

Heterogeneity of nicotinic acetylcholine receptor expression in the caudal nucleus of the solitary tract

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Abstract

The nucleus of the solitary tract (NTS) is the principal integrating relay in the processing of visceral sensory and gustatory information. In the present study, patch-clamp electrophysiological experiments were conducted using rat horizontal brainstem sections. Pre-synaptic and somatic/dendritic nicotinic acetylcholine receptors (nAChRs) expressed in neurons of the caudal NTS (cNTS) were found to be randomly distributed between pre-synaptic and somatic/dendritic sites ($\chi^2 = 0.72$, $df = 3$, $p > 0.87$, $n = 200$). Pre-synaptic nAChRs were detected by their facilitating effects on glutamatergic neurotransmission of a sub-population of cNTS neurons (categorized as “effect-positive”) upon brief picospritzer applications of 0.1–0.5 mM nicotine. These effects were resistant to inhibition by 20 nM methyllycaconitine (MLA) and 4 μ M dihydro- β -erythroidine (DH β E), and were replicated by brief picospritzer applications of 0.2–1 mM cytosine. Picospritzer applications of 0.2 mM RJR-2403, a potent agonist of $\alpha 4\beta 2$ nAChRs, did not facilitate synaptic release of glutamate in effect-positive cNTS neurons. The population of somatic/dendritic nAChRs has been found to be heterogeneous and included nAChRs that were activated by RJR-2403 and/or cytosine, or insensitive to cytosine, or inhibited by MLA. The presented results are consistent with the expression of $\beta 4$ -containing (i.e., $\beta 4^*$) nAChRs, likely $\alpha 3\beta 4^*$, in pre-synaptic terminals of effect-positive cNTS neurons. Somatic/dendritic nAChRs appear to involve both $\alpha 7$ and non- $\alpha 7$ subunits. Heterogeneity in the subunit composition of pre-synaptic and somatic/dendritic nAChRs may underlie diverse roles that these receptors play in regulation of behavioral and visceral reflexes, and may reflect specific targeting by endogenous nicotinic agents and nicotine.

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1. Introduction

The nucleus of the solitary tract (NTS) is a functionally and anatomically heterogeneous group of neurons that acts as a key integrating relay in the processing of visceral sensory and gustatory information (Lawrence and Jarrott, 1996; Contreras et al., 1982; Hamilton and Norgren, 1984). The caudal portion of NTS (cNTS) receives baroreceptor and chemoreceptor afferents from the heart and blood vessels via cranial nerves IX and X to control cardiac output. The NTS also receives visceral

afferents from various regions of the gastrointestinal tract (Sun et al., 2005; Zittel et al., 1994; Wang et al., 1999), second-order neurons of the dorsal horn, and other components of the central autonomic system, such as respiratory afferents (Boscan et al., 2002). The rostral portion of NTS (rNTS) receives mostly gustatory information directly from the oral cavity via cranial nerves VII, IX, and X (Ashworth-Preece et al., 1998; Lawrence and Jarrott, 1996; Torvik, 1956).

The presence of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) immunostaining in the NTS suggests that acetylcholine (ACh) is involved in modulation of visceral sensory and gustatory information (Armstrong et al., 1988; Ueno et al., 1993; Helke et al., 1983; Barry et al., 1993). In the rNTS region, the location of cholinergic neurons is consistent with the distribution of preganglionic parasympathetic

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neurons labeled by horseradish peroxidase applied to the chorda tympani branch of the VIIth nerve and the lingual-tonsillar branch of the IXth nerve (Contreras et al., 1980). Furthermore, both low- and high-affinity nicotinic binding sites have been demonstrated in the NTS of cats (Maley and Seybold, 1993); and electrophysiological recordings from the NTS in brainstem slices revealed a functionally heterogeneous population of nicotinic (nACh) and muscarinic (mACh) receptors (Uteshev and Smith, 2006; Ernsberger et al., 1988; Shihara et al., 1999). For the most part, nicotinic and muscarinic receptors were expressed by different cells (Shihara et al., 1999; Uteshev and Smith, 2006).

Although the main neurotransmitter released from the solitary tract appears to be glutamate (Shihara et al., 1999; Li and Smith, 1997; Wang and Bradley, 1995), some solitary tract afferents may be cholinergic, providing extrinsic cholinergic inputs to the NTS. Moreover, glutamate has been demonstrated to be the main neurotransmitter of baroreceptor afferents terminating in the NTS (Talman et al., 1980; Reis et al., 1981). However, microinjections of nicotine into the NTS elicited hypotension and bradycardia similar to that elicited by activation of baroreceptors (Kubo and Misu, 1981; Talman and Lewis, 1991). Local cholinergic interneurons may also provide cholinergic inputs within the NTS region (Kobayashi et al., 1978). Therefore, both glutamatergic and cholinergic systems seem to be involved in processing visceral sensory and gustatory information by the NTS. Additional extrinsic cholinergic inputs may arrive to the NTS from the adjacent dorsal motor nucleus of the vagus (Maley, 1996; Farkas et al., 1997) or nucleus ambiguus (Farkas et al., 1997).

2. Materials and methods

Electrophysiological patch-clamp experiments have been conducted in the Department of Anatomy and Neurobiology at the University of Tennessee Health Science Center in Memphis, TN, and in the Department of Pharmacology at the Southern Illinois University School of Medicine in Springfield, IL. The experimental results obtained in both institutions have resulted in identical conclusions.

2.1. Animals

Sprague–Dawley male or female rats (P18–25, ~100 g) were used in experiments. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (NIH 865-23, Bethesda, MD) and was approved by the Animal Care and Use Committee of the University of Tennessee and Southern Illinois University.

2.2. Electrophysiology

Rat brains were rapidly removed following decapitation and placed for 1 min in ice-cold oxygenated sucrose-based solution of the following composition (in mM): sucrose 250, KCl 2.5, NaH₂PO₄ 1.23, MgCl₂ 5, CaCl₂ 0.5, NaHCO₃ 26, glucose 10 (pH 7.4), when bubbled with carbogen (95% O₂ and 5% CO₂). The brainstem was then transferred to the brain slicer chamber (Vibratom-3000 (UT, Memphis, TN) or Vibratom-1000 (SIU, Springfield, IL), Vibratome, St. Louis, MO) and two or three horizontal brainstem slices (250 μ m thick) were cut. Slices were transferred to a storage chamber, where they were perfused at 30 °C for 30 min in an oxygenated artificial cerebrospinal fluid (ACSF) solution of the following composition (in mM): NaCl 125, KCl 2.5, NaH₂PO₄ 1.23, MgCl₂ 1, CaCl₂ 2, NaHCO₃ 26, glucose 10 (pH

7.4). In experiments conducted at SIU, Springfield, IL, the ACSF contained 35 mM NaHCO₃ to maintain pH 7.4. Slices were then perfused with an identical oxygenated ACSF at 24 °C for up to 10 h.

