Short Communication

Properties of a Novel GABA_A Receptor γ_2 Subunit Mutation Associated With Seizures

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Abstract. We recently identified a novel missense mutation of the γ_2 subunit at position 40 with serine (N40S) of the GABA_A receptor from a patient with epilepsy. Here, we report properties of the mutant receptor using the whole cell patch clamp technique. The Hill coefficient for the N40S receptor was greater than for the wild-type (WT) receptor, while the EC₅₀ and kinetics did not differ. Furthermore, the effects of diazepam, Zn²⁺, bicuculline, and pH were indistinguishable between WT and N40S receptors. These results suggest that the changes in the steepness of the concentration–response relationship for GABA in the N40S receptor may trigger epilepsy.

Keywords: epilepsy, GABA_A receptor, γ_2 subunit mutation

The γ -aminobutyric acid type A (GABA_A) receptor is a ligand-gated chloride channel that consists of five subunits, including α_{1-6} , β_{1-3} , γ_{1-3} , δ , ϵ , π , θ , and ρ_{1-3} (1, 2). Most GABA_A receptors in the central nervous system (CNS) mediate inhibitory neurotransmission, and the most common subtype is composed of α_1 , β_2 , and γ_2 subunits (3). To date, mutations in several subunits of the GABA_A receptor have been associated with epilepsy (4). A R43Q mutation in the N terminal extracellular domain of the γ_2 subunit was insensitive to functional modulation by diazepam (5). A K289M mutation in the short extracellular loop between transmembrane domains TM2 and TM3 showed smaller amplitude currents relative to wild-type (WT) GABA_A receptor current amplitudes (6). We recently identified a novel missense mutation of the γ_2 subunit that replaced asparagine at position 40 with serine (N40S) from a patient with generalized tonic-clonic seizures with childhood epilepsy (GTCS) (7). This residue is the major extracellular domain, adjacent to the first one of the two high affinity benzodiazepine (BZD) binding domains of the γ_2 subunit. In the present study,

we investigated the functional properties of N40S GABA_A receptors—expressed human embryonic kidney 293 (HEK-293) cells using the patch clamp technique.

Rat GABA_A receptor subunit α_1 , β_2 , γ_2 were individually subcloned into expression vector pcDNA3.1. HEK-293 cells were grown in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum and antibiotics in a humidified 5% CO₂ atmosphere. These cells were transiently transfected by lipofection with α_1 , β_2 , γ_2 (WT) or α_1 , β_2 , γ_2 N40S (N40S) subunits complementary DNAs with enhanced green fluorescent protein. They were used in experiments 24–48 h later.

Patch clamp recording was performed as previously described (8). Whole-cell current was measured at room temperature from single cells held at –60 mV. The data were recorded with a Multiclamp 700B (Molecular Devices, Sunnyvale, CA, USA), filtered at 1 kHz, and digitized at 10 kHz using a Digidata 1322A (Molecular Devices). Data acquisition and analysis were performed using pClamp9.2 software (Molecular Devices) and Igor Pro software (WaveMetrics, Inc., Lake Oswego, OR, USA). The pipette solution contained 140 mM CsCl, 2 mM Mg-ATP, 10 mM EGTA, 10 mM HEPES (pH 7.4 with CsOH). The extracellular solution contained: 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10

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mM glucose, and 10 mM HEPES (pH 7.4 with NaOH). The cells were lifted from the substrate to increase speed of the concentration changes. Drugs were applied for 2 s once every 2–5 min using triple-barreled glass and a Perfusion Fast-Step System SF-77 (Warner Instruments, Hamden, CT, USA). Concentration–response curves for individual cells were then fit with the Hill equation using IGOR Pro software. Activation time of GABA currents was determined from the length of time to rise from 10% to 90% of the peak current. Deactivation of GABA currents was best fit by the sum of two exponentials. Numerical data were represented as the mean $\pm$ S.E.M. Statistical significance was determined using a Student's unpaired *t*-test.

