Effects of 6-Cyano-7-nitroquinoxaline-2,3-dione on Nicotinic Receptor Subunit Transcript Expression in the Rat Brain

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ABSTRACT   The nicotinic cholinergic system exerts potent modulatory effects on glutamatergic neurotransmission, an effect mediated in part by increased glutamate release following activation of presynaptic nicotinic cholinergic receptors. Ionotropic glutamate receptor agonists also stimulate release of acetylcholine, suggesting that these neurotransmitter systems reciprocally regulate one another. We investigated an interface between the nicotinic cholinergic and glutamatergic systems by measuring nicotinic receptor subunit transcript expression following administration of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an antagonist of the AMPA and kainate subtypes of glutamate receptors. Using [^{35}S] in situ hybridization, we measured expression of $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$, $\beta 3$, and $\beta 4$ nicotinic receptor subunit transcripts in the rat forebrain. Following 7 days of treatment with vehicle or CNQX (1 mg/kg/day or 10 mg/kg/day), changes in nicotinic receptor subunit transcript expression were restricted to subunits that form heteromeric receptors. We found increased levels of transcripts for $\alpha 2$ and $\beta 2$ nicotinic receptor subunits in the hippocampus, decreased $\alpha 4$ subunit transcripts in the medial habenula and amygdala, and increased $\beta 2$ subunit transcripts in the septum and piriform cortex. We did not detect changes in expression of transcripts for the $\alpha 7$ subunit, which forms homomeric nicotinic receptors. Our findings indicate that expression of nicotinic cholinergic receptor subunit transcripts are regulated in a subunit- and region-specific fashion by CNQX, an antagonist of non-NMDA ionotropic glutamate receptors. **Synapse 52:62–72, 2004.**   © 2004 Wiley-Liss, Inc.

INTRODUCTION

Multiple studies have shown increased release of glutamate, GABA, and other neurotransmitters following presynaptic activation of nicotinic cholinergic receptors, contributing to a growing literature detailing the potent modulatory effects of the nicotinic cholinergic system (NCS) in the brain (McGehee et al., 1995; Lendvai et al., 1996; Alkondon et al., 1997; Aramakis and Metherate, 1998; Gioanni et al., 1999; Grillner and Svensson, 2000; Reuben et al., 2000; Alkondon and Albuquerque, 2001). The neuronal nicotinic cholinergic receptor (nAChR) is a ligand-gated ion channel abundantly expressed in cortical and subcortical structures (Itier and Bertrand, 2001). The native ligand for nAChRs, acetylcholine (ACh), is released from a relatively small number of cholinergic neurons that broadly innervate most areas of the brain (McGehee and Role, 1995; Dani, 2001). Nicotinic receptors are comprised of assemblies of subunits encoded by specific genes, $\alpha 2$–$\alpha 10$, and $\beta 2$–$\beta 4$ (Itier and Bertrand, 2001). These subunits can form either heteromeric ($\alpha 2$–$\alpha 6$, $\beta 2$–$\beta 4$) or homomeric ($\alpha 7$–$\alpha 10$) receptors (Picciotto et al., 2001). Several nicotinic receptors with specific combinations of subunits have been identified and characterized. The high-affinity [^{3}H]-nicotine binding site that accounts for 90% of the nicotinic sites in the brain is a pentamer comprised of obligate $\beta 2$ and $\alpha 4$ subunits in combination with $\alpha 5$, $\alpha 6$, $\beta 3$, and/or $\beta 4$ (Gotti et al., 1997; Court et al., 2000; Picciotto et al., 2001). Nicotinic receptors containing an $\alpha 3$ subunit are associated with

binding to neuronal bungarotoxin. The $\alpha7$, $\alpha8$, and $\alpha9$ subunits form pentameric homomers that bind α-bungarotoxin. Neuronal nicotinic receptors permit calcium entry into the cell following binding by two ACh molecules to a site associated with the α subunits. Nicotinic receptors desensitize following exposure to agonist, an effect whose duration depends on receptor subunit composition (Itier and Bertrand, 2001; Picciotto et al., 2001). The high-affinity [³H]-nicotine binding sites are highly expressed in the striatum and substantia nigra, with lower levels of expression in the neocortex and hippocampus (Court et al., 2000). Neuronal bungarotoxin sites are highly expressed in the hippocampus and neocortex, while α-bungarotoxin sites are expressed in the hippocampus, midbrain, neocortex, and striatum (Court et al., 2000).

A number of studies have examined interactions between the nicotinic and glutamatergic neurotransmitter systems (Ramoa et al., 1990; Rasmusson et al., 1996; Shoaib et al., 1997). Administration of nicotine to slices of rat auditory cortex enhanced NMDA-receptor mediated excitatory postsynaptic potentials, while intracerebral microdialysis experiments demonstrated increased synaptic glutamate levels following application of nicotine (Aramakis and Metherate, 1998; Gioanni et al., 1999). Other work has demonstrated increased hippocampal ACh release following application of glutamate receptor agonists by retrograde microdialysis into the medial septum or the hippocampus (Moor et al., 1996). Similar effects on ACh release were reported in the striatum, suggesting that glutamate receptors can modulate activation of the NCS (Ikarashi et al., 1998; Knauber et al., 1999). These data suggest that the nicotinic cholinergic and glutamatergic neurotransmitter systems are integrated and reciprocally regulate one another.

Glutamatergic neurotransmission involves presynaptic release of glutamate, activation of pre- and postsynaptic glutamate receptors, and tightly regulated reuptake of glutamate from the synaptic cleft (Danbolt, 2001). Glutamate receptor subtypes include three families of pharmacologically distinct ligand-gated ion channels (N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), and kainate (KA) receptors) as well as the G-protein-coupled metabotropic receptors (mGluR1–mGluR8) (Nakanishi, 1992; Hollmann and Heinemann, 1994; Bleakman and Lodge, 1998). NMDA receptor-mediated depolarization of the postsynaptic neuron is preceded by localized depolarization via activation of AMPA receptors. This localized depolarization permits removal of Mg^{2+}-mediated open channel blockade of NMDA receptors. The presynaptically localized KA receptors contribute to regulation of synaptic function by modulating neuronal glutamate release and astrocytic glutamate reuptake (Claudio et al., 2000; Delaney and Jahr, 2002).

