slices were incubated for 5 min with increasing concentrations of kainate (closed circles) or AMPA (closed triangles). DNQX (200 µM, n=4) was applied 5 min prior to stimulation with kainate (100 and 500 µM, open circles) or AMPA (100 and 500 µM, open triangles). Concentration response curves for kainate and AMPA-evoked [^{3}H]NA release were fitted to a single site Hill equation with respective apparent EC_{50} values of 31.6 µM and 28.4 µM respectively. **C** AnTx concentration response curve was carried out in the presence (open circles) or in absence (closed circles) of kynurenic acid (800 µM, n=4). Release in response to 25 µM AnTx was also determined in the presence of DNQX (200 µM, n=4, open square). **D** Hippocampal slices were stimulated with choline alone (black bar) or in the presence (grey bars) of DNQX (200 µM, n=4) or kynurenic acid (800 µM, n=4). Slices were also challenged with choline in Mg^{2+} free conditions (hatched bars) in the presence or absence of MK801 (5 µM, n=3). In A, B and C results are presented as fractional release, whereas in (D) they are expressed as a percent of choline responses in the presence of Mg^{2+}. *p<0.05, **p<0.01 statistically different from respective control, Student's t-test (A and B), one way ANOVA with *post hoc* Tukey's test (C and D).

Fig. 3. Modulation of [^{3}H]noradrenaline release from rat hippocampal slices by GABA receptors

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Rat hippocampal slices were loaded with [^{3}H]NA and released transmitter in response to drug treatment was determined by filtration, as described in the Methods. **A.** Slices were pre-incubated for 5 min with buffer alone or GABA antagonists: CGP 54626 (CGP, 1 µM) and/or bicuculline (Bic, 100 µM). Following this, slices were stimulated with GABA agonists: GABA (100 µM), baclofen (Bac, 100 µM) or muscimol (Musc, 100 µM). Results are expressed as fractional release with n = 3 to 15. **B.** Hippocampal slices were stimulated with choline (1 mM) following a 5 min pre-incubation with either buffer (control), DNQX (200 µM), bicuculline (100µM) or co-application of both antagonists. Results are expressed as a percent of choline responses, which gave a fractional release of 7.7 ± 0.6 % (n = 6). **C.** Slices were exposed either to DNQX (200 µM) or bicuculline (100 µM) before stimulation with GABA (100 µM) or glutamate (500 µM) respectively. Results are expressed as a percent of GABA control responses in absence of antagonist. *p<0.05, † p<0.05, **p<0.01 statistically different from respective control, Student's t-test (A and C) or one way ANOVA with *post hoc* Bonferroni's test (B).

Fig. 4. Effect of chronic nicotine and its withdrawal on acute stimulation of nAChR-mediated [^{3}H]NA.

Rats were exposed to either saline (control; grey bars) or nicotine (4 mg/kg/day; black bars) via osmotic minipumps for 14 days and [^{3}H]NA release was measured on day 14 or following 3 or 7 days withdrawal. Hippocampal slices were challenged by either acute 1 µM AnTx (**A)** or 1 mM choline (**B**). Results are presented as a percent of control responses at the respective timepoint. Following saline treatment, AnTx-evoked [^{3}H]NA release (fractional values) was 13.2 ± 0.8, 9.6 ± 1.0 and 8.4 ± 0.7 and choline–evoked release was 7.8 ± 1.0, 5.3 ± 0.7 and 4.1 ± 0.5 at 14 days' treatment and 3 and 7 days withdrawal, respectively. **p<0.01, statistically different from control Student's paired t-test.

Fig. 5. Model of nAChR modulation of [^{3}H]NA release in the hippocampus in relationship to glutamatergic and GABAergic influences

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The noradrenergic varicosity bears glutamate, $GABA_A$ and non-α7 nicotinic heteroreceptors. α7 nAChR are proposed to reside on glutamate afferents and a population of GABAergic interneurons (Alkondon et al., 2000a; Fabian-Fine et al., 2001). Activation of α7 nAChR on the glutamatergic terminals triggers the exocytosis of glutamate, which promotes the release of NA by acting at ionotropic GluR. Two interneurons in series are illustrated. The first GABAergic interneuron, expressing somatic α7 nAChR, impinges on another interneuron, generally considered to be also GABAergic, although another inhibitory component is not excluded. The second interneuron tonically inhibits a glutamatergic input to the noradrenergic varicosity. Hence, activation of α7 nAChR on the upstream interneuron induces the release of GABA, which in turn inhibits the tonically active interneuron resulting in a disinhibition of the excitatory input. GABA might also elicit NA release by direct depolarisation of the varicosity via $GABA_A$ receptors (Stein and Nicolls, 2003).

