Neurotransmitter GABA Activates Muscle but Not $\alpha_7$ Nicotinic Receptors

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ABSTRACT

Cys-loop receptors are neurotransmitter-activated ion channels involved in synaptic and extrasynaptic transmission in the brain and are also present in non-neuronal cells. As GABA$_A$ and nicotinic receptors (nAChR) belong to this family, we explored by macroscopic and single-channel recordings whether the inhibitory neurotransmitter GABA has the ability to activate excitatory nAChRs. GABA differentially activates nAChR subtypes. It activates muscle nAChRs, with maximal peak currents of about 10% of those elicited by acetylcholine (ACh) and 15-fold higher EC$_{50}$ with respect to ACh. At the single-channel level, the weak agonism is revealed by the requirement of 20-fold higher concentration of GABA for detectable channel openings, a major population of brief openings, and absence of clusters of openings when compared with ACh. Mutations at key residues of the principal binding-site face of muscle nAChRs ($\alpha$Y190 and $\alpha$G153) affect GABA activation similarly as ACh activation, whereas a mutation at the complementary face (eG57) shows a selective effect for GABA. Studies with subunit-lacking receptors show that GABA can activate muscle nAChRs through the $\alpha/\delta$ interface. Interestingly, single-channel activity elicited by GABA is similar to that elicited by ACh in gain-of-function nAChr mutants associated to congenital myasthenic syndromes, which could be important in the progression of the disorders due to steady exposure to serum GABA. In contrast, GABA cannot elicit single-channel or macroscopic currents of $\alpha_7$ or the chimeric $\alpha_7$-serotonin-type 3 receptor, a feature important for preserving an adequate excitatory/inhibitory balance in the brain as well as for avoiding activation of non-neuronal receptors by serum GABA.

Introduction

Nicotinic acetylcholine receptors (nAChR) are members of the Cys-loop ligand-gated ion channel superfamily that also includes glycine, serotonin-type 3 (5HT$_3$A), and GABA$_A$ receptors (Bouzat, 2012). $\alpha_7$ nAChRs are among the most abundant in the central nervous system. They contribute to cognitive functioning, sensory information processing, attention, working memory, and reward pathways. Decline or alterations of signaling involving $\alpha_7$ have been implicated in neurologic diseases, such as schizophrenia, epilepsy, autism, and Alzheimer’s disease (Kalamida et al., 2007; Thomsen et al., 2010; Wallace and Porter, 2011; Hurst et al., 2013; Wallace and Bertrand, 2013). $\alpha_7$ nAChRs localize both pre- and postsynaptically, but they dominantly operate in an extrasynaptic mode (Lendvai and Vizi, 2008). Volume transmission is also relevant in non-neuronal cells, such as lymphocytes and macrophages, in which $\alpha_7$ is involved in anti-inflammatory responses (De Rosa et al., 2009; Andersson and Tracey, 2012). Muscle nAChRs are confined at postsynaptic locations in muscle fibers and are mainly implicated in myasthenia gravis and congenital myasthenic syndromes (Engel et al., 2012).

nAChR subunits are classified as either $\alpha$, which contain a disulfide bridge important for acetylcholine (ACh) binding, or non-$\alpha$ subunits, which lack this motif (Matsuda et al., 2005). nAChRs assemble from five identical $\alpha$ subunits, such as $\alpha_7$, or from different $\alpha$ and non-$\alpha$ subunits, such as the muscle nAChR, which in the adult is composed of two $\alpha_1$, one $\beta$, one $\delta$, and one $\epsilon$ subunits. The five homologous subunits are arranged as barrel staves around a central ion-conducting pore (Bartos et al., 2009). Approximately half of each subunit is extracellular, with the remainder comprising transmembrane domains M1–M4 and a large cytoplasmic domain spanning M3 and M4 (Bouzat, 2012). The neurotransmitter binding sites are formed by three discontinuous loops of the $\alpha$ subunit, whereas the complementary face is formed by three discontinuous $\beta$-strands of the adjacent subunit, which in the muscle nAChR

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is either the $\varepsilon$ or the $\delta$ subunit. Key residues of the principal face are grouped in regions called loops A, B, and C at the principal face and loops D, E, and F at the complementary face (Brejc et al., 2001; Sine and Engel, 2006; Nys et al., 2013). Residues of the principal face are highly conserved between $\alpha7$ and $\alpha1$ subunits, whereas less conservation is found in residues located at the complementary face (Bartos et al., 2009). The potential for understanding ligand binding at the atomic structural level arose with the discovery of acetylcholine-binding protein (AChBP) (Brejc et al., 2001), a soluble protein homologous to the ligand binding domains of pentameric ligand-gated ion channels. Homology models from AChBP have helped to gain in-depth understanding of how ligands bind to an ACh binding site.