Concentration–response experiments were performed on WT or N40S receptors (Fig. 1: A, B). GABA currents were normalized to 300 μ M GABA. The average response to 300 μ M GABA was -121 ± 35 pA/pF for the WT receptor and -252 ± 110 pA/pF for the N40S receptor. The EC_{50} and Hill coefficient were $7.78 \pm 0.95 \mu$ M and 0.67 ± 0.07 for the WT receptor ($n = 17$), and $9.38 \pm 0.58 \mu$ M and 1.59 ± 0.15 for the N40S receptor ($n = 6$), respectively. There was no difference in the EC_{50} between WT and N40S receptors. The Hill coefficient was significantly different between WT and N40S receptors ($P < 0.01$).

Activation and deactivation were examined in current evoked by 300 μ M GABA for WT and N40S receptors (Fig. 2). The activation time was 161 ± 30 ms ($n = 5$) for the WT receptor and 182 ± 48 ms ($n = 5$) for the N40S receptor, respectively (Fig. 2: A, B). The deactivation phase of the currents elicited by 300 μ M GABA was fit to two exponential components (Fig. 2: A, C). The associated time constants, tau1 and tau2, for WT receptor were 469 ± 104 and 459 ± 107 ms ($n = 5$), respectively. For the N40S receptor, tau1 and tau2 were 432 ± 35 and 415 ± 37 ms ($n = 4$), respectively. Activation and deactivation

was not significantly different between WT and N40S receptors.

It has been assumed that the γ subunit of the GABA_A receptor is concerned with the sensitivity to BZDs (9). In this experiment, whole cell currents were evoked by 10 μ M GABA, a concentration that elicited a submaximal response, and the effects of various concentrations of diazepam (DZP) were determined (Fig. 3A). The average relative responses in the presence of DZP were 1.55 ± 0.18 at 0.1 μ M ($n = 6$) and 2.28 ± 0.31 at 1 μ M

Fig. 2. Kinetics of WT and N40S receptors. A) Representative traces of activation and deactivation phases of 300 μ M GABA currents for WT (black line) and N40S (gray line) receptors. B) Averaged value of the 10%–90% rise time for WT (solid bars, $n = 5$) and N40S (open bars, $n = 5$) receptors. Each value represents the mean $\pm$ S.E.M. C) Averaged values of the fast and slow deactivation time constants for WT (solid bars, $n = 5$) and N40S (open bars, $n = 4$) receptors. Each value represents the mean $\pm$ S.E.M.

Fig. 1. GABA concentration–response curves for WT and N40S receptors. A) Representative GABA concentration–current recorded in cells expressing WT and N40S receptors. B) Summary of concentration–response curves for peak GABA current obtained from WT (filled circles, $n = 17$) and N40S (open circles, $n = 6$) receptors. Currents were normalized to 300 μ M GABA. Data are fit to the Hill equation. Each value represents the mean $\pm$ S.E.M.

Fig. 3. Pharmacological properties of WT and N40S receptors. A) The effect of DZP on 10 μ M GABA-induced current for WT (solid circles, $n = 9$) and N40S (open circles, $n = 8$) receptors. Each data point was normalized to the 10 μ M GABA-activated current. Each value represents the mean $\pm$ S.E.M. B) The effect of ZnCl₂ on GABA-induced current for WT (solid circles, $n = 6$) and N40S (open circles, $n = 6$) receptors. Each data point was normalized to the 10 μ M GABA-activated current. Each value represents the mean $\pm$ S.E.M. C) The effect of pH on GABA-induced current for WT (solid bars, $n = 6$) and N40S (open bars, $n = 7$) receptors. Each data point was normalized to the 10 μ M GABA-activated current at pH 7.4. Each value represents the mean $\pm$ S.E.M. D) The effect of BMI on GABA-induced current for WT (solid circles, $n = 10$) and N40S (open circles, $n = 7$) receptors. Each data point was normalized to the 10 μ M GABA-activated current. Each value represents the mean $\pm$ S.E.M.

DZP ($n = 9$) for the WT receptor, and 1.76 ± 0.32 at 0.1 μ M ($n = 7$) and 2.44 ± 0.27 at 1 μ M DZP ($n = 8$) for the N40S receptor, respectively. There was no significant difference for potentiating responses to 10 μ M GABA induced by DZP between WT and N40S receptors.