For patch-clamp experiments, slices were transferred to the recording chamber and perfused with an oxygenated ACSF at a rate of 1.5 ml/min using a Dynamax peristaltic pump (Rainin Instrument CO, Emeryville, CA) at UT, Memphis, TN; or 1 ml/min using a 2232 Microperplex S peristaltic pump (LKB, Upsalla, Sweden) at SIU, Springfield, IL. Typically, cNTS neurons selected for patch-clamp recordings were smaller than 25 μ m in diameter. Whole-cell recordings were conducted at 24 °C. The intracellular electrode solution contained (in mM): K-gluconate 125, KCl 1, MgCl₂ 2, EGTA 1, and K-HEPES 10 (pH 7.3). In some experiments, the intracellular solution did not contain MgCl₂ and EGTA. The electrophysiological data were recorded using: a HEKA-9/2 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) at UT, Memphis, TN; or a MultiClamp-700B patch-clamp amplifier at SIU, Springfield, IL (Molecular Devices, Sunnyvale, CA). The seal resistance was >2 G Ω ; the access resistance was between 10 and 30 M Ω , and typically, was not compensated. Patches with access resistances higher than 30 M Ω were corrected by applying an additional negative suction or discarded. Data were sampled at 10 or 20 kHz and filtered at 3.33 or 6.67 kHz, respectively. Occasionally, low frequency electrical noise (60 Hz) was filtered out during off-line analysis.

Syringe pumps Pump-33 (Harvard Apparatus, Holliston, MA) at UT, Memphis, TN, or Genie Plus (Kent Scientific Corporation, Torrington, CT) at SIU, Springfield, IL, were used to add experimental drugs to the ACSF just before they entered the recording chamber. The final drug concentrations in the bath were arithmetically calculated based on the known concentrations of stock solutions and adjustable rates of all pumps. Peristaltic and syringe pumps used in this study provide exceptional stability of fluid flow and are routinely calibrated. The high degree of stability of flows generated by pumps translates into a high stability of final drug concentrations that are easily calculated. Solutions are mixed in a ~0.3 ml reservoir, ~10 s before entering the first “entrance” chamber (~1 ml), where additional mixing occurs. After the “entrance” chamber, solutions enter the main recording chamber (~2 ml), where the final mixing and application takes place. This 3-step process guarantees a complete mixture of solutions.

A picospritzer (Parker Hannifin Instrumentation, Cleveland, OH) was used for agonist applications via a pipette (4–7 M Ω) identical to that used for patch-clamp recordings. The bath solution always contained 0.1–0.5 μ M tetrodotoxin (TTX) to block Na⁺ channels. The absence of sodium action potentials was routinely tested in the current-clamp mode by applying 10–50 pA depolarizing steps from the resting potential of ~–60 mV.

In some experiments, an iontophoretic pump (Union-40, Kation Scientific, Minneapolis, MN) was used to apply nicotine or RJR-2403. The retention and ejection currents were set around –20 nA and +180 nA, respectively. The output current was monitored during experiments via a built-in current monitor. Iontophoretic pipettes that did not provide specified current amplitudes were replaced. Patch-clamp pipettes with resistances 20–60 M Ω (if filled with the K-gluconate-based internal solution) were used for iontophoretic applications of nicotine (100 mM) and RJR-2403 (50 mM). Application pipettes for picospritzer and iontophoretic applications were mounted on two Sutter 285-3 manipulators allowing applications of at least two nicotinic agents to the same neuron during the course of a single experiment. In experiments with iontophoretic applications of nicotine, 4 μ M DH β E was also added to the ACSF. In all tests involving iontophoresis, antagonists (i.e., TTX, MLA and DH β E) were added directly to the ACSF and therefore, syringe pumps were not used.

2.3. Biocytin labeling

In some experiments, the recording pipette contained 0.2% biocytin (Sigma Chemical Co., St. Louis, MO) and the neurons were filled by diffusion during patch recording. Slices were fixed in 4% buffered paraformaldehyde for at least 24 h. After fixation, slices were rinsed in phosphate-buffered saline for 15 min and then incubated for 4 h with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) at room temperature. The avidin/biotin was diluted 1:100 in PBS containing 0.5% Triton-X100 (Sigma). The slices were rinsed for 15 min and reacted with 3,3'-diaminobenzidine (DAB) from a DAB substrate kit (Vector). After rinsing, the sections were mounted on gelatin-coated

slides, dried overnight, and coverslipped using 50% glycerol. Tissue shrinkage was minimal, as the sections were not dehydrated in alcohols nor cleared in xylenes. Nineteen cNTS neurons were successfully filled to allow characterization and 3D-reconstruction using the NeuroLucida system (MicroBrightField, Inc., Williston, VT). Neuronal somal area, form factor and the number of primary branches have been measured. Form factor (ff) measures the roundness of somata, where 1.0 indicates a circle and 0.0 indicates a line ($ff = 4\pi \text{area}/\text{perimeter}^2$, where $\pi = 3.146$).

2.4. Data analysis

The liquid junction potential ($V_{LJ} \sim 13$ mV, UT, Memphis, TN; and ~ 12.9 mV, SIU, Springfield, IL) was determined using a method described in detail previously (Neher, 1992). The data presented are nominal, i.e., have not been corrected for V_{LJ} . Data are presented as means \pm SD. Statistical analysis was performed using QuickCalcs (GraphPad Software, Inc., San Diego, CA, USA) and Mathematica 2.2.3 software (Wolfram Research, Inc., Champaign, IL). In brief, let x_i and y_i be the observed and expected numbers of Type i cNTS neurons, respectively. Then, the value:

$$\chi^2 = \sum_{i=1}^{k=4} \frac{(x_i - y_i)^2}{y_i} \quad (1)$$

is distributed according to the chi-square distribution:

$$f(\lambda, k) = \frac{0.5^{k/2}}{\Gamma(k/2)} \lambda^{k/2-1} e^{-\lambda/2} \quad (2)$$

The p -value (the probability that neurons are randomly distributed among Types I–IV) is then calculated as an integral:

$$p(\lambda > \chi^2) = 1 - \int_0^{\chi^2} f(\lambda, k) d\lambda \quad (3)$$

representing the area under the tail $\chi^2 < \lambda < \infty$ of the density function $f(\lambda, k)$.

