Published in final edited form as:

Biochem Pharmacol. 2013 November 15; 86(10): 1487–1496. doi:10.1016/j.bcp.2013.09.005.

Selective potentiation of $(\alpha 4)_3(\beta 2)_2$ nicotinic acetylcholine receptors augments amplitudes of prefrontal acetylcholine- and nicotine-evoked glutamatergic transients in rats

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Abstract

Prefrontal glutamate release evoked through activation of $\alpha 4\beta 2^*$ nicotinic acetylcholine receptors (nAChRs) situated on thalamic glutamatergic afferents mediates cue detection processes and thus contributes to attentional performance. However, little is known about the respective contributions of the high sensitivity and low sensitivity (LS) stoichiometries of the $\alpha 4\beta 2$ nAChR, $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$, to these processes. In the present study we employed glutamate-sensitive microelectrodes and the $(\alpha 4)_3(\beta 2)_2$ -selective positive allosteric modulator (PAM) NS9283 to investigate the importance of the LS $\alpha 4\beta 2$ nAChR for glutamate release in the rat medial prefrontal cortex (mPFC). Firstly, the signaling evoked by physiologically relevant ACh concentrations through the $(\alpha 4)_3(\beta 2)_2$ nAChR in HEK293 cells was potentiated by NS9283, consistent with the classification of NS9283 as a PAM. In urethane-anesthetized rats, intra-prefrontal pressure ejections of NS9283 evoked glutamatergic transients. Importantly, this glutamate release was attenuated by removal of cholinergic projections to the recording area. This finding indicates that the effects of NS9283 depend on endogenous ACh, again consistent with effects of a PAM. We then conducted microdialysis to demonstrate the presence of extracellular ACh in urethane-anesthetized control rats. While detectable, those levels were significantly lower than in awake rats. Finally, the amplitudes of glutamatergic transients evoked by local pressure ejections of a low concentration of nicotine were significantly augmented following systemic administration of NS9283 (3.0 mg/kg). In conclusion, our results indicate that a LS $\alpha 4\beta 2$ nAChR PAMs such as NS9283 may enhance the cholinergic modulation of glutamatergic

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Conflict of interest

Morten Grupe, Karin Sandager-Nielsen and Morten Grunnet were employed by NeuroSearch A/S at the time of the study.

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neurotransmission in the cortex, thereby perhaps alleviating the attentional impairments common to a range of brain disorders.

Keywords

Nicotinic acetylcholine receptor; $\alpha 4\beta 2$; NS9283; positive allosteric modulator; prefrontal glutamate release

1 Introduction

The nicotinic acetylcholine receptors (nAChRs) are cation-selective ligand-gated ion channels belonging to the superfamily of Cys-loop receptors [1]. The nAChRs are membrane-bound pentameric subunit complexes, and the 16 human nAChRs subunits identified to date assemble into a considerable number of different physiological relevant subtypes characterized by distinct pharmacological, biophysical and kinetic properties and by differential expression in the central nervous system (CNS), the two predominant central receptor subtypes being the homomeric $\alpha 7$ nAChR and the heteromeric $\alpha 4\beta 2^*$ nAChR [1]. The nAChRs are involved in a wide range of important physiological processes throughout the CNS, as they in addition to their roles within the cholinergic synapse also function as heteroreceptors regulating the release of other major neurotransmitters such as glutamate, γ -aminobutyric acid (GABA) and dopamine and thus regulate the activity in these neurotransmitter systems as well [2]. For example, it is well documented that nAChRs play a pivotal role for higher cognitive functions, including domains such as learning, memory and attention [3], an importance that is further supported by the implication of nAChR in neurological pathophysiology [4–6].

A substantial amount of evidence have demonstrated a close link between $\alpha 4\beta 2^*$ nAChR signaling and synaptic glutamate release [7–14]. In the prefrontal cortex (PFC), a brain region essential for top-down control of attention [15], this link is well established [7, 8, 10, 11, 14]. Furthermore, numerous studies in both rodents [12, 16–20] and humans [21–23] have indicated a strong role for $\alpha 4\beta 2^*$ nAChRs in promoting attention. Using microelectrodes to monitor *in vivo* the release of neurotransmitters, recent studies demonstrated that transient release of glutamate and subsequently acetylcholine in the prelimbic region of medial prefrontal cortex (mPFC) is necessary for cue detection in rats performing attention tasks [24–26]. In this regard it has been proposed that stimulation of $\alpha 4\beta 2^*$ nAChRs on thalamocortical glutamatergic terminals are necessary for the generation of cholinergic transients essential for cue detection [8]. Interestingly, local ejections of $\alpha 4\beta 2^*$ preferring agonists into the mPFC evoke ‘sharp’ neurotransmitter transients similar to those observed in performing animals [7, 8, 12]. Hence, this coupling serves as a possible mechanistic explanation to the above-mentioned pro-attentional effects observed upon local ejections of $\alpha 4\beta 2^*$ selective agonists.

It is well-established that $\alpha 4\beta 2$ nAChRs in recombinant expression systems are expressed as two different subunit stoichiometries: $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$. The two receptor stoichiometries differ substantially in Ca^{2+} permeability and in their up-regulation upon nicotine exposure [27, 28], but the major functional difference is the ~100-fold higher agonist potencies exhibited by the ‘high-sensitive’ $(\alpha 4)_2(\beta 2)_3$ nAChR compared to the ‘low-sensitive’ (LS) $(\alpha 4)_3(\beta 2)_2$ nAChR [29–31]. Interestingly, studies in native rodent brain tissue have indicated functional expression of high- and low-sensitive $\alpha 4\beta 2$ nAChRs [32, 33], which could be taken as an indication of both of the two stoichiometries being expressed. However, the putative implications of this molecular diversity still remain to be

explored, mainly due to the fact that the vast majority of $\alpha 4\beta 2$ nAChR ligands currently available target both stoichiometries and/or other nAChR subtypes (e.g. [28] and [34]).

