Activation of Nicotinic Acetylcholine Receptors Increases the Frequency of Spontaneous GABAergic IPSCs in Rat Basolateral Amygdala Neurons

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ABSTRACT

The basolateral amygdala (BLA) is a critical component of the amygdaloid circuit, which is thought to be involved in fear conditioned responses. Using whole-cell patch-clamp recording, we found that activation of nicotinic acetylcholine receptors (nAChRs) leads to an action potential-dependent increase in the frequency of spontaneous GABAergic currents in principal neurons in the BLA. These spontaneous GABAergic currents were abolished by a low Ca\(^{2+}\)/high Mg\(^{2+}\) bathing solution, suggesting that they are spontaneous inhibitory postsynaptic currents (sIPSCs). Blockade of ionotropic glutamate receptors did not prevent this increased frequency of sIPSCs, nor did blockade of \(\alpha_7\) nAChRs. Among the nAChR agonists tested, cystisine was more effective at increasing the frequency of the sIPSCs than nicotine or 1,1-dimethyl-4-phenyl piperazinium iodide (DMPP), consistent with a major contribution of \(\beta_4\) nAChR subunits. The nicotinic antagonist, dihydro-\(\beta\)-erythroidine, was less effective than d-tubocurarine in blocking the increased sIPSC frequency induced by ACh, suggesting that \(\alpha_4\)-containing nAChR subunits do not play a major role in the ACh-induced increased sIPSC frequency. Although \(\alpha_{2/3/4/7}\) and \(\beta_{2/4}\) nAChR subunits were found in the BLA by RT-PCR, the agonist and antagonist profiles suggest that the ACh-induced increase in sIPSC frequency involves predominantly \(\alpha_3\beta_4\)-containing nAChR subunits. Consistent with this, \(\alpha\)-conotoxin-AuIB, a nAChR antagonist selective for the \(\alpha_3\beta_4\) subunit combination, inhibited the ACh-induced increase in the frequency of sIPSCs. The observations suggest that nicotinic activation increases the frequency of sIPSCs in the BLA by acting mainly on \(\alpha_3\beta_4\)-containing nicotinic receptors on GABAergic neurons and may play an important role in the modulation of synaptic transmission in the amygdala.
INTRODUCTION

The amygdala is a heterogeneous collection of nuclear groups (also referred to as the amygdaloid complex) located in the temporal lobe of the brain. The amygdala receives projections from several brain regions (LeDoux, 2000; McDonald, 1998). Cortical inputs reach the amygdala laterally from the external capsule; other major inputs reach the amygdala medially via the internal capsule (LeDoux et al., 1991; Romanski and LeDoux, 1993). In the amygdala, information is processed through intra-amygdaloid connections and transferred to the central amygdala, the major output station of the amygdala (Pitkänen et al. 1998). The basal nuclei of the amygdala, including the basolateral nucleus, play an important role in processing information through intra-amygdaloid connections. In aversive learning, the basolateral amygdala (BLA) receives conditioning stimuli from the lateral nucleus and relays it to the central amygdala, the output for conditioned fear responses. Thus, the BLA is thought to be a critical element in the neuronal circuits involved in fear conditioning (Davis et al. 1994, LeDoux 2000).

The amygdala receives a large cholinergic input from the nucleus basalis magnocellularis (Carlsen et al. 1985; Emson et al. 1979; Heckers and Mesulam 1994; Nagai et al. 1982; Woolfer and Butcher 1982) and a much small cholinergic projection from the lateral parabrachial nucleus in the brain stem (Woolfer and Butler 1982). Cholinergic inputs to the BLA from the nucleus basalis magnocellularis have been reported to influence aversive learning and memory (Power and McGaugh 2002; Vazdarjanova and McGaugh 1999) and to suppress kindling elicited from the amygdala (Ferencz et al. 2000). In addition to muscarinic modulation (North 1989, Washburn and Moises 1992), nicotinic activation is thought to be involved in passive avoidance learning in the amygdala (Blozovski and Dumery 1987; Riekkinen et al. 1993). Nicotine can also increase neuronal activity in the amygdala and other brain regions, an action that is consistent with nicotine’s behavioral-arousing and behavior-reinforcing properties in humans (Stein et al. 1998). However, little is known about how nicotinic activation affects neuronal activity in the amygdala. In addition, it is of interest to determine if nicotinic activation modulates GABAergic function in the amygdala, because
electrophysiological and pharmacological studies have found that the amygdala contains a powerful inhibitory GABAergic system (McDonald 1985; Takagi and Yamamoto 1981) and this GABAergic system is thought to play a crucial role in information processing in the amygdala (Lang and Paré 1998; Mahanty and Sah 1999). The aim of the present study was to investigate how nicotinic activation affects neuronal function in the BLA, to determine whether nicotinic activation modulates GABAergic transmission, and to characterize nAChR subunits which may contribute to nicotinic action in the BLA.

METHODS

Brain slices (400 μm) containing the amygdala were obtained from Sprague-Dawley rats (P7-10). Sections were cut with a Vibratome (TPI Inc., St. Louis, MO) in cold external bathing solution oxygenated with 95% O2/5% CO2. The composition of the external bathing solution was (in mM): 124 NaCl, 3KCl, 1.3 Mg2SO4, 2 CaCl2, 1.2 NaH2PO4, 25 NaHCO3 and 10 glucose. Atropine (1 μM) was added to the bathing solution in all experiments to prevent activation of muscarinic AChRs. Slices were continuously superfused at room temperature (~23°C) in a recording chamber. Agonists were applied by gravity flow through a macropipette placed about 200 μm from the recorded neuron; antagonists were applied in the bathing solution, unless otherwise indicated. The interval between agonist applications was > 4 min. BLA neurons were visualized under an Axioskop 2FS fixed-stage microscope (Carl Zeiss Inc., Thornwood, NY). For comparison, in some experiments brain slices containing hippocampus were also prepared.