To quantify the effects of nicotine on the mEPSC frequency and amplitudes in effect-positive neurons, one-tailed and two-tailed paired t -tests have been used, respectively. A one-tailed paired t -test was used to evaluate changes in the mEPSC frequency because, by definition, in effect-positive neurons, nicotine could only increase the frequency of mEPSCs.

3. Results

Horizontal brainstem sections containing the cNTS region (Fig. 1A) were used in patch-clamp experiments to investigate pre-synaptic and somatic/dendritic responses of cNTS neurons (Fig. 1B) to brief exposures to nicotine. Sodium ion channels were blocked by 0.1–0.5 μM TTX constantly present in the ACSF. The absence of sodium action potentials was routinely tested in current-clamp by applying 10–50 pA depolarizing current steps (not shown). A total of 232 cNTS neurons were investigated in this study.

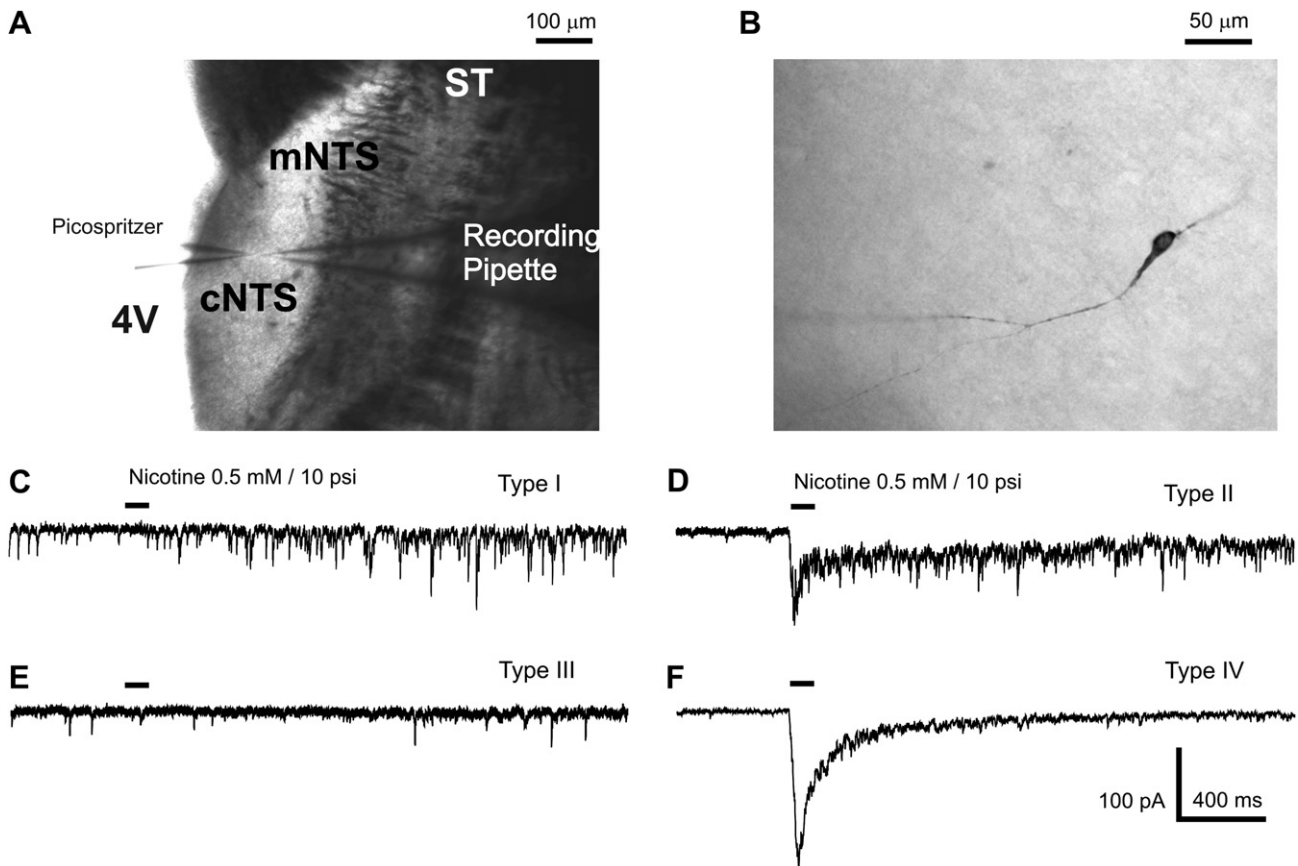


Fig. 1. Location and neuronal types of cNTS neurons. (A) An infrared image of the recording and application picospritzer pipettes during a patch-clamp experiment. Horizontal brainstem section ($\sim 250 \mu\text{m}$ thickness) were used in all experiments. The approximate location of the cNTS region relative to the solitary tract (ST), the fourth ventricle (4V) and the medial NTS region is indicated. (B) An example of a Type II cNTS neuron labeled with biocytin. Morphological analysis gave the following parameters: somal area, $199.8 \mu\text{m}^2$; form factor, 0.35; number of branches, 2.0 (C–F). Typical responses (C, D, F), or lack thereof (E), of cNTS neurons to 0.5 mM nicotine applied via a picospritzer (100 ms duration, ~ 10 psi pressure, $\sim 10 \mu\text{m}$ application distance). Four clearly distinct types of cNTS neuron have been identified (see text for details).

3.1. The effect of mEPSC facilitation

Brief picospritzer applications of nicotine (0.1–0.5 mM, 50–400 ms duration, 10 psi pressure, 10 μ m application distance) to cNTS neurons held at the membrane voltage of -60 mV in voltage-clamp were found to activate pre-synaptic and somatic/dendritic nAChRs. The activation of pre-synaptic nAChRs was detected by facilitation of miniature excitatory post-synaptic currents (mEPSCs) (Fig. 1C and D). Usually, this facilitation lasted for less than a minute, but it could be repeated as often as every minute. However, the mEPSC facilitation has been found to be most reliable when at least 3 min between applications were reserved to allow for a complete recovery from desensitization and/or residual inhibition of pre-synaptic nAChRs by nicotine (Papke et al., 2000).