Recently, the discovery of a positive allosteric modulator (PAM) selective for the LS stoichiometries of $\alpha 2^*$ and $\alpha 4^*$ nAChRs has been reported [35]. The compound NS9283 has been shown to left-shift the concentration-response relationship for ACh at the $(\alpha 4)_3(\beta 2)_2$ nAChR expressed in *Xenopus* oocytes and HEK293 cells 500- and 100-fold, respectively, thus inducing an ACh EC₅₀ at this receptor similar to that exhibited at the $(\alpha 4)_2(\beta 2)_3$ nAChR [35, 36], an effect that seems to arise predominantly from a markedly reduced deactivation rate of the NS9283-bound $(\alpha 4)_3(\beta 2)_2$ complex [36]. This increased agonist potency at the LS $\alpha 4\beta 2$ stoichiometry induced by NS9283 has been proposed to underlie the observed pro-cognitive and analgesic effects of the compound in various animal models [35, 37–39].

In the present study we have applied NS9283 and glutamate-sensitive microelectrodes implanted in anesthetized rats to investigate whether functional LS $\alpha 4\beta 2$ nAChRs are involved in glutamate release in the mPFC, and whether prefrontal agonist-evoked glutamate release transients *in vivo* can be modulated through systemic administration of this stoichiometry-selective PAM.

2 Materials and Methods

2.1 Animals

Subjects used for electrochemical and microdialysis experiments were adult male Wistar rats (Harlan Laboratories, Indianapolis, IN) weighing 300–450 g. Animals were individually housed in a temperature- and humidity-controlled environment with a 12:12 light/dark cycle (lights on at 8:00 A.M.). Food and water was available *ad libitum*. All animals were maintained in accordance with the NIH guide for the Care and Use of Laboratory Animals. Experiments were conducted in accordance with protocols approved by the University of Michigan Committee on Use and Care of Animals.

2.2 Cells

Human embryonic kidney 293 cells (CRL-1573, American Type Culture Collection, Manassas, VA) stably expressing human $\alpha 4\beta 2$ nAChRs (HEK293-h $\alpha 4\beta 2$) were cultured as described in [35]. Briefly, cells were incubated at 37°C in a humidified atmosphere with 5% CO₂ in Dulbecco's Modified Eagle Medium (Lonza, Basel, Switzerland) supplemented with Gibco® 10% fetal bovine serum and zeocin (100 µg/ml) (Invitrogen, Tåstrup, Denmark).

2.3 Drugs

NS9283 (3-[3-(3-pyridyl)-1,2,4-oxadiazol-5-yl]benzonitrile) was synthesized at NeuroSearch A/S, Ballerup, Denmark. For the electrochemistry experiments NS9283 was dissolved in 30% w/v 2-hydroxypropyl- β -cyclodextrin (HBC)/physiological saline (Sigma-Aldrich, St. Louis, MO) and sonicated with heating at 30°C (ultrasonic cleaner, model FS20H, Fisher Scientific, St. Louis, MO). For the patch-clamp electrophysiology experiments NS9283 was dissolved in DMSO and diluted to final concentrations thereof where DMSO concentrations were kept below 0.1%. Glutamate oxidase (GO; EC 1.4.3.11) was from United States Biological (Swampscott, MA). 192-IgG Saporin (192-SAP) was obtained from Advanced Targeting Systems (San Diego, CA) and dissolved in artificial cerebrospinal fluid (aCSF, pH 6.8, for recipe see 2.5). Bovine serum albumin (BSA), glutaraldehyde, metaphenylenediamine (*m*-PD), L-glutamic acid, ascorbic acid, dopamine, nicotine (nicotine hydrogen tartrate) and urethane were purchased from Sigma-Aldrich (St. Louis, MO). Nicotine was dissolved in sterile saline. The pH of solutions used for

intracranial pressure ejections (7.4–7.6) was adjusted using sodium hydroxide solution. Acetylcholine was purchased from Sigma-Aldrich (Brøndby, Denmark).

2.4 Patch-clamp electrophysiology

The HEK293-hα4β2 cells used for the patch-clamp experiments were grown on poly-D-lysine coated glass coverslips. The coverslips were placed in a recording chamber fixed at the stage of an inverted microscope (Olympus, Ballerup, Denmark). During the experiments cells were perfused with an extracellular buffer containing: 140 mM NaCl, 10 mM HEPES, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂ (pH 7.4, adjusted with NaOH). Patch pipettes, made from borosilicate capillary tubes, were pulled using a horizontal puller (Zeitz-Instrument, Munich, Germany) and backfilled with intracellular buffer containing: 120 mM KCl, 31 mM KOH, 10 mM EGTA, 10 mM HEPES, 1.785 MgCl₂ (pH 7.4, adjusted with KOH). Pipette resistances were in the range 1.5–3 MΩ when submerged in extracellular buffer. Patch-clamp experiments were performed in whole-cell configuration at 21–25°C. Data were sampled at minimum 4 kHz with Pulse software (v8.80) controlling an EPC-9 amplifier (HEKA, Lambrecht, Germany) and low-pass filtered at 2.9 kHz. Cells were held at a holding potential of –60 mV throughout experiments. Capacitive currents and series resistances were determined upon establishment of whole-cell configuration. Series resistance was compensated by 80%. Only cells with series resistance <10 MΩ were accepted for data analysis. Fast application of the drugs was managed using a double-barreled micropipette made from a theta-glass capillary (World Precision Instruments, Sarasota, FL) with a tip diameter of ~50–100 μm. Using a micromanipulator system (Eppendorf Nordic, Hørsholm, Denmark) the application micropipette was positioned in the near vicinity of the patch-clamped cell under visual guidance. This system allows control of the environment of the cell, which can be rapidly exchanged upon a fast lateral step between the buffers (with/without drug) flowing from the two barrels, controlled by a piezo-ceramic device (Burleigh Instruments, Fishers, NY). Solution exchange time of the application system was estimated by recording the open tip current caused by differences in liquid junction potentials when stepping to a solution of reduced ionic strength. The 10–90% rise time for solution exchange was ~600–800 μs. For the experiments the extracellular buffer supplemented with 1 μM ACh or 1 μM ACh + 10 μM NS9283 was applied onto the cells. All solutions were prepared on the day of the experiment from stock solutions.

