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Etomidate, propofol and the neurosteroid THDOC increase the GABA efficacy of recombinant $\alpha 4\beta 3\delta$ and $\alpha 4\beta 3$ GABA_A receptors expressed in HEK cells

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Abstract

General anesthetics, once thought to exert their effects through non-specific membrane effects, have highly specific ion channel targets that can silence neuronal populations in the nervous system, thereby causing unconsciousness and immobility, characteristic of general anesthesia. Inhibitory GABA_A receptors (GABA_ARs), particularly highly GABA-sensitive extrasynaptic receptor subtypes that give rise to sustained inhibitory currents, are uniquely sensitive to GABA_AR-active anesthetics. A prominent population of extrasynaptic GABA_ARs is made up of $\alpha 4$, $\beta 2$ or $\beta 3$, and δ subunits. Considering the demonstrated importance of GABA receptor $\beta 3$ subunits for *in vivo* anesthetic effects of etomidate and propofol, we decided to investigate the effects of GABA anesthetics on "extrasynaptic" $\alpha 4\beta 3\delta$ and also binary $\alpha 4\beta 3$ receptors expressed in human embryonic kidney (HEK) cells. Consistent with previous work on similar receptor subtypes we show that maximal GABA currents through "extrasynaptic" $\alpha 4\beta 3\delta$ receptors, receptors defined by sensitivity to EtOH (30 mM) and the β -carboline β -CCE (1 μ M), are enhanced by the GABA_AR-active anesthetics etomidate, propofol, and the neurosteroid anesthetic THDOC. Furthermore, we show that receptors formed by $\alpha 4\beta 3$ subunits alone also show high GABA sensitivity and that saturating GABA responses of $\alpha 4\beta 3$ receptors are increased to the same extent by etomidate, propofol, and THDOC as are $\alpha 4\beta 3\delta$ receptors. Therefore, both $\alpha 4\beta 3$ and $\alpha 4\beta 3\delta$ receptors show low GABA efficacy, and GABA is also a partial agonist on certain binary $\alpha\beta$ receptor subtypes. Increasing GABA efficacy on $\alpha 4\beta 3\delta$ and $\alpha 4\beta 3$ receptors is likely to make an important contribution to the anesthetic effects of etomidate, propofol and the neurosteroid THDOC.

Keywords

tonic inhibition; delta subunit; etomidate; propofol; anesthesia; neurosteroid THDOC; alcohol

Introduction

Synaptic GABA_A receptors contain γ subunits (with the $\gamma 2$ subunit being the most abundant subtype) and are thought to be located to synapses through interactions with accessory proteins

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like GABARAP (Wang et al., 1999) and gephyrin (Kneussel and Betz, 2000). This implies that GABA_A receptors without γ subunits are found outside of synapses. The presence of a γ subunit also makes GABA_ARs less sensitive to GABA, when compared to their matching $\alpha\beta$ counterparts (Baburin et al., 2008). Therefore, it is thought that activation of most γ subunit-containing “synaptic” GABA_ARs require relatively high GABA concentrations supplied by classical synaptic vesicular GABAergic neurotransmission. Given the relatively low GABA-sensitivity of γ subunit-containing receptors it seems likely that γ subunit-containing receptors are usually inactive at the low ambient “extrasynaptic” concentrations of GABA ($\leq 1 \mu\text{M}$) that are determined largely by the activity of GABA transporters (Wu et al., 2007). Tonic currents mediated by $\alpha 5$ -subunit-containing GABA_ARs in CA1 hippocampal pyramidal neurons might be an exception (Glykys et al., 2008), as they have been proposed to be composed of $\alpha 5\beta 3\gamma 2$ subunits (Caraiscos et al., 2004).

