Physiological Concentrations of Choline Activate Native $\alpha 7$ -Containing Nicotinic Acetylcholine Receptors in the Presence of PNU-120596 [1-(5-Chloro-2,4-dimethoxyphenyl)-3-(5-methylisoxazol-3-yl)-urea]

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ABSTRACT

The use of PNU-120596 [1-(5-chloro-2,4-dimethoxyphenyl)-3-(5-methylisoxazol-3-yl)-urea], a positive allosteric modulator of $\alpha 7$ nicotinic acetylcholine receptor (nAChR), may be beneficial for enhancing cholinergic therapies. However, the effects of PNU-120596 on activation of native $\alpha 7$ -containing nAChRs by physiological concentrations of choline are not known and were investigated in this study using patch-clamp electrophysiology and histaminergic tuberomammillary neurons in hypothalamic slices. In the presence of PNU-120596, subthreshold (i.e., inactive) physiological concentrations of choline ($\sim 10 \mu\text{M}$) elicited repetitive step-like whole-cell responses reminiscent of single ion channel openings that were reversibly blocked by 20 nM methyllycaconitine, a selective $\alpha 7$ nAChR antagonist. The effects of choline and PNU-120596 were synergistic as administration of 10 to 40 μM choline or 1 to 4 μM PNU-120596 alone did not elicit responses. In voltage clamp at -60 mV , the

persistent activation of $\alpha 7$ -containing nAChRs by 10 μM choline plus 1 μM PNU-120596 was estimated to produce a sustained influx of Ca^{2+} ions at a rate of 8.4 pC/min ($\sim 0.14 \text{ pA}$). In the presence of PNU-120596 in current clamp, transient step-like depolarizations ($\sim 5 \text{ mV}$) enhanced neuronal excitability and triggered voltage-gated conductances; a single opening of an $\alpha 7$ -containing nAChR channel appeared to transiently depolarize the entire neuron and facilitate spontaneous firing. Therefore, this study tested and confirmed the hypothesis that PNU-120596 enhances the effects of subthreshold concentrations of choline on native $\alpha 7$ -containing nAChRs, allowing physiological levels of choline to activate these receptors and produce whole-cell responses in the absence of exogenous nicotinic agents. In certain neurological disorders, this activation may be therapeutically beneficial, more efficacious, and safer than treatments with nAChR agonists.

There is extensive evidence to link age- and trauma-related changes in the expression and function of $\alpha 7$ nicotinic acetylcholine receptors (nAChR) to neurodegenerative and psychiatric disorders associated with cognitive and attention deficits (Scremin and Jenden, 1996; Guan et al., 2000; Nordberg, 2001; Sarter and Parikh, 2005; Martin and Freedman, 2007). By contrast, a weak sustained activation of $\alpha 7$ nAChRs by low concentrations of nicotinic agents appears to enhance cognitive performance and produce neuroprotection (Shimohama et al., 1998; Verbois et al., 2003; Buccafusco,

2004; Buccafusco et al., 2005; Guseva et al., 2008). Currently available therapeutic approaches aimed at rescuing the brain $\alpha 7$ nAChR activation include administration of $\alpha 7$ nAChR agonists (Shimohama et al., 1998; Verbois et al., 2003; Buccafusco, 2004; Buccafusco et al., 2005; Guseva et al., 2008) and positive allosteric modulators (PAMs) (Hurst et al., 2005; Faghih et al., 2007; Grønlien et al., 2007; Roncarati et al., 2008; Young et al., 2008; López-Hernández et al., 2009). Once in the brain, PAMs would be expected to enhance the responsiveness of $\alpha 7$ nAChRs to endogenous and therapeutic nicotinic agents to produce neuroprotective and cognitively beneficial effects. One of those agents is endogenous choline.

Although choline is a selective natural ligand for $\alpha 7$ nAChRs, its therapeutic significance is limited because of its low potency ($\text{EC}_{50} \sim 0.5\text{--}1.6 \text{ mM}$) (Alkondon et al., 1997; Papke and Porter Papke, 2002). Therefore, the use of PAMs

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ABBREVIATIONS: nAChRs, nicotinic acetylcholine receptors; $\alpha 7^*$, $\alpha 7$ -containing; CSF, cerebral spinal fluid; PNU-120596, 1-(5-chloro-2,4-dimethoxyphenyl)-3-(5-methylisoxazol-3-yl)-urea; DNQX, 6,7-dinitroquinoxaline-2,3-dione; AP-5, D-2-amino-5-phosphonopentanoate; TM, tuberomammillary; PAM, positive allosteric modulator; CNS, central nervous system; TTX, tetrodotoxin; MLA, methyllycaconitine; ACSF, artificial cerebral spinal fluid; NMDA, N-methyl-D-aspartate.

(e.g., PNU-120596) may be beneficial for enhancing choline-based and other cholinergic therapies. However, the effects of PNU-120596 on activation of native $\alpha 7$ nAChRs by physiological levels of choline have not been investigated.

Subthreshold (i.e., inactive) concentrations of choline (5–10 μ M) have been detected in the cerebrospinal fluid (CSF) in *in vivo* preparations under a variety of experimental conditions (Johe and Gu, 1991; Scremin and Jenden, 1991; Bertrand et al., 1996; Klein et al., 1998; Zapata et al., 1998; Rao et al., 2000; Sarter and Parikh, 2005; Parikh and Sarter, 2006). These ambient levels of choline can be elevated (3–4-fold) under conditions associated with ischemia, stroke, and substantial plasma membrane damage (Scremin and Jenden, 1991; Bertrand et al., 1996; Klein et al., 1998; Rao et al., 2000). Cell death is well known to lead to a breakdown of phosphatidylcholine, the principle plasma membrane phospholipid, into choline and diacylglycerol, providing a large source of this endogenous $\alpha 7$ nAChR agonist. Given the low ambient concentrations of choline (5–10 μ M) in the CSF under physiological conditions (Sarter and Parikh, 2005; Parikh and Sarter, 2006), it is unlikely that in the absence of cholinergic synaptic inputs or exogenous nicotinic agents, native $\alpha 7$ nAChRs are persistently activated or desensitized by endogenous choline (Uteshev et al., 2003). However, the effects may be notably different in the presence of PNU-120596, which significantly enhances the responsiveness of $\alpha 7$ nAChRs to nicotinic agents (Hurst et al., 2005; Grønlien et al., 2007; Roncarati et al., 2008; Young et al., 2008; López-Hernandez et al., 2009). PNU-120596 has been shown to increase the mean open time of $\alpha 7$ nAChR channels without producing significant changes in ion channel selectivity and single channel conductance (Hurst et al., 2005). PNU-120596 does not activate $\alpha 7$ nAChRs in the absence of nicotinic agonists. Instead, it lowers the energy barrier, allowing lower concentrations of nicotinic agonists to activate the receptor (Barron et al., 2009). The concentration-response relationships of PNU-120596 indicate that the EC_{50} value for potentiating effects of PNU-120596 falls near ~ 1.5 μ M (Grønlien et al., 2007; Young et al., 2008). This concentration of PNU-120596 is readily achievable in the CSF in *in vivo* experimental settings; the concentration of PNU-120596 detected in the brains of rats receiving 1 mg/kg *i.v.* was found to be ~ 1.5 μ M (Hurst et al., 2005). In the presence of PNU-120596, physiological levels of choline would be expected to generate a sustained activation of native $\alpha 7$ -containing (i.e., $\alpha 7^*$) nAChRs, and this activation may be more efficacious and safer than treatments with exogenous nAChR agonists. Through the use of patch-clamp electrophysiology, brain slices, and histaminergic tuberomammillary (TM) neurons of the posterior hypothalamus, this study tests the hypothesis that physiological concentrations of choline are effective in activation of native $\alpha 7^*$ nAChRs in the presence of 1 μ M PNU-120596. TM neurons of the posterior hypothalamus express high densities of native $\alpha 7^*$ nAChRs and have previously been used as an effective model in studies of kinetic and pharmacological characteristics of native $\alpha 7^*$ nAChRs (Uteshev et al., 1996, 2002, 2003; Uteshev and Knot, 2005).

