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Increased Sensitivity to Nicotine-Induced Seizures in Mice Expressing the L250T α7 Nicotinic Acetylcholine Receptor Mutation

RON S. BROIDE, RAMIRO SALAS, DAOYUN JI, RICHARD PAYLOR, JAMES W. PATRICK, JOHN A. DANI, and MARIELLA DE BIASI

Division of Neuroscience (R.S.B., R.S., D.J., R.P., J.W.P., J.A.D., M.D.B.) and Department of Molecular and Human Genetics (R.P.), Baylor College of Medicine, Houston, Texas

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ABSTRACT

High doses of nicotine, the addictive component of tobacco, induce clonic-tonic seizures in animals. Pharmacological and biochemical data have suggested that α7-containing neuronal nicotinic receptors (nAChRs) contribute to these seizures. To study potential α7 contributions, we examined α7 subunits with a Leu250-to-Thr substitution in the channel domain, which creates a gain-of-function mutation. Previous studies have shown that mice homozygous for the α7 L250T mutation (T/T) die shortly after birth, but animals heterozygous for the mutation (+/T) are viable and grow to adulthood. Hippocampal neurons from the +/T mice exhibited altered α7-type currents with increased amplitudes and slower desensitization kinetics, confirming a partial gain of function for the α7 nAChR. We found that +/T mice were more sensitive to the convulsant effects of nicotine compared with their wild-type (+/+) littermates. Furthermore, although their behavior was normal in basal conditions, +/T mice showed a unique nicotine-induced phenotype, consisting of head-bobbing and paw-tapping movements. Increased sensitivity to nicotine-induced seizures occurred despite a 60% decline in brain α7 nAChR protein levels. There were no changes in the levels of α4, α5, α6, α7, β2, and β4 mRNA, or in [^{125}I]epibatidine and [^{3}H]nicotine binding between +/T and +/+ mice. Recent data from our laboratory show that α7-null mice maintain normal sensitivity to nicotine-induced seizures. Hence, these present findings suggest that alterations in the properties rather than absence of α7 nAChRs might affect the mechanisms underlying the convulsive properties of nicotine.

The binding of nicotine to neuronal nAChRs in the nervous system produces numerous effects (Stolerman et al., 1995). These effects depend on the species studied and the dose administered. In humans, at doses obtained from cigarette smoking, nicotine can improve cognitive function, increase attention, and reduce fatigue and anxiety (Kassel and Shiffman, 1997; Newhouse et al., 1997; Levin and Rezvani, 2000). In laboratory animals, low doses of nicotine affect nociception (Jurna et al., 1993; Damaj et al., 1998; Marubio et al., 1999), cardiovascular responses (Neff et al., 1998; Marano et al., 1999), locomotor activity (Whiteaker et al., 1995; le Novere et al., 1999), thermoregulation (Lupien and Bray, 1988), and learning, memory, and attention (Levin et al., 1994; Stolerman et al., 2000). High doses of nicotine induce clonic-tonic seizures (Miner et al., 1984; Damaj et al., 1999). Understanding how stimulation of certain nicotinic cholinergic brain circuits leads to seizure becomes particularly significant in light of recent studies linking nAChR subunit genes to several forms of idiopathic epilepsies (Bertrand et al., 1998; Neubauer et al., 1998; Steinlein, 2000; Phillips et al., 2001).

Experiments conducted on several inbred mouse strains have indicated a positive correlation between sensitivity to nicotine-induced seizures and the number of α-bungarotoxin (α-BTX) binding sites in the hippocampus (Miner et al., 1984, 1985; Miner and Collins, 1989), the brain area in which nicotine-induced seizure activity may originate (Stumpf and Gogolak, 1967; Freund and Wehner, 1987). Because α7-containing nicotinic acetylcholine receptors (α7* nAChRs) represent the major binding site for α-BTX in the mammalian brain (Chen and Patrick, 1997; Orr-Urtreger et al., 1997), it was suggested that α7* nAChRs might contribute to the convulsant effects of nicotine. A recent pharmacological investigation into the mechanisms involved in nicotine-induced seizures has helped support this hypothesis (Damaj et al., 1999).

α7-mediated nAChR currents activate very rapidly and

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ABBREVIATIONS: nAChRs, neuronal nicotinic acetylcholine receptors; CNS, central nervous system; DHβE, dihydro-β-erythroidine; Cbt, cobratoxin; BTX, bungarotoxin; GABA, γ-aminobutyric acid; MLA, methyllycaconitine; Ctx, cortex; Hi, hippocampus; Hy, hypothalamus; MHb, medial habenula; SC, superior colliculus; Th, thalamus; VTA, ventral tegmental area; CPu, caudate putamen.

show fast desensitization in the presence of high concentrations of nicotine (Radcliffe and Dani, 1998; Alkondon et al., 2000; Ji et al., 2001). Studies of α7 nAChRs expressed in oocytes have shown that a Leu250-to-Thr substitution (L250T) in the channel domain increases agonist affinity and decreases the rate of desensitization, creating a gain-of-function model for this receptor (Revah et al., 1991; Bertrand et al., 1992; Orr-Urtreger et al., 2000). We have demonstrated that neonatal mice homozygous (T/T) for the α7 L250T nAChR "knock-in" mutation have a lethal phenotype (Orr-Urtreger et al., 2000). Furthermore, hippocampal neurons from T/T mice exhibit α7-type evoked currents that have properties consistent with those observed for α7 L250T nAChRs in oocytes. In contrast, heterozygous (+/T) L250T mice survive to adulthood. These mice express a partial gain of function for the α7 nAChR but are not noticeably affected by this alteration. Because of the partial gain of function imparted by the +/T L250T mutation, it was predicted that +/T mice would exhibit a higher sensitivity to nicotine-induced seizures.

To test this hypothesis, we analyzed the behavioral effects of nicotine on +/T L250T adult mice. We found that despite a 60% decline in α7 nAChR protein levels, +/T mice were more sensitive to nicotine at all doses tested than their wild-type littermates. We have recently demonstrated that mice lacking α7 nAChRs display normal sensitivity to the convulsant effects of nicotine (Franceschini et al., 2001). Taken together, these studies indicate that nicotine-induced seizures involve a complex mechanism of action. Whereas α7 nAChRs may not be required, their enhanced functional activity increases the sensitivity to nicotine-induced seizures.

Materials and Methods

Animals. All mice used in this study were back-crossed onto a C57BL/6 background for six generations. For seizure studies, male and female mice (2–4 months old; $n = 177$ for nicotine; $n = 20$ for dihydro-β-erythroidine) were housed separately in groups of two to five. One day before seizure induction, mice were weighed, marked, and transferred to the experimentation room for acclimation. Adult male mice aged 2 to 4 months were used for all other analyses. All animals had free access to food and water and were maintained on a 12-h light/dark cycle. Animals were genotyped by standard polymerase chain reaction techniques (Orr-Urtreger et al., 2000). The Institutional Animal Care and Use Committee in accordance with federal guidelines approved all procedures.

Behavioral Characterization of α7 +/T Mice in Basal Conditions. α7 +/T and wild-type littermates were evaluated on a battery of behavioral tests to assess several domains of CNS function as described previously (Paylor et al., 1998). Seventeen α7 +/T and 24 α7 +/+ mice were examined on a behavioral battery that included the following tests/assays: 1) neurological screen for simple sensory and motor function; 2) open-field test for exploratory activity and anxiety-related responses; 3) light-dark exploration box for anxiety-related responses; 4) rotarod test for motor coordination and skill learning; 5) acoustic startle response and prepulse inhibition of the startle response; 6) startle habituation; 7) Pavlovian learning using the conditioned fear test; 8) the Morris water task for spatial learning; and 9) hotplate test for analgesia-related responses.