GABA is the main inhibitor neurotransmitter in the nervous system and can exert its effects through GABA$_A$ receptors located at inhibitory synapses. However, GABA released at the synaptic cleft can diffuse and reach receptors outside of the synapse, on the soma, dendrites, and axon (Kullmann et al., 2005). Thus, GABA can be involved in both synaptic and extrasynaptic actions in the brain (Oláh et al., 2009). Moreover, GABA might even have a broader spectrum of effects because it is also present in blood at a concentration of 0.1 $\mu$M (Björg et al., 2001). Given that GABA$_A$ receptors and nACHRs belong to the same Cys-loop family, we sought to explore whether the inhibitory neurotransmitter GABA is able to activate excitatory nACHRs, thus leading to cross-talk among family members. To this end, we evaluated the action of GABA at the neuronal $\alpha7$ and muscle nACHR by macroscopic and single-channel recordings. Our results demonstrate that GABA has the ability to activate muscle, but not $\alpha7$ nACHRs, and provide insights into the structural and mechanistic differences with respect to ACh activation.

Materials and Methods

Site-Directed Mutagenesis and Expression of $\alpha7$ and Adult Muscle nACHR

BOSC 23 cells, which are modified HEK293 cells (Pear et al., 1993), were transfected with wild-type or mutant subunit cDNAs of adult muscle nACHRs: $\alpha1, \beta, \varepsilon$, and $\delta$, human $\alpha7$ or $\alpha7$-5HT$_3\alpha$ using calcium phosphate precipitation (Bouzat et al., 2002, 2004, 2008). To promote receptor expression on the cell surface, the human $\alpha7$ cDNA was cotransfected with cDNA encoding the chaperone protein RIC-3 (Williams et al., 2005). A plasmid encoding green fluorescent protein was included in all transfections because it cannot be modeled reliably (Hernando et al., 2012; Björk et al., 2001). We have not included in our analysis loop F of Lymnaea stagnalis AChBP (PDB ID 1UW6) as template. A plasmid encoding green fluorescent protein was included in all transfections to allow the identification of transfected cells under fluorescence optics. Cells were used for current measurements 48 or 72 hours after transfection. Mutant subunits were constructed using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Patch-Clamp Experiments

Single-Channel Recordings. Single-channel currents were recorded in the cell-attached configuration (Hamill et al., 1981) at room temperature. The bath and pipette solutions contained 140 mM KCl, 5.4 mM NaCl, 1.8 mM CaCl$_2$, 1.7 mM MgCl$_2$, and 10 mM HEPES (pH 7.4). For $\alpha7$-5HT$_3\alpha$, the bath solution contained low calcium (0.2 mM) to avoid open-channel block (Bouzat et al., 2008). Currents were recorded using an Axopatch 200 B patch-clamp amplifier (Axon Instruments, CA), digitized at 5-microsecond intervals, recorded using the Acquire program (Bruxton, Seattle, WA), and detected by the half-amplitude threshold criterion using the TAC 4.0.10 program (Bruxton). Channels were typically recorded at a membrane potential of $-70$ mV. Open and closed time histograms were plotted using a logarithmic abscissa and a square root ordinate and fitted to the sum of exponential functions by maximum likelihood using the TACFit program (Bruxton). Clusters or bursts of openings corresponding to a single receptor channel were identified as a series of closely spaced events preceded and followed by closed intervals greater than a specified duration ($t_{\text{crit}}$); this duration was taken as the point of intersection of the predominant closed time component and the succeeding one in the closed-time histogram (Bouzat et al., 2000). In some experiments, the tip of the pipette was filled with a solution containing ACh or GABA and the shaft with the same solution containing ACh and GABA or GABA and $\alpha$-bungarotoxin (a-BTX). Channel recordings obtained immediately after seal formation were assigned the status of control condition. As with time, the shaft solution diffuses to the tip of the pipette; channels recorded 5–6 minutes after the beginning of the recording were considered the exposed condition. Only patches in which the seal was made rapidly were used for this type of analysis.