Materials and Methods

Animals. Young male and female Sprague-Dawley rats (postnatal days 21 to 30) were used in all experiments. Animal care was in

accordance with the Institute of Laboratory Animal Resources (1996) and was approved by the Animal Care and Use Committee of Southern Illinois University.

Tissue Preparation. Three-four coronal whole-brain slices of 260- μ m thickness containing both TM hypothalamic nuclei were cut in a sucrose-rich solution at 3°C using Vibratome-1000+ tissue sectioning system (Vibratome, St. Louis, MO). The sucrose-rich solution was of the following composition: 250 mM sucrose, 3 mM KCl, 1.23 mM NaH_2PO_4 , 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose, pH 7.4, when bubbled with carbogen (95% O_2 and 5% CO_2). Slices were then transferred to a temporary storage chamber where they were maintained for ~ 30 min at 30°C in an oxygenated artificial cerebrospinal fluid (ACSF) of the following composition: 125 mM NaCl, 3 mM KCl, 1.23 mM NaH_2PO_4 , 1 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose, pH 7.4, when bubbled with carbogen. Slices were then transferred back into the storage chamber and maintained at room temperature for up to 10 h bubbled with carbogen.

PNU-120596. In this study, 1 μ M PNU-120596 was used in the majority of experiments. This concentration lies near the EC_{50} for potentiating effects of PNU-120596 in heterologous systems ($EC_{50} \sim 1.5$ μ M) (Grønlien et al., 2007; Young et al., 2008). It is noteworthy that intravenous administration of 1 mg/kg PNU-120596 has been shown to elevate the concentration of PNU-120596 in the brains of rats to similar values (~ 1.5 μ M) (Hurst et al., 2005).

Electrophysiology. For patch-clamp experiments, slices were transferred into the recording chamber perfused with ACSF. Recordings were made using a MultiClamp-700B amplifier and Digidata-1440A A/D converter (Molecular Devices, Sunnyvale, CA). Data were sampled at 10 to 50 kHz and filtered at 2 to 10 kHz, respectively. Recording pipettes were pulled using a Sutter P-97 puller (Sutter Instruments, Novato, CA). The pipette resistance was 4 to 6 M Ω . After formation of a gigaseal (> 2 G Ω), the whole-cell configuration was established. Choline and PNU-120596 were added to ACSF or pressure-applied (5–8 psi pressure) via picospritzer (Parker Hannifin, Cleveland, OH) pipettes identical to those used for recordings. The application pipette tips were positioned ~ 15 μ m away from the recorded neurons. Recordings were conducted at room temperature. The membrane voltage was maintained at -60 mV in all voltage-clamp experiments unless otherwise specified. The extracellular solution was identical to ACSF that was used for preparation. In the majority of voltage-clamp experiments, the intracellular electrode solutions contained 140 mM CsMeSO $_3$, 6 mM NaCl, 2 mM $MgCl_2$, 2 mM Mg-ATP, 0.3 mM Na-GTP, 10 mM HEPES, and 0.3 mM CsOH, pH 7.38. Recordings of spontaneous firing and action potentials were done in current-clamp using a potassium gluconate-based internal solution of the following composition: 140 mM potassium gluconate, 1 mM NaCl, 2 mM $MgCl_2$, 2 mM Mg-ATP, 0.3 mM Na-GTP, 10 mM HEPES, and 0.42 mM KOH, pH 7.38. Membrane voltages were not corrected for the liquid junction potentials: $V_{LJ}(CsMeSO_3) = 9.8$ mV and $V_{LJ}(potassium\ gluconate) = 16.2$ mV. Perfusion pump 2232-Microperpex-S (LKB, Uppsala, Sweden) was used to perfuse slices in the recording chamber at a rate of 1 ml/min. To block GABA $_A$, AMPA, NMDA, glycine/GABA $_A$, and voltage-gated Na^+ ion channels, 20 μ M gabazine, 15 μ M 6,7-dinitroquinoxaline-2,3-dione (DNQX), 50 μ M D-2-amino-5-phosphonopentanoate (AP-5), 40 μ M picrotoxin, and 0.3 μ M tetrodotoxin (TTX) were used, respectively. Although TM neurons express very low densities of NMDA receptors (V. V. Uteshev, unpublished observations), 50 μ M AP-5 was used to eliminate possible contribution of TM NMDA receptors to the effects of choline plus PNU-120596. Therefore, the effects observed in this study were resistant to the inhibiting cocktail containing 20 μ M gabazine, 15 μ M DNQX, 50 μ M AP-5, 40 μ M picrotoxin, and 0.3 μ M TTX. To study the effects of physiological levels of choline plus PNU-120596 on TM spontaneous firing, TTX was removed from ACSF.

Drugs. PNU-120596 was purchased from Tocris Bioscience (Ellisville, MO). Gabazine, DNQX, AP-5, and TTX were purchased from Ascent Scientific (Bristol, UK). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Choline-containing solutions

were freshly made before each experiment from a 1 M choline stock solution, which was kept frozen at -20°C for up to 3 months.

Analysis. The analysis of spontaneous firing was conducted using Clampfit-10.1 software program (Molecular Devices). A threshold-search event detection protocol (Clampfit-10.1) was used to evaluate the instantaneous and mean event frequencies of action potentials. To measure current net charge, choline and PNU-120596 were added to ACSF for up to 3 h, and step-like responses of TM neurons in slices were continuously recorded in voltage clamp for 10 to 20 min and analyzed offline. Net charge generated by current deviations was measured in 5-min intervals using Clampfit-10.1 (Fig. 3F).