Seizure Testing. Nicotine tartrate (Sigma, St. Louis, MO) was dissolved in physiological saline (0.9% sodium chloride) and administered in a total volume of 10 μl/g of body weight by i.p. injections. Dose groups (0.5–8 mg/kg) were designed so that on any given experimentation day, both a low and a high dose of nicotine were administered to each genotype. After injection, each mouse was placed in a 30 $\times$ 18 cm Plexiglas cage with bedding, and observed by two investigators for 5 min. During this period, animals were continuously evaluated for their response to the nicotine challenge. The experimenters were blind to the genotype of the animals tested. The effects of nicotine were dose-dependent and included sedation, Straub tail, tremors, tachypnea, back arching, rapid movement of the limbs, wild running, loss of righting response, and clonic and tonic seizures. The mice were scored independently by two experimenters on a scale from 0 to 5 (Franceschini et al., 2001). Depending on the symptoms presented the animals were scored as follows: 0 = no visible effect; 1 = sedation; 2 = tachypnea, tremors, or back arching; 3 = rapid movement of the limbs or wild run; 4 = loss of equilibrium with tonic-clonic seizure; and 5 = death. The percentage of mice exhibiting a score of 4 or 5 at each dose was calculated, and dose response curves were constructed. The data were analyzed with the Litchfield-Wilcoxon procedure to determine the confidence limits for the ED_{50}. We also examined the latency to seizure and gender susceptibility. Dihydro-β-erythroidine (DHβE; Sigma, St. Louis, MO) was dissolved in 0.9% saline (5 mg/kg) and administered i.p. Mice were observed as described above by two investigators for 10 min.

Slice Preparation and Electrophysiology. Mice (13–20 days old; $n = 52$) were anesthetized, decapitated, and their brains were immediately removed. Horizontal slices (300 μm thick) of brain were cut in ice-cold cutting solution (220 mM sucrose, 2.5 mM KCl, 30 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 10 mM dextrose, 7 mM $MgCl_2$, and 1 mM $CaCl_2$, oxygenated with 95% O_2 and 5% CO_2), and then transferred into a holding chamber containing the external solution (125 mM NaCl, 2.5 mM KCl, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 25 mM dextrose, 1 mM $MgCl_2$, and 2 mM $CaCl_2$, oxygenated with 95% O_2 and 5% CO_2). After a 30-min recovery at 35°C, slices were maintained at room temperature and were used for recordings within the subsequent 5 h. Neurons were visualized by differential interference contrast microscopy and recorded with standard patch-clamp technique in whole-cell configuration at 32 to 34°C. Atropine (1 μM) was always added to the external solution to block muscarinic receptors during the recordings. The internal solution in the recording pipettes contained the following: 115 mM K-gluconate, 20 mM KCl, 10 mM HEPES, 10 mM EGTA, 4 mM ATP (magnesium salt), 0.3 mM GTP (sodium salt), and 7 mM phosphocreatine, adjusted to pH 7.3 to 7.4 with KOH. Interneurons and pyramidal cells were voltage-clamped at -60 mV and granule cells at -80 mV. Acetylcholine (1 mM), dissolved in external solution, was delivered to the soma of interneurons and granule cells and the dendrites of pyramidal cells by a pressure injection pipette controlled by a Picospritzer (Parker Instrumentation, Chicago, IL). The Picospritzer was used to control both the pressure and the duration of the puff. The puffer pipettes had resistances of about 4 MΩ, and a 30-ms to 1-s puff with 5 to 10 psi was most commonly used. The puffer pipettes were mounted on a motorized manipulator controlled by computer. If leaks of ACh from the pipettes caused desensitization, the pipettes were withdrawn by 100 to 200 μm in between pressure applications. Moments before the application, the motorized drive moved the pipette to its final position (about 50–100 μm from the target area), ACh was puffed, and the motorized manipulator immediately withdrew the puffer pipette. This process was all done automatically by computer while visually observed on a video monitor. All genotypes were determined after collection and interpretation of the electrophysiological results.

Western Blot. Tissue for immunoblotting was processed as described previously (Orr-Urtreger et al., 2000). Briefly, α7 nAChR subunit protein was first concentrated from whole-brain homogenates by incubation with an agarose matrix coupled to α-cobratoxin (α-Cbt). After several washes, bound α7 nAChRs were eluted off the bead matrix with 6$\times$ SDS/sample buffer and the entire sample was loaded onto a 9% SDS-polyacrylamide gel. Because of the small volume of eluted α7 receptors obtained from each brain, protein analyses could not be performed. Instead, each gel was loaded with a known amount ($\approx$ 12 ng) of α7 recombinant protein, which was

determined to approximate the amount of the wild-type α7 nAChR sample loaded. Gels were transferred to nitrocellulose membranes, which were then processed for immunoblotting using an affinity-purified α7 antibody (Chen and Patrick, 1997). Proteins recognized by the antibody were visualized with the use of enhanced chemiluminescence and exposure to BioMax film (Kodak, Rochester, NY). Band intensity was quantified from film exposures in the linear range, as determined from the α7 recombinant protein band. Films showing a large discrepancy in intensity between this band and the wild-type band were not included in the final analysis. All data were examined by one-way analysis of variance, followed by Newman-Keuls post hoc comparisons.

Tissue Preparation and Histology. Mice (n = 15) were decapitated and their brains quickly removed and frozen in isopentane (−30°C, 20 s). Fresh-frozen brains were cryostat cut (20 μm) and mounted onto either gelatin-coated slides (for receptor binding, histochemical, and histological staining) or slides with an additional coating of poly-L-lysine (for in situ hybridization) kept at −20°C. Slide-mounted sections for receptor binding were stored desiccated at −20°C until use. Sections for in situ hybridization, histochemical, and histological staining were postfixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline, pH 7.4, for 1 h at 22°C. Sections were then washed in phosphate-buffered saline, air dried, and stored desiccated at −20°C until use. Slide-mounted tissue sections for histological staining were stained with cresyl-violet. Acetylcholinesterase and cytochrome oxidase histochemistry was performed as described previously (Orr-Urtreger et al., 2000).

In Situ Hybridization. Mouse DNA templates encoding the third intracellular loop of each nAChR subunit analyzed were prepared by reverse transcription-polymerase chain reaction amplification of mRNA from a mouse septal neuroblastoma cell line (SN56). Primers were chosen based on published cDNA sequences of the corresponding rat nAChR subunits. The mouse DNA templates were subcloned into pBluescript SK(−), sequenced and compared with the rat nAChR sequences for verification. The DNA region and size of each template were as reported in (Franceschini et al., 2001). cRNA riboprobes labeled with [^{35}S]UTP (PerkinElmer Life Sciences, Boston, MA) were synthesized from the mouse DNA templates, and postfixed brain sections for in situ hybridization were processed as described elsewhere (Broide et al., 1996). Briefly, slide-mounted sections were first preincubated with 1 μg/ml proteinase K for 10 min at 22°C, and then incubated for 18 h at 60°C with a hybridization solution containing ^{35}S-UTP–labeled cRNA riboprobes (1×10^7 cpm/ml) in the antisense orientation. Adjacent sections were incubated with riboprobes in the sense orientation to define nonspecific hybridization. Brain sections were then incubated with RNase A (20 μg/ml) for 30 min at 37°C, followed by high-stringency washes of decreasing salinity and a 30-min wash in 0.1× sodium chloride/sodium citrate at 60°C. Sections were dehydrated, dried in a stream of cool air, and apposed to β-max film (Amersham Biosciences, Piscataway, NJ) for 1 to 7 days at 4°C.

Receptor Autoradiography. Slide-mounted brain sections for [^{125}I]α-BTX autoradiography were processed as described previously

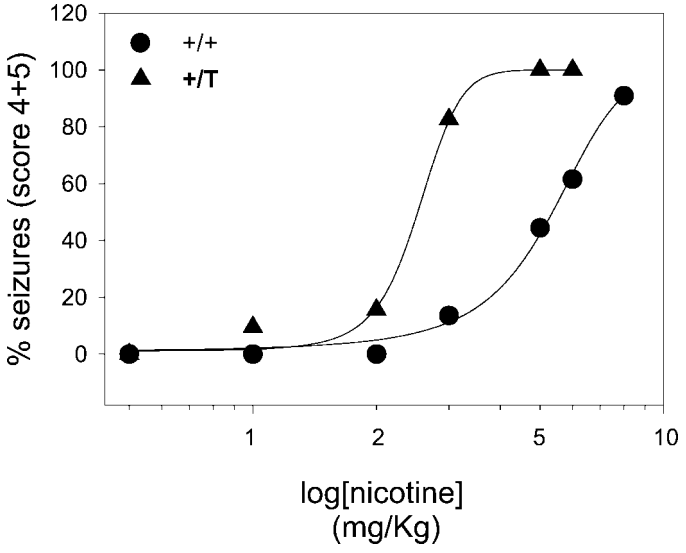

Fig. 1. Dose response curves for the convulsant effects of nicotine. Intraperitoneal injection of nicotine induced seizure activity in wild-type (+/+) and heterozygous (+/T) α7 L250T mice in a dose-dependent fashion. Data represent the percentage of mice tested that underwent seizure and either survived (score 4), or died (score 5). Twelve to 32 mice were tested for each dose.

