Acta Pharmacologica Sinica (2013) 34: 819–829
 1671-4083/13 $32.00
www.nature.com/aps

Original Article

Nicotine induction of theta frequency oscillations in rodent medial septal diagonal band *in vitro*

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Aim: This study aimed to examine the role of the nicotinic receptor (nAChR) in the generation of theta oscillations (4–12 Hz) *in vitro*.
Methods: Electrophysiological studies were performed on medial septal diagonal band area (MSDB) slices to measure theta oscillation. Immunofluorescence and confocal microscopy studies were carried out to detect α4 nAChR and β2 nAChR subunits in perfused-fixed tissue from VGluT2-GFP and GAD67-GFP transgenic mice.
Results: Application of nicotine to MSDB slices produced persistent theta oscillations in which area power increased in a dose-responsive manner. This activity was inhibited by $GABA_A$ receptor antagonists and partially by ionotropic glutamate receptor antagonists, indicating the involvement of local GABAergic and glutamatergic neurons in the production of the rhythmic activity. The nicotine-induced theta activity was also inhibited selectively by non-α7*nAChR antagonists, suggesting the presence of these receptor types on GABAergic and glutamatergic neuron populations in the MSDB. This was confirmed by immunofluorescence and confocal microscopy studies in transgenic mice in which the GABAergic and glutamatergic neurons express green fluorescent protein (GFP), showing localisation of β2 nAChR and α4 nAChR subunits, the most common constituents of non-α7*nAChRs, in both cell types in the MSDB.
Conclusion: Theta activity in the MSDB may be generated by tonic stimulation of non-α7*nAChRs.

Keywords: nicotinic receptors; medial septal diagonal band; rhythmogenesis; hippocampal theta rhythm; learning and memory; Alzheimer's disease

Acta Pharmacologica Sinica (2013) 34: 819–829; doi: 10.1038/aps.2012.198; published online 25 Mar 2013

Introduction

The medial septal diagonal band (MSDB), a rostral extension of the cortically-projecting basal forebrain system, provides major cholinergic and GABAergic projections to the hippocampus[1] and is a key generator of hippocampal theta rhythm (4–12 Hz), believed to serve as an internal timing mechanism for the processing of incoming spatial information[2, 3]. MSDB receives a cholinergic input from the pedunculopontine nucleus of the brainstem[4, 5] and a glutamatergic projection from the supramammillary nucleus (SUM) of the hypothalamus[6].

Electrical stimulation of the brainstem reticular formation increases the incidence of rhythmic burst firing in the MSDB, concomitant with a rise in the frequency and amplitude of hippocampal theta activity[7, 8]. The theta-relevant brainstem reticular activating signals may be therefore relayed to the MSDB via above-mentioned cholinergic and/or glutamatergic inputs which could be involved in causing theta oscillations in the MSDB. These oscillations may then be passed on to the hippocampus.

A major question is whether theta activity is generated in the MSDB in response to tonic activation of its circuits by these afferents, or whether patterned theta frequency input is required[6, 9].

Recently, extracellular field oscillations at theta frequency were recorded in the MSDB slices in response to tonic stimulation of ionotropic glutamate receptors, kainate receptors or metabotropic acetylcholine receptors, muscarinic receptors[10–13]. The role of the ionotropic acetylcholine receptor, nicotinic receptor (nAChR) in this theta-generating mechanism in the MSDB, however, has yet to be investigated. nAChRs are abundant in the MSDB[14, 15], but did not affect evoked and spontaneous postsynaptic responses in the MSDB[15]. Our hypothesis therefore is that tonic activation of non-α7*nAChRs, which do not desensitize as readily as α7*nAChRs[16], will produce phasic network activity in the theta frequency range in the MSDB that is mediated by local GABAergic and glutamatergic synaptic circuitry.

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Received 2012-08-10 Accepted 2012-12-20

Materials and methods

Animals

All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and with Animal ethics and administrative council of Henan province, China, and all efforts were made to minimise animal suffering and to reduce the number of animals used. Electrophysiological studies were performed on MSDB slices prepared from Wistar rats (male, 3–4 week-old, n=72). Neuroanatomical observations were also made from perfused-fixed brains of adult heterozygous VGluT2-GFP mice (male or female, 3 week-old, n=2) in which expression of vesicular glutamate transporter 2 (VGluT2), a marker for a subtype of glutamatergic neuron, is linked with expression of green fluorescent protein (GFP; MMRRC.org) and from heterozygous GAD67-GFP (Δ-neo) knock-in transgenic mice (n=2) in which glutamate decarboxylase 67 (GAD67), a marker for GABAergic neurons, is linked with expression of GFP[17]. The VGluT2-GFP mice were bred on a NIH Swiss (Harlan UK) background and GAD67-GFP (Δ-neo) mice were bred on a C57Bl6 (Harlan, UK) background. Both types of transgenic mouse were phenotyped at birth by brief observation of fluorescence in the brain (visible through the overlying tissue) under a UV lamp.

For electrophysiology, the rats were anaesthetised by intraperitoneal injection of Sagatal (sodium pentobarbitone, 100 mg/kg, Rhône Mérieux Ltd, Harlow, UK). When all pedal reflexes were abolished, the animals were perfused intracardially with chilled (5 °C), oxygenated artificial cerebrospinal fluid (ACSF) in which the sodium chloride had been replaced by iso-osmotic sucrose. This ACSF (305 mosmol/L) contained (in mmol/L): 225 sucrose, 3 KCl, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 6 $MgSO_4$, 0.5 $CaCl_2$, and 10 glucose. For extracellular field recording, two sagittal slices (450 μm) of rat brain, straddling the midline and containing the MSDB, were cut at 4–5 °C in the sucrose-ACSF, using a Leica VT1000S vibratome (Leica Microsystems UK, Milton Keynes, UK). For immunocytochemistry, rats and mice were deeply anaesthetised with an *ip* injection of urethane (12 g/kg). After loss of all pedal reflexes the animals were perfused intracardially with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). The brains were removed and placed in the same fixative for 2–3 h, and then in phosphate buffer overnight at 4 °C. The brains were either used immediately, or immersed overnight in cryoprotectant (25% sucrose, 10% glycerol in 0.05 mol/L phosphate buffer) and then frozen with powdered dry ice and stored at -45 °C.

Electrophysiological recording, data acquisition, and analysis

For extracellular field recordings, the two MSDB slices were transferred to an interface recording chamber. The slices were maintained at a temperature of 33 °C and at the interface between ACSF and warm humidified carbogen gas (95% O_2–5% CO_2). The ACSF contained (in mmol/L): 126 NaCl, 3 KCl, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 2 $MgSO_4$, 2 $CaCl_2$, and 10 glucose. The slices were allowed to equilibrate in this medium for 1 h prior to recording. Both channels of an Axoprobe 1A amplifier (Axon Instruments, Union City, CA, USA) were employed for extracellular field recordings, which were made using glass microelectrodes containing ACSF (resistance 2–5 MΩ). Data were band-pass filtered online between 0.5 Hz and 2 kHz using the Axoprobe amplifier and a Neurolog system NL106 AC/DC amplifier (Digitimer Ltd, Welwyn Garden City, UK). The data were digitized at a sample rate of 5–10 kHz using a CED 1401 plus ADC board (Digitimer Ltd). Electrical interference from the mains supply was eliminated from extracellular recordings online with the use of 50 Hz noise eliminators (HumBug; Digitimer Ltd).