The activation of somatic/dendritic nAChRs by nicotine generated whole-cell currents (Fig. 1D and F). Based on responses of 200 cNTS neurons to 0.5 mM nicotine, four neuronal types have been defined: Type I, only pre-synaptic, no somatic/dendritic responses (17%, $n = 34$; Fig. 1C); Type II, pre-synaptic and somatic/dendritic responses (13%, $n = 25$; Fig. 1D); Type III, no pre-synaptic, no somatic/dendritic responses (34%, $n = 69$; Fig. 1E); and Type IV, no pre-synaptic, only somatic/dendritic responses (36%, $n = 72$; Fig. 1F). Pre-synaptic and somatic/dendritic effects of nicotine were found to be randomly distributed among cNTS neurons tested ($\chi^2 = 0.72$, $df = 3$, $p > 0.87$, $n = 200$). In addition, 5 Type I and 14 Type II neurons were successfully reconstructed from biocytin fillings and their morphology was analyzed using the NeuroLucida system (see Section 2). The differences among mean values of the main morphological parameters of Type I and Type II neurons were found to be statistically insignificant. The mean somal areas were: $154.8 \pm 39.8 \mu\text{m}^2$ ($n = 5$, Type I) and $130.2 \pm 124.4 \mu\text{m}^2$ ($n = 12$, Type II), $p > 0.67$; the mean form factors (ff) were: 0.7 ± 0.16 ($n = 5$, Type I) and 0.58 ± 0.14 ($n = 14$, Type II), $p > 0.15$; the mean numbers of primary branches were: 2.8 ± 0.8 ($n = 5$, Type I) and 2.6 ± 1.2 ($n = 14$, Type II), $p > 0.73$. When Type I and II neurons were analyzed together, the mean values were found to be: somal area, $137.4 \pm 105.7 \mu\text{m}^2$ ($n = 17$); form factor, 0.62 ± 0.15 ($n = 19$); and number of branches, 2.6 ± 1.2 ($n = 19$). The morphologies of Type III and IV cNTS neurons have not been analyzed in the present study.

In some experiments, a leak of nicotine out of picospritzer application pipettes may have desensitized some pre- and/or postsynaptic nAChRs, causing an underestimation of the number of Type I and II neurons. This possibility, however, has been addressed in Section 3.5.

3.2. Somatic/dendritic $\alpha 7$ nAChRs

Type II currents were detected in 25 out of 200 cNTS neurons that were used for statistical analysis. In 7 of those 25 experiments, 20 nM MLA (methyllycaconitine, a selective antagonist of $\alpha 7$ nAChRs) was applied to the bath to test for the presence of $\alpha 7$ nAChRs. Somatic/dendritic nAChR-mediated responses were blocked in 5 of those 7 experiments (Fig. 2A); in the

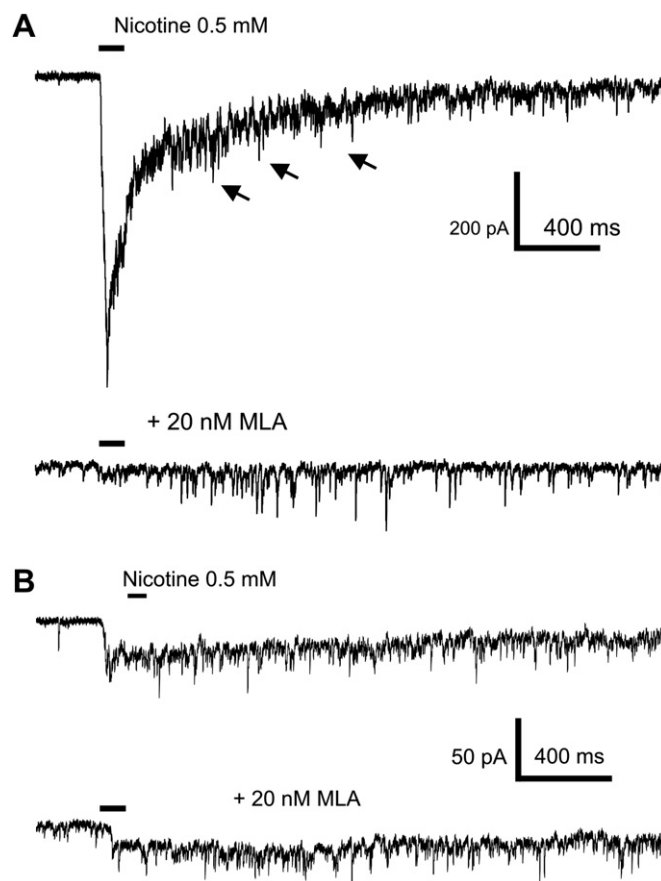


Fig. 2. Pre-synaptic nAChRs are non- $\alpha 7$. (A) In some Type II cNTS neurons, somatic/dendritic responses to nicotine (top trace) were completely blocked by 20 nM MLA, a selective $\alpha 7$ nAChR antagonist. Pre-synaptic effects of nicotine were insensitive to MLA (bottom trace). (B) In 2 Type II cNTS neurons, somatic/dendritic responses to nicotine were not completely blocked by 20 nM MLA and the MLA-insensitive current component was slower than that mediated by $\alpha 7$ nAChRs in the absence of MLA (compare with (A)). In these 2 experiments, the background frequency of mEPSCs was insignificantly low.

remaining 2 experiments, the block by 20 nM MLA was incomplete (Fig. 2B). These results indicate the expression of both $\alpha 7$ and non- $\alpha 7$ somatic/dendritic nAChRs in cNTS neurons. To eliminate $\alpha 7$ nAChR-mediated somatic/dendritic responses, 20 nM MLA was constantly present in the ACSF in the majority of experiments in this study.

3.3. Non- $\alpha 7$ nAChRs

Miniature EPSCs were blocked by 16 μ M DNQX (6,7-dinitroquinoxaline-2,3-dione, a selective AMPA receptor blocker) applied to the bath, indicating that they were mediated by AMPA receptors ($n = 4$; Fig. 3A). The facilitation of mEPSCs by nicotine was resistant to 4 μ M DH β E (dihydro- β -erythroidine, a putative selective antagonist of $\beta 2$ -containing (i.e., $\beta 2^*$) nAChRs, $n = 9$; Fig. 3B). In 5 of 9 experiments, 4 μ M DH β E was added to the bath directly, without using a syringe pump (see Section 2). Cytisine, a nicotine-like alkaloid and a potent agonist of rat $\beta 4^*$ nAChRs (Luetje and Patrick, 1991), produced similar effects of facilitation of mEPSCs ($n = 23$; Fig. 3C). Cytisine is also known to activate the rat $\alpha 7$ nAChR