The experimental results were presented as the mean $\pm$ S.D., with the exception of Fig. 4 where means $\pm$ S.E.M. were used instead of mean $\pm$ S.D. Curve fitting was done using ProStat analysis packages (Poly Software International, Pearl River, NY).

Potential Limitations. To avoid an inadvertent formation of excised patches during prolonged recordings, the membrane capacitance (72.0 ± 14.7 pF, $n = 26$) and input resistance (564.9 ± 165.7 M Ω , $n = 26$) of the recorded TM neurons were monitored in voltage clamp throughout the experiment. Formation of excised patches would be expected to associate with a considerable drop in the membrane capacitance and an increase in the input resistance. Moreover, in experiments where TTX was not used, the spontaneous firing of TM neurons was monitored in current clamp. If excised patches were to form at any point during recordings, the spontaneous firing would be expected to cease, and the resting potential would be expected to be near 0 mV. None of these changes was observed in this study, indicating that excised patches were not formed. The membrane capacitance and the input resistance were not altered by more than 10% during the course of experiments. The access resistance was <20 M Ω . Patches with higher access resistance were discarded or repaired by applying negative pressure to restore the recording quality.

Results

In voltage-clamp experiments, administration of 10 to 40 μM choline alone (in the absence of PNU-120596) or 1 to 4 μM PNU-120596 alone (in the absence of choline) via a picrosyringer (4–8 psi pressure, 1–15 s duration) did not elicit any responses in TM neurons held at -60 mV ($n = 4$; Fig. 1, A and B). By contrast, in the presence of 1 μM PNU-120596 in ACSF, pressure-administration of a mixture containing various low concentrations of choline (5–20 μM) and 1 μM PNU-120596 elicited repetitive step-like current deviations remi-

niscient of single ion channel openings (Fig. 1, C–E; membrane voltage, -50 mV). These current deviations were reversibly blocked by 20 nM methyllycaconitine (MLA), a selective antagonist of $\alpha 7$ nAChRs ($n = 11$; Fig. 1, F–H; membrane voltage, -50 mV), and were resistant to the inhibiting cocktail containing 20 μM gabazine, 15 μM DNQX, 50 μM AP-5, 40 μM picrotoxin, and 0.3 μM TTX, supporting the conclusion that GABA_A, AMPA, NMDA, glycine receptors, and voltage-gated Na⁺ ion channels are unlikely to contribute to these effects of 5 to 20 μM choline plus 1 μM PNU-120596. Therefore, these preliminary experiments demonstrate that PNU-120596 enhances the responsiveness of TM $\alpha 7$ -containing (i.e., $\alpha 7^*$) nAChRs to choline, allowing subthreshold physiological concentrations of choline (i.e., 5–10 μM) to become effective in eliciting $\alpha 7^*$ nAChR-mediated whole-cell currents.

Step-Like Current Deviations in Voltage-Clamp Experiments. To determine the effects of a prolonged exposure of native $\alpha 7^*$ nAChRs to physiological concentrations of choline and/or 1 μM PNU-120596, 10 μM choline and/or 1 μM PNU-120596 were added to ACSF for up to 3 h. Administration of choline and PNU-120596 to ACSF provides a more appropriate model of systemic drug administration than rapid pressure application. Administration of 10 μM choline alone ($n = 9$; Fig. 2A, top trace) or 1 μM PNU-120596 alone ($n = 9$; Fig. 2B, top trace) to ACSF did not elicit repetitive step-like current deviations. By contrast, the addition of 1 μM PNU-120596 to ACSF containing 10 μM choline (Fig. 2A, bottom trace) or addition of 10 μM choline to ACSF containing 1 μM PNU-120596 (Fig. 2B, bottom trace) produced robust repetitive step-like current deviations reminiscent of single ion channel openings ($n = 44$), similar to those seen in experiments with pressure application (Fig. 1). The membrane voltage in these experiments was held at -60 mV, and ACSF contained 20 μM gabazine, 15 μM DNQX, 50 μM AP-5, 40 μM picrotoxin, and 0.3 μM TTX.

The first latency time (i.e., time between the start of drug application and the first occurrence of step-like current deviations) was 16.8 ± 4.3 min ($n = 6$) upon addition of 1 μM PNU-120596 to 10 μM choline and 1.7 ± 0.5 min ($n = 6$) upon addition of 10 μM choline to 1 μM PNU-120596. Therefore,

Fig. 1. Pressure application of subthreshold physiological concentrations of choline in the presence of 1 μM PNU-120596 in slices. A and B, in the initial set of whole-cell experiments in slices, pressure applications of 10 to 40 μM choline alone (A) and 1 to 4 μM PNU-120596 alone (B) to TM neurons held in voltage clamp at -60 mV did not elicit responses. C to E, by contrast, pressure administration of 5 to 20 μM choline in the presence of 1 μM PNU-120596 elicited step-like current deviations reminiscent of single-channel openings. The membrane voltage was held at -50 mV. F to H, these responses were completely and reversibly blocked by 20 nM MLA, a selective antagonist of $\alpha 7$ nAChRs supporting the involvement of $\alpha 7^*$ nAChRs. The membrane voltage was held at -50 mV. In these experiments, ACSF contained 20 μM gabazine, 15 μM DNQX, 50 μM AP5, 40 μM picrotoxin, and 0.3 μM TTX.

Fig. 2. Synergistic effects of choline and PNU-120596. In voltage-clamp whole-cell experiments, administration of 10 μ M choline alone for $\sim$ 60 min (A, top trace) or 1 μ M PNU-120596 alone for $\sim$ 55 min (B, top trace) to ACSF did not elicit responses of TM neurons ($n = 9$). By contrast, the addition of 1 μ M PNU-120596 in the continuing presence of 10 μ M choline (A, bottom trace; 20 min after addition of 1 μ M PNU-120596) or addition of 10 μ M choline in the continuing presence of 1 μ M PNU-120596 (B, bottom trace; 22 min after addition of 10 μ M choline) resulted in step-like current deviations reminiscent of single-channel openings seen in the initial experiments with pressure application (Fig. 1). The ACSF perfusion rate was set to 1 ml/min. The volume of recording chamber was $\sim$ 2 ml. These observations confirm the synergistic effects of choline and PNU-120596 on native $\alpha 7^*$ nAChRs. C, administration of 5 μ M choline plus 1 μ M PNU-120596 to ACSF also produced similar step-like current deviations. The current trace shown in C was recorded 65 min after administration of 1 μ M PNU-120596 and 30 min after administration of 5 μ M choline. The membrane voltage was held at -60 mV. These effects were resistant to 20 μ M gabazine, 15 μ M DNQX, 50 μ M AP-5, 40 μ M picrotoxin, and 0.3 μ M TTX applied to ACSF.

the onset of effects of 10 μ M choline (in the continued presence of 1 μ M PNU-120596) was approximately 10-fold faster than the onset of effects of 1 μ M PNU-120596 (in the continued presence of 10 μ M choline). The delays in the effect onset evaluated by the first latency times reflected the method of drug administration employed in this study (drugs were added to ACSF), the speed of ACSF perfusion (1 ml/min), the volume of the recording chamber ($\sim$ 2 ml), the location of recorded neurons within the slice, and the location of the slice within the recording chamber. However, the 10-fold difference in the rates of onset of effects of choline and PNU-120596 may reflect differences in the rates of equilibration of these drugs within brain slices and are determined by biophysical characteristics of drugs, such as the drug lipophilicity, molecular size, diffusion coefficient, and receptor binding properties.