Whole-Cell Recordings. Currents were recorded in the whole-cell patch-clamp configuration at a pipette potential of $-70$ mV at room temperature. The pipette solution contained 134 mM KCl, 5 mM EGTA, 1.7 mM MgCl$_2$, and 10 mM HEPES, pH 7.3. The extracellular solution (ECS) contained 150 mM NaCl, 1.8 mM CaCl$_2$, 1 mM MgCl$_2$, and 10 mM HEPES, pH 7.3. Rapid agonist perfusion was performed, as described before (Corradi et al., 2009). Briefly, the perfusion system consisted of solution reservoirs suspended on a pole for gravity-driven flow, switching valves, a solenoid-driven pinch valve, and two tubes oriented into the culture dish. One tube contained ECS without agonist, and the other contained ECS with agonist. When the pinch valve was switched, solution flowed from only one side at a time, and switched from one side to the other. This perfusion system allowed for a rapid exchange (0.1–1 milliseconds) of the solution bathing the patch. Macroscopic currents were recorded using an Axopatch 200 B patch-clamp amplifier (Axon Instruments) and filtered at 5 kHz. Data analysis was performed using the IgorPro software (WaveMetrics, Lake Oswego, OR). Current records were aligned at the point at which the current reached 50% of maximum. Peak currents correspond to the value obtained by extrapolation of the decay current to this point. Current decays were fitted by an exponential decay function:

$$I(t) = I_0 \exp(-t/t_d) + I_c$$

(1)

where $I_0$ and $I_c$ are peak and the steady state current values, respectively, and $t_d$ is the decay time constant (Andersen et al., 2011).

EC$_{S0}$ and Hill coefficient values were calculated by nonlinear regression analysis using the Hill equation:

$$ED_{S0} = 1/[1 + (EC_{S0}/L)^{H}]$$

(2)

where EC$_{S0}$ is the agonist concentration that elicits the half-maximal response, $n_H$ is the Hill coefficient, and $L$ is the agonist concentration (Bartos et al., 2006).

Homology Modeling and Molecular Docking

Homology models of the extracellular domain of the human neuronal $\alpha7$ nACHR and the adult mouse muscle nACHR were created using the structure of the nicotine-bound Lymnaea stagnalis AChBP (PDB ID 1UW6) as template. The amino acid sequences were aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and modeling was performed using Modeler v9 v11 (http://salilab.org/modeler/) (Sali et al., 1995). We have not included in our analysis loop F of $\delta$ and $\varepsilon$ subunits because it cannot be modeled reliably (Hernando et al., 2012; Arias et al., 2013). Symmetry constraints were imposed on the Ca atoms for the same $\alpha$-type subunit. Ten models were constructed, and the one with the highest Modeler scores and the smallest percentage of amino acids in the disallowed region of the Ramachandran plot was selected for docking studies. ACh or GABA molecules were docked separately into the agonist binding sites located at $\delta$–$\varepsilon$, $\alpha$–$\varepsilon$, or each of...
the five α7–α7 interfaces using AutoDock version 4.3 (Morris et al., 2009). The ligand binding site was defined as being within 20 Å of W149. One hundred genetic algorithm runs were performed for each condition. Clustering of the results was done with AutoDock based on a root-mean-square deviation cutoff of 2.0 Å. Docking results were corroborated in four different procedures. Best docking poses for each drug and interface were plotted with Discovery Studio Visualizer 3.5 (Accelrys Software, San Diego, CA).

Statistics. Experimental data are shown as mean ± S.D. Statistical comparisons were done using the Student’s t test. \( P < 0.05 \) was considered significant.

Results

GABA Activates Muscle nAChRs. To evaluate whether the neurotransmitter GABA is able to activate muscle nAChR, we first recorded macroscopic currents in the whole-cell configuration from cells transfected with α1, β, δ, and ε subunits. At concentrations higher than 25 μM, GABA elicits macroscopic responses of adult muscle nAChR. Maximal responses elicited by 500 μM GABA are 10.5 ± 3.5% of those elicited by 500 μM ACh (Fig. 1A). Expressing the relative peak current as a function of agonist concentration results in values of EC50 of 18 ± 1.1 μM (nH = 2.7 ± 0.7, n = 3) and 268 ± 80 μM (nH = 0.7 ± 0.2, n = 3) for ACh and GABA, respectively (Fig. 1A). These results indicate that GABA has the ability to activate muscle nAChRs, although it behaves as a very low-efficacy agonist.