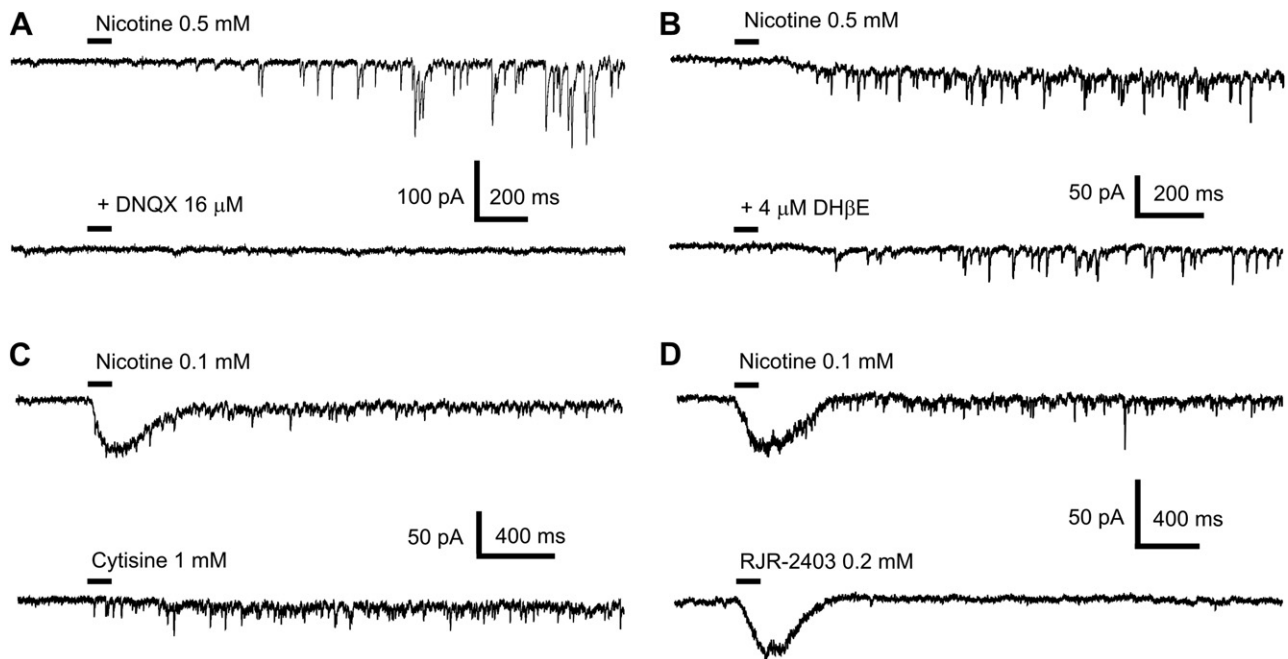


Fig. 3. Pharmacological properties of nAChRs expressed in Type I and II cNTS neurons. (A) Miniature EPSCs were blocked by 16 μ M DNQX indicating the involvement of AMPA receptors. (B) Pre-synaptic effects were resistant to 4 μ M DH β E applied to the bath indicating that pre-synaptic nAChRs are unlikely to be β 2*. (C) Pre-synaptic effects of 0.1 mM nicotine were reproduced by 1 mM cytosine applied to the same cNTS neuron. In some Type II neurons, 1 mM cytosine did not evoke somatic/dendritic responses (bottom trace). (D) Picospritzer applications of RJR-2403 (0.2 mM) did not facilitate mEPSCs in any of the effect-positive neurons tested ($n = 11$). The same neurons, however, exhibited facilitation of mEPSCs when 0.1 mM nicotine was applied (top trace) and hence, those neurons were defined as effect-positive.

subtype (Stokes et al., 2004). In the presence of 20 nM MLA, applications of cytosine produced somatic/dendritic responses in numerous experiments ($n = 18$; Fig. 5, bottom traces). However, in 11 experiments, 1 mM cytosine failed to generate somatic/dendritic responses (Fig. 3C, bottom trace). In 4 of those 11 experiments, applications of 0.1 mM nicotine to the same cNTS neurons did produce somatic/dendritic responses (Fig. 3C, top trace). Therefore, some Type II cNTS neurons may express non- α 7-non- β 4 somatic/dendritic nAChRs, possibly β 2*.

Applications of RJR-2403 (0.2 mM; 10 psi), a potent agonist of α 4 β 2 nAChRs (Papke et al., 2000) did not facilitate mEPSCs in effect-positive cNTS neurons ($n = 11$; Fig. 3D, bottom trace). The exact same pool of neurons exhibited facilitation of mEPSCs when nicotine ($n = 6$; Fig. 3D, top trace) or cytosine ($n = 5$; not shown) were applied instead of RJR-2403 via a separate picospritzer pipette (and hence, these neurons were defined as effect-positive). These results indicate that a contribution of β 2* nAChRs to the mEPSC facilitation by nicotine in effect-positive cNTS neurons is unlikely.

All pre-synaptic and somatic/dendritic nicotine-evoked effects were completely blocked by 10 μ M mecamylamine, a broad spectrum non-selective blocker of nAChRs ($n = 9$, not shown) confirming the involvement of nAChRs in the observed effects.

3.4. Quantal analysis

Thirty-two effect-positive cNTS neurons (both Type I and II) recorded for at least 30 s before and after picospritzer

applications of nicotine were used for a thorough statistical analysis. Since all neurons analyzed in this part of the study were effect-positive, only increases in the mEPSC frequency by nicotine were observed, and therefore, a one-tailed paired t-test was employed to quantify changes in the mEPSC frequency. A two-tailed paired t-test was employed to quantify changes in mEPSC amplitudes. The constant presence of 0.2–0.5 μ M TTX in these experiments eliminated sodium action potentials. The absence of sodium action potentials was routinely tested in current-clamp by applying 10–50 pA current-steps (not shown). Therefore, the observed facilitation of mEPSC frequency by nicotine was likely produced by pre-synaptically located nAChRs. Surprisingly, however, in 68.8% of effect-positive cNTS neurons, statistically significant increases in mEPSC frequency ($p < 0.0002$, $n = 32$; Fig. 4A–C) were accompanied by statistically significant increases in mEPSC amplitudes ($p < 0.003$, $n = 22$; Fig. 4D). In the remaining 31.2% of effect-positive cNTS neurons, nicotine-mediated changes in the mean mEPSC amplitudes were not statistically significant ($p > 0.4$, $n = 10$, not shown). When all 32 neurons were analyzed together, increases in both mEPSC frequency and amplitude were still statistically significant ($p < 0.0001$ and $p < 0.0004$, respectively; Fig. 4E and F).