Data were analyzed off-line using software from Spike 2 (CED, Cambridge, UK). Power spectra were generated to provide a quantitative measure of the frequency components in a stretch of recording, where power, a quantitative measure of the oscillation strength, was plotted against the respective frequency. Power spectra were constructed for 30–60 s epochs of extracellular field recordings using a fast Fourier transform algorithm provided by Spike2. The parameters used for measuring the oscillatory activity in the slice were peak frequency (Hz) and area power (μV^2). In the current study, area power was equivalent to the computed area under the power spectrum between the frequencies of 4 and 14 Hz[18] .

All statistical tests were performed using SigmaStat software (SPSS Inc, California, USA). Results are expressed as mean±standard error of mean, unless indicated otherwise. Statistical significance for comparison between two groups or between three groups was determined using tests described in the text or in the figure legends, as appropriate. Measures were considered statistically significant if P<0.05.

Drugs used for electrophysiology

All standard reagents, except where indicated, were obtained either from Sigma-Aldrich (UK) or VWR International (Lutterworth, UK). *D*-(–)-2-amino-5-phosphonopentanoic acid (D-AP5), bicuculline methochloride, SR 95531 hydrobromide (GABAzine), and 2,3,-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f] quinoxaline-7-sulphonamide (NBQX) were purchased from Tocris Cookson Ltd (Bristol, UK). Atropine sulphate, choline, dihydro-β-erythroidine (DHβE), methyllycaconitine (MLA), nicotine sulphate, and agents for the ACSF solution were obtained from Sigma-Aldrich (UK). Stock solutions, at 10^3 of the working concentration, were made up in water, except for NBQX which was dissolved in dimethylsulphoxide and stored in individual aliquots at -45 °C. Working solutions were prepared freshly on the day of the experiment.

Immunofluorescence

To confirm electrophysiological observations, immunocytochemical staining was made for the α4 nAChR and β2 nAChR subunits in perfused-fixed tissue from VGluT2-GFP and GAD67-GFP transgenic mice. Due to weakened levels of GFP-fluorescence in the VGluT2-GFP mice after fixation, the GFP in these specimens was enhanced by dual labelling for GFP immunofluorescence using an antibody to GFP. Sections were cut at 50 μm in the coronal plane using a Leica VT1000S

vibratome (Leica, Microsystems UK, Milton Keynes, UK). Unless stated otherwise, all steps were carried out at room temperature and with gentle agitation of the sections. Optimal results for antigen retrieval were obtained by treatment of the sections with ethanol and methanol solutions prior to carrying out the immunocytochemical steps[15]. This antigen retrieval process was found to reduce the background staining that is a problem with antibodies to nAChR subunits and which contributes to non-specific staining observed in knockout mice for the nAChR subunits[19]. Sections were washed in 30%, 50%, 70%, 90%, and 100% ethanol for 3–4 min each, and then placed in 100% methanol at -20 °C for 5 min. The sections were then taken through descending solutions of ethanol, and then washed in 0.1% Triton in phosphate-buffered saline (pH 7.4). The sections were then incubated for 1 h in 2% bovine serum albumin. Sections were placed in single or double antibody solutions in 2% bovine serum albumin and 0.4% sodium azide and incubated in these solutions for 1–2 d at 4 °C. The primary antibodies were omitted in controls, and sections from the hippocampus were included as positive controls for staining for the α4 and β2 nAChR receptor subunits. Primary antibody to the α4 nAChR subunit (rabbit polyclonal antibody, Santa Cruz Biotechnology) was used at a concentration of 1/600. Antibodies for the β2 nAChR receptor were used at these concentrations: 1/600 (rabbit polyclonal antibody, Santa Cruz Biotechnology, Heidelberg), 1/1000 (rat monoclonal antibody, mAb290, gift from Dr JM Linstrom, University of Pennsylvania), and 1/75 (rat monoclonal mAb270, Sigma-Aldrich). In VGluT2-GFP mice, the antibodies to the nAChR subunits were used in combination with 1/2000 chicken polyclonal anti GFP antibody (Abcam PLC, Cambridge, UK). Following several washes over the period of 1 h, the sections were incubated for 2–3 h in 1:1000 donkey secondary antibody against rabbit IgG or rat IgG conjugated to Alexa Fluor 594, on its own or combined with 1/1000 donkey anti chicken IgG conjugated to FITC (Invitrogen Life Technologies, Paisley). Sections were washed several times and mounted on "polysine" slides (Fisher Scientific UK Ltd, Loughborough, UK) and embedded in Vectamount (Vector Laboratories Ltd, Peterborough, UK). The sections were then viewed with a Zeiss LSM 510 Meta confocal microscope (Zeiss, Welwyn Garden City, UK). Stacked confocal images were taken at random through the rostro-caudal axis of the MSDB and of other areas of the brain and were analysed using the Zeiss LSM Image Browser program.

Results

Nicotine induces oscillatory activity in MSDB slices

A previous study demonstrated that the theta oscillatory activity can be recorded in midline of MSDB[11], this observation was confirmed in this study using two roving electrodes (n=8). Therefore, we performed our recording in the midline of MSDB. Bath application of 0.025–5 μmol/L nicotine to MSDB slices induced persistent oscillatory field activity, an example of nicotine (250 nmol/L) induced activity was shown in Figure 1A. This activity occurred at frequencies that ranged from 4.8 to 8.6 Hz (average: 5.8±0.6 Hz, n=47), as indicated by the power spectra (Figure 1B) and autocorrelograms of the activity (Figure 1C). The induced oscillatory field activity was also often accompanied by fast downwards signals, interpreted as extracellular action potentials recorded from one or more neuron cell bodies lying fortuitously close to the recording electrode (Figure 1A). Area power (4–12 Hz) of the oscillatory activity reached a plateau 10–20 min after application of the nicotine and remained stable for at least 30 min thereafter (Figure 1D). At a dose of 0.01 μmol/L, nicotine had no significant effect on area power, but upon application of doses of nicotine ranging from 0.025–0.5 μmol/L, area power increased in a dose-responsive manner (Figure 1E–1G). There was a statistically significant difference in the mean power among the treatment groups (ANOVA; P<0.01), but there was no significant difference in oscillation frequency between the different effective concentrations of nicotine applied (Figure 1F).