Step-like current deviations were also recorded in TM neurons when 5 μ M choline plus 1 μ M PNU-120596 were administered to ACSF ($n = 8$, Fig. 2C). However, in this study, the concentration dependence and the kinetic characteristics of current deviations were not investigated because a thorough analysis of these characteristics may require experiments utilizing isolated neurons and/or excised patches.

Figure 3, A to E, illustrates representative current traces obtained from a typical experiment under different experimental conditions. The frequency of step-like current responses elicited by administration of 10 μ M choline plus 1 μ M PNU-120596 to ACSF (Fig. 3A) was reversibly reduced during a 30-min washout period when choline was removed from ACSF; however, 1 μ M PNU-120596 remained (Fig. 3, A–C). The rates of washout of effects of choline and PNU-120596 were investigated in a separate set of experiments (Fig. 4). Step-like current deviations were completely and

reversibly blocked by 20 nM MLA ($n = 9$; Fig. 3, D and E). The experimental protocol, which was used to generate traces shown in Fig. 3 (A to E), is schematically illustrated in Fig. 3F. In these experiments, step-like current deviations were continuously recorded in voltage clamp for up to 3 h in 10- to 20-min intervals and then analyzed offline. Net charge generated by current deviations was measured at 5-min intervals (Fig. 3F). The mean net charge of $\alpha 7^*$ nAChR-mediated current deviations elicited by 10 μ M choline plus 1 μ M PNU-120596 was estimated to be 84.0 ± 55.5 pC/min ($n = 5$; membrane voltage, -60 mV). In these experiments, ACSF contained 20 μ M gabazine, 15 μ M DNQX, and 0.3 μ M TTX. The internal pipette solution contained CsMeSO₃ (see *Materials and Methods*).

The rates of washout of 10 μ M choline or 1 μ M PNU-120596 were evaluated in experiments illustrated in Fig. 4. Hypothalamic slices were incubated in 10 μ M choline plus 1 μ M PNU-120596 for up to 1 h, and then PNU-120596 (Fig. 4, A and B) or choline (Fig. 4, C and D) was removed from ACSF and the frequency of step-like current deviations (events per minute) was measured, normalized to the control value obtained 1 min before washout, and plotted as a function of time (Fig. 4, B and D). The data were fitted with a single exponential function, and the time constants were determined. The washout time constant of 1 μ M PNU-120596 (in the continued presence of 10 μ M choline in ACSF) was 16.1 min ($n = 5$; Fig. 4B; exponential fit ($R^2 = 0.93$): $y = \exp(-0.06x)$); whereas the washout time constant of 10 μ M choline (in the continued presence of 1 μ M PNU-120596 in ACSF) was 4.8 min ($n = 6$; Fig. 4D; exponential fit ($R^2 = 0.94$): $y = 1.1\exp(-0.21x)$, where x is the washout time in minutes. Therefore, the clearance of 10 μ M choline from the brain tissue (in the continued presence of 1 μ M PNU-120596

Fig. 3. Block by 20 nM MLA. Prolonged exposure of TM neurons in slices to 10 μ M choline plus 1 μ M PNU-120596 elicited sustained repetitive step-like current deviations recorded in voltage clamp (A). The frequency of current deviations was considerably but reversibly reduced when choline was removed from ACSF for up to 30 min, but PNU-120596 remained (B and C), supporting the synergistic action of choline and PNU-120596. The rates of washout of effects of choline and PNU-120596 were investigated in a separate set of experiments (Fig. 4). D to E, current deviations were completely and reversibly blocked by 20 nM MLA ($n = 9$), confirming the involvement $\alpha 7^*$ nAChRs. All current traces in A to E were obtained from the same TM neuron. F, a schematic illustration of the experimental paradigm used in the experiment illustrated in A to E. A 120-min recording was divided into 5-min intervals, and the net charge of step-like current deviations in each of those 24 intervals was measured under different experimental conditions specified in A to E using Clampfit-10.1. In F, the positions of traces shown in A to E are marked by black rhombs and corresponding letters: control 1 (A); choline washout (B), control 2 (C), + 20 nM MLA (D), and MLA washout (E). The horizontal bars above the graph illustrate the sequence and durations of corresponding phases (F). The membrane voltage was held at -60 mV. These effects were resistant to 20 μ M gabazine, 15 μ M DNQX, 50 μ M AP-5, 40 μ M picrotoxin, and 0.3 μ M TTX applied to ACSF.

in ACSF) was approximately ~ 3.4 -fold faster than the clearance of 1 μ M PNU-120596 (in the continuing presence of 10 μ M choline in ACSF). Similar to the case of effect onset (see above), these differences in the rates of clearance of choline and PNU-120596 may reflect differences in the rates of equilibration of these drugs within brain slices. The membrane voltage in these experiments was -60 mV.

The Current-Voltage Relationship. To build the current-voltage relationship of current deviations mediated by 10 μ M choline plus 1 μ M PNU-120596 in ACSF, the amplitudes of current deviations were measured in whole-cell in voltage clamp between -90 mV and $+30$ mV (Fig. 5A) and then plotted against the corresponding membrane voltages ($n = 5$; Fig. 5B). The current-voltage relationship of current deviations was inwardly rectified at negative membrane voltages (Fig. 5B). The outward current deviations were not detected (Fig. 5A, two top traces). This current-voltage relationship is typical for $\alpha 7^*$ nAChRs studied in the presence of Mg^{2+} ions in internal and external solutions (Alkondon et al., 1994; Uteshev et al., 1996; Shao and Yakel, 2000). These results support the critical role of TM $\alpha 7^*$ nAChRs in generating current deviations in the presence of 10 μ M choline and 1 μ M PNU-120596. In these experiments, ACSF contained 20 μ M gabazine, 15 μ M DNQX, 50 μ M AP-5, 40 μ M picrotoxin, and 0.3 μ M TTX.