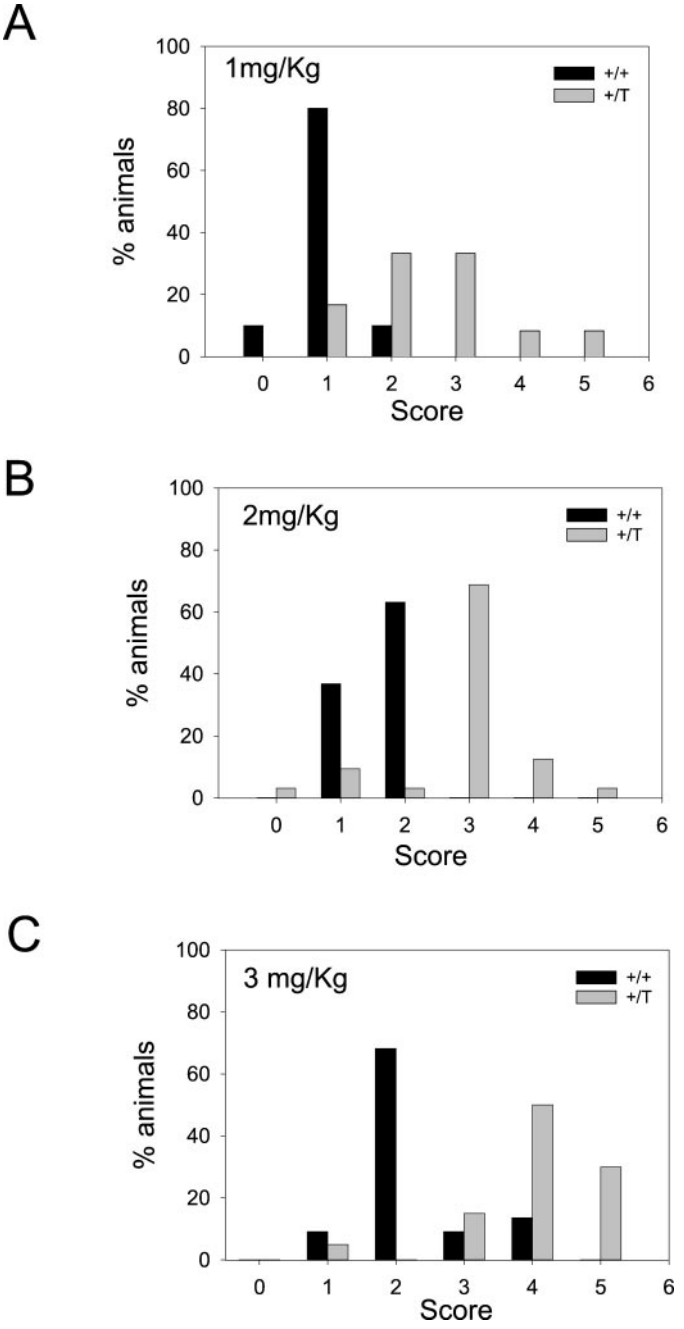

Fig. 2. Increased sensitivity to nicotine in +/T mice. Heterozygous +/T mice showed increased sensitivity to the behavioral effects of nicotine over the whole range of doses tested. Histograms represent the percentage of animals that was assigned a score from 0 to 5 after intraperitoneal injection of nicotine at 1 mg/kg (A), 2 mg/kg (B), and 3 mg/kg (C), respectively.

(Broide et al., 1996). Briefly, sections were incubated at 22°C for 2 h in buffer (50 mM Tris-base, pH 7.4, 120 mM NaCl, and 0.1% bovine serum albumin) containing 5 nM [^{125}I]α-BTX (specific activity, 10 - 20 μCi/μg; PerkinElmer). Nonspecific binding was defined on adjacent sections in the presence of 10 μM α-Cbt). Sections were then washed twice for 10 min each in ice-cold buffer, dipped once in water, air dried, and exposed to β-max film (Amersham) for 3 to 7 days.

Slide-mounted brain sections for [^{125}I]epibatidine autoradiography were incubated at 22°C for 1 h in buffer (50 mM Tris-base, pH 7.4, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, and 1 mM $MgCl_2$) containing 500 pM [^{125}I]epibatidine (specific activity, 2200 Ci/mmol; PerkinElmer). Nonspecific binding was defined on adjacent sections in the presence of 100 μM nicotine. Brain sections were then washed twice for 3 min each in ice-cold buffer, dipped once in water, air dried, and exposed to β-max film (Amersham) for 3 to 12 h.

Slide-mounted brain sections for ^{125}I-nicotine autoradiography were incubated at 22°C for 30 min in buffer (50 mM Tris-base, pH 7.4, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, and 1 mM $MgCl_2$) containing 5 nM [^{3}H]nicotine (specific activity, 69.5 Ci/mmol; PerkinElmer). Nonspecific binding was defined on adjacent sections in the presence of 10 μM nicotine. Brain sections were then washed 4 $\times$ 15 s in ice-cold buffer, dipped once in water, air dried, and exposed to ^{3}H-sensitive HyperFilm (Amersham) for 16 weeks.

Data Analysis and Statistics. X-ray films were analyzed, and quantitation was performed using computer-assisted densitometry with NIH Image software (Bethesda, MD). Relative optical densities from protein bands and discrete brain regions were measured and presented as a percentage of readings from wild-type mice. All autoradiographic data were examined by one-way analysis of variance, followed by Newman-Keuls post hoc comparisons.

Results

Heterozygous L250T Mice Are More Sensitive to Nicotine-Induced Seizures. Intraperitoneal injection of nicotine induced seizures in a dose-dependent fashion in both +/T and +/+ mice (Fig. 1), but the potency of nicotine was significantly higher in the +/T mice (ED_{50} = 2.5 mg/kg, 95% confidence limits: 2.3–2.7 mg/kg) than in their +/+ littermates (ED_{50} = 5.3 mg/kg, 95% confidence limits: 4.4–6.6 mg/kg). The latency to seizure was significantly shorter ($p < 0.01$) in the +/T mice (69.3 $\pm$ 7.6 s) than in their +/+ littermates (118.6 $\pm$ 15.6 s). In addition, the behavioral scores were higher for the +/T mice at each dose of nicotine tested (Fig. 2). No significant difference in seizure sensitivity was observed between male and female mice (data not shown).