The glutamate neurotransmitter system interacts with other neurotransmitters throughout the brain. Many of these interactions are mediated by the activities of AMPA and KA receptors. For example, AMPA receptor antagonists mediate region- and circuit-specific release of ACh, dopamine, and serotonin, while activation of serotonin or dopamine receptor subtypes modulate AMPA receptor subunit expression (Naudon et al., 1992; Meador-Woodruff et al., 1996; Moor et al., 1996; Martin-Ruiz et al., 2001; Cai et al., 2002; Chao et al., 2002; Wu et al., 2002; Ghersi et al., 2003). The effects of AMPA and KA receptor modulation on expression of nicotinic receptor expression have not previously been reported. The purpose of this study was to investigate the modulatory effects of AMPA and KA receptors on the nicotinic cholinergic system. We utilized CNQX, an AMPA and KA receptor antagonist, to investigate the regulation of brain nicotinic receptor subunit transcript expression by the AMPA and KA receptors.

Abbreviations

ACh	acetylcholine
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AMY	amygdala
Cg	cingulate cortex
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
DG	dentate gyrus
Fr	frontal cortex
GABA	gamma-aminobutyric acid
HT	hypothalamus
HPC	hippocampus
KA	kainate
MHN	medial habenula
nAChR	nicotinic cholinergic receptor
NCS	nicotinic cholinergic system
NMDA	N-methyl-D-aspartate
OT	olfactory tubercle
Par	parietal cortex
PCR	polymerase chain reaction
Pir	piriform cortex
SEP	septum
Te	temporal cortex
THA	thalamus

MATERIALS AND METHODS
Animals and tissue preparation

Three groups of 10 adult, male Sprague-Dawley rats (250 g) were treated with daily subcutaneous injections of CNQX (1 or 10 mg/kg) or vehicle (DMSO) for 7 days. These doses were selected based on previous studies that have demonstrated biochemical or behavioral changes following subchronic CNQX treatment (Healy and Meador-Woodruff, 1999; Mead and Stephens, 1999). Twenty-four hours after the last injection, brains from sacrificed animals were removed, rapidly frozen in isopentane (–30°C), and stored at –80°C until sectioned. Each brain was thawed for 30 min to a temperature of –20°C and mounted for cryostat sectioning. Ten-μm sections were mounted on polylysine-subbed microscope slides, desiccated overnight at 4°C, and stored at –80°C.

In situ hybridization

Primers for polymerase chain reaction (PCR) amplification were designed for each cholinergic receptor subunit of interest using Primer3 (http://waldo.wi.mit.edu/c/s.dll/primer/primer3) and AMPLIFY (http://www.wisc.edu/genetics/CATG/amplify/index.html; Bill Engels, Madison, WI). Unique areas of the rat $\alpha 2$ (GenBank accession #L10077, coding region 1321–1718), $\alpha 3$ (X03440, 1223–1742), $\alpha 4$ (L31620, 1114–1642), $\alpha 5$ (NM_017978, 447–906), $\alpha 7$ (NM_012832, 125–624), $\beta 2$ (U42976, 147–562), $\beta 3$ (J04636, 1153–1632), and $\beta 4$ (L31622, 1251–1747) subunits were targeted for amplification from full-length rat clones of each subunit (generously provided by Dr. Daniel Goldman, University of Michigan, Ann Arbor, MI). Due to the occurrence of significant regions of homology between sequences for the $\alpha 2$, $\alpha 4$, and $\beta 2$ subunits, we performed two-way BLAST analysis of the full-length cDNAs to confirm that our probes were designed to specifically recognize their intended targets. Following amplification, DNA was extracted and purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), subcloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, CA), and sequenced (Thermo Sequenase Radiolabeled Termination Cycle Sequencing Kit, USB, Cleveland, OH). For riboprobe synthesis, 10 μl of [^{35}S]-UTP was dried and 2.0 μl 5× transcription buffer, 1.0 μl 0.1M DTT, 1.0 μl each of 10 mM ATP, CTP, and GTP, 2.0 μl linearized plasmid DNA, 0.5 μl RNase inhibitor, and 1.5 μl T3 or T7 RNA polymerase were combined and incubated for 2 h at 37°C. Then 1.0 μl DNase (RNase-free) was added and the mixture was incubated for 15 min at room temperature (RT). Radiolabeled probe was purified with microspin chromatography columns (Bio-Rad, Hercules, CA). Two slides per level for each probe were removed from 80°C and placed in 4% (weight:vol) formaldehyde at RT for 1 h. The slides were then washed in 2× SSC (300 mM NaCl / 30 mM sodium citrate, pH 7.2) three times for 5 min each. Slides were washed in deionized water for 1 min and placed in 0.1M triethanolamine, pH 8.0 / acetic anhydride, 400:1 (vol:vol) on a stir plate for 10 min. A final wash was in 2× SSC for 5 min, followed by dehydration through graded alcohols and air-drying for 30 min. A coverslip with 30 μl of riboprobe (1 million cpm, 75% formamide buffer (75% formamide, 10% dextran sulfate, 3× SSC, 50 mM Na$_2$HPO$_4$ (pH 7.4), 10 mM dithiothreitol, 1× Denhardt's solution, 100 μg/ml yeast tRNA), 0.01M DTT) was placed on each slide. Slides were placed in a covered tray with a filter paper liner saturated with 75% formamide. After overnight incubation at 55°C, coverslips were removed and the slides were placed in 2× SSC for 5 min, followed by RNase (200 μg/ml in 10 mM Tris-HCl, pH 8.0 / 0.5 M NaCl) at 37°C for 30 min and then washed as follows: 2× SSC for 10 min at RT; 1× SSC for 10 min at RT; 0.5× SSC for 60 min at 55°C; and 0.5× SSC for 10 min at RT. The slides were dehydrated in graded ethanol solutions, air-dried, placed in X-ray cassettes, and apposed to Kodak XAR-5 film for 1–42 days, depending on the probe.

Image and data analyses

Film was developed and used for quantitative computer image analysis (NIH Image 1.61) as previously described (Meador-Woodruff et al., 1997). Tissue background readings were subtracted from duplicate left- and right-side gray scale values from specific regions of interest. Background-adjusted gray scale values were converted into optical density and subsequently averaged, providing one mean value per region per animal for each probe. Optical density values were converted to units of bound radiation from a standard curve generated from a [^{14}C] microscale standard (Amersham Life Sciences, Buckinghamshire, UK) placed on each film (Williams, 1982; Downs and Williams, 1984). All data are expressed as nanocuries per gram of tissue (nCi/g). Statistical analyses were performed using analysis of variance and, when appropriate, post-hoc analyses were by Tukey's HSD; for all tests, $\alpha = 0.05$.