Step-Like Depolarizations in Current-Clamp Experiments. In the presence of 1 μ M PNU-120596, activation of TM $\alpha 7^*$ nAChRs by 10 μ M choline was observed in both voltage-clamp (Fig. 6A) and current-clamp (Fig. 6, B and C) configurations. Traces shown in Fig. 6, A and B, were obtained from the same TM neuron 1 min apart. In current clamp, activation of individual $\alpha 7^*$ nAChRs in the presence of 10 μ M choline and 1 μ M PNU-120596 produced sustained sequences of repetitive step-like depolarizations ranging from ~ 5 mV (associated with individual $\alpha 7^*$ ion channel openings) to ~ 25 mV (associated with multiple openings) ($n = 11$; Fig. 6, B and C). These effects were resistant to 20 μ M gabazine, 15 μ M DNQX, 50 μ M AP-5, 40 μ M picrotoxin, and 0.3 μ M TTX applied to ACSF. The internal pipette solution contained CsMeSO₃ (see *Materials and Methods*). Step-like depolarizations were sufficient to occasionally activate voltage-gated conductances. For example, Fig. 6C illustrates activation of putative Ca^{2+} action potentials (arrows) at the membrane potentials near -25 mV. These experiments demonstrate that, in the presence of 1 μ M PNU-120596, subthreshold physiological levels of choline activate TM $\alpha 7^*$ nAChRs and produce sequences of step-like transient depolarizations capable of triggering voltage-gated conductances.

Fig. 4. Washout of PNU-120596 and choline. To evaluate and compare the rates of washout of PNU-120596 and choline, 1 μ M PNU-120596 and 10 μ M choline were administered to ACSF for up to 1 h, and then these compounds were removed from ACSF one at a time and the rates of washout were evaluated by measuring the number of current deviations per minute of recording. The membrane voltage was -60 mV. ACSF contained 20 μ M gabazine, 15 μ M DNQX, 50 μ M AP-5, 40 μ M picrotoxin, and 0.3 μ M TTX. **A**, current traces from one experiment just before (1), 10 min after (2), and 20 min after (3) the start of washout of 1 μ M PNU-120596. Choline (10 μ M) was continuously present in ACSF during washout of PNU-120596. **B**, analysis of PNU-120596 washout experiments obtained from $n = 5$ TM neurons. The frequency of current deviations (events per minute) was evaluated, normalized to the control value obtained 1 min before washout, and plotted as a function of time. The data were fitted with a single exponential function, and the time constants were determined. **C**, current traces from a different experiment just before (4), 5 min after (5), and 10 min after (6) the start of washout of 10 μ M choline. PNU-120596 (1 μ M) was continuously present in ACSF during washout of choline. **D**, analysis of choline washout experiments obtained from $n = 6$ TM neurons was similar to that described in **B**. The values of time constants and the exponential functions that gave the best fit for each graph are shown above the graphs, where x is the washout time in minutes. Results were presented as mean $\pm$ S.E.M.

Fig. 5. The current-voltage relationship of current deviations. The amplitudes of current deviations measured in voltage clamp between -90 mV and $+30$ mV with a step of 30 mV (**A**) were plotted against the corresponding membrane voltages to build a current-voltage relationship (**B**). The resulting current-voltage relationship was typical for $\alpha 7^*$ nAChRs studied in the presence of internal and external Mg^{2+} ions; it was inwardly rectified at negative membrane voltages and did not exhibit outward current deviations at positive membrane voltages. ACSF contained 20 μ M gabazine, 15 μ M DNQX, 50 μ M AP-5, 40 μ M picrotoxin, and 0.3 μ M TTX. Results were presented as mean $\pm$ S.D.

Modulation of Spontaneous Firing. TM neurons are native pacemakers (Uteshev et al., 1995). In the absence of choline and PNU-120596, TM neurons exhibited regular patterns of spontaneous firing with the frequency between 1.6 and 2.8 Hz (2.1 ± 0.5 Hz, $n = 5$; Fig. 7A), and when the membrane was hyperpolarized to -65 mV by injecting a small current, step-like depolarizations were not observed ($n = 5$; Fig. 7B). To investigate the effects of 10 μ M choline plus 1 μ M PNU-120596 on spontaneous firing of TM neurons, the internal CsMeSO_3 -based solution was replaced with potassium gluconate-based solution (see *Materials and Methods*), and patch-clamp recordings of spontaneous action po-

tentials were conducted at 1- to 10-min intervals in current-clamp in the absence of TTX. These experiments tested the hypothesis that activation of individual $\alpha 7^*$ nAChRs [seen in voltage-clamp experiments as step-like current deviations (Figs. 2–3, 6A, and 7C) and in current-clamp experiments as step-like depolarizations (in the presence of 0.3 μ M TTX; Fig. 6, B and C)] results in transient increases in the frequency of spontaneous firing. In the presence of 10 μ M choline plus 1 μ M PNU-120596, two modes of TM spontaneous firing were clearly seen in each recording ($n = 7$): 1) a low-frequency mode (Fig. 7D, open arrows), which was postulated to correspond to the background spontaneous firing (i.e., between

Fig. 6. Step-like voltage depolarizations recorded in current-clamp in the presence of 10 μ M choline plus 1 μ M PNU-120596. Transient step-like responses of TM $\alpha 7^*$ nAChRs were observed in both voltage-clamp (A) and current-clamp (B) whole-cell configurations. Traces shown in A and B are representative of $n = 11$ TM neurons and were obtained from the same TM neuron 1 min apart. Activation of $\alpha 7^*$ nAChRs in current-clamp elicited transient repetitive step-like depolarizations: ~ 5 mV for individual events and ~ 25 mV for simultaneous multiple events. The bottom trace in A and the top trace in B share the same time scale shown between these traces. The vertical scale bar indicates either 20 pA (for current traces shown in A) or 20 mV (for voltage traces shown in B). Occasionally, strong depolarizations were observed that resulted in putative Ca^{2+} spikes (arrows, C). These effects were resistant to 20 μ M gabazine, 15 μ M DNQX, 50 μ M AP-5, 40 μ M picrotoxin, and 0.3 μ M TTX applied to ACSF. The internal pipette solution contained CsMeSO₃. The membrane voltage in voltage-clamp experiments was -60 mV. Horizontal bars near voltage traces indicate the membrane voltage of -60 mV.

$\alpha 7^*$ nAChR channel openings; see justification below); and 2) a high-frequency mode (Fig. 7D, filled arrows), which was postulated to correspond to transient increases in spontaneous firing associated with $\alpha 7^*$ nAChR channel openings. The latter postulation was further tested and supported in later experiments (see Fig. 8). Traces shown in Fig. 7, C (voltage-clamp) and D (current-clamp), were obtained from the same TM neuron 1 min apart. The facilitating effects of 10 μ M choline plus 1 μ M PNU-120596 on spontaneous firing of TM neurons were resistant to the inhibiting cocktail containing 20 μ M gabazine, 15 μ M DNQX, 50 μ M AP-5, and 40 μ M picrotoxin but were inhibited by 20 nM MLA ($n = 4$, data not shown).