Behavioral responses of α7 +/T mice in tests that assess

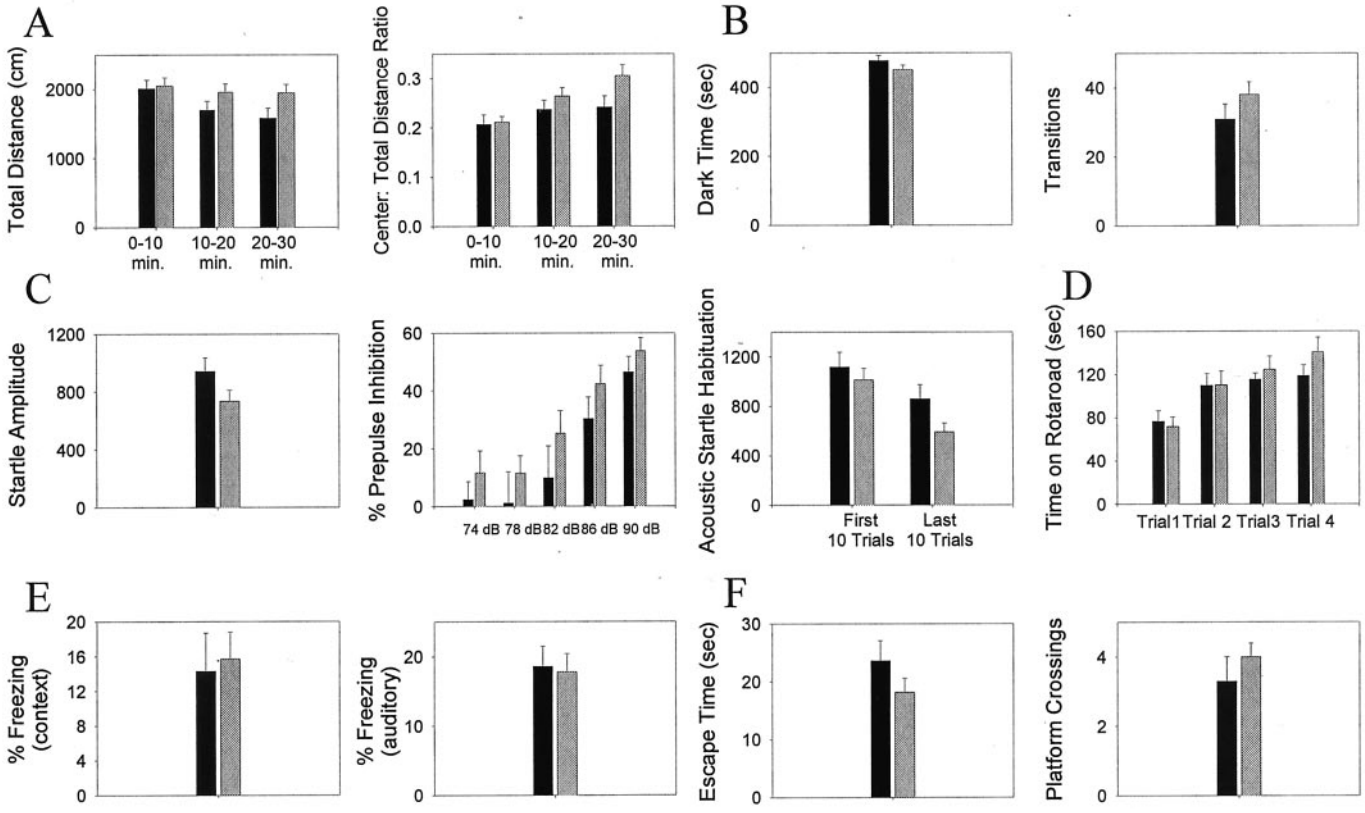

Fig. 3. Behavioral characterization of α7+/T (▨) and α7+/+ (■) mutant mice. A, the total distance (in centimeters) traveled and the center distance to total distance ratio in the open-field arena during the 30-min session was similar for α7+/T and α7+/+ mice. B, the total time (in seconds) mice were in the dark side of the light-dark box and the total number of light-dark transitions were similar for α7+/T and α7+/+ mice during the 10-min light-dark test. C, the maximum startle response (peak amplitude) to a 120-dB startle stimulus and the percentage inhibition of the startle response by different prepulse stimuli (74–90 dB) were similar for α7+/T and α7+/+ mice. D, acoustic startle habituation to repeated presentation (100 total presentation) of the 120 dB stimulus was similar α7+/T and α7+/+ mice. Data presented are the average startle response during the first block of 10 stimuli and the last 10 stimuli. D, α7+/T and α7+/+ mice spent similar amounts of time (in seconds) walking on top of a rotating rod during the four trials of the rotarod test. E, levels of fear as assessed by percentage bouts of freezing during the context test and the auditory CS test. α7+/T and α7+/+ mice displayed similar levels of freezing during both tests. F, time to locate the hidden platform during the hidden platform version of the Morris water test. Data are the escape latency (in seconds) for α7+/T and α7+/+ mice during the last block of four trials. The number of platform crossings in the training quadrant during the 60-sec probe trial given after the last training trial. Data indicate that α7+/T and α7+/+ mice used similar spatially-biased search strategies to locate the hidden platform.

several CNS functions in basal conditions were indistinguishable from those of α7 +/+ mice (Fig. 3). Nevertheless, the +/T mice exhibited a unique nicotine-induced phenotype consisting of two distinct stereotypic movements. The first stereotypic movement was usually apparent within 30 s after injection and involved a continuous side-to-side head movement, referred to as "head bobbing". Head bobbing was observed after injections of low or high doses of nicotine in nearly all +/T mice, but was never detected in +/+ littermates. The second stereotypic movement, which usually followed head bobbing, consisted of a rapid alternate tapping of the forepaws in a crisscross pattern. This tapping movement was best observed at low doses of nicotine (1–3 mg/kg) in +/T mice and would often last for more than 10 to 20 min. A variation of this movement involved a tapping of one forepaw, along with clutching of the other.

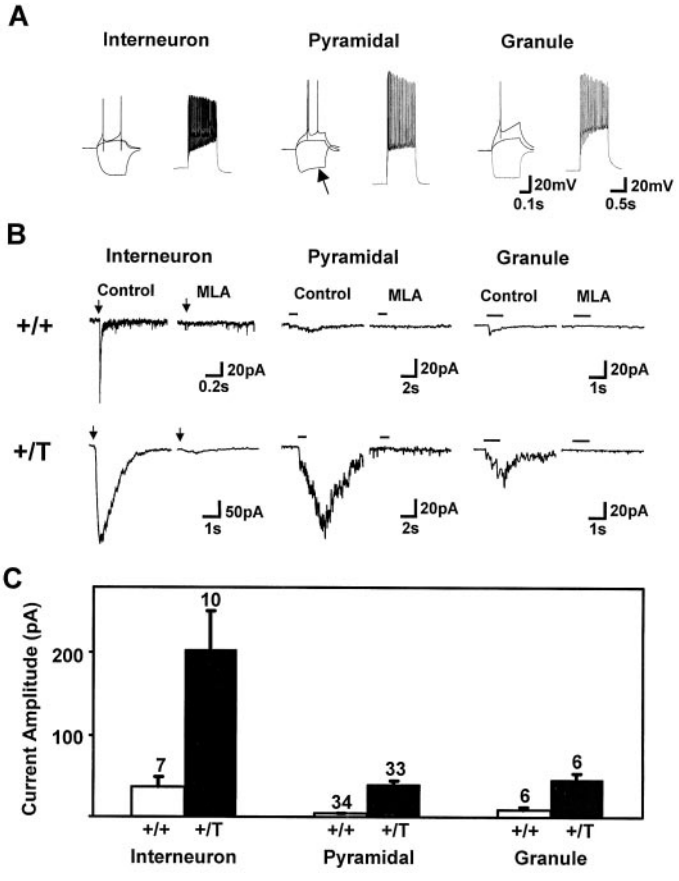

Fig. 4. Nicotinic currents in hippocampal neurons from wild-type (+/+) and heterozygous (+/T) L250T mice. A, electrophysiological characteristics were used to identify the three different cell types. The arrow indicates the voltage "sag" seen with pyramidal neurons in response to injection of hyperpolarizing current. The currents that were injected to elicit the responses were as follows (pA): CA1 interneuron, −50, 10, 20 (left), and 180 (right); CA1 pyramidal cell, −200, 50, 80 (left), 300 (right); granule cell, −150, 20, 50 (left), and 150 (right). Membrane properties were the same for both +/+ and +/T mice. B, ACh-induced currents are shown to be sensitive to the α7-specific inhibitor, MLA (20 nM). The ACh-induced currents were recorded from CA1 interneurons, CA1 pyramidal cells, and granule cells from +/+ and +/T mice as indicated. ACh (1 mM) was delivered to the soma of interneurons for 10 ms (arrows above the traces) and to the dendrites of pyramidal neurons and granule cells for 1 s (black bars above the traces). C, the average current amplitudes from +/+ and +/T mice are compared. The number of neurons that expressed MLA-sensitive nAChR currents is indicated above the data bars.