GABA_A receptors assembled from α_1 , β_2 , and γ_2 subunits are less sensitive to inhibition by ZnCl₂ (10). We tested the blocking action of ZnCl₂ (Fig. 3B). The currents elicited by 10 μ M GABA were inhibited to $86.8\% \pm 5.12\%$ at 1 μ M ($n = 6$), $94.3\% \pm 5.76\%$ at 10 μ M ($n = 6$), and $55.6\% \pm 3.19\%$ at 100 μ M ZnCl₂ ($n = 5$) in the WT receptor, and $96.4\% \pm 8.12\%$ at 1 μ M ($n = 6$), $94.0\% \pm 11.7\%$ at 10 μ M ($n = 6$), and $56.7\% \pm 4.17\%$ at 100 μ M ZnCl₂ ($n = 6$) in the N40S receptor. The inhibitory effects of ZnCl₂ were not affected by the mutation.

It is known that GABA-activated currents for the GABA_A receptor were sensitive to external pH (11). In our results, the ratios of the 10 μ M GABA-induced current were 0.78 ± 0.05 at pH 6.5 ($n = 6$) and 0.18 ± 0.05 at pH 6.0 ($n = 6$) in the WT receptor and 0.78 ± 0.04 at pH 6.5 ($n = 7$) and 0.13 ± 0.05 at pH 6.0 ($n = 6$) in the N40S receptor, respectively (Fig. 3C). Again, the mutation did not affect the enhancement or inhibition of responses by

protons.

Finally, we examined the ability of bicuculline methiodide (BMI) to block gating of WT and N40S receptors (Fig. 3D). The currents elicited by 10 μ M GABA were inhibited to $89.6\% \pm 3.25\%$ at 0.1 μ M ($n = 8$), $61.1\% \pm 4.57\%$ at 1 μ M ($n = 9$), and $21.8\% \pm 2.99\%$ at 10 μ M BMI ($n = 10$) in the WT receptor and $84.3\% \pm 2.68\%$ at 0.1 μ M ($n = 6$), $53.3\% \pm 3.52\%$ at 1 μ M ($n = 7$), and $26.9\% \pm 3.10\%$ at 10 μ M BMI ($n = 7$) in the N40S receptor, respectively. The inhibitory effects of BMI were also not affected by the mutation.

Previous reports show that GABA binds at the interface between α_1 and β_2 subunits following channel opening (12). Our result showed that WT and N40S receptors had similar EC₅₀ values. The EC₅₀ reflects both the affinity of the agonist for its binding site and the ability of the channel to gate (13). Therefore, it is considered that the affinity for GABA is little affected by the mutation of the γ_2 subunit at position 40. On the other hand, the Hill coefficient changed between WT and N40S receptors. It has also been shown that the Hill coefficient of the mutation in the delta subunit that replaced glutamine at position 177 with alanine was bigger than that of the wild-type

(14). Colquhoun has reported that the steepness of the curve (value of the Hill coefficient) is influenced by some factors (13). One is the concerted change in conformation. If subunits are independent of the conformation change, then no cooperativity at all would be expected for agonist binding, and only a low Hill slope would be predicted for the response. Consequently, our result suggests that the mutation replacing a highly conserved asparagine in the γ_2 subunit of the GABA_A receptor may change the receptor conformation. It is well known that the γ_2 subunit is an important site in regulating the BZD sensitivity to GABA_A receptors (9). It has been reported that BZDs bind at the interface between α_1 and γ_2 subunits and potentiate the effects of GABA (9). N40S is near the high affinity BZD-binding domain that is arginine at residue 43 of the γ_2 subunit. A R43Q mutation shows the insensitivity to functional modulation by DZP (5). This mutation decreased the side chain volume and increased its hydrophobicity (using the hydrophobicity scales of Kyte and Doolittle) (15). The N40S mutation also decreased the side chain volume and increased hydrophobicity. However, there was no change in the character for DZP sensitivity, pH, Zn²⁺, and BMI between WT and N40S receptors, suggesting that position 40 of the γ_2 subunit participates little in the kinetics and the BZD sensitivity of the GABA_A receptor.

In summary, we found that a novel human GABA_A receptor γ_2 subunit mutation (N40S) identified in a patient with GTCS causes changes in the steepness of the concentration–response relationship for GABA. In this case, the responses of N40S receptors for a low concentration of GABA are smaller than those for the WT receptor. This condition may easily occur during the excitation of neurons by the reduction of the CNS inhibitory system at rest.

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