In some experiments, single BLA neurons were isolated from BLA slices using an enzyme-free mechanical dissociation procedure, as described by Akaike and Moorhouse (2003). Briefly, BLA slices were transferred to a 35 mm dish (coated with poly-D-lysine) with an external recording buffer containing (in mM): 140 NaCl, 5 KCl, 10 HEPES, 2 MgCl2, 2 CaCl2, 0.025 AP5 (DL-2-amino-5-phosphonopentanoic acid), 0.04 CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) and 10 glucose (pH 7.40). A fire-polished glass micropipette was placed on the surface of the BLA. The tip of the pipette was vibrated
horizontally at 6-8 Hz for ~2 min. The isolated neurons were allowed to settle to the bottom of the dish for 10-15 min. The neurons were visualized on an inverted microscope.

Patch-pipettes were made using a two-stage microelectrode puller (PC-10, Narishige, Japan) and had resistances of 6-8 MΩ after filling with solution containing (in mM): 140 CsCl or KCl, 10 HEPES, 5.5 BAPTA, 2.0 MgCl2, and 2.0 Mg-ATP. Whole-cell current was measured from a holding potential of ~60 mV using conventional patch-clamp techniques (Axopatch 200B, Axon Instruments, Foster City, CA). Data were acquired using pClamp8 software through a Digidata 1200 interface (Axon Instruments) and plotted with SigmaPlot (SPSS Inc., Chicago, IL). For brain slice experiments, continuous recording was collected for 25 s and drugs were applied for 15 s; for isolated BLA neurons, continuous recording was collected for 15 s and drugs were applied for 8 s. Spontaneous synaptic currents were initially detected using MiniAnalysis Software (Synaptosoft Inc., Decatur, GA) with threshold criteria of 20 pA; smaller events (down to 10 pA) were manually detected during visual inspection of traces. Spontaneous events were collected in 50 ms and 5 pA bins for cumulative event interval and amplitude plots, respectively. Cumulative distributions of spontaneous events were fitted by the Hill equation, \( P=1/[1+(X/X_{0.5})^n] \), where \( P \) is the cumulative probability, \( n \) is the Hill slope, \( X \) is the event interval or amplitude, and \( X_{0.5} \) is the interval (or amplitude) at \( P=0.5 \). Average data are expressed as mean ± SE; significance was analyzed by the Kolmogorov-Smirnoff two sample test (K-S test) for the cumulative distributions of sIPSCs and \( t \)-test or ANOVA were used to examine significant differences, as indicated.

In some experiments, neurons were filled passively with 0.4% Lucifer yellow. At the end of the physiological experiment, the slice was fixed with 4% paraformaldehyde for 1 hr at 4°C. The slices were dehydrated in graded alcohol, mounted on coverslips and imaged with a laser scanning confocal microscope (LSM 5 Pascal, Carl Zeiss Inc.).

Bicuculline (BIC), dihydro-β-erythroidine (DHβE) and methyllycaconitine (MLA) were purchased from RBI/Sigma (Natick, MA). Alpha-bungarotoxin (α-BgTx), acetylcholine (ACh), γ-amino-n-butyric acid (GABA), DL-2-amino-5-phosphonopentanoic acid (AP5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), cytisine, 1,1-dimethyl-4-phenyl-
piperazinium (DMPP), nicotine, tetrodotoxin (TTX), d-tubocurarine (dTc) and atropine were purchased from Sigma-Aldrich (St.Louis, MO). Alpha-conotoxin-AuIB (AuIB) was prepared as described previously (Luo et al. 1998).

Reverse transcript-polymerase chain reaction (RT-PCR) was used to assay messenger RNA coding for nicotinic receptor subunits. For this RNA assay, a 20 gauge needle was used to punch out the BLA from amygdala brain slices and total RNA was isolated from the pooled BLA of four rats. First strand cDNA was synthesized from 5 μg total RNA in a 50 μl reaction volume with reverse transcriptase. The PCR reactions contained 2 μl of cDNA and 100 pmol forward/reverse primers in a 40 μl volume. The PCR conditions consisted of 94°C for 2 min and 40 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 30 s. The RT-PCR result was confirmed in another group from the pooled BLA of four rats. As a control for genomic contamination, reactions were also run without reverse transcriptase. The primer pairs for amplifying cDNA regions were designed to span an intron-exon border on the basis of the genomic information available. The forward/reverse primer sets (in bp) used to amplify specific nACh receptor subunit mRNA were the following (starting from 5’ end): GAG-GAG-GAA-GAA-GAT-GAA-AAC/CAG-GAA-TGCTAGG-AAG-GA for α2 (336); ATC-GCA-TCT-TTC-TCT-TCT-GGG-TTT-T/TAT-GGA-GAT-CCT-GCA-CTA-ATG-G, α3 (363); CCTCCC-TGG-CTG-GCT-GGT-ATG-AT/TTG-GGA-CTC-GGC-CTG-CAA-CTG-TAT, α4 (230); GAG-ATG-GAA-TCC-TGA-CGA-AT/ATTAG-AGG-ATG-CA/CAG-CA, α5 (396); CTT-CGT-GTT-CCA-GCA-GAT-AA/TAT-AAA-ACA-TGGG0TCA-GCC-TC, α6 (409); GAA-ATG-CGC-AGA-TAA-GAA-GAG-AT/GCG-CAT-AGC-AAA-GGC-AGA-C, α7 (357); CCG-GGA-AGC-AGT-GGA-TGG/TGA-GGA-GCT-GCA-AAT-GAA-TGA-GAC, β2 (263); CCA-TGG-CAA-AAA-GAT-CAG-AGG-TT/GTC-ATC-AGG-GCT-TGG-CAC-TAC-TT, β3 (223); GCC-GTG-TGG-GAA-GCT-GAC-TGT-T/GAA-GGC-GGG-CTG-GTG-GTG-GAC, β4 (270). The primers for α5/6 were adapted from Sudweeks and Yakel (2000). For comparison, medial habenular nucleus was also isolated to assay mRNA coding for nicotinic receptor subunits; the mRNA nAChR subunit expression pattern in the medial habenular nucleus has been reported by Sheffield et al. (2000).
RESULTS

Nicotinic activation increases the frequency of spontaneous GABAergic currents in BLA principal neurons

This study focused particularly on the BLA pyramidal-type principal neurons (Fig. 1A), which are the predominant neuronal type in the BLA. In this study, we analyzed 85 neurons in the BLA that met the criteria described by Washburn and Moises (1992) for BLA principal neurons, namely the injection of a depolarizing current pulse generated only one or a few spikes before the cell fell silent for the duration of the pulse (Fig. 1B). For these neurons, when a Cl⁻ based internal solution was used for recording, the local application of ACh increased the frequency of spontaneous inward currents (Fig. 1C, top). These spontaneous inward currents were abolished by the GABAA receptor antagonist, bicuculline (20 μM) (Fig. 1C, bottom), indicating that the currents are GABAergic [Note also that in the presence of bicuculline, ACh did not activate a detectable current in these neurons (Fig. 1C, bottom)]. The ACh-induced increase in the frequency of these GABAergic currents was reversibly abolished by an external bathing solution containing low Ca²⁺ and high Mg²⁺ (Fig. 1D).