Heterozygous L250T Mice Display Normal Sensitivity to Dihydro-β-erythroidine. The nicotinic antagonist DHβE has been shown to act as an agonist and evoke ionic currents from α7 L250T homopentameric nAChRs (Bertrand et al., 1992; Orr-Urtreger et al., 2000). However, whether the compound also acts as an agonist at nAChRs containing both wild-type and α7 L250T subunits is unknown. To determine whether DHβE exhibits agonist-like properties at α7 L250T nAChRs in +/T mice by inducing seizures, we injected +/+ (n = 10) and +/T (n = 10) mice i.p. with a single dose of DHβE (5 mg/kg). DHβE did not induce tonic or clonic seizures in the +/T mice, and both groups of mice displayed the same biphasic behavioral pattern. At first, the mice manifested increased locomotor activity, including restless digging and sniffing, along with an increase in whole body tremors. This was usually followed by a second phase, consisting of partial to full sedation, along with occasional dyspnea. Death occurred in 3 of 10 +/T and 5 of 10 +/+ mice. Because the effects of DHβE on +/+ and +/T mice were similar, we did not pursue these experiments further.

Heterozygous L250T Mice Have Altered α7-Type Currents. Hippocampal circuits are thought to participate in the convulsant effects of nicotine (Stumpf and Gogolak, 1967; Freund and Wehner, 1987), and α7* nAChRs are highly expressed throughout the hippocampus (Albuquerque et al., 1997; Radcliffe and Dani, 1998; Ji and Dani, 2000). To examine the potential cellular mechanisms underlying the increased sensitivity of +/T mice to nicotine, we characterized and compared α7-type currents in hippocampal slices from +/T and +/+ mice. Currents were recorded from three different cell types in the hippocampus: CA1 interneurons, pyramidal cells, and granule cells from the dentate gyrus. The cell types were identified by their location and by their membrane properties, which are summarized in Fig. 4A and Table 1. Electrophysiological characteristics of GABA interneurons were as follows (Schwartzkroin and Mathers, 1978; Lacaille et al., 1987; Ji and Dani, 2000): holding potentials more positive than −65 mV, relatively high firing frequency, high input resistance, and no sag in the response to a hyperpolarizing current step. Pyramidal neurons had holding potentials more negative than −65, slower firing rates, lower

TABLE 1

Different membrane properties of CA1 interneurons, CA1 pyramidal neurons, and dentate granule cells

Data are presented as the mean ± S.E.M.

Cell type	n	Vm	Rm	Maximum firing rate	Sag ratio[a]
		mV	*MΩ*	*Hz*	*%*
CA1 Interneuron (a)	7	−62.1 ± 0.8	540 ± 52	89.3 ± 13.3	7 ± 2
CA1 Pyramidal (b)	10	−67.9 ± 0.9	230 ± 22	30.4 ± 3.4	39 ± 4
Dentate granule (c)	6	−82.0 ± 1.3	51 ± 87	32.7 ± 2.8	3 ± 2

[a] The sag ratio was determined by measuring the voltage response to a hyperpolarizing current and then calculating the percent difference in the response at the end of the current injection compared with the maximum response.

input resistance, and a sag in the voltage response to an hyperpolarizing current step (Fig. 4A, arrow). Dentate granule neurons had more negative holding potentials, relatively slow firing rates, and showed no sag after a hyperpolarizing current step.

All three cell types examined expressed ACh-induced nAChR currents that were sensitive to 20 nM MLA, an antagonist of α7* nAChRs (Fig. 4B). Examining the currents suggests kinetic differences in the nAChRs from these cell types. However, these apparent kinetic differences may reflect the experimental conditions and the low density and distribution of nAChRs on pyramidal and granule cells. Agonist applications in the slice are very susceptible to the local tissue density, the spreading of the agonist after application, and the distribution of the receptors. Therefore, these apparent kinetic differences are at least partially produced by the experimental situation, and may not reflect profoundly different nAChR kinetics among the cell types.

Rapid application of 1 mM ACh produced currents that were larger in interneurons than those seen in pyramidal or granule cells (Fig. 4C). To eliminate the possibility that ACh was causing currents indirectly by inducing the release of other neurotransmitters, we inhibited glutamate and GABA receptors. Blockade of glutamatergic and GABA-ergic activity by the addition of 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (20 mM), 2-amino-5-phosphonovalerate (50 mM), and bicuculline (20 mM) to the bathing solution did not significantly change the amplitude of the ACh-induced currents. The α7 nAChR currents in all three cell types increased dramatically by 5- to 10-fold in the +/T mice compared with the corresponding cell type in +/+ neurons (Fig. 4, B and C). Furthermore, the ACh-induced currents lasted longer in the slices from +/T mice, as would be expected with the L250T mutation in the α7 subunit (Revah et al., 1991; Bertrand et al., 1992; Orr-Urtreger et al., 2000).

Heterozygous L250T Mice Show Apparently Normal Neuroanatomical Structure. Brains from +/T mice exhibited normal anatomical structure compared with their +/+ littermates. In the hippocampus, histological analysis using Nissl and acetylcholinesterase-staining revealed normal layering within all subregions (Fig. 5, A-D). We were also interested in determining whether adult +/T mice showed defined barrel structures in their somatosensory cortex because T/T L250T neonatal mice exhibit abnormal development in this region of the brain (Orr-Urtreger et al., 2000). Thus, +/T mice were analyzed for the presence of barrels using cytochrome oxidase histochemistry. Sections through the somatosensory cortex of +/T mice showed characteristic barrel-like structures that were indistinguishable from those in +/+ littermates (Fig. 5E, F).

Heterozygous L250T Mice Have Reduced Levels of α7 nAChRs. Previous results have shown a decrease in [^{125}I]α-BTX binding and α7 nAChR protein levels in brains of neonatal +/T and T/T mice (Orr-Urtreger et al., 2000). To determine whether a similar reduction occurred in adult +/T mice, we examined and compared the distributions of α7 nAChR mRNA and [^{125}I]α-BTX binding sites in the brains of +/+ and +/T mice, using in situ hybridization and receptor autoradiography. Strong labeling for α7 mRNA and [^{125}I]α-BTX binding was observed in such regions as the hippocampus (Hi), hypothalamus (Hy), amygdala (Fig. 6), and the superior (SC) and inferior colliculus (IC) (data not shown). More moderate levels were detected in the cortex (Ctx) and caudate putamen (CPu). Low levels of mRNA and binding were found throughout most of the thalamus (Th). We observed a similar pattern of α7 mRNA and [^{125}I]α-BTX binding distribution in the brains of +/+ and +/T mice (Fig. 6A, B).

Mean levels of specific α7 mRNA expression and α-BTX binding site density in the cortex, CA1, and DG regions of the Hi, CPu, and the SC were determined by quantitative analysis of autoradiographic images of brain sections. As shown in Table 2, the levels of α7 mRNA were not significantly different between +/T mice and +/+ littermates within any of the regions examined. However, a significant reduction in [^{125}I]α-BTX binding was observed throughout +/T brains compared with +/+ littermates (Fig. 6, A and B; Table 2). Levels of [^{125}I]α-BTX binding were reduced by approximately 50% in the Ctx, CA1, and DG, about 35% in the SC, and by as much as 65% in the CPu of +/T mouse brains ($P < 0.001$) compared with +/+ littermates (Table 2). Levels of hybridization with the sense probe, and [^{125}I]α-BTX binding in the presence of 1 μM α-Cbt were low and similar to background levels (data not shown).