Certain GABA_AR subtypes such as those containing δ subunits are excluded from synapses and show an extrasynaptic or perisynaptic localization (Farrant and Nusser, 2005). In contrast to the effects of $\gamma 2$ subunits, incorporation of δ subunits into functional receptors is believed to increase GABA potency ($\text{EC}_{50} < 1 \mu\text{M}$) when compared to matching $\alpha\beta$ GABA_ARs. This high GABA potency allows δ subunit-containing receptors to open at low ambient GABA concentrations, a feature that (together with slow desensitization) makes δ subunit-containing receptors uniquely suited to mediate a constant (tonic) inhibitory activity that can potently suppress neuronal excitation. The importance of ambient GABA levels and tonic GABA currents in the control of normal and pathological neuronal activity (Richerson, 2004) as well as the finding that extrasynaptic GABA_ARs are important mediators of alcohol (Hancher et al., 2005; Wallner et al., 2006a) and gaboxadol (Chandra et al., 2006) actions, makes it likely that extrasynaptic receptors play an important role in controlling neuronal excitability even in the absence of exogenous modulators. In addition, many medications that are used to control hyperexcitability phenomena like convulsions, epilepsy, and tremor directly or indirectly (e.g., by increasing extrasynaptic GABA) augment tonic inhibition (Richerson, 2004).

It has been shown in neurons in slices that tonic GABA currents mediated by δ subunit-containing receptors are distinguished from their synaptic counterparts by several pharmacological features: 1) they are enhanced by nanomolar concentrations of the neurosteroid THDOC (Stell et al., 2003); 2) they are enhanced by ethanol at low inebriating (3–30 mM) concentrations (Hancher et al., 2005; Wei et al., 2004); and 3) they are activated by high nanomolar concentrations of the GABA-analog THIP/Gaboxadol (Chandra et al., 2006; Cope et al., 2005). Given that THDOC, ethanol, and THIP have anesthetic-like actions (lead to “loss of righting reflex” in rodents at appropriate doses), the high potency and efficacy of these drugs on δ subunit-containing receptors suggests that δ subunit-containing receptors can mediate functional effects of GABA_AR-active anesthetics. In addition, while δ subunit-containing receptors are uniquely sensitive to GABA, it has been shown in a number of studies on recombinant δ subunit-containing receptors that etomidate, propofol, THDOC and barbiturates lead to increases in peak currents even at saturating GABA concentrations (Bianchi and Macdonald, 2003; Feng et al., 2004; Wallner et al., 2003; Wohlfarth et al., 2002; Zhelezнова et al., 2008). This indicates that δ subunit-containing receptor subtypes, while showing high potency for GABA, have a low GABA efficacy, likely because of inefficient coupling of GABA binding to pore opening. In other words, GABA can be considered a partial agonist on these low efficacy GABA_AR subtypes (Bianchi and Macdonald, 2003; Wallner et al., 2003). The low GABA efficacy on δ subunit-containing receptors seen in recombinant systems (particularly with $\alpha 1$ subunits and in the oocyte expression system) explains some of the difficulty in expressing these receptors in recombinant systems, with one recent report stating that $\alpha 1\beta 2\delta$ receptors expressed in oocytes are essentially silent receptors which can be “recruited” by tracazolol and the neurosteroid THDOC (Zhelezнова et al., 2008).

Besides δ subunit-containing receptors, there is evidence for additional GABA_AR subtypes formed by receptors composed of binary $\alpha\beta$ combinations without δ , γ , or ε subunits in the pentamer. Particularly interesting in this respect are biochemical data showing that only 7% of $\alpha 4$ subunits are associated with δ subunits, whereas around 50% of $\alpha 4$ subunit-containing receptors do not contain γ or δ subunits (Bencsits et al., 1999). It seems likely that the majority of these $\alpha 4$ subunit-containing receptors without γ or δ subunits are composed of $\alpha 4$ and β subunits alone (Bencsits et al., 1999). Such binary, $\alpha\beta$ receptor subtypes also have been proposed to be present in cultured hippocampal neurons where they mediate certain forms of tonic inhibition (Mortensen and Smart, 2006) and it seems likely that most of the GABA response recorded from neurons derived from $\gamma 2$ subunit knock-out mice is mediated by $\alpha\beta$ receptor subtypes (Günther et al., 1995).