2.5 Microdialysis

2.5.1 Implantation of guide cannula for the measurement of mPFC ACh release—Animals underwent surgery for implantation of a guide cannula in the right mPFC. All surgeries were performed under aseptic conditions. Anesthesia was induced with 4–5% isoflurane using an anesthesia machine (Anesco/Surgivet, Waukesha, WI). Animals' heads were shaved using electric clippers and cleaned with alcohol wipe. Animals were then placed in a stereotaxic frame (model No. 962; David Kopf Instruments, Tujunga, CA). Anesthesia was maintained with 2% isoflurane carried via oxygen at a flow rate of 0.6 L/min. A unilateral guide cannula (model MAB 4.151c; cannula o.d.: 0.38 mm; Sci Pro; Sanborn, NY) was implanted in the right mPFC (measured from bregma: AP: +3.0 mm; ML: –0.7 mm; DV: –0.6 mm below dura). The guide cannulas were equipped with stainless steel stylets to prevent clogging. A headstage to hold the guide cannula in place was constructed with surgical screws implanted in the skull and dental cement. Neosporin was applied to the area around the headstage. On the day of surgery and 24 hrs post-surgery animals were given injections of an antibiotic (amikacin, 250 mg/ml, 0.1 ml, i.p.) and an analgesic (buprenorphine, 0.01 mg/kg/ml, i.p.). Following surgery animals were returned to their home cages.

2.5.2 Microdialysis procedures and quantification of ACh concentrations—

Two days prior to microdialysis sessions animals were tethered and individually habituated for 2–4 hrs to their respective chambers in which the ACh collection would take place. For the microdialysis sessions the stylet was removed and a concentric microdialysis probe (model MAB4; membrane o.d.: 0.24 mm; membrane length: 3.0 mm; Sci Pro; Sanborn, NY) was inserted into the guide cannula. Animals were placed in their chambers and aCSF (pH 6.8 ± 0.1 , containing the following (in mM): 126.5 NaCl, 27.5 NaHCO₃, 2.4 KCl, 0.5 Na₂SO₄, 0.5 KH₂PO₄, 1.2 CaCl₂, 0.8 MgCl₂, 5.0 dextrose) was perfused at a rate of 2.0 μ L/min. Before dialysate collection the probes were perfused for at least 120 min to allow ACh efflux to stabilize. Dialysates were collected over 8 min periods. During the first three 8 min collections, animals were awake but motionless, whereas during the last three 8 min collections, animals were kept active/awake by tactile stimulation. Animals were then anesthetized with urethane (~2.0 g/kg, i.p.) and 6 collections were performed. In order to mimic a typical electrochemistry experiment the anesthetized animals were placed in stereotaxic frames and dialysate collections were started ~100 min following full anesthesia onset, representing the duration of craniotomy, microelectrode preparation and stabilization of baseline current (see below). The body temperature of the animals was maintained at 37°C using a heating system (T/Pump, model TP500; Mul-T-Pad, model TP22B; Gaymar, Orchard Park, NY). ACh levels were quantified by HPLC-ECD as previously described [40]. The detection limit of this system was 2 fmol/15 μ l.

2.6 Electrochemical recordings of glutamatergic transients *in vivo*

Glutamate-sensitive microelectrodes were prepared as previously described [7]. Briefly, extracellular glutamate release was measured using ceramic-based glutamate-sensitive microelectrodes and fixed-potential amperometry (Quanteon, Nicholasville, KY). Each microelectrode had four platinum recording sites, arranged in pairs side by side. In order to measure changes in glutamate concentrations the bottom pair was coated with GO crosslinked with a BSA/glutaraldehyde solution. The upper pair was coated with BSA/glutaraldehyde solution alone and served as background sentinel sites. *m*-PD was electropolymerized for ~60 min onto the surface of the recording sites to block electroactive interferents. Microelectrodes coated with GO were calibrated as previously described [7]. Prior to *in vivo* glutamate recordings, *in vitro* calibration of the microelectrode allowed calculation of slope (sensitivity for glutamate detection), limit of detection (LOD), ratio of selectivity for glutamate and ascorbic acid, and linearity R^2 . Microelectrodes used in glutamate recordings ($n = 23$) had in average ($\pm$ SEM) a slope of 8.8 ± 0.4 pA/ μ M, LOD of 543 ± 55 nM, selectivity of 68 ± 7 , and linearity R^2 of 0.999 ± 0.0003 .

Animals were anesthetized with urethane (~2.0 g/kg, i.p.) and placed in a stereotaxic frame (model 962; David Kopf Instruments, Tujunga, CA). The body temperature of the animals was maintained at 37°C using a heating system (T/Pump, model TP500; Mul-T-Pad, model TP22B; Gaymar, Orchard Park, NY). A craniotomy was performed over the region of interest and the dura removed. An Ag/AgCl reference electrode was implanted in the opposite hemisphere. For pressure ejections of drug solutions single barrel glass capillaries (1.0 $\times$ 0.58 mm, 6 in.; A-M Systems, Carlsborg, WA) were pulled using a micropipette puller (Model #51210; Stoelting, Wood Dale, IL). The micropipette (inner tip diameter ~15 μ m) was attached to the microelectrode using sticky wax (Kerr, Romulus, MI) with the tip centered between the two pairs of recording sites, ~70 μ m from the surface of the microelectrode. Following loading of the micropipette with drug solution (see below) the microelectrode/micropipette assembly was slowly lowered into the right mPFC (measured from bregma: AP: +3.0 mm; ML: -0.7 mm; DV: -2.6 to -3.2 mm below dura) using a microdrive (MO-10; Narishige International, East Meadow, NY). A fixed potential of +0.7 V relative to the Ag/AgCl reference was applied to the microelectrode. The amperometric

recordings were made at 1 Hz, and data was digitized using a FAST-16 recording system (Quanteon LLC, Nicholasville, KY). Experiments were initiated following 45–60 min of stabilization of baseline current. Currents were self-referenced as previously described [7].