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TABLE 1

Effects of tetrodotoxin (1 μM) on evoked [^{3}H]NA release

	[^{3}H]noradrenaline release (fractional release)				
Stimulus	**Veratridine 50 μM**	**AnTx 1 μM**	**Choline 1 mM**	**GABA 100 μM**	**Glutamate 500 μM**
control	88.3 ± 13.6	11.9 ± 1.4	12.1 ± 2.5	9.6 ± 1.5	13.0 ± 0.6
+ TTX	4.4 ± 1.4*	-0.8 ± 0.7*	-2.3 ± 1.4*	-3.1 ± 1.4*	0.7 ± 1.3*
	(n=4)	(n=8)	(n=3)	(n=4)	(n=4)

*statistically different from respective control, p<0.01, Student's t-test

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TABLE 2

Effects of chronic nicotine treament and its withdrawal on brain [^{3}H]epibatidine ([^{3}H]epi) and [^{125}I]αbungarotoxin ([^{125}I]αBgt) binding sites

	[^{3}H]epi binding sites (fmol/mg)			**[^{125}I]αBgt binding sites (fmol/mg)**		
Treatment	MiniPump	Withdrawal		MiniPump	Withdrawal	
	14 days	3 days	7 days	14 days	3 days	7 days
Saline	99.7 ± 8.2	90.4 ± 9.0	81.0 ± 10.9	117.1 ± 5.0	109.2 ± 4.2	102.4 ± 6.1
Nicotine	137.1 ± 15.7*	128.4 ± 5.6*	119.0 ± 9.1*	114.6 ± 7.3	112.9 ± 6.4	105.2 ± 11.7
	(n=16)	(n=16)	(n=16)	(n=16)	(n=16)	(n=16)

*statistically different from respective control, $p<0.05$, one way Anova

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TABLE 3

Effects of antagonists on choline-evoked [^{3}H] NA release following chronic nicotine treatment and its withdrawal

	Inhibition of choline-evoked [^{3}H]NA release (%)					
Antagonist	**DNQX (200 μM)**			**Bicuculline (100 μM)**		
Treatment	MiniPump	Withdrawal		MiniPump	Withdrawal	
	14 days	3 days	7 days	14 days	3 days	7 days
Saline	49.0 ± 4.5	56.4 ± 12.4	50.1 ± 11.1	85.7 ± 7.5	89.8 ± 6.2	84.5 ± 3.5
Nicotine	52.7± 10.9	40.9 ± 18.0	58.6 ± 8.6	82.3 ± 9.0	87.1 ± 15.8	84.8 ± 6.6
	(n=12)	(n=7)	(n=8)	(n=12)	(n=7)	(n=8)

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TABLE 4

Effects of chronic nicotine treament and its withdrawal on [^{3}H] NA release evoked by AMPA or GABA

	[^{3}H] NA release (fractional release)					
Stimulus	**AMPA (100μM)**			**GABA (100 μM)**		
Treatment	MiniPump	Withdrawal		MiniPump	Withdrawal	
	14 days	3 days	7 days	14 days	3 days	7 days
Saline	11.1 ± 1.1	10.6 ± 1.7	8.0 ± 1.6	7.1 ± 0.9	6.5 ± 1.1	4.8 ± 1.3
Nicotine	13.1 ± 1.1	12.9 ± 2.2	10.1 ± 1.9	7.3 ± 0.8	7.2 ± 1.1	7.6 ± 2.9
	(n=15)	(n=7)	(n=8)	(n=15)	(n=7)	(n=8)

