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Reduced effect of propofol at human α1β2(N289M)γ2 and α2β3(N290M)γ2 mutant $GABA_A$ receptors†

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Background. Propofol is an i.v. anaesthetic commonly used during general anaesthesia and intensive care. It is known that the second transmembrane segment of the β subunit in the $GABA_A$ receptor is an important target for the effects of propofol; however, this has not been investigated in human receptors. The aim of this study was to investigate the effect of propofol on *human* β2 and β3 $GABA_A$ subunits with point mutations corresponding to the N265M mutation in the rat β2 and β3 subunits.

Methods. Asparagine-to-methionine replacement at amino acid position 289 and 290 (N289M and N290M) in the β2 and β3 $GABA_A$ receptor subunits, respectively, was accomplished by site-directed mutagenesis. Thereafter, subunits for three human wild-type (α1β2γ2, α2β2γ2, and α2β3γ2) and two mutant $GABA_A$ receptor channels [α1β2(N289M)γ2 and α2β3(N290M)γ2] were introduced into *Xenopus* oocytes and studied with two-electrode voltage clamp.

Results. The mutant receptors left-shifted the GABA concentration–response curve. In comparison with the wild-type receptors, both the positive modulatory and the agonistic effects of propofol were strongly reduced in potency and amplitude at both mutated $GABA_A$ channels.

Conclusions. We demonstrate that N289M or N290M mutation in *human* $GABA_A$ β2 and β3 subunits increases sensitivity to GABA, which is in contrast to the corresponding rat N265M mutation. Furthermore, the N289M and N289M mutations reduce both the potentiation of GABA-induced currents and the direct effect of propofol on channels incorporating either of the mutated subunits, which confirms earlier findings concerning the corresponding mutation in rat receptors and knock-in mice.

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Propofol (2,6-diisopropylphenol) is a common i.v. anaesthetic used for sedation, in general anaesthesia and in intensive care medicine. Although the exact mechanism(s) behind anaesthesia are not known, it is accepted that anaesthetics target several different receptors and neuroanatomical structures in order to produce immobility, unconsciousness, and amnesia.[1 2] The $GABA_A$ receptor has been identified as an important target for general anaesthetics and for propofol in particular.[1 2] The $GABA_A$ receptor belongs to the cys-loop ligand-gated superfamily of ion channels[3] and mediates most of the inhibitory fast synaptic transmission in the central nervous system. The

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$GABA_A$ receptor consists of five subunits arranged around a central anion-conducting pore. Each subunit has four transmembrane (TM) domains with TM2 facing the pore. Activation of the receptor by binding of GABA causes an inward flow of chloride ions through the channel and hyperpolarization of the cell.

In the brain, most $GABA_A$ receptor subtypes are composed of α, β, and γ or δ subunits, and the stoichiometry is believed to be 2:2:1 ($\alpha:\beta:\gamma/\delta$).[4] The most common $GABA_A$ receptor subtype in the central nervous system is α1β2γ2 (60%) followed by α2β3γ2 (15%).[5] Previous studies have confirmed that propofol positively modulates the GABAergic response and directly activates the $GABA_A$ chloride channel at concentrations higher than those needed for modulation.[6–8] It was later demonstrated that alteration of a single amino acid in the $GABA_A$ receptor could greatly alter the receptor's sensitivity to anaesthetics[9 10] and that the α6β3(N289M)γ2L receptor had a reduced sensitivity to propofol.[11] In addition, it was confirmed that this asparagine-to-methionine mutation at amino acid 265 (N265M) in the TM2 of the β2 and β3 subunits reduced both the modulatory and the direct effect of propofol at rat benzodiazepine-sensitive α1β2(N265M)γ2 and α2β3(N265M)γ2 $GABA_A$ receptors.[12 13] Furthermore, the modulatory effect of propofol was reduced in the rat α1β2(M286W)γ2 and α2β3(M286W)γ2 $GABA_A$ receptors but abolished in the combined rat/human α1β2(M286W)γ2S receptor.[12–14] In addition, the agonist effect of propofol was reduced both in the rat and combined rat/human α1β2(M286W)γ2 receptor but not in the α2β3(M286W)γ2 receptor.[12–14] Interestingly, the same studies demonstrate that the direct action of the neurosteroid alphaxalone was reduced in the α1β2(N265M)γ2 but not in the α2β3(N265M)γ2 receptor, whereas the effect was unchanged in the α1β2(M286W)γ2 receptor and increased in the α2β3(M286W)γ2 receptor.[12 13] Taken together, this means that anaesthetic drugs have distinct effects on each $GABA_A$ receptor subtype and that a change in an individual amino acid can profoundly alter functional affinity. Consequently, no generalizations can be made based on previous results and each receptor subtype/mutation must be investigated individually.