2.6.1 Experimental design—Drug solutions were ejected into mPFC through the micropipette using a pressure ejection system (Picospritzer III; Parker Hannifin, Fairfield, NJ). A stereoscope fitted with a reticule was used to monitor ejection volumes. Glutamatergic signals were evoked by pressure ejecting solutions with nicotine and NS9283. The concentrations for drug ejections were calculated on the basis of the ejected volume of nicotine (1 μ M and 20 μ M) and NS9283 (10 μ M) solutions. The effect of systemic administration of NS9283 (3 mg/kg, base weight, i.p.) and 30% w/v HBC-saline (vehicle) on nicotine-evoked glutamatergic signals was investigated by comparing glutamatergic signals before and after the administration (30 min post injection, corresponding to maximum NS9283 plasma concentration in male Wistar rats [35]) within each animal. Intracranial pressure ejections were separated by at least 5 min. Pressure ejections with KCl (70 mM KCl, 2.5 mM CaCl_2 , 75 mM NaCl) was used to confirm glutamate release at the end of electrochemistry experiments with local ejections of NS9283 and HBC-saline.

2.7 Lesion of prefrontal cholinergic neurons

Loss of cholinergic neurons in right mPFC was produced by bolus-infusing animals ($n = 5$) with the choline-selective immunotoxin 192-SAP (200 ng/ μ l, 0.8 μ l/site) into the mPFC (measured from bregma: AP: +3.0 mm; ML: –0.7 mm; DV: –2.5 mm below dura). The needle was left in position for 10 min to foster absorption of the toxin. To ensure toxin effect, electrochemical recordings in lesioned animals were conducted two weeks after the 192-SAP infusions. Left mPFC was left unlesioned in order to serve as a positive glutamate release control in electrochemistry experiments.

2.8 Histochemical and immunohistochemical methods

Following electrochemical recordings and microdialysis experiments urethane-anesthetized animals were transcardially perfused with ice-cold 0.1 M PBS followed by formalin (Fisher Scientific, St. Louis, MO) or 4% paraformaldehyde in 0.1 M PBS (pH 7.4) (for Nissl- or choline acetyltransferase (ChAT)-staining, respectively). Brains were removed and stored in their respective fixative overnight and then transferred to 30% sucrose 0.1 M PBS and allowed to sink. Coronal sections (40 μ m) surrounding the site of interest were sliced using a freezing microtome (Leica CM 2000R; Leica Microsystems Inc., Chantilly, VA) and stored in 0.1 M PBS or in cryoprotectant solution at –20 °C until further processing. The slices surrounding the microelectrode and dialysis probe were mounted onto gelatin-coated glass slides and allowed to dry before Nissl-staining for verification of probe placement.

2.8.1 ChAT staining—Prefrontal cholinergic depletion upon infusion of 192-SAP into the mPFC was assessed *post hoc* on two 192-SAP lesioned animals and one sham lesioned animal in parallel brain sections processed for immunohistochemical detection of ChAT-containing neurons with a Vectastain Elite ABC kit (PK-6105; Vector Laboratories, Burlingame, CA) and a primary antibody (polyclonal goat anti-choline acetyltransferase; Millipore, Temecula, CA). For incubation and rinse periods an orbital shaker was used. Sections were first rinsed in 0.1 M phosphate buffer (pH 7.4) three times for 5 min each, followed by 30 min incubation in 0.3% peroxide. They were then rinsed again in 0.1 M phosphate buffer two times for 5 min followed by incubation in a 1.5% normal blocking serum with 0.2% Triton X for 1 h. After, tissue was immediately transferred to incubate in the primary antibody (goat anti-ChAT made in rabbit; 1:250) overnight at 4°C. The next day sections were rinsed in 0.1 M PBS with 0.2% Triton X three times for 5 min each followed by 2 hr incubation with the biotinylated secondary antibody (biotinylated rabbit anti-goat;

1:200; supplied in the Vectastain Elite ABC kit). Sections were then rinsed three times again for 5 min each in 0.1 M phosphate buffer with 0.2% Triton X, incubated in the avidin-biotin complex (1:25) for 30 min, and rinsed in 0.1 M phosphate buffer three times for 5 min each. Next, tissue was rinsed in a peroxidase substrate solution (0.4% DAB (diaminobenzidine) and 0.19% nickel (II) chloride in 0.1 M phosphate buffer). Ten microliters of 30% hydrogen peroxide were added immediately before use. After having reached the desired color (5 min), sections were rinsed with 0.1 M phosphate buffer three times for 5 min each. The sections were then mounted onto gelatin-coated glass slides and allowed to dry overnight before dehydration in an ascending alcohol series (70, 90, and 100%) and defatting in xylene before coverslipping.

2.9 Data analysis and statistics

Glutamatergic signals were analyzed using FAST software (Version 4.4; Quanteon LLC, Nicholasville, KY) with respect to peak signal amplitude and signal decay rate (t_{80} : time required for signal to decline by 80% from peak amplitude). The averages of two-four responses per drug manipulation per animal were used for statistical analyses. Statistical differences between group means were assessed with paired Student's *t*-test and repeated measures one-way ANOVA followed by Tukey's *post hoc* comparison (multiplicity adjusted *p* value) using GraphPad Prism as indicated in the Results section (v4.03, GraphPad Software Inc., San Diego, CA). In the patch-clamp experiments currents were baseline-subtracted by the average current 250 ms prior to compound application and quantified by measuring peak current amplitude. Where applicable, statistical errors of averaged data are given as mean $\pm$ S.E.M. values with the number of determinations (*n*).