GABA receptors containing $\beta 3$ subunits are important mediators of etomidate and propofol anesthetic effects *in vivo*, because it has been shown that mice with the knock-in point mutation $\beta 3N265M$ lack the immobilizing effects of etomidate and propofol in response to painful stimuli (Jurd et al., 2003). In contrast, a similar knock-in point mutation at the homologous position in the $\beta 2$ subunit that drastically reduces etomidate effects in $\beta 2$ subunit-containing receptors ($\beta 2N265S$, a change in $\beta 2$ to the residue present in the “etomidate-insensitive” $\beta 1$ subunit (Belelli et al., 1997)), reduces the sedative but not the immobilizing and hypnotic etomidate effects (Reynolds et al., 2003). One factor that may account for the importance of $\beta 3$ subunit-containing receptors in mediating anesthetic actions is the association of $\beta 3$ subunits with extrasynaptic receptors, a finding supported by the correlation of alcohol sensitivity of $\beta 3$ subunit-containing receptors in recombinant systems (Wallner et al., 2003) with alcohol sensitivity of native tonic δ -receptor-mediated GABA currents (Hancher et al., 2005; Wei et al., 2004). Another factor may be that $\beta 3$ subunit-containing GABA_AR subtypes could be key components in specific neuronal circuits that are particularly important in mediating anesthetic actions (Grasshoff et al., 2006).

Here we compare receptors formed by $\alpha 4\beta 3$ subunits alone with those formed by $\alpha 4\beta 3\delta$ subunits. We find that $\alpha 4\beta 3$ receptors show only slightly lower GABA sensitivity when compared to $\alpha 4\beta 3\delta$ receptors ($\alpha 4\beta 3$ GABA $EC_{50} = 1.1 \mu M$, $\alpha 4\beta 3\delta$ GABA $EC_{50} = 0.5 \mu M$), a finding consistent with the notion that binary $\alpha 4\beta 3$ receptors present in native neurons could mediate unique forms of tonic inhibition. Further we show that peak GABA responses, not only on $\alpha 4\beta 3\delta$, but also on binary $\alpha 4\beta 3\delta$ receptors are enhanced by GABA_AR-active anesthetics etomidate, propofol, and THDOC. Thus, the suggestion that these anesthetics act by increasing GABA efficacy can be extended to include binary $\alpha 4\beta 3$ receptors and possibly other “binary” $\alpha\beta$ GABA_AR subtypes. Our results are consistent with the hypothesis that binary $\alpha\beta$ receptors could contribute not only to tonic GABA currents, but that such receptors could potentially make important contributions to the actions of GABA_AR-active anesthetics.

Results

$\alpha 4\beta 3$ and $\alpha 4\beta 3\delta$ receptors expressed in HEK cells are sensitive low “extrasynaptic” [GABA]

GABA_ARs composed only with α and β subunits may exist on native neurons and are readily expressed in recombinant systems; indeed evidence suggest that they are often a contaminating species when $\gamma 2$, δ or ε subunit-containing receptors are the desired subject of study (Baburin et al., 2008; Olsen et al., 2007). Most native δ subunit-containing GABA_ARs are composed with $\alpha 4$ or $\alpha 6$, and either $\beta 2$ or $\beta 3$ subunits. Considering that $\beta 3$ has been implicated as important for anesthetic actions and low dose ethanol sensitivity, we decided to compare the GABA sensitivity and modulation by GABA anesthetics of $\alpha 4\beta 3$ and $\alpha 4\beta 3\delta$ GABA_ARs.