RESULTS
$\alpha 2$ Subunit expression and regulation

$\alpha 2$ subunit transcripts were expressed in the dentate gyrus (DG) and throughout the hippocampal subfields (Fig. 1). In the HPC, we found a main effect for treatment ($F_{(2,105)} = 74.9$, $P < 0.0001$) and region ($F_{(4,105)} = 6.23$, $P < 0.0001$), but we did not find a treatment by region interaction (Fig. 2). Post-hoc analysis revealed increased $\alpha 2$ subunit transcript expression in the higher-dose CNQX treatment group vs. the lower-dose CNQX ($P = 0.0001$) and vehicle-treated groups ($P = 0.0001$) (Fig. 2). The lower dose CNQX-treated group was not significantly different from the vehicle-treated group. Significantly higher levels of $\alpha 2$ subunit transcripts were detected in the DG compared to the four hippocampal subfields (Fig. 2).

$\alpha 4$ Subunit

$\alpha 4$ subunit transcripts were differentially expressed in several areas of the rat brain, including the medial habenula and the amygdala (Fig. 3). In the HPC, we detected a main effect for region ($F_{(4,95)} = 13.2$, $P < 0.0001$), but not treatment or treatment by region interaction (Fig. 2). Significantly higher levels of $\alpha 4$ subunit transcripts were detected in the CA1 subfield vs. the other hippocampal subfields and DG (Fig. 2). We found a main effect for treatment in the medial habenula (MHN) ($F_{(2,15)} = 8.56$, $P < 0.003$) and in the amygdala (AMY) ($F_{(2,15)} = 7.32$, $P < 0.006$) (Fig. 4). Post-hoc analysis revealed significantly lower levels of $\alpha 4$ subunit transcripts in the MHN and AMY for both the higher ($P = 0.019$, $P = 0.009$, respectively) and
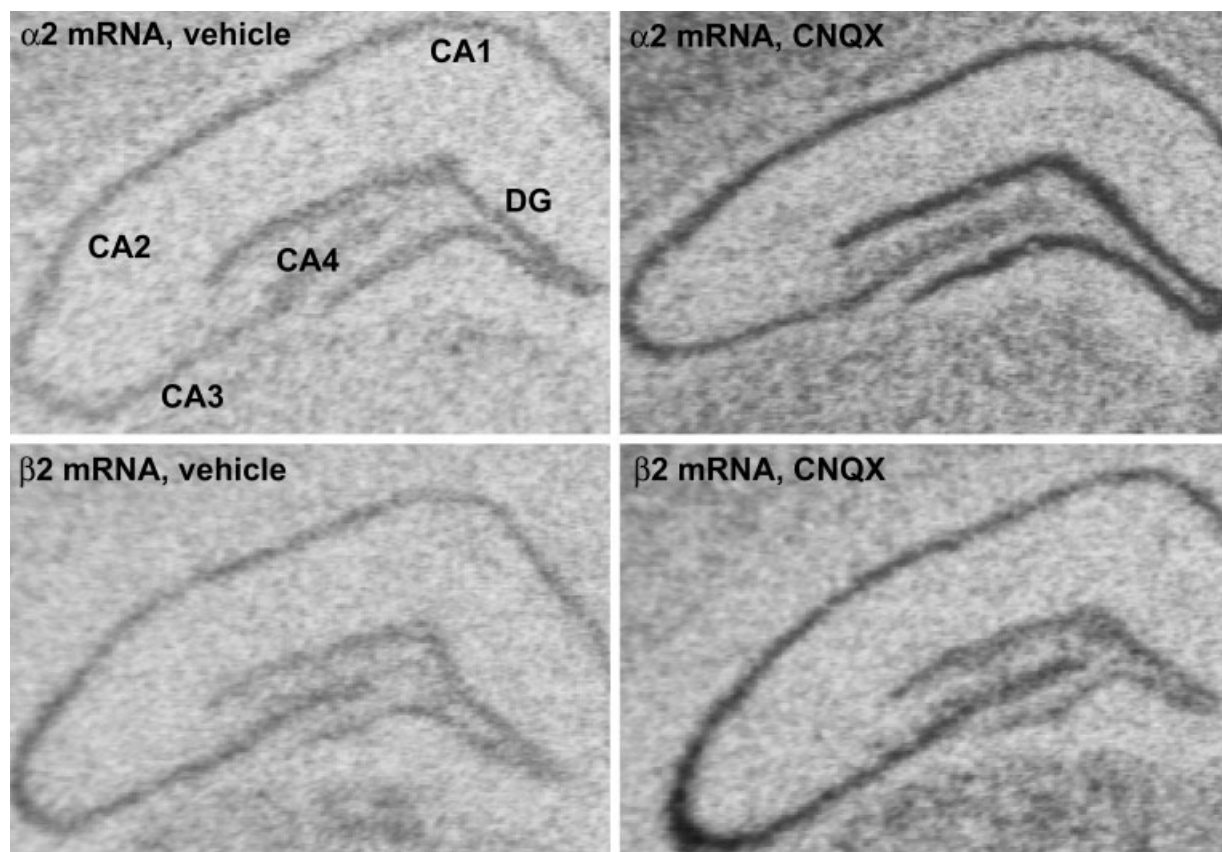
Fig. 1. In situ hybridization using [^{35}S]-labeled antisense probes for the α2 and β2 nicotinic receptor subunits in the dentate gyrus (DG) and hippocampal subfields CA1, CA2, CA3, and CA4 of rats treated for 1 week with vehicle or 10 mg/kg/day CNQX. Sections probed for α2 and β2 were exposed to film for 42 and 9 days, respectively.

lower ($P = 0.002$, $P = 0.005$) dose CNQX treatment groups vs. the control group (Fig. 4). We did not detect changes in α4 subunit transcript expression in the cingulate cortex (Cg), parietal cortex (Par), thalamus (THA), hypothalamus (HT), frontal cortex (Fr), septum (SEP), HPC, or piriform cortex (Pir) (Fig. 4).

α5 Subunit

No treatment effects on α5 subunit mRNA expression were detected in the Fr, SEP, Cg, MHN, or temporal cortex (Te) (Fig. 5).

α7 Subunit

In the HPC, there was a main effect for region (F = 102.8, df 4, 105, $P < 0.0001$), but not treatment, nor was there a treatment by region interaction (Fig. 2). Post-hoc analysis revealed significantly lower levels of mRNA expression in CA1 vs. the other CA subfields and the DG, as well as lower levels of expression in CA2 vs. CA3 and CA4, lower levels in CA3 vs. CA4, and higher levels in CA3 and CA4 vs. DG (Fig. 2). There were no treatment effects on α7 subunit mRNA expression in the THA, HT, AMY, Par, Cg, Pir, Fr, or SEP (Fig. 6).