3 Results

3.1 NS9283 potentiates signal evoked by physiologically relevant ACh concentrations through $(\alpha 4)_3(\beta 2)_2$ nAChR *in vitro*

Prior to exploring the modulation of glutamate release by NS9283 *in vivo*, we investigated whether the compound was indeed suitable as a pharmacological tool for this purpose. Since NS9283 has no intrinsic effect at $(\alpha 4)_3(\beta 2)_2$ nAChR signaling [35, 36], any receptor response caused by NS9283 is dependent on the presence of endogenous ACh and/or a co-administered agonist. In a recent study, the tonic or resting concentration of ACh in the rat mPFC has been determined to be $\sim 1 \mu\text{M}$ [41]. Hence, we addressed whether NS9283 can evoke a response at the $\alpha 4\beta 2$ nAChR *in vitro* in the presence of this ACh concentration. This was done using HEK293 cells stably expressing human $\alpha 4\beta 2$ nAChR (HEK293-h $\alpha 4\beta 2$) and the whole-cell patch-clamp technique with an ultra-fast application setup. As displayed in Fig. 1A, application of $1 \mu\text{M}$ ACh (1 s) evoked an inward current response at the receptor in this set-up. The average peak current amplitude calculated from two responses per cell was $69 \pm 11 \text{ pA}$ ($n = 7$). In the same recordings the patch-clamped cells were subsequently incubated with $1 \mu\text{M}$ ACh in order to reach a level of steady state activation/desensitization. Following $\sim 40 \text{ s}$ of ACh incubation, NS9283 ($10 \mu\text{M}$) was applied on top of ACh, resulting in an inward current response, see Fig. 1B. The average NS9283-evoked peak current amplitude was $78 \pm 15 \text{ pA}$ ($n = 7$), corresponding to 112% of the average peak amplitude of $1 \mu\text{M}$ ACh responses recorded in each cell prior to the 40 s ACh incubation ($t(6) = 1.42$, $p = 0.21$). Baseline currents (average current 250 ms prior compound application) were $-67.3 \pm 5.8 \text{ pA}$ and $-75.7 \pm 12.4 \text{ pA}$ for ACh and NS9283, respectively (data not shown). This difference was not significantly different ($t(6) = 0.63$, $p = 0.55$), and thus considered negligible. In summary, this demonstrates that application of NS9283 can evoke current through the receptor in the presence of an ACh concentration similar to the reported ACh tone in rat mPFC.

3.2 Urethane-anesthesia lowers prefrontal ACh levels

Before testing for NS9283-evoked responses *in vivo* we wanted to confirm the presence of a tonic extracellular concentration of ACh in mPFC. This was addressed with microdialysis in animals that were awake but motionless (inactive), active as well as anesthetized. Collections of dialysates during the latter were initiated 100 min following full anesthesia in order to mimic the electrochemistry experiments presented below. Collection of dialysates revealed significant state-dependent differences in ACh levels as determined by repeated measures one-way ANOVA ($F_{(2,12)} = 12.47$, $p = 0.0147$). Mean ACh values $\pm$ SEM (fmol/15 μ l) were 8.7 ± 1.4 , 4.6 ± 0.9 and 2.1 ± 0.3 for active, inactive and anesthetized animals, respectively (Fig. 2). *Post hoc* comparisons revealed a significant difference in ACh levels between the active and anesthetized state ($p = 0.026$) and inactive and anesthetized ($p = 0.036$) but not active and inactive ($p = 0.136$).

3.3 NS9283 evokes glutamate release in mPFC

We next investigated whether local ejection of NS9283 in mPFC could evoke glutamate release. This was addressed using microelectrodes coated with glutamate oxidase implanted into the mPFC of anesthetized rats. As can be seen in Fig. 3A, local ejections of NS9283 (400 pmol) evoked a robust glutamate release. The average peak amplitude was 4.9 ± 0.9 μ M and t_{80} decay rate was 2.1 ± 0.2 s ($n = 5$). Local ejections of vehicle (HBC-saline) did not evoke glutamate release ($n = 3$) (see representative trace in Fig. 3A). At the end of each recording glutamate release at the recording site was verified with local ejection of 70 mM KCl (see representative release trace in Fig. 3B). The average KCl-evoked glutamate release was 18.9 ± 4.7 μ M at an average ejection volume of 135 nL ($n = 8$).

3.4 NS9283-evoked glutamate release in mPFC requires intact cholinergic fibers

Given that the NS9283-evoked glutamate release in mPFC is likely to be dependent on the presence of ACh we hypothesized that selective removal of cholinergic efferents to the prefrontal region would abolish the NS9283-evoked glutamate release in the region. This would also confirm that the effects on glutamate release observed from local injections of the PAM were mediated selectively through the cholinergic projections.