To determine whether the decrease in [^{125}I]α-BTX binding sites in +/T mouse brains was due to a reduction in α7 nAChR subunit protein levels, we performed Western blot analysis. Immunoblots of protein extracts prepared by α-Cbt affinity chromatography from +/+ and +/T mouse brains were probed with antibodies to the N-terminal portion of the α7 subunit (Chen and Patrick, 1997). As demonstrated in Fig. 5C, a major immunoreactive protein band of 57 kDa was

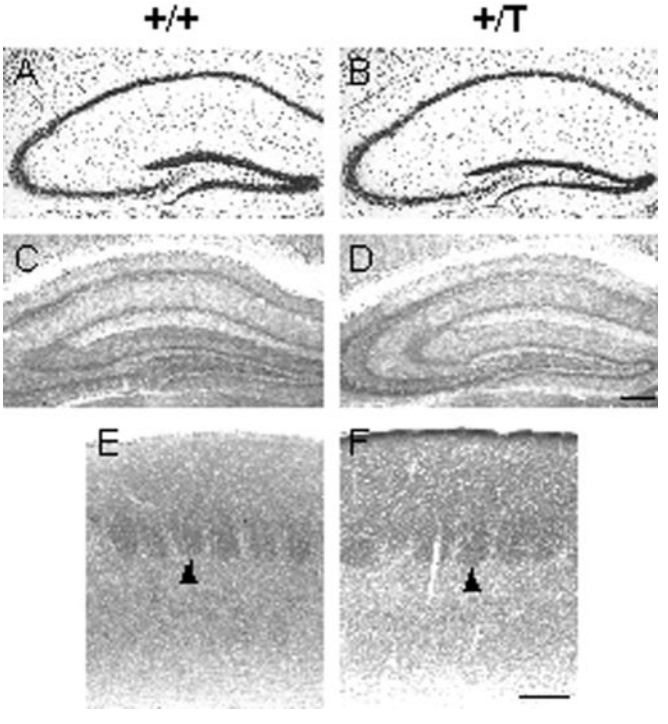

Fig. 5. Heterozygous L250T mice exhibit normal neuroanatomy. A and B, photomicrographs of transverse brain sections magnified to show the hippocampus of wild-type (+/+) and heterozygous (+/T) L250T mice. Sections were histologically stained with cresyl-violet. C and D, adjacent sections histochemically stained for acetylcholinesterase. E and F, magnified brain sections depicting the primary somatosensory cortex were histochemically stained for cytochrome oxidase. Arrowheads point to whisker barrels in layer IV of the cortex. Scale bars, 100 μm.

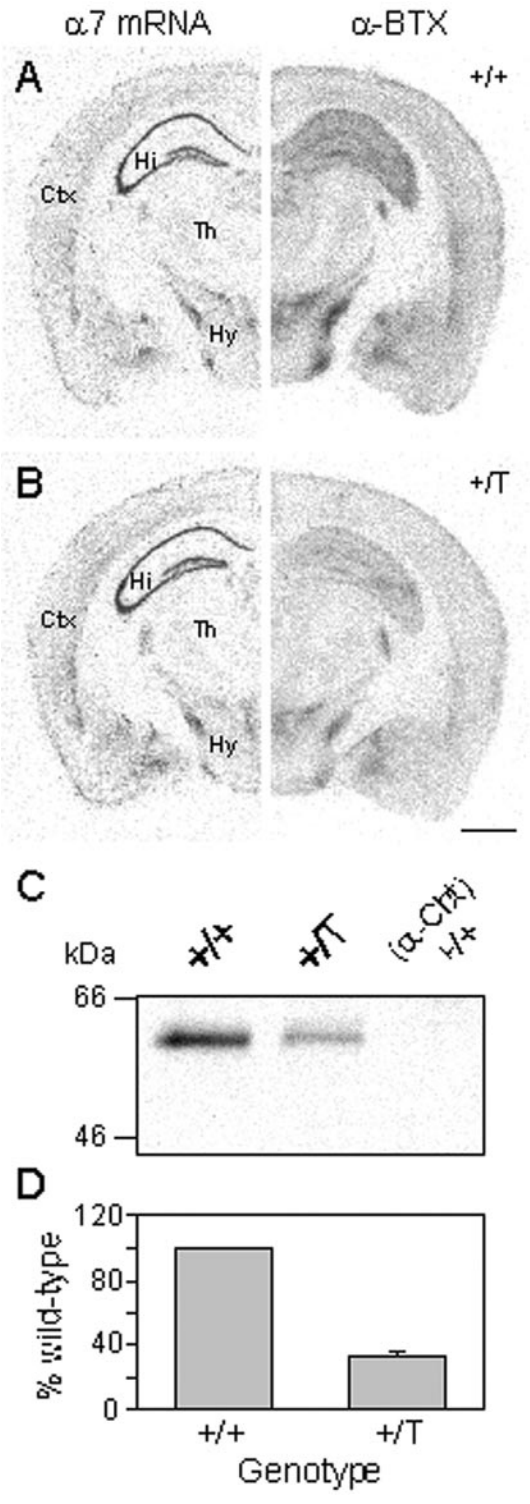

Fig. 6. Heterozygous L250T mouse brains show reduced levels of α7 nAChRs. A and B, autoradiographic images of adjacent transverse brain sections at the level of the hippocampus from wild-type (+/+) and heterozygous (+/T) L250T mice. Sections show the distributions of α7 nAChR mRNA (α7 mRNA) and [^{125}I]α-BTX binding sites (α-BTX). Ctx, cortex; Hi, hippocampus; Hy, hypothalamus; Th, thalamus. Scale bar, 1 mm. C, Western blot analysis of α7 nAChR proteins purified from brains of +/+ and +/T mice. As a control, the binding of proteins to the affinity column was blocked by preincubation with α-cobratoxin (α-Cbt). Protein size markers are indicated. D, densitometric analysis of Western blots showing levels of α7-subunit protein expression from +/T animals as a percentage of the +/+ controls. Data represent the mean ± S.E.M. for four animals each.

detected in extracts from both genotypes and was absent when protein extracts were preincubated with α-Cbt. Quantitative densitometry of the immunoreactive 57-kDa bands demonstrated that α7 nAChR subunit protein levels were reduced by approximately 65% in the brains of +/T mice compared with their +/+ littermates (Fig. 6D).

Heterozygous L250T Mice Have Normal Levels of Non-α7 nAChR Subtypes. Recent studies have suggested that in addition to α7, the α4, α5, and α6 nAChR subunits may play a role in nicotine-induced seizures (Stitzel et al., 1998, 2000). Hence, the increased sensitivity to nicotine in the +/T mice might depend not only on the α7 L250T mutation, but also on changes in expression levels of other nAChR subunits. To test this hypothesis, we performed in situ hybridization experiments to examine the mRNA distribution patterns and levels for the α4, α5, α6, β2, and β4 nAChR subunits (Fig. 7). Levels of α4 mRNA expression were highest throughout the Th, medial habenula (MHb), SN, and ventral tegmental area (VTA), with low to moderate mRNA levels observed in the Ctx, Hi, and Hy. A strong hybridization signal for α5 mRNA was detected in the interpenduncular nucleus (IPn), with more moderate mRNA levels observed in the Ctx, Hi, SN, and VTA. High levels of α6 mRNA expression were seen in the SN and VTA, with moderate mRNA levels in the SC. The strongest signal for β2 mRNA was found throughout the Th, Hi, and MHb, with moderate mRNA levels observed in the Ctx, CPu, SN, and olfactory bulb. Expression of β4 mRNA was restricted to the MHb, IPn, and olfactory bulb. There were no statistically significant differences between +/T and +/+ mice in the relative abundance of α4, α5, α6, β2, and β4 subunit transcripts (Table 2).

Finally, to examine the levels of nonα7 nAChR subtypes in α7 +/T mice, we performed receptor autoradiography using 500 pM [^{125}I]epibatidine. This concentration of epibatidine has been demonstrated to bind with equal affinity to two major nAChR subtypes, probably composed of α3, α4, β2, and β4 subunits (Zoli et al., 1998; Whiteaker et al., 2000). High levels of [^{125}I]epibatidine binding were observed throughout the Th, SC, MHb, and IPn of +/+ and +/T mouse brains, with more moderate levels in the Ctx and CPu (Fig. 8A-D). Low levels of binding were found in the Hp. As with subunit mRNA expression levels, no significant difference in the distribution or levels of [^{125}I]epibatidine binding was demonstrated between +/T and +/+ mouse brains (Fig. 8A-D; Table 2). In addition, no changes were observed in the binding of [^{3}H] nicotine to brain slices from α7 +/T and +/+ mice (Fig. 8, E-H)

Discussion

The present study demonstrates that mice heterozygous for the α7 L250T nAChR mutation have a 2-fold higher sensitivity to nicotine-induced seizures than their +/+ littermates. Such increased sensitivity was evident for all behavioral effects of nicotine; +/T mice received higher scores at all nicotine doses tested. In both oocytes and hippocampal cultures, L250T receptors have been shown to have higher affinity for agonists and reduced rates of desensitization (Revah et al., 1991; Orr-Urtreger et al., 2000). We now show that neurons in hippocampal slices from +/T mice express α7-type currents with qualitative properties similar to those found in tissue culture. Because +/T α7-type currents display an in-

termediate profile between +/+ and T/T currents, it seems that +/T mice maintain a partial gain of function for the α7 nAChR compared with T/T mice (Orr-Urtreger et al., 2000). This hypothesis is supported by the increased sensitivity of +/T mice to nicotine compared with that of their +/+ littermates.