Nicotine-mediated increases in the mEPSC frequency in effect-positive cNTS neurons were always transient and decayed to the background frequency observed before the application of nicotine (Fig. 4C, right histogram). The decay time constants varied from neuron to neuron between 1.2 and 81.2 s with the mean value of 10.8 ± 15.6 s ($n = 32$).

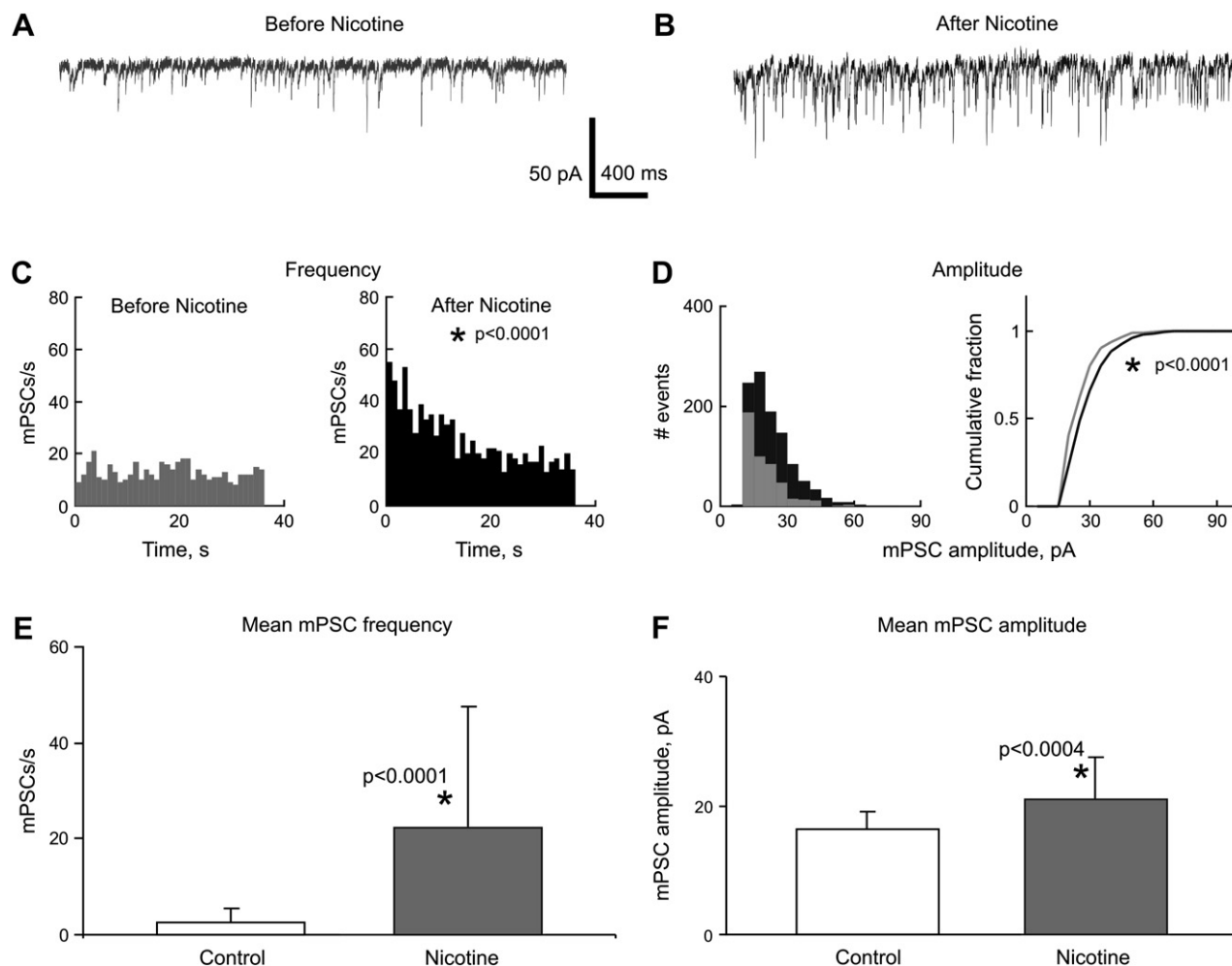


Fig. 4. Quantal analysis. (A,B) Current traces from an effect-positive NTS neuron before and after nicotine application under control conditions. In 68.8% of analyzed effect-positive neurons, the increase in mEPSC frequency was accompanied by a statistically significant increase in the mean mEPSC amplitude ($p = 0.003 \pm 0.006$, $n = 22$). In the remaining 31.2% of neurons, nicotine-mediated changes in the mean mEPSC amplitudes were not statistically significant ($p = 0.40 \pm 0.23$, $n = 10$). (C,D) Frequency and amplitude histograms built using quantal events recorded from the same neuron as those shown in (A,B) before (gray) and after (black) nicotine application. (E,F) Mean frequency and amplitude histograms built using data obtained from all 32 tested neurons. Increases in both mEPSC frequency and amplitudes were found to be statistically significant ($p < 0.0001$ and $p < 0.0004$, respectively).

3.5. Ionophoretic agonist application

A leak of nicotine from a picospritzer pipette may have desensitized some pre- and/or post-synaptic nAChRs, for instance, high potency $\alpha 4\beta 2$, and thus, potentially may have led to an underestimation of the number of effect-positive cNTS neurons and the types of nAChRs involved in mediating the effect. Therefore, in 8 experiments with effect-positive cNTS neurons, ionophoretic applications of nicotine (100 mM, $n = 4$) or RJR-2403 (50 mM, $n = 4$) were conducted in combination with picospritzer applications of 0.2 mM cytosine to determine whether the results are dependent on the method of agonist application. Cytosine applications were used to define cNTS neurons as effect-positive or -negative. In these experiments, 0.1–0.2 μ M TTX and 20 nM MLA were always present in the ACSF. The absence of action potentials was tested in current-clamp by applying 10–50 pA current steps. In experiments with ionophoretic applications of nicotine,

4 μ M DH β E was also added to the ACSF. In all tests involving iontophoresis, antagonists (i.e., TTX, MLA and DH β E) were added to the ACSF directly, i.e., syringe pumps were not used.

Ionophoretic applications of nicotine and picospritzer applications of cytosine facilitated mEPSCs in four effect-positive cNTS neurons ($n = 4$; Fig. 5A, top and bottom traces, respectively). In contrast, ionophoretic applications of RJR-2403 failed to facilitate mEPSCs in a different group of four effect-positive cNTS neurons (Fig. 5B, top trace) that exhibited mEPSC facilitation to picospritzer applications of cytosine ($n = 4$; Fig. 5B, bottom trace).