To test this, the cholino-selective immunotoxin 192-SAP [42] was infused into the right hemisphere above the recording region in mPFC, while keeping the left hemisphere intact to serve as a positive control. As shown in Fig. 4A, local ejections of NS9283 in the left hemisphere resulted in dose-dependent glutamate release, whereas no glutamate release was observed upon local ejections of the PAM in the right lesioned hemisphere. Average peak signal amplitudes of ejections of 400 and 800 pmol NS9283 in left and right hemisphere are displayed in Fig. 4B: left hemisphere, 400 pmol: 3.3 ± 0.7 μ M, 800 pmol: 5.8 ± 1.7 μ M ($n = 2$); right hemisphere, 400 pmol: 0.8 ± 0.2 μ M, 800 pmol: 0.7 ± 0.2 μ M ($n = 5$). Glutamate release was confirmed at the end of each recording in both hemispheres with local ejections of 70 mM KCl. Average peak amplitude in right hemisphere reached 16.4 ± 4.2 μ M at an average ejection volume of 177 nL ($n = 5$) and in left hemisphere 31.9 ± 22.4 μ M at an average ejection volume of 143 nL ($n = 2$). The similar KCl-evoked glutamate release detected in right unlesioned hemisphere, see 3.3, indicates that glutamate release capacity in mPFC was not altered by the infusion of 192-SAP. *Post hoc* assessment of the 192-SAP-induced lesion in mPFC using immunohistochemical detection of ChAT-positive fibers revealed a loss of cholinergic fibers in the lesioned hemisphere (Fig. 4C) as compared to the intact contralateral hemisphere (Fig. 4D) and sham (not shown). In summary, these findings indicate that NS9283-evoked glutamate release is dependent on intact cholinergic fibers.

3.5 Systemic administration of NS9283 potentiates amplitude of nicotine-evoked glutamatergic transients in mPFC

The established pharmacological profile of NS9283 and the observed NS9283-evoked glutamate release in the mPFC suggest the presence of $(\alpha 4)_3(\beta 2)_2$ nAChRs situated on glutamatergic neurons in the mPFC. Local ejections of nicotine have previously been demonstrated to evoke glutamate release in rat mPFC via $\alpha 4\beta 2^*$ nAChRs [7]. Additionally, nicotine potency at the $3\alpha:2\beta$ subunit stoichiometry of $\alpha 4\beta 2$ nAChR is dramatically increased in the presence of NS9283 *in vitro* [35]. Together, these findings make nicotine a suitable nicotinic agonist for the investigation of modulation of *in vivo* glutamate release.

Firstly, nicotine-evoked glutamate release in mPFC was probed at four concentrations: 10, 40, 2000 and 4000 pmol. At concentrations 40 pmol local nicotine ejections consistently evoked robust glutamate release ($n = 2-5$), however, at 10 pmol nicotine-evoked glutamate release was only observed in 3 of 5 recordings. Representative glutamate release traces evoked by 10, 40 and 2000 pmol nicotine are displayed in Fig. 5A with average peak amplitudes of $1.2 \pm 0.2 \mu\text{M}$ ($n = 3$), $3.1 \pm 0.7 \mu\text{M}$ ($n = 5$), and $5.9 \pm 0.6 \mu\text{M}$ ($n = 2$), respectively (see inset in Fig. 5A). Glutamate release at 4000 pmol nicotine was similar to that of 2000 pmol (peak amplitude: $6.0 \pm 0.1 \mu\text{M}$, $n = 2$, data not shown), indicating that the nicotine-evoked glutamate release had reached maximum amplitude at 2000 pmol.

In the same recordings the effect of NS9283 on nicotine-evoked glutamate release was investigated. This was addressed by comparing glutamate release before and after systemic administration (i.p.) of NS9283. Nicotine-evoked glutamate release was measured 30 min after NS9283 administration (NS9283, 3 mg/kg, i.p.: $T_{\text{max}} = 15-30$ min, $t_{1/2} = 1.6$ hr [35]), separated by at least 5 min intervals. From extensive *in vitro* characterization we know that NS9283 alters only agonist potency and not efficacy at $\alpha 4\beta 2$ nAChRs, resulting in agonist potentiation within a specific agonist concentration interval with no detectable potentiating effects at sufficiently low and high agonist concentrations [35, 36]. As reported above, glutamate release was inconsistent at 10 pmol nicotine ejections and at maximum at 2000 pmol nicotine. We therefore mainly focused on potential modulation of glutamate release by NS9283 at 40 pmol nicotine, as consistent glutamate release with an average peak amplitude in between those of 10 and 2000 pmol was observed at this dose (Fig. 5A). Following systemic administration of NS9283, a 48% potentiation of glutamate peak amplitude was observed for 40 pmol nicotine (40 pmol + NS9283: $4.5 \pm 0.6 \mu\text{M}$; $t_{(4)} = 4.065$, $p = 0.015$) (Fig. 5B). Modulation by NS9283 at 10 and 2000 pmol nicotine was also probed, but no apparent effect on glutamate release was observed (10 pmol + NS9283: $1.6 \pm 0.7 \mu\text{M}$, $n = 3$ (only the three recordings displaying glutamate release were analyzed here, see above); 2000 pmol + NS9283: $5.3 \pm 0.8 \mu\text{M}$, $n = 2$) (data not shown). Representative release traces for 40 pmol nicotine before and after NS9283 administration are displayed in Fig. 5C. Systemic administration of vehicle had no potentiating effect on glutamate peak amplitude at 40 pmol nicotine ($n = 3$). In contrast, peak amplitudes decreased 16% following vehicle administration (40 pmol: $4.4 \pm 0.8 \mu\text{M}$, 40 pmol + vehicle: $3.7 \pm 0.6 \mu\text{M}$) (data not shown). No apparent change in signal decay rate, t_{80} , was observed for any of the nicotine concentrations following NS9283 administration (data not shown). Microelectrode placement in mPFC was confirmed in coronal Nissl-stained sections (Fig. 5D). In summary, this demonstrates that systemically administered NS9283 potentiates submaximal glutamate release at low nicotine concentrations in mPFC.