Ionotropic glutamate and GABA receptors mediate nicotine-induced oscillatory activity in the MSDB slice

Oscillatory activity induced by nicotine (250 nmol/L) was examined after addition of the ionotropic glutamate receptor antagonists D-AP5 (50 μmol/L) and NBQX (20 μmol/L) or the ionotropic $GABA_A$ receptor antagonists GABAzine (10 μmol/L) and bicuculline (20 μmol/L).

Addition of NBQX largely reduced nicotine-induced oscillatory activity (Figure 2A), but D-AP5 had no substantial effect on nicotine-induced oscillatory activity (Figure 2B). Substantial reduction of the nicotine-induced oscillatory activity was achieved with GABAzine (Figure 2C). The results so far therefore indicate that tonic activation of nAChRs is able to generate theta frequency-like activity in MSDB slices, and that this activity is mediated by both ionotropic glutamatergic and GABAergic receptor neurotransmission, *ie* suggesting involvement of local GABAergic and glutamatergic circuitry in the nicotine-induced oscillatory activity. Our result showed AMPA receptor antagnoist NBQX but not NMDA receptor antagonist D-AP5 largely reduced nicotine induced theta oscillation. This is in agreement with previous findings that kainate induced theta oscillations were not mediated by NMDA receptor activation (Garner *et al* 2005), suggesting that nicotine and kainate activate similar local network circuitry involving in AMPA receptor activation.

Nicotine-induced oscillatory activity in the MSDB slice is mediated by non- α7*nAChR subtypes

Oscillatory activity induced in the MSDB slice by 250 nmol/L nicotine was blocked by 0.3 μmol/L dihydro-β-erythroidine (DHβE), which in low concentrations is an antagonist for the α4β2 nAChR[20] (Figure 3A–3D). Pretreatment of the slices with 0.3 μmol/L DHβE alone had no significant effect (3.4%±5.3%) on area power of the baseline activity, and addition of 250 nmol/L nicotine caused a small but non-significant increase in the area power (16%±9.7%, P>0.05 *vs* DHβE alone, Figure 3E). Substitution of the bathing medium with 250 nmol/L nicotine significantly increased area power by

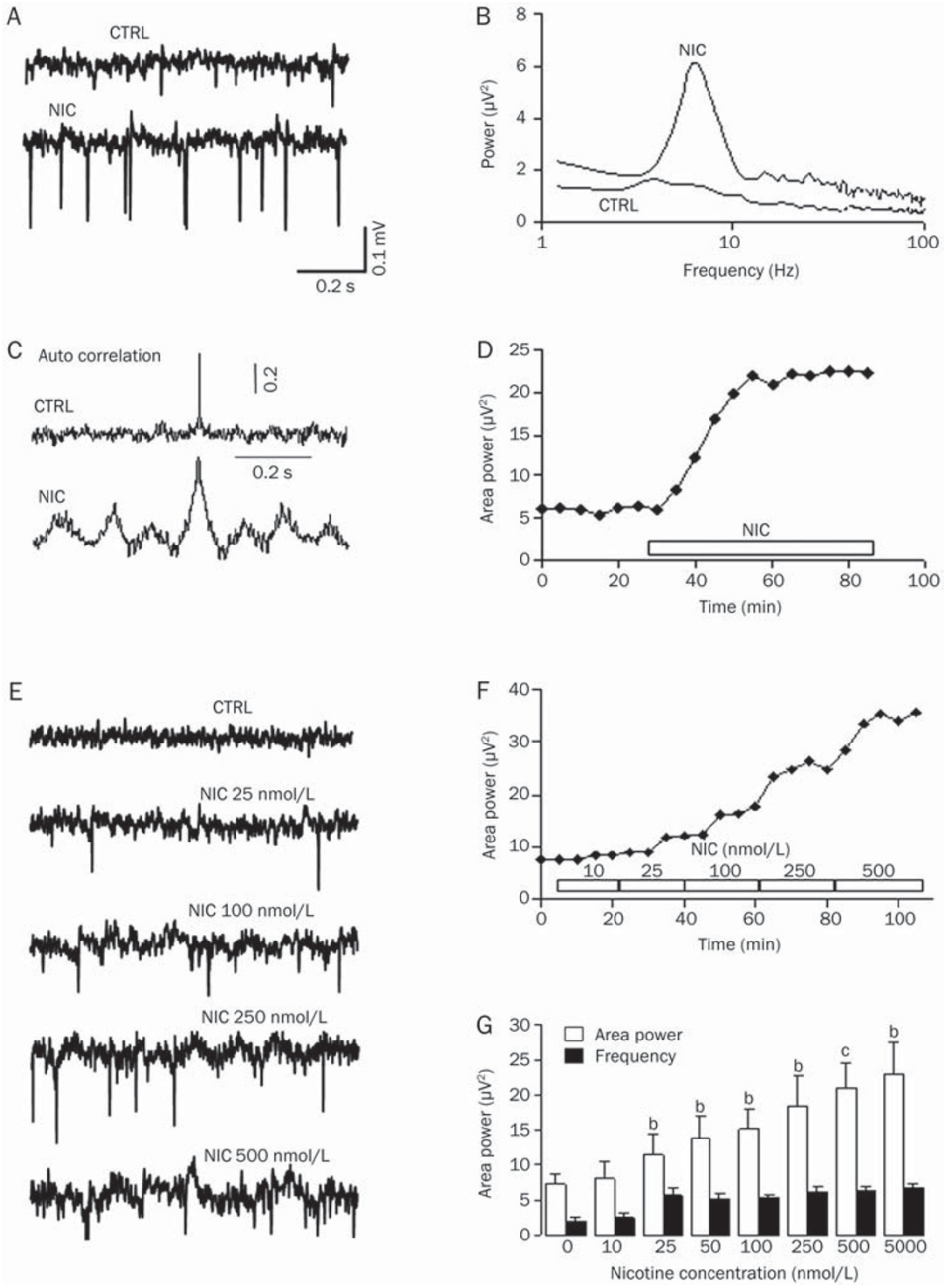

Figure 1. Nicotine induces oscillatory activity in MSDB slices. A) Representative extracellular recordings of oscillatory field activity before and after application of nicotine (250 nmol/L) to an MSDB slice. B) Corresponding power spectra of episodes of the oscillations shown in A. C) Corresponding autocorrelograms of the oscillations shown in A. D) Time course of the action of 250 nmol/L nicotine on area power (4–14 Hz) corresponding to the experiment shown in A. E) Representative extracellular recordings of oscillatory field activity before and after application of increasing concentrations of nicotine to an MSDB slice. F) Example time course of the effects of application of increasing concentrations of nicotine on area power (4–14 Hz) in an MSDB slice. G) Change in area power (4–14 Hz) with concentrations of nicotine; results were pooled from experiments in which successive administration of higher doses of nicotine was made in the same slice, or by application of various concentrations of nicotine to different slices (n=19–20 for 50 nmol/L and 500 nmol/L groups. n=12 for 5 μmol/L groups and n=6–7 slices for the remaining groups. ^{b}P<0.05, ^{c}P<0.01, for area power in comparison with control, ANOVA, *post hoc* Student-Newman-Keuls test). There was no significant difference in oscillation frequency between concentrations of nicotine applied (ANOVA, *post hoc* Student-Newman-Keuls test).