The ACh-induced increase in the frequency of spontaneous GABAergic currents was found to be concentration-dependent (Fig. 2). The average percentage increase in the number of spontaneous GABAergic currents induced by ACh concentrations of 10, 30 and 100 μM was 165 ± 59% (p<0.05), 403 ± 65% (p<0.01) and 661 ± 105% (p<0.01), respectively (n=9, t-test, Fig. 2A). For further analysis, the spontaneous GABAergic events were collected in 50 ms bins for event interval plots and 5 pA bins for amplitude plots. Fig. 2B shows that ACh caused concentration-dependent reductions in GABAergic event intervals that were significant (p<0.05, p<0.01 and p<0.001 for ACh 10, 30 and 100 μM, respectively, K-S test, compared with control). ACh produced a reduction in event amplitudes only at the highest concentration of 100 μM (p<0.05, K-S test). To determine if nicotinic activation has a direct effect on postsynaptic GABAₐ receptor-mediated responses, GABA responses were directly activated by the exogenous
application of 7.3 µM GABA in the presence of 1 µM TTX. We found that 100 µM ACh had no significant effect on the current activated by the application GABA in BLA principal neurons. In four cells tested, the average current activated by 7.3 µM GABA was 262 ± 39 pA in the absence of ACh and 264 ± 36 pA in the presence of 100 µM ACh ($p>0.05$, $t$-test).

*ACh-induced increase in the frequency of spontaneous GABAergic currents is action potential-dependent*

Figure 3A shows that the increased frequency of the spontaneous GABAergic currents induced by ACh was abolished by the pre-application of 1 µM TTX, a blocker of voltage-gated Na$^+$ channels. In addition, in the presence of 1 µM TTX, ACh did not activate detectable current in these neurons (Fig. 3A).

In this study, we recorded three neurons that were identified as interneurons on the basis of electrophysiological properties described by Szinyei et al. (2000), namely the injection of a depolarizing current generated repetitive firing with little adaptation (Fig. 3B, *inset*). In these interneurons, ACh induced both an increase in the frequency of spontaneous inward currents and an ACh-activated inward current (Fig. 3B, *top*). In addition, in these interneurons, TTX (1 µM) abolished the ACh-induced spontaneous inward currents, but not the ACh-activated inward current (Fig. 3B, *bottom*). Bicuculline (20 µM) also abolished the ACh-induced increase in the frequency of spontaneous inward currents, but not the ACh-activated inward current ($n=4$, not shown), indicating that the spontaneous inward currents are GABAergic and the ACh-activated inward current is not.

The effect of nicotine on the spontaneous GABAergic currents in BLA principal neurons was also examined. In the control external bathing solution, the bath application of 10 µM nicotine for 2 min caused a significant increase in the frequency of the spontaneous GABAergic currents (Fig. 3C, *top*). In the presence of 1 µM TTX, this effect of nicotine was abolished, even when the neurons were exposed to nicotine for a prolonged period of time (Fig. 3C, *bottom*).
Figure 3D shows that a combination of the glutamate receptor antagonists, 25 μM AP5 (2-amino-5-phosphonopentanoic acid) and 40 μM CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), did not significantly affect the ACh-induced increase in the frequency of spontaneous GABAergic currents in the BLA principal neurons. On average, for a 25 s continuous recording, 100 μM ACh alone caused a 795 ± 125 % increase in the number of spontaneous GABAergic currents and an 824 ± 228 % increase in the presence of AP5/CNQX (25/40 μM) (p>0.5, n=6, t-test). Glutamatergic blockade also had no significant effect on cumulative distribution of spontaneous GABAergic event intervals induced by ACh (p>0.05, K-S test, Fig. 3D, bottom).

Taken together, the preceding observations suggest that the spontaneous inward currents induced by nicotinic activation are GABAergic spontaneous inhibitory postsynaptic currents (sIPSCs).

**RT-PCR analysis of nAChR subunits in BLA**

To determine the nAChR subunit mRNA expression in the BLA, total RNA was isolated from pooled BLA punches and the transcripts were assayed by RT-PCR for putative nAChRs using primer sets specific for nAChR subunits α2-7 and β2-4. Figure 4A shows that in the BLA the PCR products for α2/3/4/7 (336/363/230/357) and β2/4 (263/270) subunits were well detected. Thus, in the BLA, nAChRs could consist of α2-4, 7 and β2/4 subunits. We also ran RT-PCR using the same primers but RNA samples from the medial habenular nucleus (MH); the RT-PCR products detected in this brain region have been reported previously by Sheffield et al. (2000). In our experiments, the PCR products for α3/4 (363/230) and β2/3/4 (263/223/270) subunits were well detected in the MH (Fig. 4B).

*Are high or low affinity nAChRs responsible for the ACh-induced increase in sIPSC frequency?*
Neuronal nAChRs have been classified into two major categories on the basis of their permeability to Ca\(^{2+}\) and their sensitivity to alpha-bungarotoxin [\(\alpha\)-BgTx] (Role and Berg 1996). One category of nAChR, the \(\alpha_7\)-containing subtype, is highly permeable to Ca\(^{2+}\), blocked by \(\alpha\)-BgTx and desensitizes rapidly upon agonist activation (cf. Severance et al. 2004). The nAChRs in the other category, non-\(\alpha_7\)-containing nicotinic receptors, are less permeable to Ca\(^{2+}\), insensitive to \(\alpha\)-BgTx, and they have high affinity for nicotine compared with \(\alpha_7\)-containing nAChRs. Since our RT-PCR analysis indicated that mRNA encoding for the \(\alpha_7\) subunit of AChRs is present in the BLA, we examined whether \(\alpha_7\)-containing nAChRs may be involved in the ACh-induced increase in sIPSC frequency in BLA neurons.