4 Discussion

The proposed coupling between activation of $\alpha 4\beta 2^*$ nAChRs and glutamate release in the mPFC [7, 8, 10, 11] formed the basis for the present study. Using glutamate-sensitive electrodes and local ejections and systemic administrations of the stoichiometry-selective

PAM NS9283 we investigated the potential involvement of $3\alpha:2\beta$ stoichiometry $\alpha 4\beta 2$ nAChR in glutamate release in mPFC. To date only few studies have addressed the roles of the $3\alpha:2\beta$ and $2\alpha:3\beta$ $\alpha 4\beta 2$ stoichiometries in native brain tissue, but these seem to indicate the expression of both stoichiometries in cortex and thalamus [43, 44] as well as in Renshaw cells in mouse spinal cord [33]. It is important to note that NS9283 in addition to its $3\alpha:2\beta$ $\alpha 4\beta 2$ nAChR activity also has activity at the $3\alpha:2\beta$ stoichiometry of $\alpha 4\beta 4$, $\alpha 2\beta 2$ and $\alpha 2\beta 4$ nAChRs [35]. However, although we cannot completely exclude contributions from these subtypes to our findings, the limited cortical expression of $\alpha 2$ [45] and $\beta 4$ [46] mRNA in the adult rat strongly suggests that these contributions are negligible. In the light of this and the abundant expression of $\alpha 4\beta 2$ nAChR [1], we ascribe the evoked and potentiated glutamate release in mPFC from NS9283 to its activity at the $(\alpha 4)_3(\beta 2)_2$ nAChR.

Before embarking on these studies we used patch-clamp electrophysiology to verify that fast application of NS9283 evokes a response through $\alpha 4\beta 2$ receptors in the presence of a physiologically relevant ACh concentration (Fig. 1B). In contrast to other nicotinic PAMs [47, 48] NS9283 is not capable of reactivating desensitized receptors [36]. Thus, our results indicate that at a low micromolar ACh concentration where a population of $\alpha 4\beta 2$ receptors is at a steady state level of activation and desensitization the current response of activated receptors can be potentiated by NS9283. This is not necessarily surprising as the employed ACh concentration falls within the $\alpha 4\beta 2$ ACh window current [36].

The patch-clamp experiment as well as the local *in vivo* ejections of NS9283 were designed based on the assumed presence of a low endogenous ACh concentration in the prefrontal extracellular space, and this presence was demonstrated using microdialysis. Interestingly, urethane-anesthesia of the rats significantly lowered prefrontal ACh concentrations compared to those in unanesthetized animals. A similar effect on ACh levels in the frontal cortex has been observed with anesthesia induced by isoflurane [49] and pentobarbitone [50], whereas ketamine-anesthesia has been reported to increase ACh levels [50]. The endogenous ACh values detected in this study do not correspond to the assumed ACh concentration [41], but we believe this discrepancy may be explained by the inherent limitations of the microdialysis technique compared to enzyme-coated microelectrodes. The latter samples directly from the extracellular space with high temporal resolution, whereas the spatial and temporal resolution of microdialysis is compromised by the dependence on diffusion of analytes through a semipermeable membrane over minutes, thus representing both analyte release, degradation and diffusion, as well as by the occurrence of gliosis and edema [41, 51].

The fact that NS9283 evoked robust glutamate release in mPFC strongly indicate expression of $(\alpha 4)_3(\beta 2)_2$ nAChRs in this region, and this finding is supported by the observed activity of NS9283 at native rat nicotinic receptors in thalamocortical neurons *ex vivo* [35]. Interestingly, the NS9283-evoked glutamate release was abolished in 192-SAP lesioned hemispheres, indicative of the presence of an endogenous mPFC ACh tone contributing to the observed release. In contrast, a previous study has argued against the presence of an ACh tone sufficient to mediate dopamine release upon local delivery of the $\alpha 7$ nAChR PAM PNU-120596 [52]. A possible explanation for this apparent discrepancy could be the use of microdialysis as detection method as well as retrograde diffusion over 30 min as compound delivery method. As discussed above, enzyme-coated microelectrodes in combination with a sub-second compound delivery system as used in this study appear more suitable for detecting transient changes in neurotransmitter levels.

A key finding of the current study was the *in vivo* potentiation of nicotine-evoked glutamate release in mPFC following systemic NS9283 administration. Analogously, systemic administration of PNU-120596 has previously been demonstrated to potentiate dopamine

release in the prefrontal cortex *in vivo* [52]. Interestingly, in rats performing a sustained attention task the amplitudes of prefrontal glutamate release transients during cue detection appear to be positively related to attentional performance [24]. Thus, we speculate that augmented glutamate release could be a contributing mechanism to cognition enhancement through positive allosteric modulation of $\alpha 4\beta 2$ nAChR [35]. The recording of neurotransmitter release in performing animals [24–26] would be helpful in addressing this. The findings of the present study indicate that only submaximal glutamate release evoked by low concentrations of nicotine is potentiated by NS9283. In regards, we note that the maximum nicotine-evoked glutamate release was not due to limitations of the instruments as KCl-evoked glutamate release reached markedly higher amplitudes, see 3.3. Firstly, this indicates that NS9283 does not alter the efficacy of glutamate release through nicotinic receptors. Secondly, it suggests that the pharmacological window for increasing glutamate release through allosteric modulation of $\alpha 4\beta 2$ nAChR may be limited in healthy rats. Additionally, a robust increase in prefrontal ACh release during attentional performance [40] would most likely reduce the window even further. Thus, a ‘ceiling effect’ could be part of the explanation to the modest effects of NS9283 on attentional performance in healthy rats compared to its robust effects in compromised animals in other cognitive areas [35]. Analogously, several behavioral studies have highlighted that pharmacological effects of nicotinic compounds could only be detected under specific experimental conditions, be it aged, pharmacologically impaired, or performance-screened animals [17–19, 53].