To reveal the basis underlying partial activation of muscle nAChR by GABA, we performed single-channel recordings (Fig. 1B). Single-channel openings are readily detected at GABA concentrations as low as 1 μM GABA. Activity appears mainly as isolated openings flanked by long closings (Fig. 1B). For GABA concentrations lower than 1 μM, the frequency of openings was markedly low and many patches appeared silent. To unequivocally confirm that the detected channel activity arises from GABA-activated nAChRs and not from endogenous GABA receptors, we recorded single channels from cells transfected only with green fluorescent protein (GFP) cDNA, whereas no channels are observed in cells transfected with GFP and nAChR-subunit cDNAs. Whereas no channels are observed in the patch containing 1 mM GABA and 1 μM α-BTX (Fig. 2; Table 1). GABA (1 μM–10 mM) does not elicit detectable single-channel activity (n = 14) in good agreement with previous report in HEK cells (McCann et al., 2006). This result demonstrates that the detected channel activity arises from nAChR and that GABA-activated channels are blocked by α-BTX.

Activation by GABA of Mutant Muscle nAChRs. We next evaluated how mutations at the principal and complementary faces of the binding site that affect ACh activation also affect activation by GABA.

The amino acid αY190 located at loop C of the principal face of the binding site has been shown to be essential for muscle (Sine et al., 1994) and α7 (Rayes et al., 2009; Andersen et al., 2013) nAChR activation. The mutation αY190F decreases ACh affinity and the efficacy of activation (Chen et al., 1995; Purohit and Auerbach, 2011; Williams et al., 2011). At the single-channel level, the changes are evidenced by the absence of clusters at high ACh concentrations and brief opening events (Fig. 2; Table 1). GABA (1 μM–10 mM) does not elicit detectable single-channel activity (n = 14) in good agreement with the reduced activation shown for ACh (Table 1).

αG153S, located at loop B of the principal side, is a key residue for ACh activation of mammalian muscle and neuronal α7 nAChRs (Grutter et al., 2003; Jaday et al., 2013). Single-channel kinetic analysis of αG153S nAChR revealed a markedly decreased rate of ACh dissociation, which causes the mutant nAChR to open repeatedly forming bursts (Sine et al., 1995; Rayes et al., 2004) (Fig. 2). In the presence of GABA, kinetic changes are qualitatively similar to those observed in the presence of ACh. Channel openings appear in clear bursts (0.1–500 μM GABA) (Fig. 2). The mean durations of the slowest open component (1.7 milliseconds; Table 1) and of bursts (4.53 ± 1.69 milliseconds, relative area = 0.26 ± 0.16, n = 4) are briefer than those determined in the presence of ACh (3.7 milliseconds [Table 1] and 10.18 ± 0.35 milliseconds [relative area = 0.61 ± 0.05, n = 3] for mean open and burst durations, respectively, \( P < 0.01 \)).

Position 57 of loop D of the complementary face of the binding site has been shown to be a determinant of drug selectivity (Bartos et al., 2006, 2009). Recordings from nAChR containing the mutant α57Q subunit show increased open durations for GABA-activated channels, but not for ACh-activated channels with respect to wild-type nAChRs (\( P > 0.01, n = 3 \)) (Table 1). For receptors carrying the δ57Q mutation, no detectable differences in the mean open duration for both GABA and ACh are observed (Table 1) (\( P > 0.05, n = 5 \)).

Overall, binding site mutations at the principal face affect similarly GABA and ACh activation, whereas position 57 of the ε subunit located at the complementary face changes GABA, but not ACh activation.
To gain more insights into GABA activation, the importance of the α/δ binding-site interface was evaluated by studying activation of nAChR lacking the ε subunit. In the absence of this subunit, nAChRs contain two α/δ binding-site interfaces instead of one α/δ and one α/ε interface as in wild-type nAChR. These receptors express well in cells and are activated by ACh (Bouzat et al., 1994). ACh-elicited openings are more prolonged than those of wild-type nAChR (Fig. 3A). The mean duration of the slowest open component for ACh-activated channels is 5.27 ± 1.37 milliseconds (relative area = 0.77 ± 0.13, n = 3) instead of ~1 millisecond for wild-type nAChR. GABA (10 μM) also elicits single-channel currents from α2βδ2 receptors (Fig. 3A), indicating that this agonist is capable of activating nAChR through the α/δ interface. In agreement with ACh-elicited activity, the duration of the slowest open component (1.60 ± 0.40 milliseconds, relative area = 0.10 ± 0.05, n = 4) is longer than that of wild-type nAChR when GABA is the agonist (P < 0.05). Although spontaneous activity of α2βδ2 receptors has been reported (Zhou et al., 1999), we were not able to record clearly detectable openings in this study in the absence of agonist. This
difference may be due to differences in receptor expression because the open probability of ligand-free activated receptors is very low. Thus, we can ensure that the detected openings arise from GABA-activated nAChR.