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Figure 1

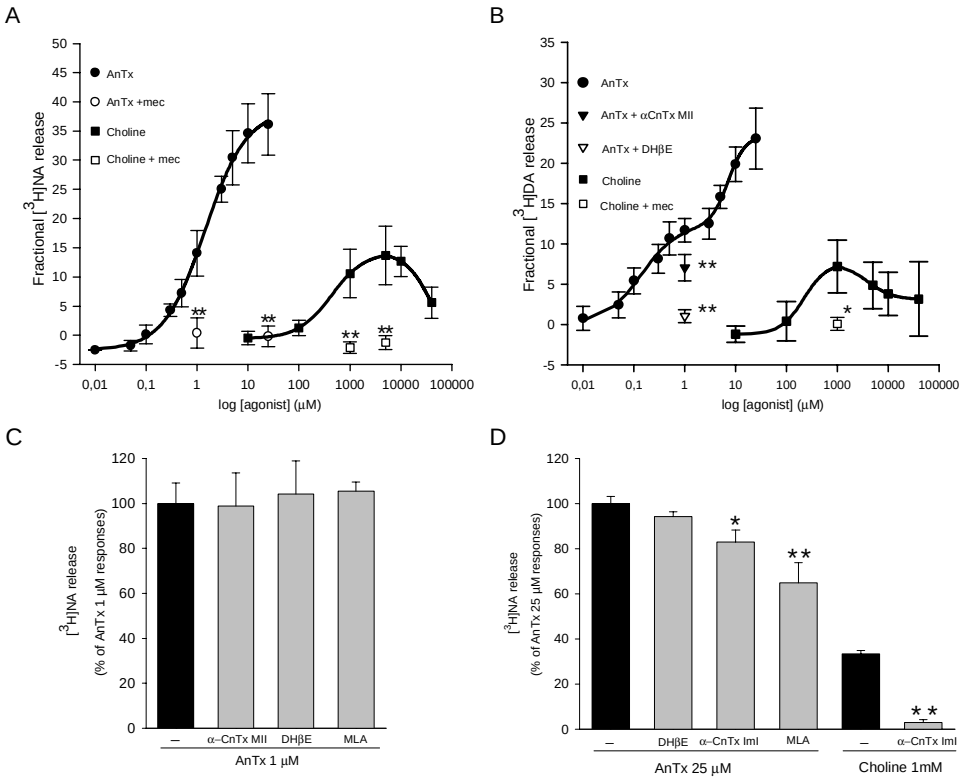

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Figure 2

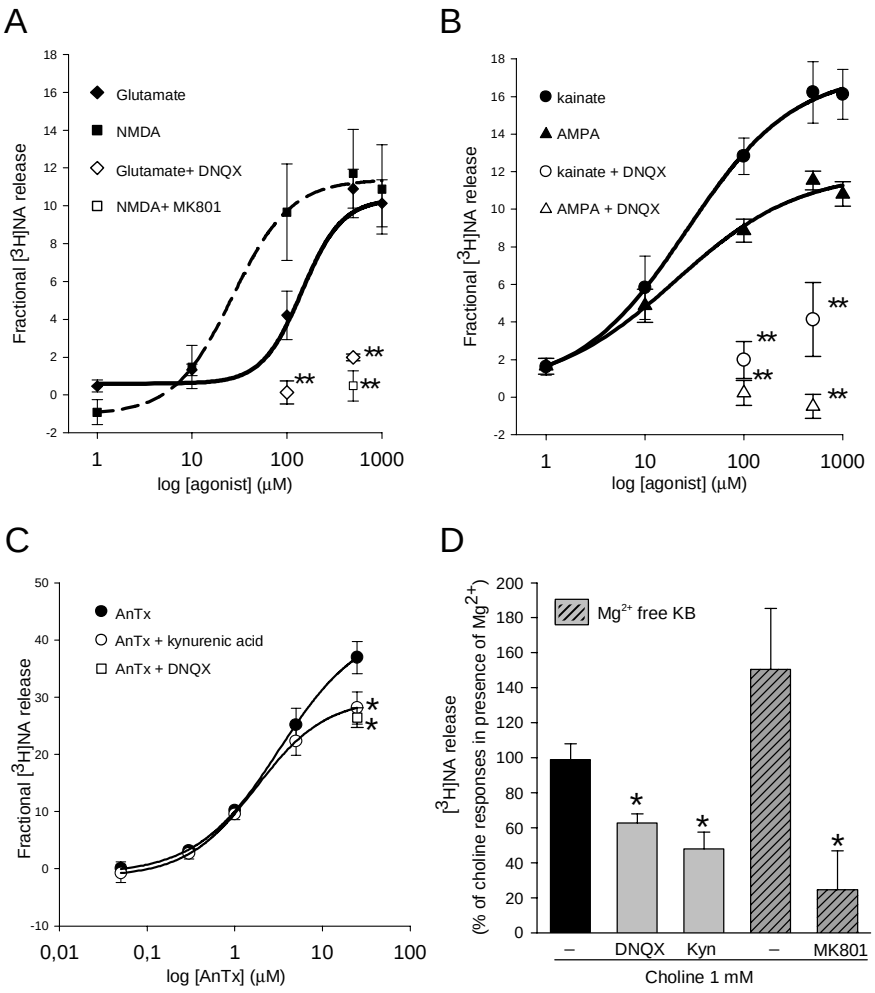