47%±9.6% (n=11, P<0.05 *vs* DHβE with nicotine, repeated measures ANOVA on rank; Figure 3E). This work suggests that the induction of persistent oscillatory activity in MSDB slices by bath-applied nicotine is mediated by activation of non-α7*nAChRs. Coupled with the results described in the previous section, this is in consistent with our recent work showing

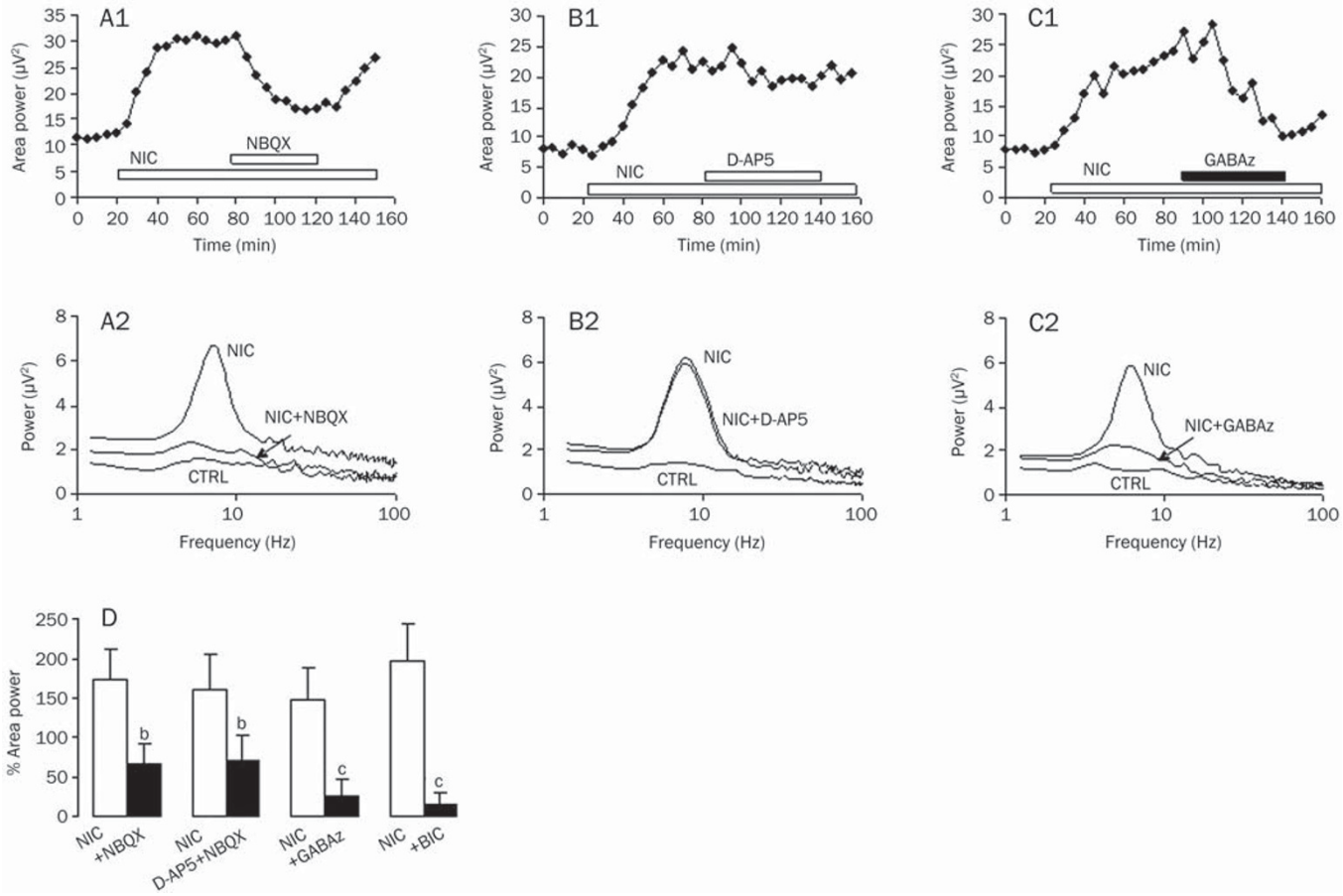

Figure 2. Ionotropic glutamate and GABA receptors mediate nicotine-induced oscillatory activity in the MSDB slice. A1) Time course of the effects of the addition of 250 nmol/L nicotine, followed by 20 μmol/L NBQX on area power of oscillatory activity in an MSDB slice. A2) Corresponding power spectra of the episodes of the oscillations shown in A1. B1) Example time course of the effects of the addition of 250 nmol/L nicotine, followed by D-AP5 (50 μmol/L) on area power of oscillatory activity in an MSDB slice. B2) Corresponding power spectra of the episodes of the oscillations shown in B1. C1) Example time course of the effects of the addition of 250 nmol/L nicotine, followed by 10 μmol/L GABAzine on area power of oscillatory activity in an MSDB slice. C2) Corresponding power spectra of the episodes of the oscillations shown in C1. D) Effects of NBQX (20 μmol/L), D-AP5 (50 μmol/L)+NBQX (20 μmol/L), or of bicuculline (20 μmol/L) or GABAzine (10 μmol/L) on theta oscillatory activity induced by 250 nmol/L nicotine (n=7 all groups. ^{b}P<0.05, ^{c}P<0.01 *vs* NIC. Student t test).

the presence of somatodendritic, non-α7*nAChRs receptors on GABAergic neurons in the MSDB[15].

There is no significant involvement of α7*nAChRs in nicotine-induced oscillatory activity in the MSDB slice

The effects of 100 nmol/L methyllycaconitine (MLA), an α7*nAChR antagonist, was tested on nicotine-induced oscillatory activity in the MSDB slice. MLA did not have a significant effect on oscillatory activity pre-induced by 250 nmol/L nicotine (n=4, P>0.05, paired t-test, data not illustrated). Pretreatment of slices with MLA did not significantly affect basal field activity (Figure 4A2, 4A3), but application of 250 nmol/L nicotine in the presence of MLA caused a significant increase in the area power (Figure 4A). Substitution of medium with 250 nmol/L nicotine increased the area power further, but this was not significantly different from the previous (Figure 4A). To further examine the possible involvement of α7*nAChR in nicotine-induced oscillatory activity in the MSDB, the effects of the bath application of choline, a selective α7*nAChR agonist was examined. Choline (5 mmol/L) on its own did not induce a significant level of oscillatory activity in the MSDB slice (Figure 4B), but substitution of the choline with 500 nmol/L nicotine produced robust oscillatory field activity indicating that the lack of effect of the choline was not due to a defective slice preparation (Figure 4B). These results suggest that α7*nAChRs do not play a significant role in the induction of persistent oscillatory activity in MSDB slices by bath-applied nicotine.