β2 Subunit

The β2 subunit mRNA was expressed in multiple regions of the rat brain, including the septum and piriform cortex (Fig. 7). In the HPC, we detected a main effect for CNQX treatment (F = 20.3, df 2, 105, $P < 0.0001$) and region (F = 22.8, df 4, 105, $P < 0.0001$), but no treatment by region interaction (Figs. 1, 2). Post-hoc analysis revealed significantly higher levels of mRNA expression in both the higher-dose ($P = 0.0003$) and lower-dose CNQX treatment groups ($P = 0.0001$) vs. the control group, and higher levels of mRNA expression in the higher-dose vs. lower-dose CNQX treatment group ($P = 0.014$). Post-hoc analysis for region revealed significantly lower levels of mRNA expression in CA1 vs. the other hippocampal subfields, lower levels of expression in DG vs. CA2, CA3, and CA4, and higher levels in CA3 vs. CA4 (Fig. 2). We also found a main effect for treatment in the SEP (F = 3.63, df 2, 20, $P = 0.045$) and Pir (F = 10.8, df 2, 20, $P = 0.001$) (Fig. 8). Post-hoc analysis revealed higher levels of β2 subunit expression in the SEP ($P = 0.035$) for the higher-dose CNQX treatment group vs. the vehicle-treated group. Post-hoc analysis also revealed higher levels of expression in the Pir for the higher-dose CNQX treatment
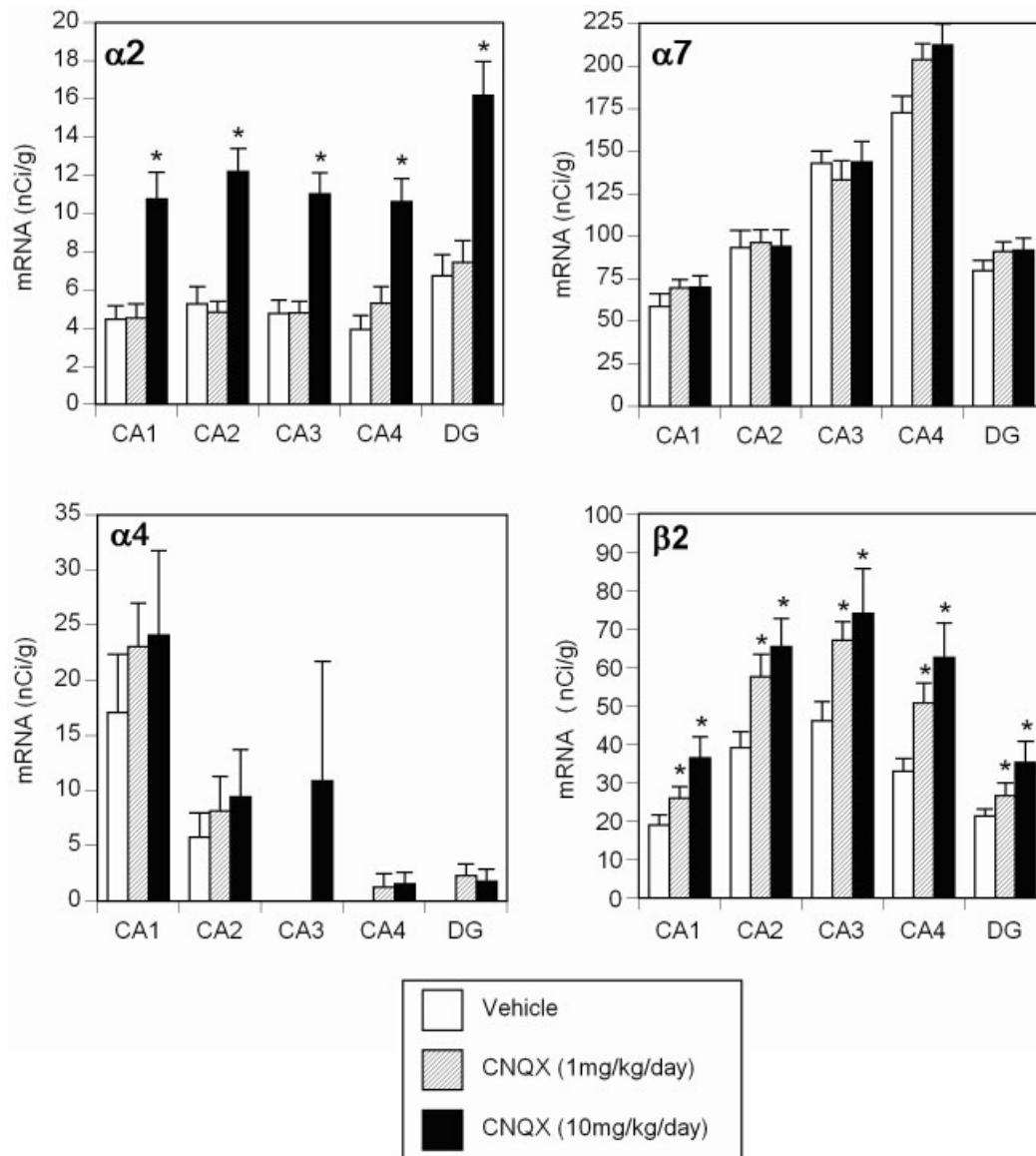
Fig. 2.   α2, α4, α7, and β2 nicotinic receptor subunit transcript expression in the dentate gyrus (DG) and hippocampal subfields following 7 days of CNQX treatment. Expression of α2 (10 mg/kg/day) and β2 (1 mg/kg/day and 10 mg/kg/day) transcripts in rats treated with CNQX was significantly elevated compared to the vehicle group. There were 10 rats in each treatment group. *$P < 0.05$.

group vs. both the vehicle ($P = 0.001$) and lower-dose CNQX treatment groups ($P = 0.013$). We did not detect a treatment effect in the Fr, olfactory tubercle (OT), MHN, Cg, Par, THA, HT, or AMY (Fig. 8).

α3, β3, and β4 Subunits

α3, β3, and β4 subunit transcripts were restricted to the MHN. We did not detect an alteration in any of these subunit transcripts following CNQX treatment (Fig. 9).