The mutation eT264P at the M2 domain of the e subunit, which has been first described in a patient suffering from a slow-channel congenital myasthenic syndrome, leads to gain of function (Ohno et al., 1995; Engel et al., 2012). In the absence of ACh, spontaneous activity of the mutant eT264P nAChR is evidenced by the presence of brief openings (Fig. 3B), being the mean duration of the longest duration open component of 1.5 ± 0.3 milliseconds (relative area = 0.05 ± 0.01, n = 3). In the presence of ACh (1 μM) or GABA (0.1–100 μM), significantly prolonged openings are observed (Fig. 3B). Open time histograms are fitted by three components, and the slowest one, which is not detected in the absence of agonist, shows a mean duration of 20.62 ± 7.84 milliseconds for 1 μM ACh (relative area = 0.56 ± 0.08, n = 3) and 28.45 ± 3.45 milliseconds for 0.1 μM GABA (relative area = 0.81 ± 0.05, n = 3). Thus, GABA behaves very similar to ACh in this M2 mutant receptor.

GABA Cannot Activate α7 nAChR. We evaluated whether GABA is able to activate human α7 and the high conductance form of the chimeric α7-5HT3A receptor, which is a good model for studies of α7 because it shows high surface

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**TABLE 1**

Open durations of wild-type and mutant muscle nAChR activated by ACh and GABA

Single-channel recordings were performed from cells expressing muscle wild-type receptors or receptors containing the indicated mutant subunit. Open duration components were obtained from the open time histograms. Data are shown as mean ± S.D. for at least three recordings for each condition. For αY190F, single-channel activity in the presence of GABA was not detected in 14 different patches from GFP-transfected cells (green cells).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Agonist</th>
<th>μM</th>
<th>Open 1 (ms) (Relative Area)</th>
<th>Open 2 (ms) (Relative Area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>ACh</td>
<td>1</td>
<td>0.99 ± 0.10 (0.70 ± 0.12)</td>
<td>0.24 ± 0.07 (0.30 ± 0.13)</td>
</tr>
<tr>
<td></td>
<td>GABA</td>
<td></td>
<td>0.76 ± 0.12 (0.31 ± 0.04)</td>
<td>0.10 ± 0.03 (0.69 ± 0.04)</td>
</tr>
<tr>
<td>αY190F</td>
<td>ACh</td>
<td>60</td>
<td>0.58 ± 0.17 (0.11 ± 0.06)</td>
<td>0.19 ± 0.05 (0.89 ± 0.06)</td>
</tr>
<tr>
<td></td>
<td>GABA 1–10,000</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>αG153S</td>
<td>ACh</td>
<td>1</td>
<td>3.76 ± 0.29 (0.67 ± 0.07)</td>
<td>0.36 ± 0.04 (0.32 ± 0.07)</td>
</tr>
<tr>
<td></td>
<td>GABA</td>
<td></td>
<td>1.68 ± 0.40 (0.39 ± 0.157)</td>
<td>0.18 ± 0.02 (0.60 ± 0.157)</td>
</tr>
<tr>
<td>eG57Q</td>
<td>ACh</td>
<td>1</td>
<td>0.88 ± 0.0 (0.18 ± 0.12)</td>
<td>0.32 ± 0.03 (0.82 ± 0.12)</td>
</tr>
<tr>
<td></td>
<td>GABA 1000</td>
<td>1.91 ± 0.33 (0.11 ± 0.01)</td>
<td>0.24 ± 0.03 (0.89 ± 0.01)</td>
<td></td>
</tr>
<tr>
<td>δD57Q</td>
<td>ACh</td>
<td>1</td>
<td>1.18 ± 0.25 (0.67 ± 0.08)</td>
<td>0.32 ± 0.06 (0.33 ± 0.08)</td>
</tr>
<tr>
<td></td>
<td>GABA 1000</td>
<td>1.55 ± 0.59 (0.06 ± 0.02)</td>
<td>0.16 ± 0.02 (0.93 ± 0.01)</td>
<td></td>
</tr>
<tr>
<td>aY190F</td>
<td>ACh</td>
<td>60</td>
<td>0.58 ± 0.17 (0.11 ± 0.06)</td>
<td>0.19 ± 0.05 (0.89 ± 0.06)</td>
</tr>
<tr>
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<td>nd</td>
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<td></td>
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</tbody>
</table>