First, we tested the effect of methyllycaconitine (MLA) on the ACh-induced increase in sIPSC frequency. MLA has been reported to be a selective antagonist of \(\alpha_7\)-containing nACh receptors at low nM concentrations (Alkondon et al. 1992; Alkondon and Albuquerque 1993; Gray et al. 1996). Figure 5A shows that 30 nM MLA appeared to have little effect on the increased frequency of spontaneous GABAergic currents induced by 100 µM ACh. On average, ACh caused a 723 ± 103% increase in the number of sIPSCs per 25 s in the presence of 10-30 nM MLA compared to an increase of 794 ± 125% with ACh alone (\(p>0.5\), n=10; paired \(t\)-test).

Second, we examined the effect of bath application of \(\alpha\)-BgTx on the ACh-induced increase in sIPSC frequency. On average, ACh alone caused a 506 ± 56 % increase in the number of sIPSCs per 25 s before the application of \(\alpha\)-BgTx and a 536 ± 62% increase after a 10 min bath application of 100 nM \(\alpha\)-BgTx (not shown, \(p>0.5\), n=5; paired \(t\)-test). To make sure of adequate drug delivery, we also tested the effect of 100 nM \(\alpha\)-BgTx on the ACh-induced increase in sIPSC frequency in neurons freshly isolated from the BLA using an enzyme-free procedure described by Akaike and Moorhouse (2003). Because of the enzyme-free isolation procedure, these freshly isolated neurons have functioning synaptic boutons. In agreement with a previous study in another brain region (Léna et al, 1993), we found that sIPSC frequency in the isolated BLA neurons can be increased by ACh (Fig. 5B, top). Superfusion of these isolated BLA neurons with
100 nM α-BgTx for 8 min did not inhibit the ACh-induced increase in sIPSC frequency (Fig. 5B, bottom). In these isolated BLA neurons, on average, 100 µM ACh produced a 232 ± 54 % increase in the number of sIPSc per 15 s prior to α-BgTx and a 223 ± 52 % increase after exposure to 100 nM α-BgTx for 8 min (p>0.5, n=5; paired t-test).

What nAChR subunits are involved in the ACh-induced increase in sIPSC frequency?

Two approaches were used to elucidate the nAChR subunits in the BLA that may be involved in the ACh-induced increase in sIPSC frequency in the BLA.

First, we examined the sensitivity of sIPSCs frequency to various nicotinic agonists. Previous studies have found that that DMPP is much less efficacious than cytisine or nicotine in activating β4-containing nAChR subunits (Wong et al. 1995; Quick et al. 1999). On the other hand, cytisine has been reported to be much less efficacious than DMPP or nicotine in activating β2-containing nAChR subunits (Luetje and Patrick 1991; Papke and Heinemann 1994). As illustrated in Fig. 6A, we found that, of three nicotinic agonists tested, cytisine was the most effective agonist in increasing sIPSC frequency in the BLA and DMPP was the least effective. On average, for a 25 s continuous recording, 10 µM cytisine and 10 µM nicotine produced an 808 ± 120 % (n=7) and a 407 ± 85% (n=5) increase in the number of sIPSCs, respectively, whereas 10 µM DMPP caused only a 135 ± 29 % increase (n=7). There were significant differences among these agonists: p<0.001 for cytisine vs. DMPP; p<0.01 for nicotine vs. DMPP; and p<0.05 for cytisine vs. nicotine (ANOVA). Fig. 6B shows that cytisine was the most effective agonist in reducing spontaneous GABAergic event intervals.

Second, we studied the sensitivity of the ACh-induced increase in sIPSC frequency in the BLA to different antagonists. Previous studies on recombinant nAChRs have reported that d-tubocurarine (dTC) preferentially inhibits α3β4 and α3β2 subunit combinations, whereas dihydro-β-erythroidine (DHβE) preferentially inhibits non- α3β4 nAChRs (Wong et al. 1995; Harvey et al. 1996; Xiao et al. 1998). Figure 7A illustrates that in our experiments in the BLA, dTC inhibited the ACh-induced increase in sIPSC frequency
more effectively than DHβE. On average, for a 25 s continuous recording, the application of 100 µM ACh for 15 s increased the number of sIPSCs by 411 ± 60% (n=6). In the presence of 1 µM dTC or 1 µM DHβE, 100 µM ACh increased the GABAergic events by 119 ± 37% (p<0.05, n=6; paired t-test) and 277 ± 74% (p<0.001, n=6; paired t-test), respectively. Although both antagonists significantly reduced the increase in sIPSC frequency induced by ACh, dTC was more effective than DHβE at reducing ACh-induced GABA release (p<0.01, ANOVA). For further analysis, we plotted the cumulative distributions of event intervals and amplitudes. Both antagonists significantly suppressed the ACh-induced reduction in GABAergic event intervals (Fig. 7B, top, p<0.01 and p<0.001 for DHβE and dTC, respectively; K-S test), but the effect of dTC of was significantly greater than that of DHβE (p<0.05, t-test, n=6), judged by the event interval at P0.5. The antagonists, dTC and DHβE, did not significantly affect the amplitude of the ACh-induced sIPSCs (Fig. 7B, bottom, p>0.05, K-S test).