DISCUSSION

In the present study we detected differential regulation of nicotinic cholinergic receptor subunit transcripts fol-

lowing treatment with the AMPA and the kainate receptor antagonist CNQX (Table I). We found elevated levels of β2 subunit transcripts in the HPC, SEP, and Pir, decreased levels of α4 subunit transcripts in the AMY and MHN, and increased levels of α2 subunit mRNA in the HPC (Table I). Our findings suggest that gene expression of nAChR subunits can be regulated by non-NMDA ionotropic glutamate receptors in a subunit- and region-specific fashion. These changes in nAChR subunit expression likely reflect alterations of subunit stoichiometry of the nAChRs. Since the nAChRs have distinct functional characteristics based on their subunit composition, alterations in subunit expression affect nAChR activity in the synapse.
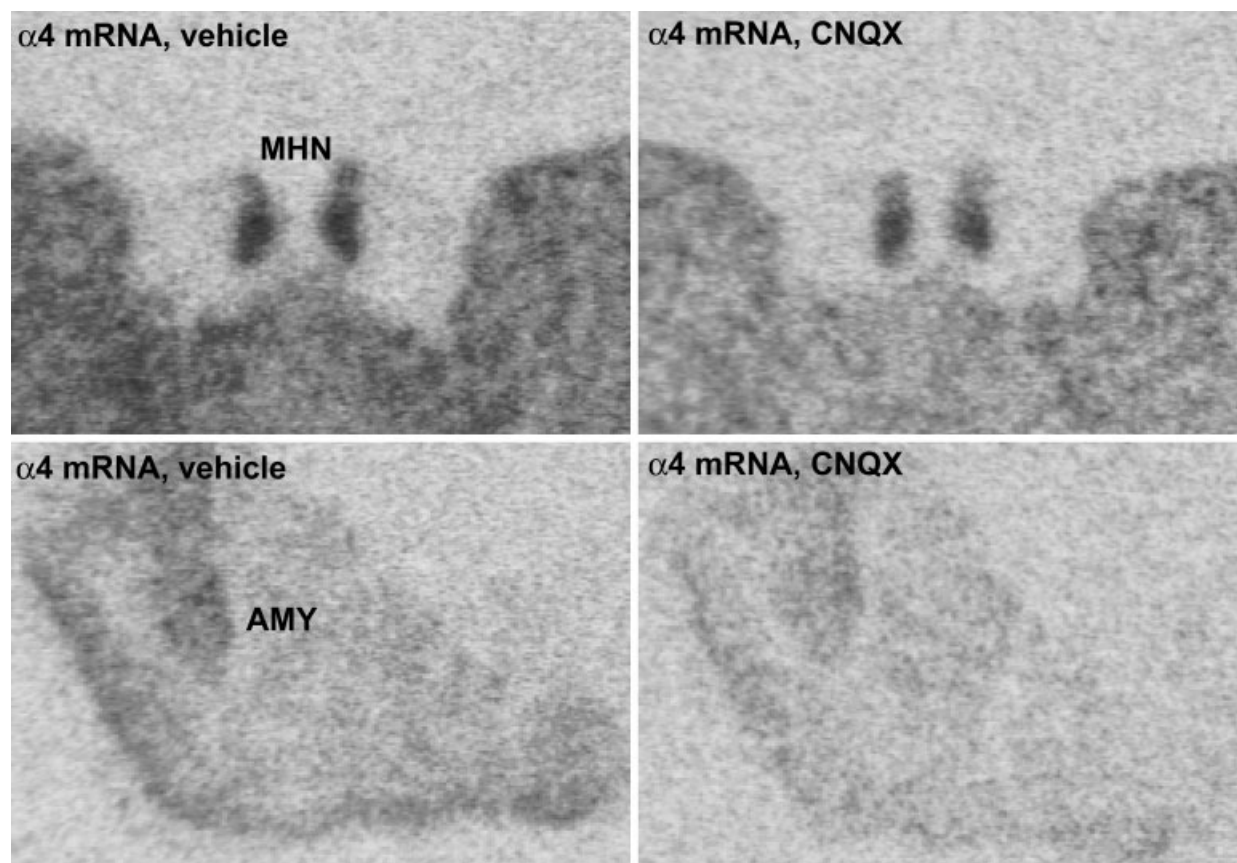
Fig. 3. In situ hybridization using [^{35}S]-labeled antisense probes for the α4 nicotinic receptor subunit in the medial habenula (MHN) and amygdala (AMY) of rats treated for 1 week with vehicle or 10 mg/kg/day CNQX. Sections probed for α4 were exposed to film for 6 days.

Numerous studies have demonstrated enhanced release of glutamate following activation of presynaptic nAChRs, a process likely mediated by direct calcium influx or localized depolarization and activation of voltage-dependent channels (McGehee et al., 1995; Aramakis and Metherate, 1998; Gioanni et al., 1999). nAChR-mediated calcium influx activates protein kinases and phosphatases, modifying synaptic functions such as neurotransmitter release (McGehee et al., 1995; Drew and Werling, 2001). Interestingly, our data suggest that the interface of the NCS and glutamatergic neurotransmission is not limited to nAChR-mediated neurotransmitter release. We found alterations in α2, α4, and β2 nAChR subunit transcript expression following treatment with CNQX, suggesting that AMPA and KA receptor activity may directly participate in the modulation of nAChR function by regulating the expression of nAChR subunit genes.

There are several mechanisms that could account for glutamatergic regulation of nAChR expression. Expression of axon-terminal nAChRs acting as autoreceptors could be affected by antagonism of somatodendritic AMPA receptors coexpressed on cholinergic neurons (Kumamoto and Murata, 1995; Dani, 2001). Alternatively, expression of subunits that comprise postsynaptic high affinity (fast) nAChRs may be altered secondary to diminished synaptic ACh levels, since AMPA-mediated hippocampal ACh release can be blocked by CNQX treatment (Moor et al., 1996). Other possible mechanisms include an indirect CNQX-mediated decrease in glutamatergic tone in neuronal circuits that include cells expressing nAChRs. This is supported by reports that ACh becomes the primary excitatory neurotransmitter in the absence of glutamate excitation in primary hypothalamic neuronal cultures (Belousov et al., 2001, 2002). Interestingly, we detected increased expression of the obligate β2 subunit transcripts in the HPC, SEP, and Pir, a finding consistent with past observations of augmented nicotinic cholinergic function following attenuation of glutamatergic activity by CNQX (Belousov et al., 2001, 2002).