A typical example of TM spontaneous firing frequency distributions as a function of time is illustrated in Fig. 7E; the distribution of instantaneous frequencies (i.e., frequencies defined by pairs of neighboring action potentials) is shown as black dots, whereas the distribution of mean frequencies (defined as the number of action potentials per 1 s) is shown as a frequency histogram with a 1-s bin size. Figure 7, D and

E, presents the same set of data with the same time scale. The analysis of firing frequencies indicates that, in the presence of 10 μ M choline plus 1 μ M PNU-120596, transient depolarizations associated with $\alpha 7^*$ nAChR channel openings increased the instantaneous frequency of TM spontaneous firing (Fig. 7E, black dots) from 1.61 ± 0.65 Hz ($n = 7$; the low-frequency mode only; Fig. 7, D and E, open arrows) to 2.67 ± 0.80 Hz ($n = 7$; the high-frequency mode only; Fig. 7, D and E, filled arrows), a statistically significant 66% increase ($p = 0.002$; paired t test; $n = 7$). The mean event frequency, defined as the mean frequency of action potentials measured over prolonged intervals of time (i.e., >1 min), was also increased in the presence of $\alpha 7^*$ nAChR channel openings from 2.03 ± 0.80 Hz ($n = 6$; the low-frequency mode only; Fig. 7, D and E, open arrows) to 2.74 ± 0.83 Hz ($n = 6$; both the low- and the high-frequency modes; Fig. 7, D and E, open and filled arrows), a statistically significant 35% increase ($p < 0.02$; paired test; $n = 6$).

It is interesting that the frequency of the low-frequency mode, 2.03 ± 0.8 Hz ($n = 6$; Fig. 7, D and E, open arrows), and the control frequency of spontaneous firing recorded in the absence of choline and PNU-120596, 2.1 ± 0.5 Hz ($n = 5$; Fig. 7A), were found to be statistically similar ($p > 0.86$, unpaired t test). Therefore, the low-frequency mode of TM spontaneous firing (i.e., spontaneous firing in between $\alpha 7^*$ nAChR channel openings; Fig. 7, D and E, open arrows) appears to be indistinguishable from the control spontaneous firing (i.e., spontaneous firing in the absence of choline and PNU-120596 in ACSF; Fig. 7A). This finding demonstrates that, in the absence of (or between) $\alpha 7^*$ nAChR channel openings, a mixture of 10 μ M choline plus 1 μ M PNU-120596 does not significantly alter the properties of TM spontaneous firing.

Additional evidence linking TM $\alpha 7^*$ nAChR openings and transient increases of TM spontaneous firing comes from current-clamp experiments with injections of prolonged small hyperpolarizing currents ($n = 3$; Fig. 8). When a continuous hyperpolarizing current (~ -40 pA) was injected into the recorded TM neuron during a prolonged interval of increased frequency (the moment of injection is marked by an asterisk; Fig. 8A and inset), it resulted in cessation of spontaneous firing allowing detection of the final portion of the underlying step-like depolarization (Fig. 8A, filled triangle, and inset). Therefore, in these elusive events, the effect of a prolonged depolarization was observed as both an increase in spontaneous firing at the beginning of depolarization (Fig. 8A, open arrowhead, and inset) and a depolarizing square-like step at the end of depolarization (Fig. 8A, filled triangle, and inset). Subsequent transient step-like depolarizations are seen between two dashed lines (Fig. 8A, inset). These observations further support the conclusion that, in the presence of 10 μ M choline plus 1 μ M PNU-120596, transient increases in the frequency of TM spontaneous firing (Figs. 7D and 8A) are associated with step-like depolarizations observed in current-clamp experiments and thus correspond to individual openings of TM $\alpha 7^*$ nAChRs recorded in voltage-clamp (Figs. 2, 3, 6A, and 7C) and current-clamp (Figs. 6, B and C, and 8, A and B) whole-cell experiments.

In a separate set of experiments, spontaneous firing ceased when the neuronal membrane was hyperpolarized by injecting a small continuous negative current (-5 pA; Fig. 8B and insets). Under these slightly hyperpolarized experimental conditions, activation of individual TM $\alpha 7^*$ nAChRs trig-

Fig. 7. Activation of TM $\alpha 7^*$ nAChRs by 10 μ M choline plus 1 μ M PNU-120596 enhances spontaneous firing of TM neurons in current clamp. The spontaneous firing of TM neurons was native as current injections were not applied (i.e., 0 pA). Horizontal bars indicate -65 mV. A to B, in current clamp, in the absence of PNU-120596 and choline, TM neurons exhibited regular patterns of spontaneous firing with the mean frequency of 2.1 ± 0.5 Hz ($n = 5$) (A). In these control experiments, when the membrane voltage was hyperpolarized to -65 mV by injections of a small current (B), step-like depolarizations were not observed ($n = 5$) (B). Recordings in A and B were obtained from the same TM neuron 1 min apart. In $n = 7$ experiments, after the sustained repetitive activation of TM nAChRs was observed in voltage-clamp upon administration of 10 μ M choline plus 1 μ M PNU-120596 (C), current-clamp recordings were conducted using the same TM neuron (D). In current clamp, activation of TM $\alpha 7^*$ nAChRs resulted in transient repetitive increases in the frequency of spontaneous firing of TM neurons (C and D, filled arrows). Traces shown in C and D were obtained from the same TM neuron 1 min apart. The framed inset illustrates a portion of recording containing one transient excitation at a higher time resolution. E, analysis of the voltage trace shown in D. A histogram of event frequencies (solid line, bin size = 1 s) is overlaid with a scattered plot of instantaneous frequencies. D and E share the same time scale. The left and right ordinates correspond to the event frequency histogram and instantaneous frequencies, respectively. A similar analysis was conducted in $n = 7$ TM neurons (see text). In the presence of 10 μ M choline and 1 μ M PNU-120596, transient depolarizations mediated by $\alpha 7^*$ nAChRs increased the instantaneous frequency of TM spontaneous firing (black dots) from ~ 1.61 (the low-frequency mode only; $n = 7$, D-E, open arrows) to ~ 2.67 Hz (the high-frequency mode only; $n = 7$; D-E, filled arrows), a statistically significant 66% increase ($p = 0.002$, paired t test, $n = 7$). The mean event frequency of TM spontaneous firing, defined as the mean frequency of action potentials measured over prolonged intervals of time (>1 min), was also increased from 2.03 ± 0.80 Hz ($n = 6$; the low-frequency mode only; D-E) to 2.74 ± 0.83 Hz ($n = 6$; both the low- and the high-frequency modes; D-E, open and filled arrows), a statistically significant 35% increase ($p < 0.02$; paired test; $n = 6$). These effects were resistant to 20 μ M gabazine, 15 μ M DNQX, 50 μ M AP-5, and 40 μ M picrotoxin applied to ACSF. TTX was not used in these experiments. The internal pipette solution contained potassium gluconate. The frequencies of the low-frequency mode (2.03 ± 0.8 Hz, $n = 6$; D-E, open arrows) and the control spontaneous firing (2.1 ± 0.5 Hz, $n = 5$; A) were found to be statistically similar ($p > 0.86$).

gered short trains of action potentials, which occurred on top of each step-like depolarization ($n = 9$, open arrows; Fig. 8B and bottom inset). Occasionally, however, those transient depolarizations failed to trigger action potentials or triggered only a single action potential per depolarization (filled arrows; Fig. 8B and top inset). These current-clamp experiments demonstrate that, in the presence of PNU-120596, subthreshold physiological levels of choline may enhance the excitability of $\alpha 7^*$ nAChR-expressing neurons by activation of $\alpha 7^*$ nAChRs. Furthermore, in the presence of 1 μ M PNU-120596, a single opening of an individual $\alpha 7^*$ nAChR ion channel appeared to transiently depolarize the entire neuron and facilitate spontaneous firing.