The gain of function in +/T mice is also suggested by the reduction in α7 nAChR expression that was observed in comparison to their +/+ littermates. A negative feedback regulatory response may compensate for the increase in α7 nAChR activity within L250T mutant mice by altering protein synthesis. However, this reduction in α7 expression is only partial (≈50 to 65% reduction) compared with the reduction (≈80 to 90% reduction) in T/T mice (Orr-Urtreger et al., 2000) or −/T L250T mice (Broide et al., 2001). This result is most likely to arise because of the presence of wild-type subunits in +/T mice that are lacking in T/T and −/T mice. Both the T/T and the −/T phenotypes are lethal, whereas the presence of a wild-type allele in the +/T mice seems to provide a protective mechanism for these animals (Broide et al., 2001). The expression of wild-type subunits in +/T L250T receptors also confers an intermediate pharmacological profile compared with T/T receptors. In fact, we could not elicit seizures after injections of DHβE, an agonist at α7 L250T homopentameric nAChRs. Our in vivo results thus confirm previous in vitro experiments in which DHβE evoked currents from T/T hippocampal cultures but not from +/T cultures (Orr-Urtreger et al., 2000).

Role of α7 nAChRs in Nicotine-Induced Seizures. The results of this study show that α7 nAChRs are capable of influencing nicotine-induced seizures. We have previously demonstrated, however, that mice lacking α7 nAChRs show normal sensitivity to nicotine (Franceschini et al., 2001). One possible way to interpret these findings is to consider the desensitization properties of α7 nAChRs. At lower nicotine concentrations, like those found in smokers, the majority of α7 nAChRs are most probably not desensitized. This has recently been demonstrated for α7 nAChRs expressed in both *Xenopus laevis* oocytes and on rat hippocampal neurons (Alkondon et al., 2000; Fenster et al., 1997). At the high nicotine concentrations required to elicit seizures, α7 nAChRs may exist in a desensitized state (Briggs and McKenna, 1998; Alkondon et al., 2000). Hence, wild-type α7 nAChRs would most probably be inactive during nicotine-induced seizures (Franceschini et al., 2001). Conversely, α7 nAChRs in the +/T mice might still be active in the presence of high doses of nicotine because of the slower desensitization kinetics. Activation of α7 L250T nAChRs might therefore amplify or modify the cellular mechanisms served by α7 nAChRs. This would most probably alter the balance between inhibitory and excitatory circuits in the hippocampus (Ji and Dani, 2000), resulting in a net excitation and increased sensitivity to nicotine-induced seizures.

Recent investigations on gene polymorphisms of inbred mouse strains with different sensitivity to nicotine have identified yet other nAChR subunits (α4, α5, and α6) that may play an important role in regulating the sensitivity to nicotine (Stitzel et al., 1998, 2000). Therefore, another possible interpretation of our data is that +/T mice are more sensitive to nicotine because of changes in the expression levels of other, more relevant neuronal nAChR subtypes as a result of the expression of α7 L250T mutant nAChRs. However, we failed to find any difference in the mRNA expression patterns or levels of the α4, α5, α6, β2, and β4 nAChR subunits in the

TABLE 2

Density of [^{125}I]α-bungarotoxin and [^{125}I]epibatidine binding, and α4, α5, α6, α7, β2, and β4 mRNA expression in various regions of wild-type (+/+), and heterozygous (+/T) mouse brains

Data represent the mean ± S.E.M. for three to five animals and are presented as a percentage of wild-type controls.

Label	Wild-Type									
	Cortex	Hippocampus		DG	Caudate Putamen	Thalamus	Medial Habenula	IPn	Substantia Nigra	Superior Colliculus
		CA1	CA2							
	%									
[^{125}I]α-BTX										
+/+	100 ± 7.2	100 ± 6.2		100 ± 8.9	100 ± 7.5					100 ± 4.2
+/T	56 ± 4.6[a]	54.7 ± 2.8[a]		55.2 ± 4.9[a]	32.9 ± 3.3[a]					65.7 ± 3.7[a]
[^{125}I]Epi										
+/+	100 ± 6.0				100 ± 2.5	100 ± 4.6	100 ± 2.7	100 ± 5.6	100 ± 6.1	100 ± 4.3
+/T	99.0 ± 6.5				96.9 ± 7.4	99.6 ± 2.3	99.1 ± 2.7	95.9 ± 1.6	107.5 ± 5.9	106.7 ± 1.4
α4 mRNA										
+/+	100 ± 3.9	100 ± 5.6	100 ± 9.2		100 ± 7.7	100 ± 4.9	100 ± 0.8		100 ± 0.9	100 ± 3.5
+/−	102 ± 5.9	113 ± 9.2	98 ± 12.5		109 ± 17.4	103 ± 3.0	91 ± 3.5		95 ± 3.8	94 ± 7.3
α5 mRNA										
+/+	100 ± 6.2	100 ± 7.0					100 ± 6.4	100 ± 6.8	100 ± 6.3	
+/T	119.7 ± 8.7	98.7 ± 2.4					86.6 ± 11.5	102.4 ± 5.3	102.6 ± 5.9	
α6 mRNA										
+/+									100 ± 1.3	100 ± 5.7
+/T									103 ± 4.5	98 ± 9.7
α7 mRNA										
+/+	100 ± 6.8	100 ± 3.8	100 ± 2.4	100 ± 4.3	100 ± 9.5					100 ± 6.6
+/T	97 ± 8.4	101 ± 3.7	104 ± 3.7	109 ± 5.9	109 ± 11.4					97 ± 8.7
β2 mRNA										
+/+	100 ± 5.4	100 ± 10.0	100 ± 7.5	100 ± 4.4		100 ± 2.5	100 ± 2.1		100 ± 3.0	100 ± 2.8
+/T	82.2 ± 8.4	99.2 ± 13.8	98.1 ± 8.7	100.7 ± 5.3		82.5 ± 8.3	87.0 ± 6.6		88.2 ± 7.3	82.8 ± 10.5
β4 mRNA										
+/+							100 ± 2.1			
+/T							94 ± 3.5			

[a] $P < 0.01$; significantly different from wild-type (+/+) by Newman-Keuls test.

brains of +/T mice compared with their +/+ littermates. Furthermore, we found no difference in the expression patterns or levels of [^{125}I]epibatidine and [^{3}H]nicotine binding to non–α7 nAChR subtypes between +/T and +/+ mice. These observations suggest that the other nAChR subtypes found in the brain are not affected by and probably do not contribute to the increased nicotine sensitivity of +/T mutant mice.

α7 nAChRs in the Hippocampus. Numerous electrophysiological studies have demonstrated the functional expression of α7* nAChRs in the hippocampus. These receptors have been found on presynaptic terminals of pyramidal neurons (Gray et al., 1996) and postsynaptic regions of interneurons (Alkondon et al., 1997; Ji and Dani, 2000), where they regulate neurotransmitter release. Recently, α7 nAChR-mediated currents have been detected on other hippocampal cell types, where they can alter circuit excitability and influence synaptic plasticity (Ji and Dani, 2001). In the present study, we were able to detect α7-type nAChR currents from CA1 interneurons and pyramidal cells as well as dentate gyrus granule cells of +/T hippocampal slices. Furthermore, these currents were much larger in amplitude, with slower desensitization kinetics than the corresponding +/+ α7 currents. These data help to confirm the expression of α7 nAChRs on different cell types in the hippocampus, where they probably modulate both excitatory and inhibitory mechanisms.