Following treatment with CNQX, we detected increased levels of α2 transcripts in the HPC, increased β2 transcripts in the HPC, SEP, and Pir, and decreased α4 transcripts in the MHN and AMY. In contrast, we did not detect changes in α7 mRNA expression in the HPC, AMY, Pir, or SEP with CNQX treatment. The α7 nicotinic subunit exclusively participates in the formation of a homomeric receptor, while α2, α4, β2, and
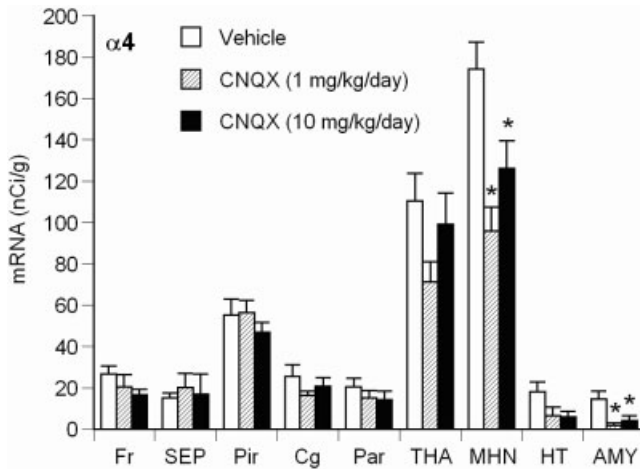
Fig. 4. α4 nicotinic receptor subunit transcript expression in the frontal cortex (Fr), septum (SEP), piriform cortex (Pir), cingulate cortex (Cg), parietal cortex (Par), thalamus (THA), medial habenula (MHN), hypothalamus (HT), and amygdala (AMY) following 7 days of CNQX treatment. Expression of α4 transcripts in rats treated with 1 mg/kg/day and 10 mg/kg/day CNQX was significantly decreased in the MHN and in the AMY compared to the vehicle group. There were 10 rats in each treatment group. *$P < 0.05$.
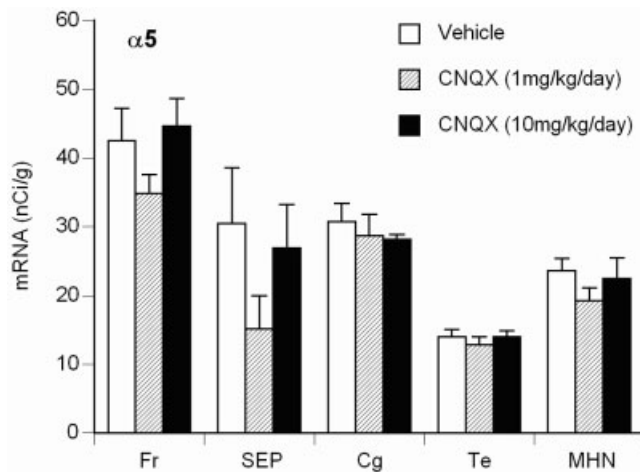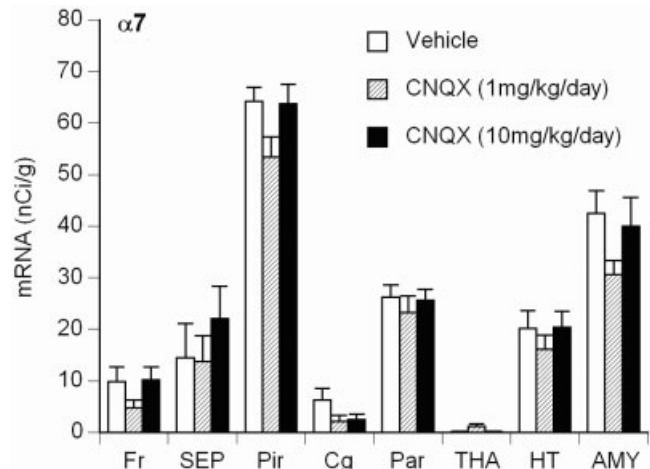

Fig. 6. α7 nicotinic receptor subunit transcript expression in the frontal cortex (Fr), septum (SEP), piriform cortex (Pir), cingulate cortex (Cg), parietal cortex (Par), thalamus (THA), hypothalamus (HT), and amygdala (AMY) following 7 days of CNQX treatment. Expression of α7 transcripts did not differ between CNQX- and vehicle-treated groups. There were 10 rats in each treatment group.

Fig. 5. α5 nicotinic receptor subunit transcript expression in the frontal cortex (Fr), septum (SEP), cingulate cortex (Cg), temporal cortex (Te), and medial habenula (MHN) following 7 days of CNQX treatment. Expression of α5 transcripts did not differ between CNQX- and vehicle-treated groups. There were 10 rats in each treatment group.

other subunits contribute to the formation of heteromeric nicotinic receptor subtypes (Itier and Bertrand, 2001; Picciotto et al., 2001). Thus, our findings suggest that inhibition of KA and/or AMPA receptors only affects expression of a subset of pharmacologically distinct nAChRs. Several studies have demonstrated electrophysiological differences between heteromeric (α4β2) and homomeric (α7) nAChRs in the rat hippocampus. α7 selective ligands had no effect on excitatory postsynaptic current (EPSC), while α3β2-specific compounds, or low concentrations of nicotine itself, modulate EPSCs in hippocampal slices, suggesting a

non-α7 mechanism (Alkondon and Albuquerque, 2002). In a related study, mediation of inhibition of CA1 pyramidal neurons was three times stronger by α4β2 vs. α7 receptors. (Alkondon and Albuquerque, 2001). Differences between heteromeric and homomeric nAChRs are not limited to the HPC. The α4β2 antagonist dihydro-β-erythrodine (DHβE) attenuated the excitatory effects of nicotine on dopaminergic neurons in the ventral tegmental area, while the α7 antagonist methyllycaconitine (MLA) did not (Grillner and Svensson, 2000).

The differences in neurophysiological function between heteromeric and homomeric nAChRs may underlie discrete behavioral tasks. For example, Levin et al. (2002) evaluated the effects on specific memory tasks of antagonism of α4β2 vs. α7 containing nAChRs in the rat. Reference memory was impaired following administration of the α7 antagonist MLA by cannula in the HPC, but not the α4β2 antagonist DHβE (Felix and Levin, 1997; Levin et al., 2002). In contrast, working memory was impaired following administration of either MLA or DHβE, suggesting that some cognitive functions involve differential activation of nAChR subtypes. The discrete functional roles of the α7 and α4β2 nAChRs in the HPC are consistent with our findings of differential nAChR transcript expression following CNQX treatment, and support our hypothesis that expression of phenotypically distinct nicotinic receptor subtypes is differentially regulated by CNQX, a modulator of non-NMDA ionotropic glutamate receptors.