For comparison, we examined the ACh-induced increase in sIPSC frequency in pyramidal neurons in the CA1 region of hippocampal brain slices, where it has been proposed that α4β2-containing nAChRs play a major role in ACh-induced sIPSC frequency (Alkondon et al. 1999; Alkondon and Albuquerque 2001). As illustrated in Fig. 6 C/D, in contrast to the BLA neurons, in the CA1 hippocampal pyramidal neurons DMPP was the most effective agonist in increasing sIPSC frequency. On average, in the CA1 neurons, 10 µM DMPP produced a 432 ± 49% increase in the number of sIPSCs per 25 s (p<0.01, n=6; t-test), whereas 10 µM nicotine and 10µM cytisine caused only a 118 ± 32% and an 86 ± 34% increase, respectively (p>0.05, n=6; paired t-test). There is a significant difference between the effect of DMPP and cytisine on sIPSC frequency (p<0.01, ANOVA). With respect to antagonist action in the hippocampus, we found that in the CA1 neurons, 100 µM ACh alone produced an average 503 ± 130% increase in the number of sIPSCs/25 s (n=5). On the other hand, in the hippocampal CA1 neurons, in the presence of 1 µM dTC or 1 µM DHβE, ACh increased the average number of sIPSC/25 s by 403 ± 135 % (p<0.05, n=6; paired t-test) and 103 ± 50%, respectively (p>0.05, n=6; paired t-test) [not shown]. Thus, the pharmacology of the ACh-induced increase in
sIPSC frequency in CA1 hippocampal pyramidal neurons differs significantly from that in BLA principal neurons.

To test whether $\alpha_3\beta_4$ subunits may be involved in the ACh-induced increase in sIPSC frequency in the BLA, we examined the effect of $\alpha$-conotoxin-AuIB (AuIB), a 15-amino acid peptide that selectively inhibits this subunit combination (Luo et al. 1998). As illustrated in Fig. 8A, after superfusion with 1 µM AuIB for 4 min, on average, 100 µM ACh produced only a 20 ± 54% ($p>0.05$) increase in the number of sIPSCs/25 s, compared to a 581 ± 81% increase prior to AuIB ($p<0.05$, $n=5$; $t$-test). AuIB also suppressed the ACh-induced decrease in the IPSC event intervals as shown in Fig 8B ($p<0.01$, $K$-$S$ test).

DISCUSSION

We used the whole-cell patch-clamp technique to study nicotinic AChR actions in BLA neurons of rat brain. In the BLA principal neurons, in the presence of atropine, the local application of ACh increased the frequency of spontaneous inward currents. These inward currents were blocked by the GABA$_A$ receptor antagonist, bicuculline, but they were not affected by the glutamate antagonists, AP5/CNQX, indicating that these ACh-induced spontaneous inward currents are GABAergic. On the other hand, ACh did not significantly affect the postsynaptic current directly activated in these neurons by the exogenous application of GABA. A low Ca$^{2+}$ (0.1 mM) and high Mg$^{2+}$ (6 mM) external bathing solution abolished the ACh-induced spontaneous inward currents suggesting that these ACh-induced spontaneous GABAergic currents are sIPSCs.

**Cholinergic action in the BLA**

The BLA receives a major cholinergic input from the basal forebrain (Carlsen et al. 1985; Heckers and Mesulam 1994; Woolfer and Butcher 1982). In the amygdala, the immunoreaction for choline acetyltransferase (ChAT) is greatest in the BLA and the
majority of the synapses formed by ChAT-immunoreactive terminals are symmetric synaptic contacts with unlabeled terminals (serial synapses) that in turn form asymmetric synapses with dendritic structures (Li et al. 2001). Thus, ACh release from cholinergic terminals in the BLA could modulate neuronal activity via muscarinic and/or nicotinic receptors located on BLA neurons. Electrical stimulation of the cholinergic input has been reported to elicit a slow depolarization in the BLA (Moises et al. 1995). Intracellular recording indicates that the activation of muscarinic receptors on BLA neurons can produce a brief hyperpolarization followed by a prolonged depolarization (Washburn and Moises 1992). In addition, there are clear differences between muscarinic and nicotinic modulation in the BLA. Muscarinic modulation is reported to be mainly via postsynaptic M-receptors, which inhibit potassium conductances in the principal neurons (North 1989). On the other hand, we found that nicotinic modulation of principal neurons is primarily via increased sIPSC frequency due to activation of nAChRs. In electrophysiologically identified interneurons, we found that ACh directly activated an inward current in the presence of TTX and atropine. ACh also increased sIPSC frequency from terminals on freshly isolated principal neurons. In addition, in a cell-attached configuration, we found that ACh application did not increase the firing rate of BLA principal neurons (unpublished observations). These data suggest that in the BLA, activation of nAChRs on the soma and terminals of GABAergic interneurons can increase the frequency of sIPSCs in BLA principal neurons. The activation of nicotinic receptors has been reported to increase glutamate release in the brain (Gray et al. 1996; Guo et al. 1998; McGhee et al. 1995); however, in the BLA we found that the glutamate antagonists, CNQX/AP5, did not significantly affect the ACh-induced increase in sIPSC frequency, suggesting that increased glutamate release activating ionotropic glutamate receptors does not appear to be involved in the ACh-induced increase in sIPSC frequency.

*ACh-induced increase in sIPSC frequency is action potential-dependent*
In the chick brain slice, the application of nicotinic agonists has been found to induce an increase in IPSCs that is TTX-insensitive (Zhu and Chippinelli 2002). In the BLA, on the other hand, we found that TTX blocked the ACh-induced increase in sIPSC frequency, even when the application of the nicotinic agonist was prolonged. Thus, the modulation of sIPSCs in the present study appears to be predominantly, if not exclusively, action potential-dependent. In other studies, such action potential-dependent transmitter release has been attributed to “preterminal” nAChRs (Léna et al, 1993; McMahon et al. 1994). In our experiments, the observation of an ACh-induced inward current in the BLA interneurons could explain an ACh-induced increase in the firing rate of these interneurons that would result in an action potential dependent increase in sIPSC frequency.