Discussion

The deficiency in the expression and function of $\alpha 7$ nAChRs may be associated with certain pathological aspects of schizophrenia, Alzheimer's disease, traumatic brain in-

jury, and other neurodegenerative disorders (Scremin and Jenden, 1996; Guan et al., 2000; Nordberg, 2001; Olincy and Stevens, 2007; Martin and Freedman, 2007), whereas activation of $\alpha 7^*$ nAChRs by low concentrations of nicotinic agents is thought to enhance cognitive performance and is neuroprotective (Shimohama et al., 1998; Verbois et al., 2003; Buccafusco, 2004; Buccafusco et al., 2005; Olincy and Stevens, 2007; Guseva et al., 2008). Some of the presently available therapeutic approaches aimed at rescuing the brain $\alpha 7$ nAChR activation include PAMs (e.g., PNU-120596) (Hurst et al., 2005; Faghih et al., 2007; Grønlien et al., 2007; Roncarati et al., 2008; Young et al., 2008; López-Hernández et al., 2009). Specifically, intravenous administration of 1 mg/kg PNU-120596 has been shown to result in beneficial effects on the auditory gating deficit in rats (Hurst et al., 2005). The concentration of PNU-120596 detected in the brains of rats receiving 1 mg/kg intravenously was found to be ~ 1.5 μ M (Hurst et al., 2005), thus near the EC_{50} for potentiating effects of PNU-120596 ($EC_{50} \sim 1.5$ μ M) (Grøn-

Fig. 8. The effects of individual step-like depolarizations in current clamp. **A**, when a hyperpolarizing current (~ -40 pA) was injected in the recorded TM neuron (the injection time is marked by *) during a prolonged interval of increased frequency (the interval between open and filled triangles), it resulted in cessation of spontaneous firing, allowing detection of the final portion of underlying step-like depolarization ($n = 3$; filled triangle). Therefore, a prolonged depolarization was observed as both an increase in spontaneous firing in the beginning of depolarization (open triangle) and a depolarizing step at the end of depolarization (filled triangle). Subsequent step-like depolarizations are seen between two dashed lines (see inset). The inset illustrates this transition process at a higher resolution. In these experiments, ACSF contained $20 \mu\text{M}$ gabazine, $15 \mu\text{M}$ DNQX, $50 \mu\text{M}$ AP-5, and $40 \mu\text{M}$ picrotoxin. The internal solution was potassium gluconate-based (see *Materials and Methods*). **B**, to visualize individual depolarizations, a small continuous hyperpolarizing current (-5 pA) was injected into the recorded neuron resulting in cessation of spontaneous firing. Under these silent conditions, transient depolarizations triggered short trains of action potentials (**B**, open arrows). However, occasionally, depolarizations did not trigger action potentials or triggered only a single action potential per depolarization (**B**, filled arrows). Horizontal bars indicate the membrane voltage of -65 mV.

lien et al., 2007; Young et al., 2008). Using slightly lower concentrations, this study tested and confirmed the hypothesis that $1 \mu\text{M}$ PNU-120596 enhances the effects of subthreshold concentrations of choline, allowing physiological levels of choline (5 – $10 \mu\text{M}$) to become effective in activation of native $\alpha 7^*$ nAChRs in the absence of exogenous nicotinic agents.

Activation of TM $\alpha 7^*$ nAChRs was not detected when 10 to $40 \mu\text{M}$ choline or 1 to $4 \mu\text{M}$ PNU-120596 was administered alone, supporting the synergistic nature of the effects of choline and PNU-120596 on TM nAChRs (Figs. 1 and 2). By contrast, upon administration of $10 \mu\text{M}$ choline plus $1 \mu\text{M}$ PNU-120596, repetitive step-like deviations were observed in both voltage-clamp and current-clamp whole-cell experiments (Figs. 3–8). These current/voltage deviations appeared to be mediated by openings of individual $\alpha 7^*$ nAChR ion channels because these deviations were not observed in the absence of choline and/or PNU-120596, were reversibly

blocked by 20 nM MLA, and were resistant to the inhibiting cocktail containing $20 \mu\text{M}$ gabazine, $15 \mu\text{M}$ DNQX, $50 \mu\text{M}$ AP-5, $40 \mu\text{M}$ picrotoxin, and $0.3 \mu\text{M}$ TTX. Moreover, the current-voltage relationship was inwardly rectified, which is a typical feature of $\alpha 7^*$ nAChRs in the presence of Mg^{2+} ions (Alkondon et al., 1994; Uteshev et al., 1996; Shao and Yakel, 2000).

Remarkably, in current-clamp experiments, individual openings of TM $\alpha 7^*$ nAChRs produced step-like depolarizations (~ 5 mV) capable of triggering voltage-gated conductances (Fig. 6, B and C), generating action potentials (Fig. 8B), and increasing the frequency of spontaneous firing (Figs. 7, D and E, and 8A). Although any kind of depolarization would be expected to promote spontaneous firing, the fact that these effects were driven by subthreshold physiological levels of choline and that activation of a single TM $\alpha 7^*$ nAChR was sufficient for altering the behavior of the entire neuron points to a high potency of PNU-120596 and its po-

tential therapeutic value. The observed high susceptibility of TM membrane potentials to prolonged openings of single $\alpha 7^*$ nAChR ion channels is supported by the high-input resistance of TM neurons $564.9 \pm 165.7 \text{ M}\Omega$ ($n = 26$).

An increase in the excitability of TM neurons mediated by physiological choline in the presence of PNU-120596 may promote release of histamine, link cholinergic and histaminergic systems, and emphasize the close resemblance of the behavioral effects of nicotine and histamine: elevated alertness and suppressed food intake. The results of this study can be generalized to other brain regions because the properties of TM $\alpha 7^*$ nAChRs (Uteshev et al., 1996, 2002) are similar to the properties of $\alpha 7^*$ nAChRs expressed elsewhere in the brain (Albuquerque et al., 1997; Frazier et al., 1998; Uteshev and Smith, 2006). However, the effects of PNU-120596 on neuronal behavior may be weaker in brain regions that express lower densities of $\alpha 7^*$ nAChRs than the TM.