To our knowledge, the effect of mutations on the affinity and actions of propofol at the two most common *human* $GABA_A$ receptors in the central nervous system, namely the α1β2γ2 and α2β3γ2subtypes, has never been investigated before. The aim of this study was therefore to investigate the effect of propofol on *human* $GABA_A$ receptors containing the β2(N289M) and β3(N290M) mutations, corresponding to the rat β2(N265M) and β3(N265M) mutations. More specifically, here we examine the α1β2(N289M)γ2 and α2β3(N290M)γ2 $GABA_A$ receptor subtypes. Furthermore, we investigate the effect of propofol after replacement of one subunit in order to compare the most prevalent $GABA_A$ receptor subtypes, α1β2γ2, α2β3γ2, and α2β2γ2.

Methods

Clones

The human $GABA_A$ receptor subunits α1, α2, β2, β3, and γ2 were cloned from a human cDNA library. GenBank (Bethesda, MD, USA) access numbers for the cDNA nucleotide sequences are: NM_000806.2 (α1), NM_000807 (α2), NM_000813 (β2), NM_000814 (β3), and NM_000816.1 (γ2). The cDNAs were subcloned into different expression vectors, pKGem (AstraZeneca, Wilmington, DE, USA) (α1, α2, β2, and γ2) and pBSTA (University of California, Irvine, CA, USA) (β3).

Introduction of a mutation in the human $GABA_A$ β2 and β3 subunits corresponding to N265M in the rat sequences[12 13] resulted in an amino acid change, N289M and N290M, respectively, in the human sequences (Fig. 1). Mutagenesis was performed using a QuickChange® II site-directed mutagenesis kit (Stratagene, cat no. 200523) according to the manufacturer's instructions. Primer sequences for mutagenesis were as follows with the mutated nucleotides underlined: h$GABA_A$ β2(N2989M) forward: (OAPC-2599) CACAATGACCACAATCATGACCCACCT CCGGGAAACTC, h$GABA_A$ β2(N289M) reverse: (OAPC -2600) GAGTTTCCCGGAGGTGGGTCATGATTGTGGT CATTGTG, h$GABA_A$ β3(N290M) forward: (OAPC-2597) GCTGACAATGACAACCATCATGACCCACCTTCGGGA GAC, h$GABA_A$ β3(N290M) reverse: (OAPC-2598) GTCT CCCGAAGGTGGGTCATGATGGTTGTCATTGTCAGC, GAGTTTCCCGGAGGTGGGTCATGATTGTGGTCATT GTG. mRNA was transcribed *in vitro* using the mMessage mMachine® T7 kit (Ambion, Austin, TX, USA) and analysed using a bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Numbering of the amino acid sequences was done using the SWISSPROT database.

Xenopus oocyte injection

The study was approved by the local animal ethics committee at Karolinska Institutet, Stockholm, Sweden. Preparation and injection of oocytes and the electrophysiological recordings were done as previously described.[15–17] Briefly, *Xenopus laevis* oocytes were isolated by partial ovariectomy from frogs anaesthetized with 0.2% Tricaine. The ovaries were mechanically dissected into smaller lumps and digested in OR-2 buffer (in mM, NaCl 82.5, KCl 2, $MgCl_2$ 1, HEPES 5, pH adjusted to 7.5 with NaOH) containing 1.5 mg ml^{-1} collagenase (Type 1A, Sigma, St Louis, MO, USA), for 60 min in order to remove the follicular epithelia from the oocytes. After 1–4 h, the oocytes were injected with 50–625 ng mRNA in a total volume of 50 nl per oocyte. The α1β2γ2, α2β2γ2, α2β3γ2, α1β2(N289M)γ2, and α2β3(N290M)γ2 $GABA_A$ receptor subtypes were injected at a ratio of 1:1:10. The oocytes were maintained in Leibovitz L-15 medium (Sigma) diluted 1:1 with Millipore filtered H_2O (Billerica,