In general, even in experiments where only picospritzer applications were used to deliver nicotinic agonists, the tips of application pipettes were only barely ($< 20 \mu$ m) inserted in the brain slice and in several experiments, where neurons were located just under the surface of the slice ($< 10 \mu$ m), applications were made with the tip positioned just above the investigated neuron ($< 10 \mu$ m) and completely outside of the

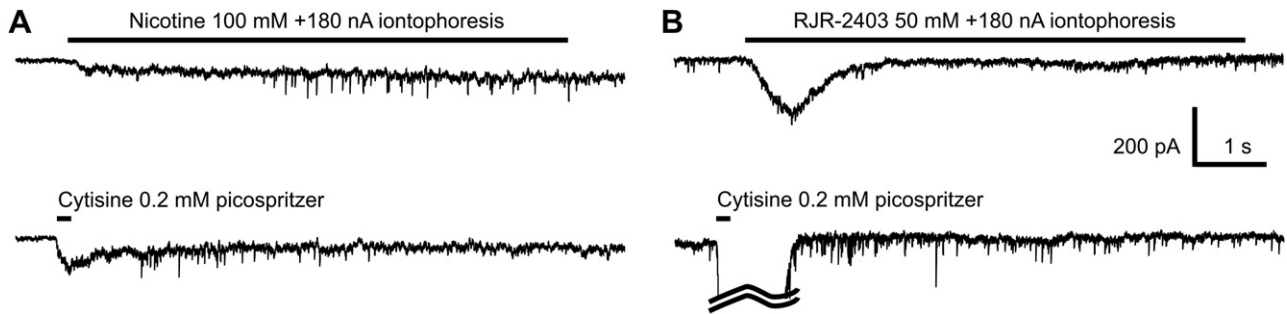


Fig. 5. Iontophoretic agonist application. In some experiments, iontophoresis was employed instead of a picospritzer to eliminate a possibility of a leak of nicotine or RJR-2403 from application pipettes. (A) Iontophoretic applications of nicotine (100 mM, +180 nA) facilitated the mEPSC frequency (top trace) in a neuron that also exhibited a facilitation of mEPSC frequency upon picospritzer applications of 0.2 mM cytosine (bottom trace). (B) In a different experiment, iontophoretic applications of RJR-2403 (50 mM, +180 nA) did not facilitate the mEPSC frequency (top trace) in a neuron that exhibited such a facilitation upon picospritzer applications of 0.2 mM cytosine (bottom trace). The somatic/dendritic response to cytosine was truncated (curved lines).

brain slice. Moreover, in all experiments, application pipettes were always positioned behind the investigated neuron, relative to the perfusion flow. Therefore, in the event of a leak of nicotine from the application pipette, nicotine would have had to diffuse against perfusion flow to be able to desensitize the majority of pre-synaptic nAChRs that contributed to the effect. All of the above considerations suggest that a leak of nicotine from picospritzer application pipettes would have produced only a negligible influence, if any, on the presented results.

4. Discussion

The presented study demonstrates that nicotine facilitates synaptic release of glutamate in a subpopulation (i.e., “effect-positive”) of cNTS neurons in the brainstem. Nicotinic AChRs that mediate these effects of nicotine are most likely located on pre-synaptic glutamatergic terminals and not somata of neighboring cNTS neurons, because experiments were conducted in the constant presence of 0.1–0.5 μ M TTX to block sodium action potentials. The absence of action potentials was routinely tested in current-clamp by applying 10–50 pA current steps. The presented experimental results are consistent with the pre-synaptic expression of $\beta 4^*$ nAChRs, likely $\alpha 3\beta 4^*$, in effect-positive cNTS neurons. This conclusion is based on the observations that the facilitation of mEPSCs by nicotine: (1) is not blocked by 4 μ M DH β E (Fig. 3B); (2) is not triggered by RJR-2403, a potent agonist of $\alpha 4\beta 2$ nAChRs, applied either via a picospritzer (0.2 mM; Fig. 3D) or iontophoresis (50 mM, +180 nA; Fig. 5B); and (3) can be replicated by picospritzer applications of cytosine (0.2 mM; Figs. 3C and 5A,B).

Applications of DH β E (4 μ M) to the bath would be expected to block $\alpha 3\beta 2$ ($IC_{50} = 0.41$), $\alpha 4\beta 2$ ($IC_{50} = 0.37$) and $\alpha 4\beta 4$ ($IC_{50} = 0.19$) receptors (Harvey et al., 1996). DH β E is slightly less effective for blocking $\alpha 2\beta 2$ ($IC_{50} = 1.3$ μ M) and $\alpha 2\beta 4$ ($IC_{50} = 2.3$ μ M) nAChRs (Harvey et al., 1996), but at 4 μ M, DH β E would still be expected to significantly inhibit both of those receptor subtypes. In the experiments presented in this study, 4 μ M DH β E failed to block mEPSC facilitation. Moreover, the $\alpha 2$ nAChR subunit has not been detected in the NTS area of the brainstem (Wada et al., 1989). Therefore, pre-synaptic effects of nicotine in cNTS neurons

are unlikely to be mediated by $\alpha 3\beta 2$, $\alpha 4\beta 2$, $\alpha 4\beta 4$, $\alpha 2\beta 2$ and $\alpha 2\beta 4$ nAChRs. On the other hand, the IC_{50} of DH β E for $\alpha 3\beta 4$ is 23.1 μ M (Harvey et al., 1996). Therefore, 4 μ M DH β E would be expected to have only a minor inhibitory effect on $\alpha 3\beta 4$ nAChR which is consistent with the presented observations (Fig. 3B).