We detected increased α2 and β2 subunit transcript expression in the HPC following treatment with CNQX, suggesting either an increase in nicotinic receptors containing these subunits or an alteration in receptor stoichiometry. In addition, we did not detect
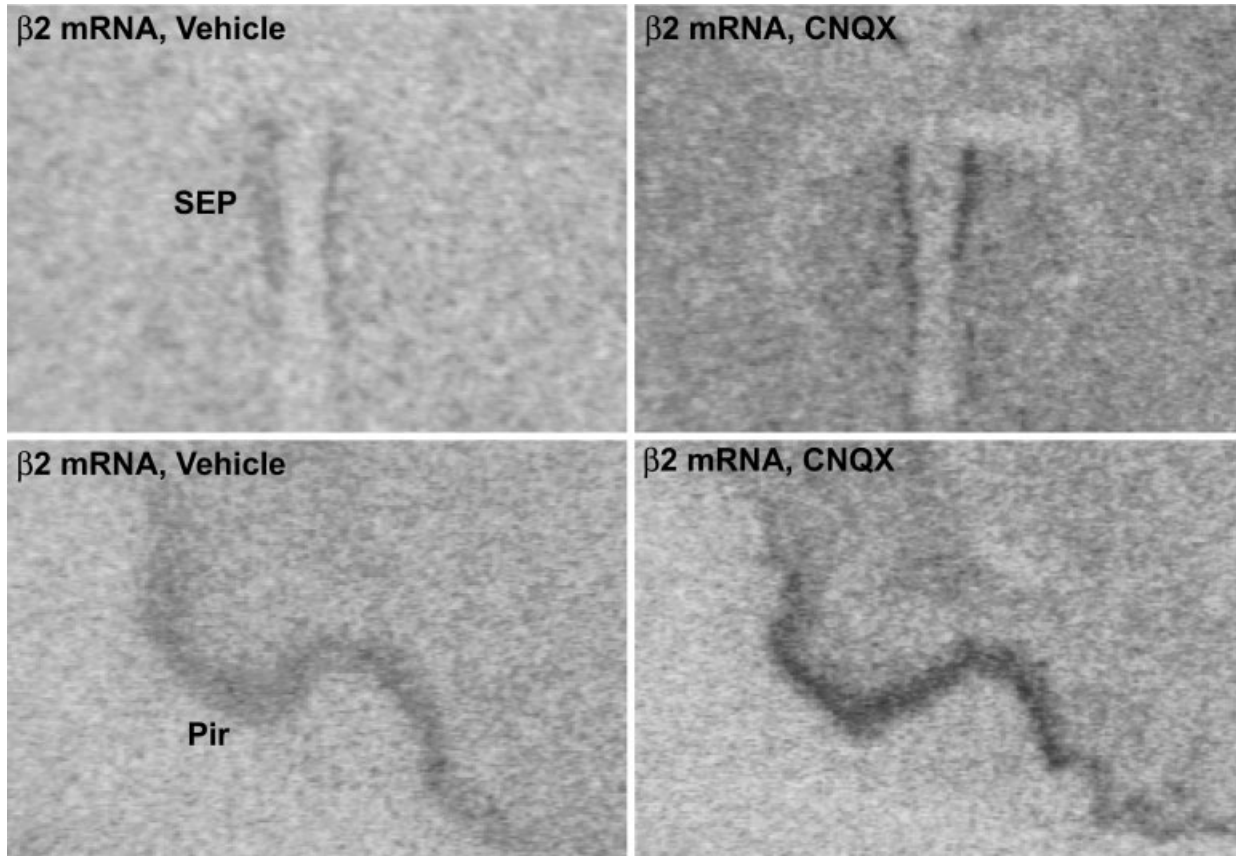
Fig. 7. In situ hybridization using [^{35}S]-labeled antisense probes for the β2 nicotinic receptor subunit in the septum (SEP) and piriform cortex (Pir) of rats treated for 1 week with vehicle or 10 mg/kg/day CNQX. Sections probed for β2 were exposed to film for 9 days.
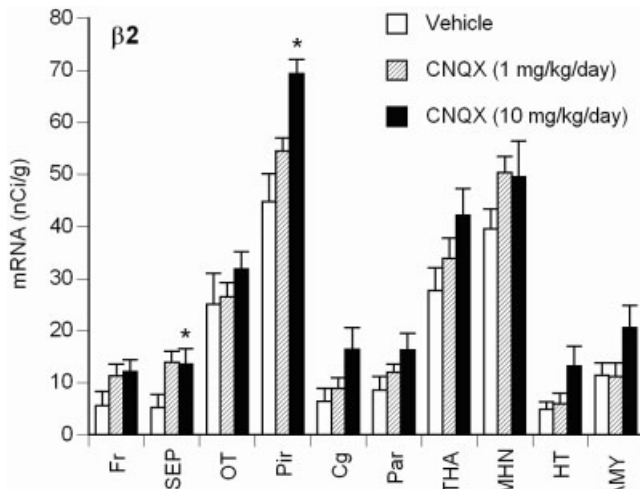

Fig. 8. β2 nicotinic receptor subunit transcript expression in the frontal cortex (Fr), septum (SEP), olfactory tubercle (OT), piriform cortex (Pir), cingulate cortex (Cg), parietal cortex (Par), thalamus (THA), medial habenula (MHN), hypothalamus (HT), and amygdala (AMY) following 7 days of CNQX treatment. Expression of β2 transcripts in rats treated with 10 mg/kg/day CNQX was significantly increased in the SEP and in the Pir compared to the vehicle group. There were 10 rats in each treatment group. *$P < 0.05$.

changes in α4 subunit mRNA levels, suggesting that alterations in nAChR expression following CNQX treatment are limited to a subset of heteromeric receptor subunits. In the HPC, expression of α2, α3, and α4 subunits has been correlated with medium, fast, and slow peak rise times, respectively, measured by whole cell recordings of interneurons in the stratum oriens and stratum radiatum of the HPC (Sudweeks and Yakel, 2000). The authors of that study suggested that α2-containing receptors were both a major component of the total nAChR population in the stratum oriens and functionally distinct from α3- or α4-containing receptors (Sudweeks and Yakel, 2000). Thus, an increase in hippocampal α2 subunit expression, regulated indirectly by CNQX via modulation of AMPA and KA receptor activity, may lead to a change in nAChR-mediated currents.

We detected decreased α4 subunit transcript expression in the AMY following treatment with CNQX, suggesting an interaction with the glutamatergic system in this region. α4 knockout mice have been used to investigate the contributions of the α4 subunit to nicotinic receptor function (Marubio and Changeux, 2000). High-affinity nicotine binding was significantly attenuated in α4 knockout mice, confirming that this

subunit is central for generation of type 2 ($\alpha4\beta2$) nicotinic currents in many brain areas. $\alpha4$ knockouts also displayed a decrease in antinociception, an effect localized to the raphe magnus and the thalamus based on patch clamp recordings (Marubio and Changeux, 2000). Other work suggests that the NCS contributes to performance of both passive avoidance and water maze testing via circuitry that involves both the amygdala and the nucleus basalis, while implantation of nicotinic receptor-modulating compounds in the AMY decreased FSH release (Piva et al., 1980; Riekkinen et al., 1993).