Immunoreactivity for the α4 nAChR and β2 subunits is present in GABAergic and glutamatergic neurons in the MSDB of GFP transgenic mice

To confirm the presence of non-α7*nAChR subtypes on GABAergic and glutamatergic neurons in the MSDB, immunocytochemical staining was carried out for the α4 nAChR and β2 nAChR subunits, the most common constituents of non-

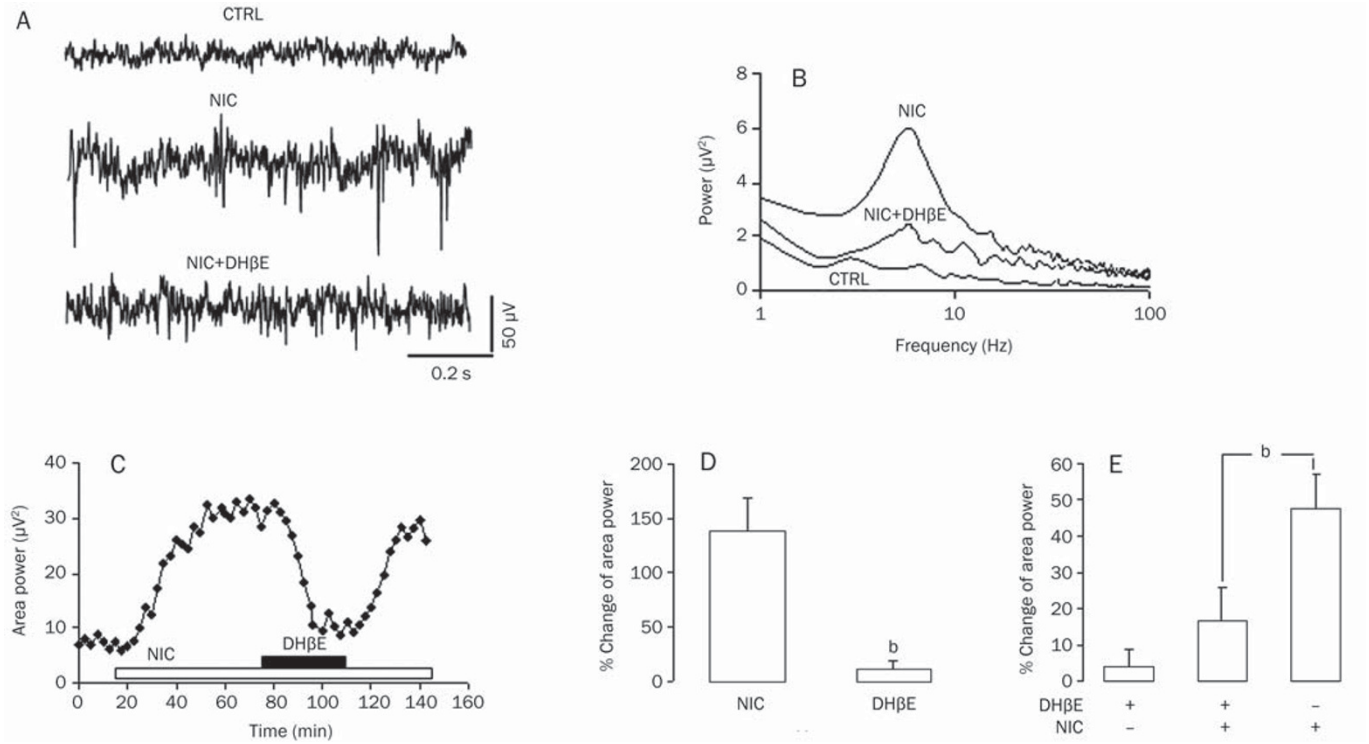

Figure 3. Nicotine-induced oscillatory activity in the MSDB slice is mediated by non-α7*nAChR subtypes, some of which are present on glutamatergic neurons. A) Representative traces of nicotine-induced oscillations showing that these oscillations are blocked by 0.3 μmol/L DHβE. B) Corresponding power spectra of episodes of the oscillations shown in A. C) Example time course of the action of 250 nmol/L nicotine, followed by 0.3 μmol/L DHβE on area power, for the experiment shown in A. D) Percentage change in area power with respect to control after addition of 250 nmol/L nicotine, followed by 0.3 μmol/L DHβE ($^bP<0.05$, $n=5$, Student *t*-test). E) Percentage change in area power with respect to control after addition of 0.3 μmol/L DHβE, followed by 250 nmol/L nicotine, followed by washout of the DHβE.

α7*nAChR subtypes in the CNS. This work was performed on tissue from GAD67-GFP and VGluT2-GFP mice, to allow for identification of the GABAergic and glutamatergic neuronal populations in the MSDB by the presence of GFP. No significant labelling was observed in the tissue upon omission of the primary antibodies (Figure 5A). As a positive control, subunit labelling was observed in interneurons of CA1 of the hippocampus but not in the pyramidal cell layer (Figure 5B), in keeping with the electrophysiological studies that have shown that a proportion of interneurons in CA1 possess non-α7*nAChR-like responses to puffed ACh[21] . No labelling of the hippocampal interneurons was present when the primary antibody was omitted (Figure 5C). In the MSDB, the α4 nAChR subunit was present in GAD67-GFP neurons (Figure 5D) and in VGluT2-GFP neurons (Figure 5E). Similar observations were also made for the β2 nAChR subunit (example not illustrated). In GAD67-GFP transgenic mice, the total neuronal number for α4 and β2 nAChR subunit antibody group is 226 and 343, respectively (Figure 5F1). The percentage of positive staining neurons for α4 nAChR antibody only (GAD67-GPF negative), GAD67-GFP only (nAChR staining negative) and α4 nAChR antibody and GAD67-GFP is 14%, 32%, and 54%, respectively (Figure 5F2, open bars). The similar results were obtained for β2 nAChR antibody staining (Figure 5F1, 5F2, filled bars). The percentage of the neurons with both nAChR antibody and GAD67-GFP positive over all GAD67-GFP positive neurons is 63% for both α4 nAChR and β2 nAChR antibody groups.