It is important to note that the results of the present study do not in any way rule out a role for the high-sensitivity ($\alpha 4$)₂($\beta 2$)₃ nAChR in prefrontal glutamate release. This stoichiometry has previously been attributed a role in dopamine release in striatum [54], and recently it was proposed to be involved in prefrontal glutamate release in mice based on the observed effects of low concentrations of an $\alpha 4\beta 2^*$ selective agonist on excitatory postsynaptic currents [55]. Based on the different functional properties exhibited by the two stoichiometries *in vitro* [28, 30, 56], it has been proposed that at a pre- and/or extrasynaptic location the high-sensitive 2 α :3 β stoichiometry would be suitable for detecting low ACh concentrations in volume transmission, whereas the low-sensitive 3 α :2 β stoichiometry could be involved in detecting brief high-concentration ACh pulses postsynaptically, thus contributing to wired neurotransmission [27, 33, 56]. Interestingly, the compound used in this study robustly increases the ACh sensitivity at 3 α :2 β receptors to that of 2 α :3 β receptors [35, 36]. Thus, the pro-cognitive effects of NS9283 in various animal models [35] may arise from the increased fraction of high-sensitive receptors in the $\alpha 4\beta 2$ population induced by the compound. Although it cannot be concluded from the present study whether 3 α :2 β receptors are to a larger extent localized at presynaptic or postsynaptic sites, our findings suggest that the activation of this stoichiometry can trigger glutamate release. In summary, our studies are consistent with the hypothesis that both $\alpha 4\beta 2$ stoichiometries are involved in prefrontal neurotransmission, which future studies hopefully will help to elucidate further.

Acknowledgments

This study was supported by NIH grant MH080332 and The Ministry of Science, Innovation and Higher Education, Denmark, PhD grant 10-084289.

Abbreviations

192-SAP	192-IgG saporin
ACh	Acetylcholine
aCSF	Artificial cerebrospinal fluid BSA, Bovine serum albumin

BSA,	Bovine serum albumin
ChAT	Choline acetyltransferase
CNS	Central nervous system
ECD	Electrochemical detection
Glut	Glutamate
GO	Glutamate oxidase
HBC	2-hydroxypropyl- β -cyclodextrin
HEK293-hα4β2	HEK293 cells stably expressing human α 4 β 2 nAChR
HPLC	High-performance liquid chromatography
LOD	Limit of detection
LS	Low sensitivity
<i>m</i>-PD	<i>Meta</i> -phenylenediamine
mPFC	Medial prefrontal cortex
nAChR	Nicotinic acetylcholine receptor
PAM	Positive allosteric modulator

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Fig. 1. Fast application of NS9283 evokes current through $\alpha 4\beta 2$ nAChR in the presence of ACh *in vitro*

Average current traces ($n = 7$) of voltage-clamped HEK293-h $\alpha 4\beta 2$ cells upon ultra-fast application of 1 μ M ACh (gray bar; left panel), followed by ~ 40 s incubation with 1 μ M ACh and ultra-fast application of 1 μ M ACh + 10 μ M NS9283 (black bar; right panel). The average baseline current 250 ms prior to ACh and NS9283 application, respectively, was subtracted from the elicited currents. Timescale (s) has been offset corrected to compound application.

Fig. 2. Prefrontal ACh levels in inactive, active and urethane-anesthetized rats

ACh levels were monitored every 8 min by collecting dialysates through a microdialysis probe lowered into the mPFC and quantified via HPLC-ECD. During the active and inactive states, three collections were performed, whereas ACh under urethane-anesthesia was monitored for a longer interval to mimic a typical electrochemical session (six collections; $n = 5$ animals, $*p < 0.05$, repeated measures one-way ANOVA). Bars represent the mean $\pm$ S.E.M. of 5 animals.

Fig. 3. Local ejections of NS9283 evoke glutamate release in mPFC

Real time glutamate release in anesthetized rats was measured with enzyme-coated microelectrodes implanted in the mPFC. Glutamate release was evoked by local serial pressure ejections separated by at least 5 min. Signal peak amplitudes were averaged from two to four ejections in each animal.

A) Representative, self-referenced traces of NS9283- and HBC-saline-evoked glutamate release (ejection volume, 40 nL). Timescale has been offset corrected to drug ejection.

B) Representative, self-referenced traces of KCl-evoked (70 mM, ejection volume: 110 nL) glutamate release. Timescale has been offset corrected to KCl ejection.

Fig. 4. NS9283-evoked glutamate release requires intact cholinergic fibers

A) Representative, self-referenced traces of NS9283-evoked glutamate release in the lesioned right hemisphere (top panel) and intact left hemisphere (bottom panel) of a 192-SAP lesioned animal. Timescale has been offset corrected to drug ejection.

B) Average peak amplitude of NS9283-evoked glutamate release in left intact (light gray bars, $n = 2$) and right 192-SAP lesioned hemisphere (dark gray bars, $n = 5$). Bars represent the mean $\pm$ S.E.M.

C) and D) ChAT-immunostained sections of mPFC of left and right hemisphere, respectively, of a unilaterally lesioned rat (right hemisphere). ChAT-positive cortical interneurons (arrows) were not affected by the lesion [57].

Fig. 5. NS9283 potentiates nicotine-evoked glutamate release in mPFC

A) Representative, self-referenced traces of nicotine-evoked glutamate release. Timescale has been offset corrected to nicotine ejection. Inset: average peak amplitude of nicotine-evoked glutamate release. Bars represent the mean $\pm$ S.E.M. of 2–5 animals.

B) Average peak amplitude of glutamate release evoked by local ejections of 40 pmol nicotine before (light gray bars) and after (dark gray bars) systemic (i.p.) administration of NS9283. Symbols connected by lines represent individual experiments. Bars represent the mean $\pm$ S.E.M. of 5 animals. * $p < 0.05$, paired t -test. Glutamate release at 10 and 2000 pmol was not altered significantly following NS9283 administration (data not shown).

C) Representative, self-referenced traces of nicotine-evoked (40 pmol) glutamate release before (gray trace) and after (black trace) systemic (i.p.) NS9283 administration. Timescale has been offset corrected to nicotine ejection.

D) Nissl-stained coronal brain section displaying microelectrode placement in mPFC (circled).