Cytisine is known to be at least 100-fold more effective in activation of $\beta 4^*$ nAChRs than $\beta 2^*$ nAChRs (Luetje and Patrick, 1991; Papke and Heinemann, 1994; Fenster et al., 1999). In the present study, brief applications of 0.2–1 mM cytosine via a picospritzer replicated pre-synaptic effects of nicotine (Figs. 3C and 5A,B). In contrast, 0.2 mM RJR-2403 applied via a picospritzer (Fig. 3D, bottom trace) or iontophoretically (Fig. 5B, top trace) failed to elicit mEPSC facilitation. RJR-2403 is a relatively potent and effective agonist of a number of $\beta 2^*$ and $\beta 4^*$ nAChRs (Papke et al., 2000): $\alpha 4\beta 2$ ($EC_{50} = 16$ μ M), $\alpha 3\beta 2$ ($EC_{50} = 150$ μ M) and $\alpha 4\beta 4$ ($EC_{50} = 50$ μ M), but not $\alpha 3\beta 4$ ($EC_{50} = 347$ μ M) or $\alpha 3\beta 2\alpha 5$ ($EC_{50} = 360$ μ M) (Papke, 2002; Papke et al., 2000). Moreover, RJR-2403 has a significantly lower efficacy for $\alpha 3\beta 4$ than for other non- $\alpha 7$ nAChRs (Papke et al., 2000). Therefore, $\alpha 4\beta 2$, $\alpha 3\beta 2$ and $\alpha 4\beta 4$ nAChRs may be safely excluded from the list of potential contributors to the observed facilitation of mEPSCs in cNTS neurons. In addition, $\alpha 3\beta 2\alpha 5$ may be excluded because it is poorly activated by cytosine (Nelson et al., 2001). Taken together, these results are consistent with the pre-synaptic expression of $\beta 4^*$ nAChRs, most likely $\alpha 3\beta 4^*$, that mediate facilitation of glutamatergic mEPSCs in a sub-population of cNTS neurons in the brainstem. The expression of somatic/dendritic nAChRs appears to be more heterogeneous and involves both $\alpha 7$ and non- $\alpha 7$ nAChRs (Figs. 2, 3 and 5). However, in the present study somatic/dendritic effects of nicotine have not been studied in detail.

Four clearly distinct types of cNTS neuron have been identified on the basis of both pre-synaptic and somatic/dendritic responses to nicotine (Fig. 1C–F). Statistical analysis utilized the χ^2 test statistic (see Section 2) and determined that nAChR expression was distributed randomly between pre-synaptic and somatic/dendritic sites ($\chi^2 = 0.72$, $df = 3$, $p > 0.87$, $n = 200$). Type I and II neurons were generally found to be small (somal area, 137.4 ± 105.7 μm^2 , $n = 17$), elongated (form factor,

0.62 ± 0.15 , $n = 19$) with only 2–3 primary branches (number of branches, 2.6 ± 1.2 , $n = 19$). Differences in the main morphological parameters of Type I and II neurons were found to be insignificant. However, Type I and II neurons were significantly smaller ($p < 0.017$), more elongated ($p < 0.003$) and had fewer branches ($p < 0.034$) than neurons from the rostral NTS region analyzed previously using identical techniques (somal area, $204.3 \pm 106.9 \mu\text{m}^2$, form factor, 0.72 ± 0.12 , and number of branches, 3.15 ± 0.93 , ($n = 74$) (data were re-analyzed from Uteshev and Smith, 2006).

In 68.8% of effect-positive cNTS neurons, the increase in mEPSC frequency by nicotine was accompanied by an increase in mEPSC amplitudes despite the presence of $0.5 \mu\text{M}$ TTX. In the remaining 31.2% of effect-positive neurons, nicotine significantly increased only the frequency of mEPSCs. This finding may indicate that two different mechanisms of facilitation of synaptic release of glutamate exist in cNTS neurons, or that two different populations of effect-positive neurons exist in the cNTS, or the presence of TTX-insensitive voltage-gated sodium channels in a sub-population of neurons that synapse with effect-positive cNTS neurons. A thorough comparison of the pharmacology, kinetics and, perhaps, calcium dependence of mEPSC facilitation may be necessary to resolve this uncertainty. Ultimately, understanding the effects of nicotine in cNTS may require a detailed investigation of mechanisms of potentiation of mEPSC amplitudes. Some of those general mechanisms have been discussed in detail elsewhere and must be considered in future studies including multi-vesicular release (Gordon and Bains, 2005) and direct modulation of AMPA receptors by nicotine (Neff et al., 1998).

Although, $\beta 2^*$ and $\alpha 7$ are the most common types of nAChRs in the CNS, mounting evidence supports the idea that functional $\beta 4^*$ nAChRs are equally abundant, or even predominant in selected brain regions, and that their involvement in regulation of neuronal functions and neurotransmission may have important physiological consequences (Mulle et al., 1991; Fenster et al., 1997; Takeda et al., 2003; Dhar et al., 2000).

The observed diversity of nAChRs in the cNTS may be physiologically significant. The pharmacological properties of $\beta 2^*$, $\beta 4^*$ and $\alpha 7$ nAChRs are quite different (Fenster et al., 1997; Luetje and Patrick, 1991; Gotti et al., 1997; Briggs and McKenna, 1998; Papke et al., 2000). For example, nicotine has been shown to cause significant residual inhibition in $\alpha 4\beta 2$ and $\alpha 7$, but not $\alpha 3\beta 4$ nAChRs (Papke et al., 2000). Micromolar concentrations of choline, a selective endogenous agonist of $\alpha 7$ nAChRs (Alkondon et al., 1997; Papke et al., 1996), have been shown to activate $\alpha 7$ nAChRs or reduce the responsiveness of $\alpha 7$ nAChRs to ACh (Uteshev et al., 2003; Alkondon and Albuquerque, 2006). Choline however, has been reported to inhibit $\beta 4^*$ nAChRs expressed in *Xenopus* oocytes (Zwart and Vijverberg, 2000; Gonzalez-Rubio et al., 2006). Therefore, the effects of choline on pre-synaptic and somatic/dendritic nAChRs would be expected to be opposite. Also different are the biophysical properties of $\beta 2^*$, $\beta 4^*$ and $\alpha 7$ nAChRs (Cachelin and Jaggi, 1991; Fenster et al., 1997; Gerzanich et al., 1994; Bertrand et al., 1992). For instance, the desensitization kinetic of $\alpha 7$ nAChRs is considerably faster

than that of $\beta 2^*$ and $\beta 4^*$ nAChRs (Fenster et al., 1997). Therefore, pre-synaptic and somatic/dendritic nAChRs in cNTS neurons would be expected to respond to identical nicotinic stimuli differently.

Differences in pharmacological properties of pre-synaptic and somatic/dendritic nAChRs presents an opportunity to pharmacologically separate and individually investigate pre-synaptic and somatic/dendritic effects of nicotine in a brain region involved in regulation of cardiovascular and other visceral reflexes that is susceptible to nicotine toxicity (Mendelowitz, 1999). Differences in the subunit composition of nAChRs expressed in cNTS neurons pre-synaptically and somatic/dendritically may underlie different roles that these receptors play in regulation of behavioral and visceral reflexes and may reflect a specific targeting by endogenous nicotinic agents and nicotine.

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