In VGluT2-GFP transgenic mice, the total number of neurons for α4 and β2 nAChR subunit antibody groups is 140 and 255, respectively (Figure 5G1). The percentage of positive staining neurons for α4 nAChR antibody only (VGlut2-GFP negative), VGluT2-GFP only (nAChR staining negative) and α4 nAChR antibody and VGluT2-GFP is 56%, 24%, and 20%, respectively (Figure 5G2, open bars). The percentage of positive staining neurons for β2 nAChR antibody only (VGluT2-GFP negative), VGluT2-GFP only (nAChR staining negative) and β2 nAChR antibody and VGluT2-GFP is 60%, 30%, and 10%, respectively (Figure 5G2, filled bars). The percentage of neurons with both nAChR antibody and VGluT2-GFP positive over all VGlut2-GFP positive is 45% for α4 nAChR and 25% for β2 nAChR subunit. Therefore, quantification of the labelling indicated both GABAergic and glutamatergic neuron types were labelled for each of the α4 nAChR and the β2 nAChR subunits (Figure 5F, 5G). These results, coupled with the pharmacological data, suggest that activation of non-α7*nAChRs by nicotine in the MSDB may have a effect on both GABAergic and glutamatergic circuitry in the MSDB slice preparation.

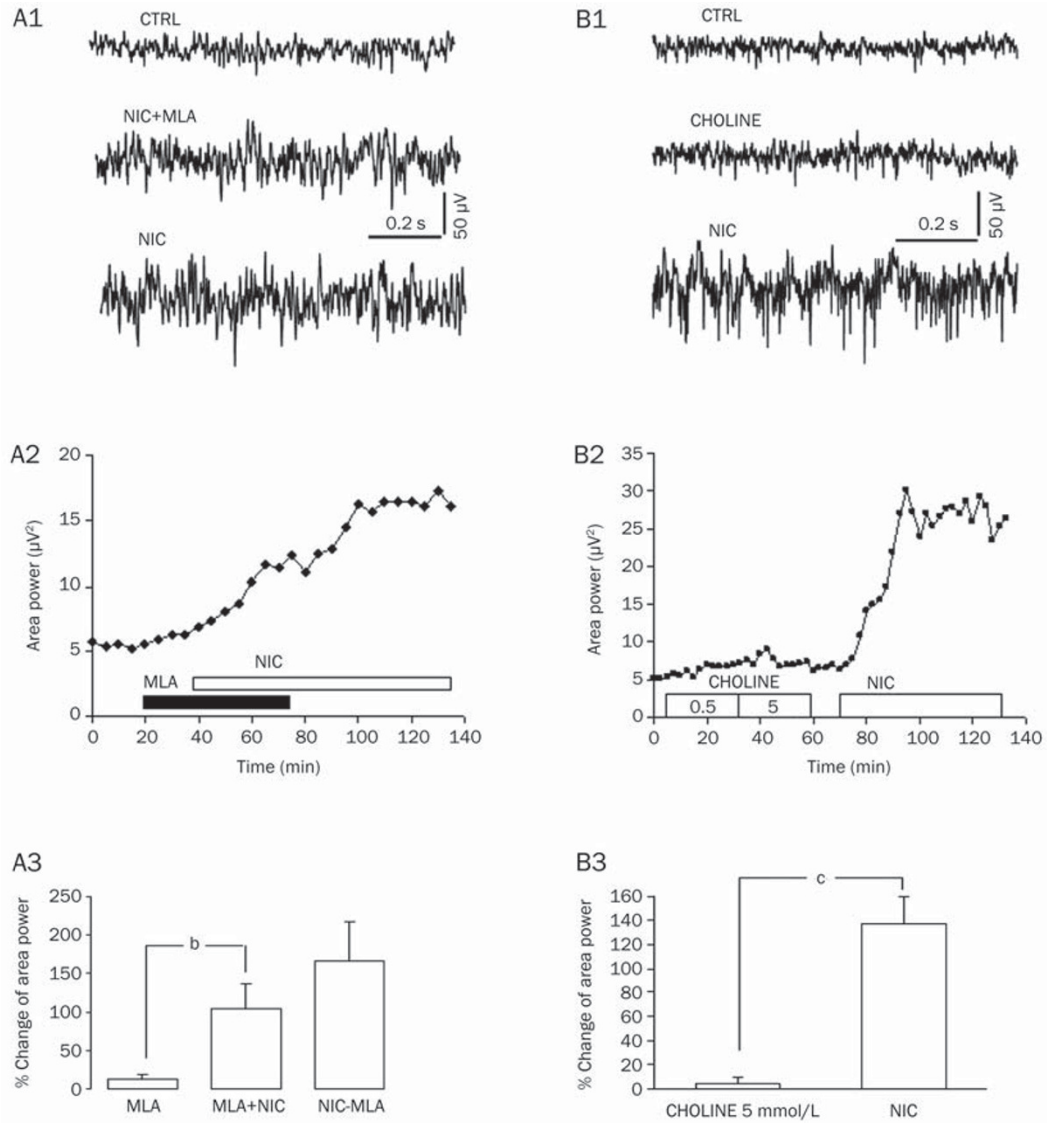

Figure 4. There is no significant involvement of α7*nAChRs in nicotine-induced oscillatory activity in the MSDB slice. A1) Example traces of nicotine-induced oscillatory activity in the presence of 100 nmol/L methyllycaconitine (MLA), a reversible α7*nAChR antagonist, and after removal of the MLA and retention of the nicotine. A2) Example time course of the nicotine-induced oscillatory activity in the presence of MLA and after washout of the MLA and in the presence of nicotine. A3) Percentage changes in area power with respect to control after addition of MLA, followed by 250 nmol/L nicotine, followed by washout of the MLA. There was a significant difference of area power between application MLA and then nicotine, but not between the latter and the area power in the presence of nicotine after washout of the MLA (n=5. ^{b}P<0.05, repeated measures ANOVA). B1) Example traces of oscillatory field activity firstly in the presence of 5 mmol/L choline, a specific α7*nAChR agonist, followed by washout of the choline and application of 250 nmol/L nicotine. B2) Example time course of oscillatory field activity firstly in the presence of choline, followed by washout of the choline and application of 250 nmol/L nicotine. B3) Percentage changes in area power with respect to control after addition of 5 mmol/L choline, followed by washout of the choline and application of 250 nmol/L nicotine. The area power generated by choline was not significantly different from the control, but application of nicotine caused an increase in area power that was significantly different from both control and the choline-induced activity (n=7. ^{c}P<0.01, repeated measures ANOVA).