RT-PCR analysis

Analysis of mRNA has been widely used to study receptor diversity in the brain, including nAChRs (Klink et al. 2001; Wada et al. 1989). Our RT-PCR analysis indicates that the nAChRs in the BLA could contain α_{2,4,7} and β_{2/4} subunits. In the present study, negative results or weak responses for α_{5/6} and β_{3} products in the BLA do not appear to be due to a poor efficiency of primers. The efficiency of the primers for the α_{5/6} subunits has been previously demonstrated (Sudweeks and Yakel 2000) and we detected β_{3} product in the medial habenular nucleus. This suggests that there are very few, if any, copies of α_{5/6} and β_{3} mRNAs in the BLA. In the medial habenular nucleus (MH), we found that the PCR products for nAChR subunits α_{3/4} and β_{2/3/4} were well detected. Sheffield et al. (2000) also detected PCR products for α_{3/4} and β_{2/3/4} nAChT subunits in the MH. In addition, Sheffield et al. (2000) detected PCR products for the α_{5/6/7} nAChR subunits in the MH, which we did not observe. This difference may be due to the fact that some of the animals used by Sheffield et al. (2000) were older than the ones we used; viz. Sheffield et al. (2000) used 7-21 day-old rats, whereas we used 7-10 day-old rats.
However, since high affinity nAChRs are thought to be pentameric structures composed of α and β subunits often with a stoichiometry of two α and three β subunits, mRNA data do not provide a complete description of the nAChR subunit combinations present in various types of neurons in the brain. The finding of an mRNA provides an index of a possible expressed protein. On the other hand, in the current study, negative results with RT-PCR suggest the absence or very few copies of mRNAs. In addition, several precautions are needed to interpret RT-PCR results. One is to exclude genomic contamination with a negative cDNA control, especially after many cycles of PCR. Thus, it is important to run RT-PCR reactions without reverse transcriptase to control for genomic contamination. In addition, primers were chosen to span an intron-exon border if detailed genomic information was available. Finally, the presence of an mRNA does not always indicate the existence of an expressed protein. Thus, other approaches are also needed to determine nACh receptor protein expression in various brain regions.

**α7-containing nAChRs in the BLA**

In the central nervous system, MLA and α-BgTx have been reported to be selective antagonists at α7-containing nAChRs (Alkondon et al. 1992, Gray et al. 1996). Since we found that the ACh-induced increase in sIPSC frequency was not inhibited by MLA or α-BgTx, the α7-containing nAChRs do not appear to play a major role in the ACh-induced increase in sIPSC frequency in the BLA. However, RT-PCR analysis indicated the presence of mRNAs coding for putative α7 nAChRs in the BLA. Thus, there could be α7-containing nAChRs in the BLA. How do we explain this apparent discrepancy? The α7-containing nAChRs are reported to desensitize very rapidly (Couturier et al. 1990a; Zhang et al. 1994); it is therefore possible that in our experiments the α7-containing nAChRs desensitize rapidly and thus do not contribute significantly to the ACh-induced increase in sIPSC frequency (cf. Baranzagi et al. 2001). On the other hand, it is also possible that the α7-containing nAChRs are located mainly on the presynaptic terminals of non-GABAergic fibers and thus do not significantly contribute to the modulation of GABAergic sIPSC frequency in the BLA.
Profiles of high affinity nAChRs in the BLA

By RT-PCR analysis, in addition to $\alpha_7$ containing nAChRs, $\alpha_4\beta_2/\alpha_3\beta_2/\alpha_2\beta_2/\alpha_4\beta_4$/\$ are possible $\alpha\beta$ subunit combinations of nAChRs that could contribute to the ACh-induced increase in the frequency of spontaneous GABAergic currents in the BLA. In addition, more complex combinations of subunits are also possible. We found that the rank order of nicotinic agonists to increase sIPSC frequency was cytisine>nicotine>DMPP, suggesting that $\beta_4$ is a major $\beta$ subunit involved in the ACh-induced increase in sIPSC frequency in the BLA (Papke and Heinemann 1994). In addition, in the BLA we found that dTC produced a stronger inhibition of the ACh-induced increase in sIPSC frequency than DH$\beta$E; whereas DH$\beta$E inhibited the ACh-induced increase in sIPSC frequency to a much greater extent than dTC in the CA1 region of hippocampus, indicating that different populations of nAChR modulate the ACh-induced increase in sIPSC frequency in the BLA compared to the hippocampus. In studies on recombinant receptors, human $\alpha_2\beta_4$-containing nAChRs are reported to display a sensitivity to dTC and DH$\beta$E (Chavez-Noriega et al. 1997) that is similar to the sensitivity we observed in the BLA. Considered together, the data suggest that $\alpha_3\beta_4$ combination could be the main functional subunits of nAChRs involved in the ACh-induced increase in sIPSC frequency in the BLA. This possibility is supported by the finding that AuIB, a selective antagonist at $\alpha_3\beta_4$-containing nAChRs, inhibited the ACh-induced increase in sIPSC frequency in the BLA. However, we were unable to determine if the ACh-induced inward current in BLA interneurons may be mediated by $\alpha_3\beta_4$-containing nAChRs because recordings from BLA interneurons were obtained so infrequently.

In addition to $\alpha_3\beta_4$ subunits, it is possible that other nAChR subunits may also be involved in the ACh-induced increase in sIPSC frequency in the BLA. It has been reported that individual neurons can express more than one nAChR subunit combination (Alkondon and Albuquerque 1993; Moss and Role 1993). Indeed, combinations of three
or even four nAChR subunits have been reported (Champtiaux et al. 2003; Conroy and Berg 1998; Nai et al. 2003).

**Diversity of nAChRs in the CNS**

The most abundant nAChR, which accounts for most of the high affinity nicotine binding in rat CNS, is reported to be made from the $\alpha_4$ and $\beta_2$ gene products (Schoepfer et al. 1988; Whiting et al. 1987, 1991). In general, $\alpha_3\beta_4$ subunits have been reported to mainly make up ganglionic nAChRs (Couturier et al. 1990b; Deneris et al. 1991). However, some studies suggest that the $\alpha_3\beta_4$ combination contributes to the functional nAChRs in medial habenula neurons (Quick et al. 1999; Zoli et al. 1998) and the $\alpha_3\beta_4$ combination has also been proposed to modulate glutamate release in hippocampal CA1 neurons (Alkondon and Albuquerque 2002). The present findings are thus consistent with other studies of nicotinic receptor diversity in the CNS.