Heterozygous L250T Mice Display a Unique Nicotine-Induced Phenotype. Mutant +/T mice were examined in a battery of behavioral tests aimed at assessing several CNS functions. The behavior of +/+ and +/T mice was indistinguishable in basal conditions, but in the presence of nicotine the +/T mice displayed a unique nicotine-induced phenotype. This phenotype was particularly evident at lower doses of nicotine and consisted of head-bobbing and paw-tapping movements. The stereotypic movements sometimes

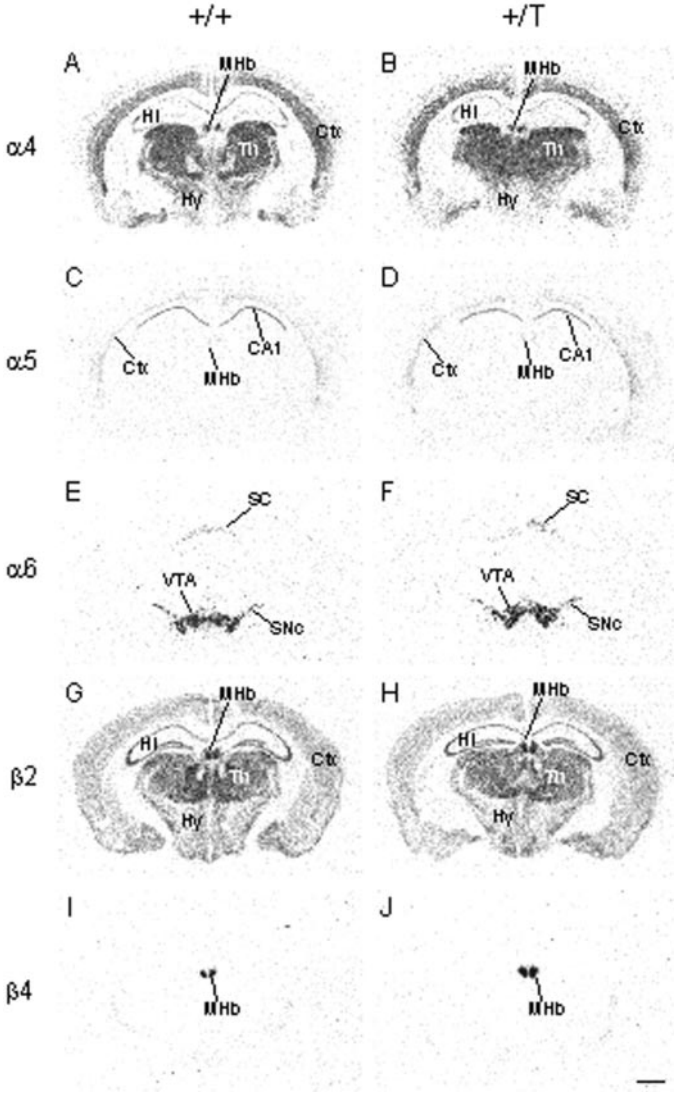

Fig. 7. Heterozygous L250T mouse brains show normal mRNA levels of non-α7 nAChR subunits. Autoradiographic images of adjacent transverse brain sections at the level of the hippocampus and substantia nigra from wild-type (+/+) and heterozygous (+/T) L250T mice. Sections show the distributions of mRNA transcripts for the α4 (A, B), α5 (C, D), α6 (E, F), β2 (G, H), and β4 (I, J) nAChR subunits. Ctx, cortex; Hi, hippocampus; Hy, hypothalamus; MHb, medial habenula; SC, superior colliculus; SNc, substantia nigra compacta; Th, thalamus; VTA, ventral tegmental area. Scale bar, 1 mm.

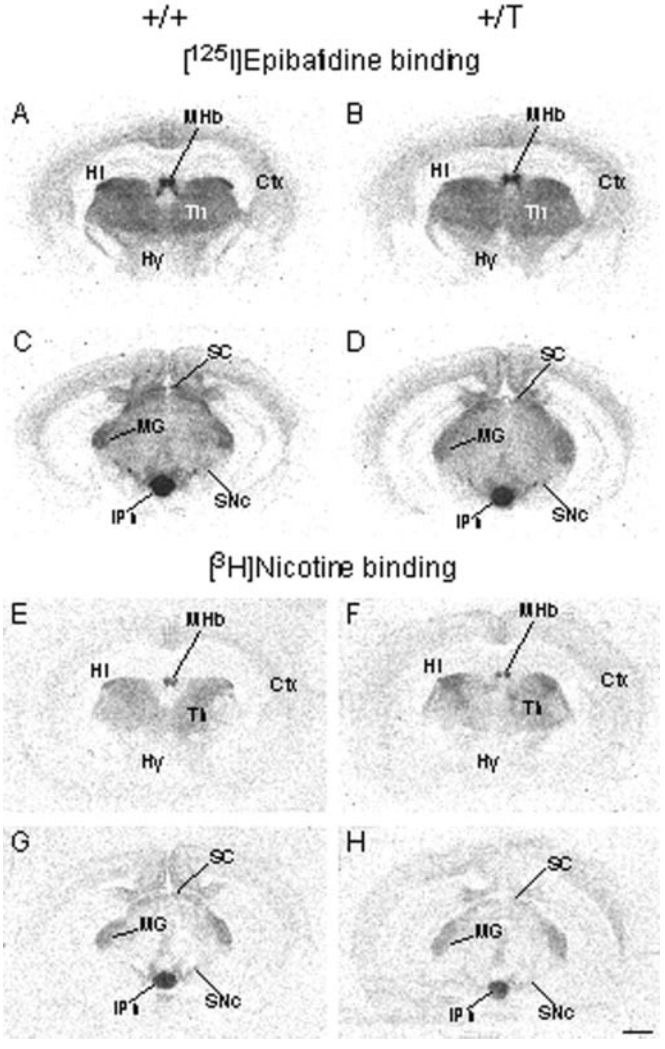

Fig. 8. Heterozygous L250T mouse brains show normal levels of [^{125}I]epibatidine and [^{3}H]nicotine binding. Autoradiographic images of adjacent transverse brain sections at the level of the hippocampus (A, B, E, F) and substantia nigra (C, D, G, H) from wild-type (+/+) and heterozygous (+/T) L250T mice. Sections show the distributions of [^{125}I]epibatidine (A-D) and [^{3}H]nicotine (E-H) binding sites. Ctx, cortex; Hi, hippocampus; Hy, hypothalamus; IPn, interpenduncular nucleus; MG, medial geniculate; MHb, medial habenula; SC, superior colliculus; SNc, substantia nigra compacta; Th, thalamus. Scale bar, 1 mm.

lasted for long periods of time, even after the animal was returned to its home cage. This finding may suggest a role for α7 nAChRs in locomotor activity. Increasing evidence indicates that nicotine stimulates locomotor activity in animals by acting on nAChRs found along the mesostriatal dopaminergic pathway (Whiteaker et al., 1995; Louis and Clarke, 1998; Wonnacott et al., 2000). Although studies have demonstrated the involvement of α6 nAChR subunits in nicotine-elicited locomotion (le Novere et al., 1999), the role of α7 nAChRs seems to be negligible (Grottick et al., 2000; Kempsill and Pratt, 2000). Thus, activation of α7 L250T nAChRs in +/T mouse brains may trigger unique mechanisms that help augment the role of these receptors in the mesostriatal pathway. Identification of these mechanisms may lead to a better understanding of the normal physiological function of α7 nAChRs.

In conclusion, our data suggest that mutations that modify the biophysical and pharmacological properties of α7* nAChRs at the cellular level, such as the α7 L250T mutation, may also modify brain responses in vivo. The +/T mice become more sensitive to nicotine-induced seizures, and they exhibit novel behavioral responses when exposed to lower doses of nicotine. The absence of α7* nAChRs, in contrast, does not modify the sensitivity to nicotine-induced seizures and does not alter behavioral responses to nicotine (Franceschini et al., 2001). These observations could be relevant for human pathologies, such as idiopathic epilepsies in which mutations of nAChR subunits underlie the genetic cause (Neubauer et al., 1998; Steinlein, 2000; Phillips et al., 2001). A recent investigation of a mouse model possessing a gain-of-function mutation in the α4 nAChR subunit has also reported altered brain function and behavior (Labarca et al., 2001). Therefore, animals bearing nAChR point mutations, such as the α7 L250T mice, represent an important tool for the investigation of brain nicotinic cholinergic mechanisms.

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Address correspondence to: Dr. Mariella De Biasi, Division of Neuroscience, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. E-mail: debiasi@bcm.tmc.edu