Discussion

The current study showed that bath-application of nicotine at nanomolar concentration is able to elicit persistent and dose-responsive oscillatory activity in the MSDB slice, and within the *in vivo* theta frequency range of 4–12 Hz. This activity was similar in appearance and magnitude to that produced by bath application of nanomolar concentrations of kainate to the MSDB slice[11] and which has been correlated with the presence of compound inhibitory synaptic potentials at theta frequency[10]. Although it has been shown previously that application of nanomolar concentrations of kainate to hippocampal slices elicits oscillatory activity in the gamma frequency range[22], application of same concentrations of kainate to MSDB slices induces oscillatory activity in the theta

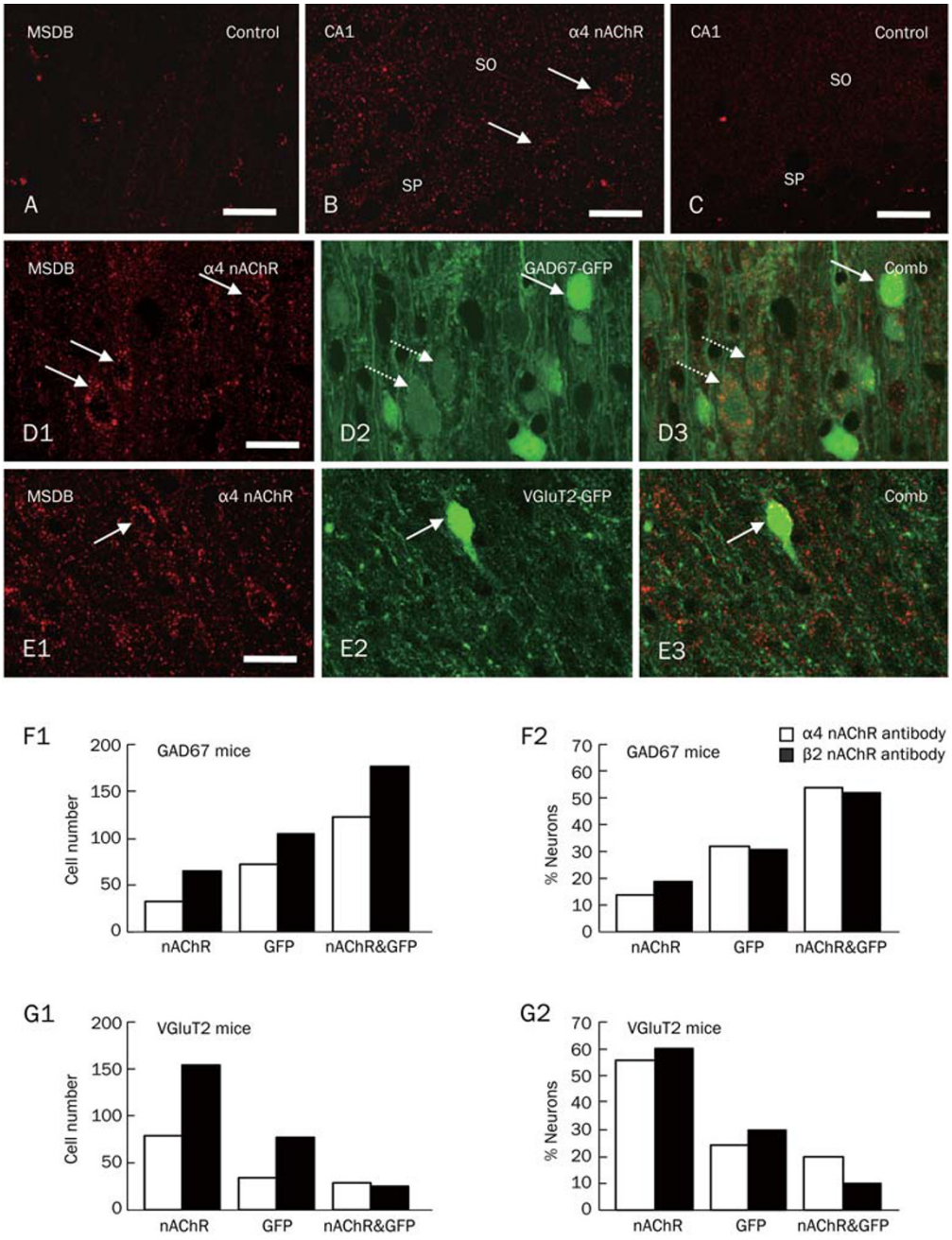

Figure 5. Immunoreactivity for the α4 and β2 nAChR subunits is present in GABAergic and glutamatergic neurons in the MSDB of GFP transgenic mice. A) Negative control, showing lack of immunofluorescence in MSDB from VGluT2-GFP mouse, after omission of the primary antibody to the α4 nAChR subunit in the staining protocol. B) Positive control, showing the presence of immunofluorescence for the α4 nAChR subunit in hippocampal interneurons (arrowed) in the striatum oriens (SO), but not in the pyramidal neurons of the striatum pyramidale (SP) of the CA1 region in the hippocampus from the VGluT2-GFP mouse. C) Negative control for (B), showing lack of immunofluorescence in CA1 from hippocampus of VGluT2-GFP mouse, after omission of the antibody to the α4 nAChR subunit in the staining protocol. D) Dual fluorescence confocal image showing presence of the α4 nAChR subunit in GFP+ve soma (arrowed) and GFP-ve soma (arrowed, dashed arrows) in the MSDB of the GAD67-GFP mouse. E) Dual fluorescence confocal image showing presence of the α4 nAChR subunit in GFP+ve soma (arrowed) and GFP-ve soma (arrowed) in the MSDB of the VGluT2-GFP mouse. F) Proportions of MSDB neurons showing labelling for the α4 or β2 nAChR subunits in GAD67-GFP; F1) the total number of positive staining neurons for α4 and β2 nAChR subunit antibody groups in GAD67-GFP. F2) The percentage of positive staining neurons for α4 and β2 nAChR subunit in GAD67-GFP. The open bars refer to the staining for the α4 nAChR subunit, and the filled bar charts refer to staining for the β2 nAChR subunit. G) Proportions of MSDB neurons showing labelling for the α4 or β2 nAChR subunits in VGluT2-GFP mice. G1) the total number of positive staining neurons for α4 and β2 nAChR subunit antibody groups in VGlut2-GFP. G2) The percentage of positive staining neurons for α4 and β2 nAChR subunit in VGluT2-GFP.

frequency range[11]. The kainate-induced rhythm reported by Garner *et al* was not affected by either muscarinic or nicotinic antagonists and the activity reported in this study was not completely blocked by the AMPA/kainate blocker which suggest that different agonists may generate different networks of neurons to produce the same output such as theta oscillations[11]. This has been shown to be the case in the hippocampus for carbachol and DHPG (a selective agonist of group I metabotropic glutamate receptors)-evoked gamma frequency oscillations[23]. Therefore, induction of theta rhythm is likely determined by local neuronal circuits activated by specific receptor agonists in the MSDB. In the current study it was likely that the oscillatory field activity induced by nicotine in the MSDB slice represents network oscillations produced by activation of local GABAergic and glutamatergic circuitry, since the oscillatory activity was inhibited by ionotropic GABAergic and partially inhibited by glutamatergic receptor antagonists. This is an important conclusion about the collaboration between glutamatergic and GABAergic neurons in the generation of theta oscillatory activity in the MSDB that was not so easy to test in the previous experiments involving kainate. Compared with kainate induced theta oscillation, the extracellular oscillatory field activity induced by nicotine in the MSDB was of relatively small amplitude, and is most likely due to the lack of neuronal array in parallel as they are in the hippocampus. Nevertheless, oscillatory activity induced locally by carbachol in the MSDB in an *in vitro* septohippocampal preparation has been shown to be powerful enough to drive theta activity in the attached hippocampus[13].