**Functional implications**

The present study suggests that $\alpha_3\beta_4$-containing nAChRs are mainly responsible for the ACh-induced increase in sIPSC frequency in the BLA. Other studies suggest that $\alpha_4\beta_2$-containing nAChRs are widely expressed in other brain regions, including the hippocampus. This difference implies that selective activation or inhibition of different subtypes of nAChRs may be important in the localized regulation of brain function and could be potential sites of therapeutic value for the treatment of diseases associated with nervous system function. In addition, in animal studies, nicotine has been reported to reduce fear and stress and to have anxiolytic effects (George et al. 2001; Szyndler et al. 2001). Thus, inhibition of BLA principal neurons by ACh-induced increase in sIPSC frequency may be involved in those mechanisms. The basolateral amygdala is thought to play a critical role in fear conditioning (Davis et al. 1994). As illustrated in Fig. 9, the BLA principal neurons receive a glutamatergic projection from the lateral nucleus (LA) (Smith and Paré 1994), the main input of the amygdala for fear conditioned stimuli.
(LeDoux 2000). The BLA principal neurons then project to the medial sector of the central nucleus (CEm) (Collins and Paré 1999), the output for fear conditioned responses (Hitchcock et al. 1989). Thus, nicotinic activation of GABAergic sIPSCs in the BLA may be involved in the modulatory control of fear-related information processing in the amygdala.
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FIGURE LEGENDS

FIG. 1.  Activation of nicotinic acetylcholine receptors in basolateral amygdala (BLA) principal neurons increases the frequency of spontaneous inward currents. A: a confocal microscope image of a Lucifer yellow filled BLA pyramidal-shaped neuron. B: in current-clamp mode, injection of depolarizing currents generates a characteristic membrane response of only one or a few spikes, indicating that the recordings are from BLA principal neurons (Washburn and Moises 1992). The injection of negative current produced a “sag” in the hyperpolarizing response. C: in voltage-clamp mode, the local application of 100 µM acetylcholine (ACh), in the presence of 1 µM atropine, increased the frequency of spontaneous inward currents (top trace). This increased frequency of spontaneous inward currents was blocked by the GABA_A receptor antagonist, bicuculline (BIC, 20 µM) (lower trace), indicating that the currents are GABAergic. D: The ACh-induced increase in the frequency of spontaneous GABAergic currents (top) (a 532 ± 64% increase, n=5, p<0.05; t-test) was abolished by a low Ca^{2+} (0.1 mM) and high Mg^{2+} (6 mM) external bathing solution (middle) (only an 11 ± 75 increase, p>0.05, n=5; t-test); this effect was reversed after returning to the control external bathing solution (bottom).

FIG. 2.  The ACh-induced increase in the frequency of spontaneous GABAergic currents is concentration-dependent. A: increase in spontaneous GABAergic currents by ACh concentrations of 10, 30 and 100 µM, as indicated. B: cumulative distributions of event intervals (left) and amplitudes (right) were constructed from 25 s recordings and fitted with the Hill equation. Intervals at P_{0.5} were 416 ± 23, 228 ± 24, 141 ± 16 and 93 ± 11 ms for control and ACh concentrations of 10, 30 and 100 µM, respectively (p< 0.01, n=8, ANOVA). The event amplitudes at P_{0.5} were 32 ± 4.0, 36 ± 5.4, 28 ± 2.5 and 23 ± 1.3 pA for control and ACh concentrations of 10, 30 and 100 µM, respectively. The event amplitude was only decreased by the highest ACh concentration of 100 µM (p< 0.05, n=8, K-S-test).
FIG. 3.  Tetrodotoxin (TTX), but not the glutamate receptor antagonists AP5 plus CNQX (AP5/CNQX), blocks the increased frequency of GABAergic currents induced by ACh. A: the increased frequency of GABAergic currents induced by 100 μM ACh (top trace) was abolished by 1 μM TTX (middle trace). The bottom trace was recorded 15 min after beginning TTX washout. B: recording from a BLA interneuron, identified in current-clamp by the generation of repetitive firing with little adaptation in response to the injection of depolarizing current (inset). In these interneurons, in voltage-clamp mode, ACh induced both a postsynaptic inward current and increased spontaneous inward currents (top); TTX (1 μM) abolished the ACh-induced spontaneous inward currents, but not the postsynaptic inward current (bottom). C: top, average nicotine-induced GABAergic current frequency (n=4); bottom, average nicotine-induced GABAergic current frequency in the presence of 1 μM TTX (n=4). D: top traces, ACh-induced GABAergic currents were not blocked by the combined application of glutamate antagonists AP5 and CNQX (25/40 μM); bottom, cumulative distributions of GABAergic event intervals constructed from five neurons; the combination of AP5 and CNQX had no significant effect on the cumulative distribution of GABAergic event intervals (p>0.05, K-S test).

FIG. 4.  RT-PCR analysis of total RNA isolated from rat basolateral amygdala (BLA) and medial habenular nucleus (MH). Total RNA was pooled from four rats. A: and B: are from BLA and MH, respectively. The sizes for RT-PCR products were 336 (α2), 363(α3), 230 (α4), 396 (α5), 409 (α6), 357 (α7), 263 (β2), 223 (β3), and 270 (β4) bp, respectively. A: Upper panel. The subunits detected in BLA were α2/3/4/7 and β2/4. The product coding for the α6 subunit was not detected in BLA. The products coding for α5 and β3 were very light in BLA. B: Upper panel. The subunits detected in MH were α3/4 and β2/3/4. The products coding for the α2/5/6/7 subunits were not detected in MH. Lower panel for both A & B: reactions run without reverse transcriptase to control for genomic DNA-contamination.
FIG. 5. The α7 nAChR antagonists, methyllylcacoitine (MLA) and alphabungarotoxin (α-BgTx), do not block the ACh-induced increase in sIPSC frequency. A: top, the frequency of sIPSCs was increased by the application of 100 µM ACh (bar); bottom, pretreatment of the slice for 5 min with MLA (30 nM), a selective antagonist at α7-containing nACH receptors, did not block the effect of 100 µM ACh on the ACh-induced increase in sIPSC frequency. B: top, increase in the frequency of sIPSCs induced by the application of 100 µM ACh (bar); bottom, application of α-BgTx (100 nM) for 8 min did not block the ACh-induced increase in the frequency of sIPSCs; traces recorded from a BLA neuron isolated by mechanical dissociation without enzymes (see METHODS).