nd, not detected.

Fig. 2. Activation of mutant muscle nAChRs by GABA. Left, channels were recorded from BOSC 23 cells expressing nAChR containing the mutant αY190F, αG153S, eG57Q, or δD57Q subunits. Currents are shown at a bandwidth of 7 kHz with channel openings as upward deflections. Right, open and closed time histograms of nAChRs carrying the specified mutant subunit. The data are representative of four to six recordings for each condition. Membrane potential: −70 mV.
expression and channel activity is easily detected (Rayes et al., 2005; Andersen et al., 2011). In cells transfected with $\alpha_7$ (Fig. 4A) or $\alpha_7$-5HT$_3A$ (Fig. 4B), macroscopic currents are elicited by 300 $\mu$M ACh (Bouzat et al., 2008). In contrast, application of 1–10 mM GABA to cells showing ACh responses does not generate detectable macroscopic currents (Fig. 4). No single-channel activity from cells expressing $\alpha_7$ ($n = 18$) or $\alpha_7$-5HT$_3A$ receptors ($n = 20$) is detected in the presence of 100–1000 $\mu$M GABA. In contrast, typical single-channel openings with mean durations of $\sim$0.28 milliseconds for $\alpha_7$ (100 $\mu$M ACh) and $\sim$9.5 milliseconds for $\alpha_7$-5HT$_3A$ (500 $\mu$M ACh) (Rayes et al., 2005; Bouzat et al., 2008) are detected (Fig. 4, A and B). In the $\alpha_7$-5HT$_3A$ receptor, we tested whether a mutation at position 153 of the extracellular domain ($\alpha_7$G153E), which has been shown to increase efficacy in muscle and $\alpha_7$ nAChRs (Bartos et al., 2006; Jadey et al., 2013), allows detectable single-channel activity elicited by GABA. In contrast to the results observed in muscle nAChRs, channel activity was not detected in the presence of GABA in $\alpha_7$-5HT$_3A$ carrying the G153E mutation (500 and 1000 $\mu$M, $n = 13$) (Fig. 4C).

We also explored whether the presence of GABA affects $\alpha_7$ activity. To this end, we recorded $\alpha_7$-5HT$_3A$ channels in the presence of 300 $\mu$M ACh in the tip of the pipette and 300 $\mu$M ACh plus 10 mM GABA in the shaft. Under these conditions, competition of GABA should be evidenced as a marked reduction of the frequency of channel openings with time of recording due to the diffusion of GABA to the pipette tip. The time-dependent decrease in channel activity (67%), which was measured by the decrease in the number of openings/min between minutes 1 and 5 of the recording, was not markedly different from that observed for control conditions—only ACh present (61%)—thus evidencing that GABA does not antagonize ACh action at $\alpha_7$ receptors.

Docking of GABA into nAChRs. To gain insights into how GABA may interact with nAChRs, we performed in silico studies. For the adult muscle nAChR, docking of ACh in both $\alpha$–e and $\alpha$–d interfaces resulted in only one energetically favorable model, with best binding energy (BBE) of $\sim$5.0 kcal/mol. We observed the well known cation–π interaction between the positively charged group of ACh and the aromatic ring of aW149 (Dougherty, 2007). ACh also showed the possibility to form a hydrogen bond between its carbonyl group and the polar backbone hydrogen of δL121 or eL119, a hydrogen bond with δ–e–Y104, and to interact with W55 at the complementary face (Fig. 5A for $\alpha$/$\epsilon$).