Administration of CNQX also decreased $\alpha4$ subunit mRNA expression in the MHN. The MHN links the limbic forebrain with diencephalic structures and mediates diverse neurophysiological functions, including sensory gating, reward pathways, and stereotyped motor behaviors (Ellison, 1994). One study demonstrated that heteromeric nAChRs accounted for 75% of nicotine-induced currents in the medial MHN, a population that may include $\alpha4$-containing receptors (Quick et al., 1999). Interestingly, amphetamine and cocaine induced degeneration of neurons in the lateral MHN, while a different study demonstrated that administra-

tion of high levels of nicotine tartrate led to neurotoxicity in the medial MHN (Carlson et al., 2001; Ellison, 2002). In both of these studies, neuropathologic changes were observed in the fasciculus retroflexus, an output tract of the MHN. These studies implicate the NCS in the MHN as a possible substrate for stimulant-mediated pathophysiology. Our data suggest a direct interface between the glutamatergic and nicotinic cholinergic systems in the MHN.

Increases in $\beta2$ subunit transcript expression in the SEP following CNQX treatment may be explained by previous work that has demonstrated expression of NMDA, AMPA, and KA receptors on cholinergic neurons in the SEP (Kumamoto and Murata, 1995). Attenuation of AMPA receptor activation with CNQX would decrease NMDA receptor activation, decreasing calcium influx, altering intracellular signaling pathways, and generation of excitatory postsynaptic currents. Interestingly, administration of CNQX by microdialysis into the medial septum decreased ACh release, suggesting that increased $\beta2$ subunit expression is secondary to diminished release of ACh (Moor et al., 1996). However, 7 days of intravenous administration of the nAChR agonists nicotine and/or mecamylamine did not alter $\beta2$ mRNA expression in the SEP, suggesting that regulation of subunit expression involves more than an alteration in the synaptic level of neurotransmitter or agonist (Pauly et al., 1996).

While expression of subunits that comprise both hetero- and homomeric nAChRs has been demonstrated in the Pir, we only detected changes in $\beta2$ transcript expression in this region (Wada et al., 1989). To our knowledge, the interface of the glutamatergic and nicotinic cholinergic systems in the Pir has not previously been examined. In the Pir, ACh release enhanced long-term potentiation, likely contributing to associative memory function (Barkai and Hasselmo, 1997; Patil et al., 1998; Linster and Hasselmo, 2001). More work is needed to assess the relevance of increased $\beta2$ subunit expression in the Pir following CNQX treatment.

A limitation of this study is that we only measured nAChR subunit transcript expression. For example, while we did not detect a change in $\alpha7$ subunit mRNA expression, there may have been a change in $\alpha7$ subunit translation or $\alpha7$ receptor mobilization to the cell
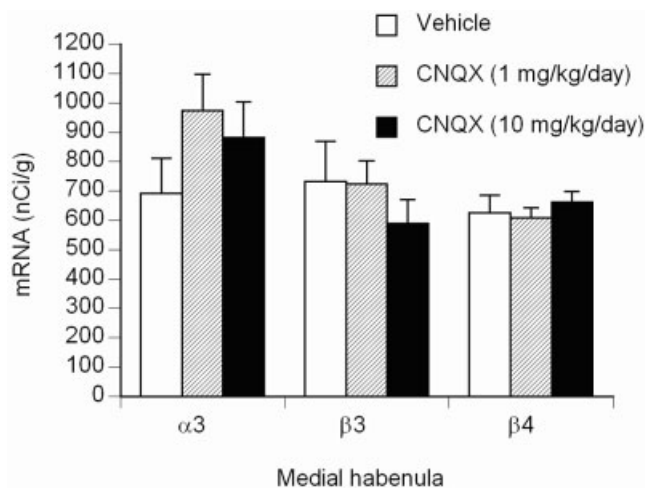

Fig. 9. $\alpha3$, $\beta3$, and $\beta4$ nicotinic receptor subunit transcript expression in the medial habenula (MHN) following 7 days of CNQX treatment. Expression of these transcripts did not differ between CNQX- and vehicle-treated groups. There were 10 rats in each treatment group.

TABLE I. Summary of changes in nicotinic receptor subunit expression following treatment with CNQX

Region		HPC	Fr	SEP	Pir	Cg	Par	THA	MHN	HT	AMY	Te	OT
Heteromeric α subunits	$\alpha2$	↑	–	–	–	–	–	–	–	–	–	–	–
	$\alpha3$	–	–	–	–	–	–	–	–	–	–	–	–
	$\alpha4$	nc	nc	nc	nc	nc	nc	nc	↓	nc	↓	–	–
	$\alpha5$	–	nc	nc	–	nc	–	–	nc	–	–	nc	–
Heteromeric β subunits	$\beta2$	↑	nc	↑	↑	nc	nc	nc	nc	nc	nc	–	nc
	$\beta3$	–	–	–	–	–	–	–	nc	–	–	–	–
	$\beta4$	–	–	–	–	–	–	–	nc	–	–	–	–
Homomeric subunit	$\alpha7$	nc	nc	nc	nc	nc	nc	nc	–	nc	nc	–	–

Abbrieviations: increased (↑) or decreased (↓) transcript expression, no change detected (nc), not measured (–), amygdala (AMY), cingulate cortex (Cg), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), hypothalamus (HT), hippocampus (HPC), medial habenula (MHN), olfactory tubercle (OT), parietal cortex (Par), piriform cortex (Pir), frontal cortex (Fr), septum (SEP), temporal cortex (Te), thalamus (THA).

surface following CNQX treatment. Furthermore, an alteration in transcript expression does not necessarily indicate an alteration in expression of functional receptors. Despite these limitations, our findings may be conservatively interpreted to indicate an important interaction between these excitatory neurotransmitter systems.

This study demonstrates alterations in nAChR subunit transcript expression following treatment with CNQX, an AMPA and KA receptor antagonist, suggesting a regulatory interface between the nicotinic cholinergic and glutamatergic neurotransmitter systems. Changes in transcript expression were confined to a subset of nAChR subunits associated with heteromeric receptor channels, supporting the hypothesis that expression of phenotypically distinct nicotinic receptor subtypes is differentially regulated by CNQX via modulation of non-NMDA ionotropic glutamate receptors.

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