FIG. 6. Nicotinic agonists differentially increase the frequency of sIPSCs in BLA principal neurons and CA1 hippocampal pyramidal neurons. Among the nAChR agonists tested, cytisine was the most effective agonist in increasing sIPSC frequency in the BLA neurons (left panel), whereas DMPP was the most effective agonist in the CA1 hippocampal pyramidal neurons (right panel). Left panel, A: effect of the nicotinic agonists, DMPP, nicotine and cytisine, on the frequency of sIPSCs; all traces are from the same BLA neuron. In the records from this BLA neuron, the number of agonist-induced sIPSCs was 28 for DMPP (top), 58 for nicotine (middle) and 155 for cytisine (bottom). B: the cumulative distributions of event intervals for each agonist. The intervals at P0.5 were 360 ± 55 for DMPP, 222 ± 38 for nicotine and 83 ± 12ms for cytisine (p<0.05, n=8; ANOVA). Right panel, all traces in C are from the same CA1 hippocampal pyramidal neuron. In the records from this CA1 neuron, the number of agonist-induced sIPSCs was 78 for DMPP (top), 28 for nicotine (middle) and 14 for cytisine (bottom). D: the cumulative distributions of event intervals for each agonist. The intervals at P0.5 were 137 ± 45 for DMPP, 366 ± 37 for nicotine and 524 ± 32 ms for cytisine (p<0.05, n=7; ANOVA).

FIG. 7. Antagonist action on the frequency of ACh-induced sIPSCs. A: effect of the nicotinic antagonists, dTC and DHβE, on the frequency of ACh-induced sIPSCs; top, increase in the frequency of sIPSCs induced by 100 µM ACh; middle, this increase was
suppressed by 1 µM dTC; bottom, dihydro-β-erythroidine (DHβE, 1µM) was less effective than dTC at reducing the frequency of the ACh-induced sIPSCs. All traces are from the same neuron. In this neuron, the number of sIPSCs was 177 for ACh alone, 59 for ACh + dTC, and 150 for ACh + DHβE. B: the cumulative distribution of event intervals and amplitudes. The event intervals at P0.5 were 90 ± 14, 252 ± 59 and 130 ± 30 ms for ACh (100 µM), ACh plus 1 µM dTC (p<0.01, n=6, paired t-test) and ACh plus 1 µM DHβE, respectively (p<0.05, n=6; ANOVA). The event amplitudes at P0.5 were 34 ± 9.9 pA, 30 ± 7.9, and 30 ± 8.4 pA for ACh (100 µM), ACh plus 1 µM dTC, and ACh plus 1 µM DHβE, respectively (p>0.05, n=6; ANOVA).

FIG. 8. The selective nicotinic α3β4 antagonist, α-conotoxin-AuIB (AuIB), inhibits the increased frequency of the ACh-induced sIPSCs. A: top, sIPSCs in the presence of 100 µM ACh; middle, inhibition of ACh-induced sIPSCs by the application of 1µM AuIB; bottom, recovery of ACh-induced sIPSCs 15 min after beginning AuIB washout. ACh (100 µM) was present throughout the three traces in A. AuIB was pre-applied for 4 min before co-application with ACh. B: The cumulative distribution of GABAergic event intervals; intervals at P0.5 were 139 ± 22 ms for 100 µM ACh and 283 ± 56 ms for ACh plus AuIB (p<0.05, n=6; t-test).

FIG. 9. Schematic diagram of the BLA neuronal circuit showing the potential role of ACh-induced increased frequency of GABAergic sIPSCs in information processing in this amygdaloid circuit. The BLA principal neuron receives an excitatory input from the lateral nucleus (LA) and then projects an excitatory output to the central amygdaloid nucleus (CEM) (Smith and Paré 1994; Paré et al., 1995; Pitkänen et al., 1995). Nicotinic activation of mainly α3β4-containing receptors on GABAergic interneurons would increase the frequency of GABAergic sIPSCs in the BLA principal neurons, which would play an inhibitory role in controlling impulse traffic between the input and output of this amygdaloid circuit.
FIG. 1/Zhu et al.
FIG. 2/Zhu et al.

A

Control

ACh 10 µM

ACh 30 µM

ACh 100 µM

B

Cumulative Probability

Event Interval (s) Event Amplitude (pA)

0.0 0.5 1.0

0 1 2 3

0 50 100 150

Cumulative Probability

Event Interval (s) Event Amplitude (pA)

Control ACh 10 µM ACh 30 µM ACh 100 µM

0 1 2 3

0 50 100 150
FIG. 3/Zhu et al.

ACh 100 µM

TTX 1 µM

wash

ACh 100 µM

nicotine 10 µM

AP5/CNQX (25/40 µM)

IPSCs/5 sec

Time (s)

Cumulative Probability

Event Interval (s)
FIG. 4/Zhu et al.

A

BLA

bp M α2 α3 α4 α5 α6 α7 β2 β3 β4

RT (+)

RT (-)

B

MH

RT (+)

RT (-)
ACh 100 µM

ACh 100 µM + MLA 30 nM

A

B

ACh 100 µM

α-BgTx 100 nM

ACh 100 µM

200 pA

1 s

100 pA

2 s
FIG. 6/Zhu et al.

A. BLA
- DMPP 10 µM
- Nicotine 10 µM
- Cytisine 10 µM

B. Cumulative Probability

C. CA1

D. Cumulative Probability

Event Interval (s)

0.0 0.5 1.0

Cululative Probability

0 1 2 3 4

Event Interval (s)

0 1 2 3 4
FIG. 7/Zhu et al.

A

ACh 100 µM

ACh + dTC 1 µM

ACh + DHβE 1 µM

B

Cumulative Probability

Event Interval (s)

0.0 0.5 1.0 1.5 2.0

0.0 0.5 1.0

0.0 50 100 150

Event Amplitude (pA)

0.0 0.5 1.0

ACh 100 µM

ACh + dTC

ACh + DHβE
ACh 100 µM

ACh + AulB 1 µM

ACh after AulB washout

B

Cumulative Probability

Event Interval (s)
To $C_{E_M}$

Excitation

Glutamatergic Principal Neuron

GABAergic Interneuron

$\alpha_3\beta_4$

Cholinergic Input

From Basal Forebrain

Glutamatergic Input

From LA