The onset of effects of $10 \text{ }\mu\text{M}$ choline (in the continued presence of $1 \text{ }\mu\text{M}$ PNU-120596) was ~ 10 -fold faster than the onset of effects of $1 \text{ }\mu\text{M}$ PNU-120596 (in the continued presence of $10 \text{ }\mu\text{M}$ choline); whereas the clearance of effects of $10 \text{ }\mu\text{M}$ choline (in the continued presence of $1 \text{ }\mu\text{M}$ PNU-120596) was ~ 3.4 -fold faster than the clearance of effects of $1 \text{ }\mu\text{M}$ PNU-120596 (in the continued presence of $10 \text{ }\mu\text{M}$ choline). These differences may reflect different rates of equilibration of choline and PNU-120596 within the brain tissue and may be determined by differences in the drug lipophilicity, molecular size, diffusion coefficient, and receptor binding properties.

One limitation of this study is that it investigates relatively short-term effects of PNU-120596 ($< 3 \text{ h}$). Chronic effects of prolonged administration of PNU-120596 have not been studied and may include reduced potency for activation of $\alpha 7^*$ nAChRs due to desensitization and potential side-effects resulting from activation of voltage-gated conductances. Conversely, chronic administration of PNU-120596 may result in a near complete removal of $\alpha 7^*$ nAChR desensitization and thus may cause neurotoxic effects due to excessive accumulation of Ca^{2+} ions in the cytosol.

Estimating the Influx of Ca^{2+} Ions and the Probability of $\alpha 7^*$ nAChR Channel Opening in the Presence of PNU-120596. $\alpha 7$ nAChRs are highly permeable to Ca^{2+} ions (Castro and Albuquerque, 1995; Uteshev, 2010). The persistent activation of native $\alpha 7^*$ nAChR ion channels by physiological levels of choline would be expected to result in a sustained influx of Ca^{2+} ions. The permeability ratio of TM $\alpha 7^*$ nAChRs ($P_{\text{Ca}}/P_{\text{Na}}(\text{TM } \alpha 7) \sim 5.9$; Uteshev, 2010) is very similar to that estimated for cultured hippocampal neurons ($P_{\text{Ca}}/P_{\text{Na}}(\text{hippocampus } \alpha 7) \sim 6.1$) (Castro and Albuquerque, 1995). These values translate into the fractional Ca^{2+} current, $P_{\text{f}}^{\text{est}}(\alpha 7) \sim 10\%$, at -60 mV (Uteshev, 2010). PNU-120596 has been shown to not significantly alter the Ca^{2+} permeability of $\alpha 7$ nAChRs (Hurst et al., 2005). Therefore, in the presence of endogenous choline and $1 \text{ }\mu\text{M}$ PNU-120596, Ca^{2+} ions would be expected to be responsible for $\sim 10\%$ of net charge produced by persistently active $\alpha 7$ nAChR ion channels. In this study, the mean net charge of TM $\alpha 7^*$ nAChR-mediated responses elicited by $10 \text{ }\mu\text{M}$ choline plus $1 \text{ }\mu\text{M}$ PNU-120596 was estimated to be $\sim 84 \text{ pC/min}$ (Fig. 3F). Therefore, given the 10% fractional Ca^{2+} current, Ca^{2+} ions would be expected to enter TM neurons at a rate of 8.4 pC/min , which translates into a sustained Ca^{2+} current $\sim 0.14 \text{ pA}$. This rate of Ca^{2+} influx mediated by physiological

concentrations of choline in the presence of $1 \text{ }\mu\text{M}$ PNU-120596 may correspond to a near optimal level of activation of native $\alpha 7^*$ nAChRs because $1 \text{ }\mu\text{M}$ PNU-120596 in the CSF of rats has been attributed to therapeutically beneficial effects (Hurst et al., 2005). These values, however, may be higher in brain regions that express lower densities of $\alpha 7^*$ nAChRs compared with those seen in histaminergic TM neurons.

In the absence of PNU-120596, the mean open time of TM $\alpha 7^*$ nAChRs is extremely short, $\sim 80 \text{ }\mu\text{s}$ (V. V. Uteshev, unpublished observations), thus similar to values determined for putative $\alpha 7^*$ nAChRs in cultured hippocampal neurons ($\sim 100 \text{ }\mu\text{s}$) (Mike et al., 2000). TM neurons express high densities of $\alpha 7^*$ nAChRs (Uteshev et al., 1996), and yet the presented results suggest that on average less than one $\alpha 7^*$ nAChR channel is constantly open in TM neurons in the presence of $10 \text{ }\mu\text{M}$ choline and $1 \text{ }\mu\text{M}$ PNU-120596. Specifically, the mean amplitude of current deviations observed upon administration of $10 \text{ }\mu\text{M}$ choline plus $1 \text{ }\mu\text{M}$ PNU-120596 was estimated to be $\sim 5.1 \text{ pA}$ ($n = 5$; Fig. 5B, -60 mV). Therefore, on average, only $NP_{\text{open}} \sim 0.27$ ($= 1.4 \text{ pA}/5.1 \text{ pA}$) $\alpha 7^*$ nAChR ion channels are open at any given time because the mean sustained current under these conditions is $\sim 1.4 \text{ pA}$ ($= 84 \text{ pC}/60 \text{ s}$; see above). This observation supports the extremely low probability of $\alpha 7$ nAChR channel openings. If a typical TM neuron expresses $N = 10,000$ $\alpha 7^*$ nAChRs, then in the presence of $10 \text{ }\mu\text{M}$ choline plus $1 \text{ }\mu\text{M}$ PNU-120596, the probability of opening of an individual TM $\alpha 7^*$ nAChR ion channel would be $P_{\text{open}} = 0.000027$ and the probability of $\alpha 7^*$ nAChR channel opening in the absence of PNU-120596 may be significantly lower.

In summary, this study demonstrates that $1 \text{ }\mu\text{M}$ PNU-120596 enhances the effects of subthreshold concentrations of choline on native $\alpha 7$ -containing nAChRs, allowing physiological levels of choline to activate native $\alpha 7$ -containing nAChRs, enhance neuronal excitability, and produce whole-cell responses in the absence of exogenous nicotinic agents. A single opening of an individual $\alpha 7$ -containing nAChR ion channel appeared to transiently depolarize the entire neuron, activate voltage-gated conductances, and facilitate spontaneous firing. Activation of $\alpha 7$ -containing nAChRs by low concentrations of nicotinic agents is thought to produce positive cognitive effects and neuroprotection. Therefore, this study supports the hypothesis that, in the presence of PNU-120596, sustained activation of $\alpha 7$ -containing nAChRs by physiological levels of choline may be more efficacious and have less side effects associated with administration of exogenous nicotinic agonists. It is intriguing to speculate that the therapeutic effects of PNU-120596 may be enhanced by a high-choline diet (Guseva et al., 2008), ischemia, stroke, or other conditions associated with plasma membrane damage and increase in the ambient levels of choline in the CSF (Scremin and Jenden, 1991; Bertrand et al., 1996; Klein et al., 1998; Rao et al., 2000).

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