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Figure 3

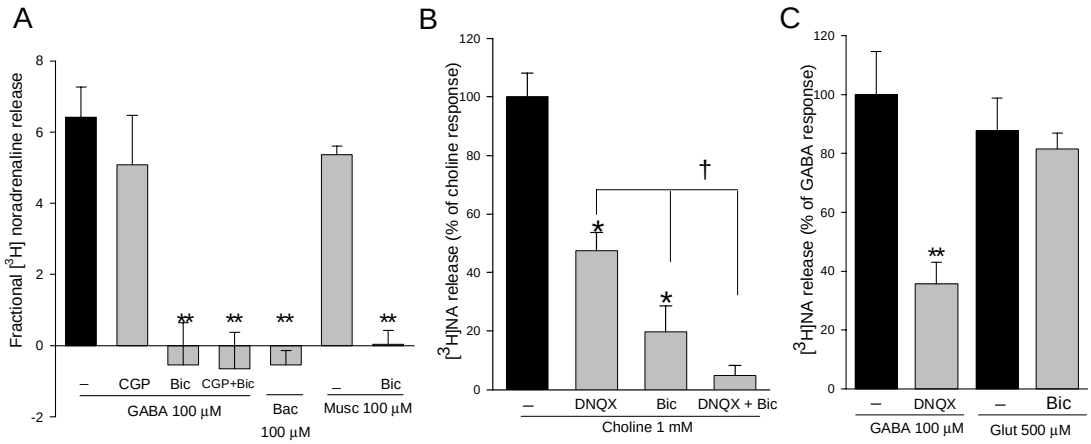

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Figure 4

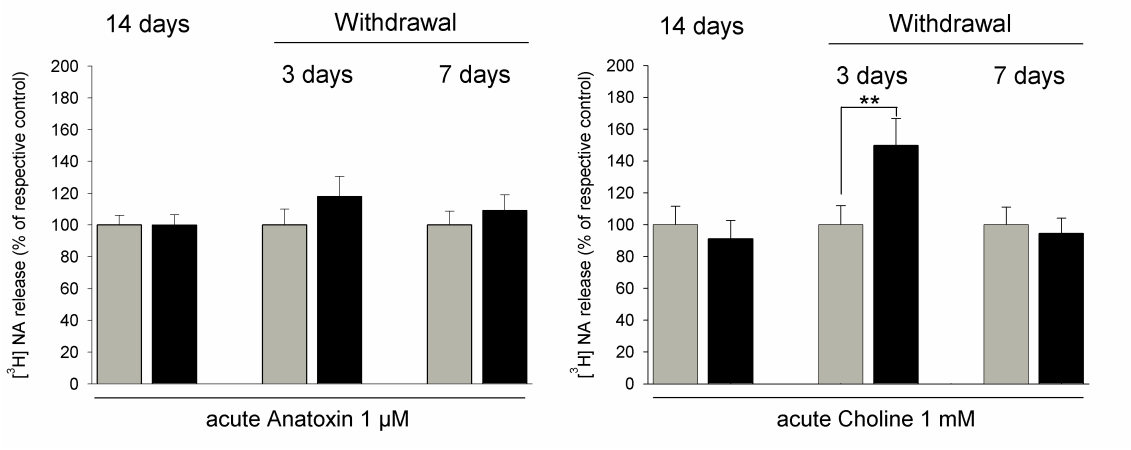

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Figure 5