For GABA, two plausible models were detected for the $\alpha$–e interface, named model 1 (Fig. 5B) and model 2 (Fig. 5C), with BBE of $\sim$3.5 and $\sim$3.0 kcal/mol, respectively. The probability to adopt one orientation or the other appears to be similar. For the $\alpha$–d interface, all docking runs were clustered into only one group with a similar orientation and BBE as that of model 1. Model 1 is able to reproduce most of the features of ACh interaction (Fig. 5B). The carboxyl group of GABA shows the potential to form a hydrogen bond with Y104 and δL121 or eL119, a hydrogen bond with δ–ε–Y104, and to interact with W55 at the complementary face (Fig. 5D). All interactions are similar to those observed for muscle interfaces, with the exception of the hydrogen bond with Y104, which is phenylalanine in $\alpha_7$. In contrast, GABA shows two plausible models, with similar...
orientations to those in α–ε interface. However, the probability to adopt model 2 conformation is twofold higher than that of model 1. In model 1 orientation, it shows the potential to form the cation−π interaction and the hydrogen bond with W149 (Fig. 5E). GABA may also form hydrogen bonds with L119 and with N107, which is not observed in our model of muscle nAChR interfaces. In model 2 orientation, the majority of key interactions are similar to those observed in the muscle nAChR (Fig. 5F; Supplemental Fig. 1).

**Discussion**

In this study, we demonstrate that GABA behaves as a partial agonist of the muscle nAChR, but cannot activate the neuronal α7 nAChR.
Macroscopic current recordings show that maximal peak currents of muscle nAChRs elicited by GABA are only 10% of those elicited by ACh, and dose-response curves show that the EC₅₀ is 15-fold higher for GABA than ACh, indicating that GABA is a low-efficacy agonist of the muscle nAChR. At the single-channel level, the mechanistic basis of this weak agonistic efficacy is revealed by the following: 1) the requirement of ~20-fold higher concentration of GABA than ACh (1 μM instead of 50 nM for ACh) (Spitzmaul et al., 2004) for detectable reliable channel activity; 2) the presence of a major population of brief openings; and 3) the absence of clusters at all GABA concentrations, in contrast to what is observed with ACh or other agonists (Bouzat et al., 2000).

We explored whether GABA requires similar residues to those shown for efficient ACh activation in the muscle nAChR. We demonstrated that key residues of the principal face, Y190 located in loop C and G153 located in loop B, are also required for GABA activation of muscle nAChR. The effects of αY190F and αG153S mutations on muscle nAChR activation have been well characterized (Sine et al., 1995; Salamone et al., 1999; Grutter et al., 2003; Rayes et al., 2004; Mukhtasimova et al., 2009; Purohit and Auerbach, 2011, 2013; Jady et al., 2013). In parallel with the changes observed for ACh, GABA responses are increased in αG153S mutant and are negligible in αY190F mutant.

Position 153 at loop B of the binding site has been shown to be associated with a slow-channel syndrome (Sine et al., 1995), to be involved in the high efficacy of activation of nematode nAChRs by anthelminthic drugs (Rayes et al., 2004), to affect gating in neuronal nAChR (Grutter et al., 2003), and to affect agonist efficacy of muscle nAChR (Purohit and Auerbach, 2011). GABA is capable of eliciting bursts of openings, which are significantly prolonged in the αG153S when compared with the wild-type nAChR. Nevertheless, open and burst durations are briefer than those determined in the presence of ACh. This result indicates that these positions, Y190 and G153, are not determinants of GABA partial agonism, but they are important for GABA as well as for ACh activation.

Our study reveals that the residue at position 57 located in loop D of the complementary face of the α/ε binding site is a key determinant of the action of GABA at muscle nAChR. The mutation εG57Q selectively increases the efficacy of GABA activation, as evidenced by significantly increased open channel durations and by the presence of bursts. As described before (Bartos et al., 2009), ACh activation is not modified in the mutant εG57Q with respect to the control. The equivalent mutation in the δ subunit does not affect either GABA or ACh activation. These results suggest that GABA binds to the α/ε interface, and that εG57 is involved in its activation. An alternative explanation could be that GABA does not bind to the α-ε interface except in the presence of the εG57Q mutation. Under this hypothesis, the major proportion of brief openings detected in wild-type nAChRs would correspond to openings of single-ligated receptors (α/δ interface). The mutation would lead to more prolonged openings—corresponding to biligated receptors—by allowing binding of GABA to the α/ε interface. For this to occur, GABA must be able to bind to the α/δ interface. To test this possibility, we studied receptors lacking the ε subunit because, unfortunately, it is not possible to detect channel openings from δ-lacking receptors (Bouzat et al., 1994; Zhou et al., 1999). We found that GABA is capable of mediating activation through the α/δ interface. In the presence of GABA, single-channel currents from αβδε occur in bursts of prolonged openings, which are significantly different from those detected in the absence of agonist, thus indicating that GABA can activate the receptor through the α/δ interface. The mutation εG57Q has been shown to increase significantly the efficacy of morantel activation (Bartos et al., 2009). Thus, the present results reinforce the role of position 57 as a determinant of selectivity for a broader spectrum of drugs (Shimomura et al., 2002; Bartos et al., 2006).