Nicotine-induced theta activity in the MSDB slice is mediated by non-α7* nAChRs

The results of the current study indicated a clear involvement of non-α7*nAChRs, probably of the α4β2*nAChR-type rather than α7*nAChRs, in mediating nicotine-induced rhythmogenic activity in the MSDB *in vitro*. Immnunofluorescence and the application of pharmacological agents to the MSDB slices also suggested the presence of α4β2*nAChRs on local GABAergic and glutamatergic neurons in MSDB, and with possibly greater abundance of the receptors on the GABAergic neurons. This is in agreement with previous electrophysiology and immunocytochemical studies suggesting the presence of non-α7*nAChRs on GABAergic neurons[15, 24] and electrophysiological studies indirectly indicating the presence of these receptors on glutamatergic neurons in the MSDB[14]. In the hippocampus the majority of GABAergic interneurons express postsynaptic α7*nAChRs[20], whilst in the MSDB this receptor subtype seems to be confined mainly to the cholinergic neurons, which do not apparently take part in conventional synaptic transmission[15]. Non-α7*nAChRs desensitise less readily than α7*nAChRs, and in MSDB the former respond to concentrations as high as 20 μmol/L with apparently little desensitisation[14]. It is likely therefore that the effect of nicotine on promoting theta frequency activity in the MSDB, and its apparent ineffectiveness in the hippocampus[25] is related to the different desensitising properties of the predominant nAChR subtypes present in these two areas. These studies, however, do not preclude the contribution of presynaptic receptors in promoting the nicotine-induced oscillatory activity in the MSDB slice, but so far little is known about the presynaptic distribution of nAChRs in the MSDB and which specific neuron cell types they are associated with.

Inhibition of ionotropic GABAergic transmission does not promote paroxysmal activity in the MSDB in the presence of nicotine

In our study we have shown that $GABA_A$ receptor antagonists such as bicuculline inhibit nicotine-induced oscillatory activity in the MSDB slice. In such preparations exposed to other agonists such as carbachol, the addition of bicuculline promotes paroxysmal theta activity, interpreted as being due to release of intrinsic glutamatergic neurons from local inhibition[12]. These contrasting results may reflect differences in the proportional distribution of nAChR and muscarinic receptors on GABAergic and glutamatergic neurons in the MSDB. Certainly with respect to non-α7*nAChRs, we have shown by immunocytochemistry that these appear to be more abundant on the GABAergic rather than the glutamatergic neurons of the MSDB. These studies therefore suggest a possible therapeutic value for specific nAChR agonists in restoring some of the effects of a basal forebrain cholinergic innervation depleted in Alzheimer's disease. However, it is still not known whether the cholinergic drive to the MSDB arises from the intrinsic cholinergic neurons, or those from the pedunculopontine nucleus, which are not affected in Alzheimer's disease.

Nicotine promotes oscillatory field activity but not rhythmic burst firing in the MSDB *in vitro*

The hippocampal theta rhythm *in vivo* is tied in which the presence of rhythmic burst firing activity recorded by extracellular electrodes in the midline regions of the MSDB[8, 26]. Extrinsic, non-cortical inputs to the septum seem to control the incidence of the burst firing[7-9], but there has been controversy as to whether this activity in the MSDB is generated intrinsically by tonic input or is driven by theta-patterned input from elsewhere, most notably the SUM[6] . Evidence for the intrinsic ability of the MSDB to generate theta activity is suggested by the retention of rhythmic burst firing at theta frequency in the MSDB following undercutting the septum, or lesion to the fornix or SUM[27–29]. Spontaneous rhythmic burst firing at 3 Hz was also reported to occur in the MSDB slice of the ground squirrel and to be mediated by activation of nAChRs[30] , but such activity has not been described previously in rodent MSDB *in vitro*.

Basal forebrain glutamatergic neurons may play a role in relaying external rhythmogenic inputs to the MSDB

The results so far indicate that tonic activation of nAChRs is able to generate theta frequency-like activity in MSDB slices, and that this activity is mediated by both ionotropic glutamatergic and GABAergic receptor neurotransmission, *ie* suggesting involvement of local GABAergic and glutamatergic

circuitry in the nicotine-induced oscillatory activity. It is noteworthy that nicotine induced oscillations were reduced but not completely abolished by the ionotropic glutamate receptor blocker, NBQX, suggesting that glutamatergic system enhances the theta frequency activity but is not essential for its generation. The lack of robustness of rhythmic burst firing activity in the MSDB *in vitro* also suggests the importance of a patterned theta component from elsewhere that is removed from the conventional slice preparation[31]. Whether these components arise directly from the SUM or from medium-range connections from nearby regions that are excluded from the slice preparations is not known. This is especially important with respect to the role of the newly discovered glutamatergic neurons in the basal forebrain area in which the MSDB is embedded. Although the septal region is seen as a relay station between its ascending inputs and the hippocampus, it is of interest that the non-cortical inputs to the MSDB that arise from the SUM[6, 32–34], dorsolateral nucleus of the lateral septum[35, 36] and the pedunculopontine nucleus of the brainstem[37] terminate in many regions lateral and caudal to the MSDB, some of which host the newly discovered populations of VGluT2-GFP-positive neurons[14, 38–40]. This suggests that the basal forebrain region is a highly complex structure and that the main function of the glutamatergic neurons of the basal forebrain is to receive some of the ascending input and transmit this information to the MSDB via local short-range and medium-range connections. The exact manner in which these inputs are processed by the basal forebrain glutamatergic neurons will require future investigation.

Clinical significance

Theta oscillation is asssociated with higher brain function such as learning and memory[41]. The interruption of these oscillatory activities were implicated in neurodegenerative diseases such as Alzheimer's Disease (AD)[42], which the loss or atrophy of the cholinergic neurons are known[43]. Induction of theta oscillations in MSDB by nicotine suggests that nAChR may be a potential therapeutic target in AD treatment. Disorder in network oscillatory activity has also been documented in neuropsychological disorders such as schizophrenia, and the new nAChR agonists have recently been developed and demonstrated to be effective in the treatment of cognitive impairments associated with schizophrenia[44]. Therefore, this study has provided additional evidence to support clinical application of nAChR agonists in these neuropsychological disorders.

Acknowledgements

This work was supported by National Natural Science Foundation of China (grant No 31070938, No 81271422) and the UK Medical Research Council (grant No G0500823).

Author contribution

Cheng-biao LU designed and performed research; Cheng-zhang LI performed research; Zaineb HENDERSON performed immunohistochemistry experiments; Dong-liang LI analyzed data; Cheng-biao LU wrote the paper.

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