Docking studies showed only one possible way of interaction of ACh and of GABA at the α/δ interface, but two of GABA at αε and α7/α7 interfaces. Models 1 and 2 differ in the orientation of the GABA molecule, which can form the H-bonds and cation-π interactions with key residues of the binding site (Y93, W149 of the principal face and Y104, N107 or L119/L121 of the complementary face), similar to those observed for ACh and other agonists. In light of these observations, the partial agonism of GABA at muscle nAChRs may be explained by its ability of adopting multiple orientations at the binding site, as reported previously for other partial agonists (Hibbs et al., 2009). Considering our experimental evidence that GABA can activate nAChR through the αδ subunit interface, we can suggest that model 1 corresponds to the most probable orientation associated with activation. The apparent controversy between our experimental results, which reveal lack of functional interaction of GABA with α7, and our docking results, which show GABA orientations at α7 similar to those at αε, may have different explanations. A possible explanation is related to the prevalence of model 2 orientation in α7, in which GABA does not occupy the same space as ACh within the binding pocket, and it may therefore neither be able to elicit receptor activation nor to compete with ACh. It could be also possible that, although the overall orientations of GABA appear similar in αε and α7/α7 interfaces, differences in specific potential interactions may be involved in the differential selectivity. Also, loop F, which could not be included in our homology models, may be involved in the differential actions of GABA because it has been shown to be a determinant for drug selectivity and partial agonism (Stokes et al., 2004; Hibbs et al., 2009). Alternatively, other residues located outside the binding site, but involved in coupling agonist binding to channel opening, may influence the capability of GABA to activate muscle, but not α7 nAChRs. Although conclusions from our docking results should be considered with caution, they open doors for further research.

Plasma GABA concentration in healthy individuals is about 0.1 μM (Bjork et al., 2001). It has been proposed that peripheral lymphocytes, which have a complete GABAergic system (Dionisio et al., 2011), could be a source of plasmatic GABA. The lack of α7 activation by GABA is therefore important because this receptor is present in blood cells (Wessler and Kirkpatrick, 2008; De Rosa et al., 2009), and it is therefore continuously exposed to serum GABA. Even at higher GABA concentrations, α7 cannot be activated by GABA, which is also important to avoid cross-talk at the synaptic level by spillover of GABA, which may diffuse locally (Fabian-Fine et al., 2001; Zago et al., 2006). It would be interesting to explore whether other neuronal nAChRs, such as α4β2, can be activated by GABA.
Serum GABA concentration is not high enough to activate muscle nACHr. However, we found that mutant muscle nACHr associated with slow-channel congenital myasthenic syndromes (αG153S and εT264P mutants) are activated by 0.1 μM GABA, a concentration reached in plasma. For both gain-of-function receptors, single-channel activity in the presence of GABA is significantly different from that observed in the absence of neurotransmitter. Moreover, the mean duration of both bursts and openings is similar for ACh- and GABA-activated εT264P nACHr. Thus, slow-channel congenital myasthenic syndrome nACHr at the neuromuscular junction are likely to be activated by steady exposure to serum GABA. This continuous channel activity may contribute to the pathophysiology of the disease. Interestingly, a similar effect has been proposed for serum choline at nACHr from slow-channel congenital myasthenic syndromes (Zhou et al., 1999).

In conclusion, GABA cannot activate neuronal α7 receptors, a feature extremely important for preserving an adequate excitatory/inhibitory balance. In contrast, muscle nACHr are partially activated by GABA, and mutations at the principal face of the binding site affect GABA activation in the same manner as ACh activation. This activity elicited by GABA in the progression of congenital myasthenic syndromes associated with gain-of-function mutations.

Author Contributions

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References

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