Figure 1 shows that receptors formed by $\alpha 4\beta 3\delta$ subunits in HEK cells are half-maximally activated at a GABA concentration of ~ 500 nM, an EC_{50} only slightly higher than our previous

(lower) estimate (~ 200 nM) of extrasynaptic [GABA] in cerebellar granule cells in perfused slices (Santhakumar et al., 2006). Binary $\alpha 4\beta 3$ receptors also respond to low GABA concentrations, although slightly less potently ($EC_{50} = 1.1 \mu M$, 300 nM is GABA $\sim EC_{20}$ at binary $\alpha 4\beta 3$ receptors). This small, yet highly significant shift in GABA-sensitivity indicates that δ subunit expression indeed leads to increased GABA-sensitivity and this is opposite to the reduced GABA sensitivity generally observed with γ subunit incorporation.

We showed previously with recombinant expression in oocytes that expression of the δ subunit makes $\alpha 4\beta 3\delta$ and $\alpha 6\beta 3\delta$ receptors sensitive to inebriating (3–30 mM) concentrations of ethanol, whereas $\alpha 4\beta 3$ receptors are insensitive to EtOH at these same low concentrations (Hancher et al., 2005; Wallner et al., 2003). We therefore investigated in $\alpha 4\beta 3\delta$ -transfected HEK cells if GABA responses are ethanol sensitive. Figure 2 show representative recordings from $\alpha 4\beta 3\delta$ - and $\alpha 4\beta 3$ -transfected cells in which various pharmacological features (sensitivity to 30 mM ethanol, 1 μM β -CCE, and 1 μM Zn^{2+} , co-applied with GABA) were tested. As shown, currents evoked by application of 300 nM GABA from $\alpha 4\beta 3\delta$ -transfected cells are enhanced by 30 mM EtOH and by 1 μM of the β -carboline β -CCE, whereas 300 nM GABA responses in all $\alpha 4\beta 3$ -transfected cells were insensitive to 30 mM EtOH and 1 μM β -CCE. Furthermore, 1 μM Zn^{2+} led to a significant (~70%) inhibition of GABA currents in $\alpha 4\beta 3$ receptors, whereas EtOH- and β -CCE-sensitive 300 nM GABA responses measured in $\alpha 4\beta 3\delta$ transfected cells were not inhibited by 1 μM Zn^{2+} .

Given the observation that δ subunit-containing receptors in recombinant expression show low GABA efficacy, we decided to test the hypothesis that incorporation of the δ subunit confers low GABA efficacy and that this in turn allows for hypnotic/anesthetic modulator-induced increases in GABA efficacy. To test for increases in GABA efficacy by anesthetics we evoked GABA responses with a saturating (100 μM) GABA concentration alone, or with 100 μM GABA co-applied with either 3 μM etomidate, 3 μM propofol, or 1 μM THDOC. The concentrations of etomidate, propofol, and THDOC were chosen to match frequently used concentrations applied to expressed $\alpha 1\beta 3\delta$, $\alpha 6\beta 3\delta$ and $\alpha 1\beta 2\gamma 2$ receptors (Bianchi et al., 2002; Bianchi and Macdonald, 2003; Feng and Macdonald, 2004; Rusch et al., 2004; Wallner et al., 2003; Wohlfarth et al., 2002) and tonic GABA currents (Herd et al., 2008). Figure 3 shows that, as expected from previous work on $\alpha 1$ - and $\alpha 6$ subunit-containing receptors, co-application of anesthetics indeed leads to a significant enhancement of $\alpha 4\beta 3\delta$ receptors. However, to our surprise, etomidate, propofol, and THDOC also increased peak current responses in $\alpha 4\beta 3$ receptors, with no significant difference in the amount of peak current enhancement when compared to $\alpha 4\beta 3\delta$ receptor currents (Fig. 3).

To assess whether etomidate and propofol effects on $\alpha 4\beta 3$ receptors are mediated by the potential transmembrane sites, defined by mutations (like $\beta 3N265M$) in α and β subunits (Belelli et al., 1997; Mihic et al., 1997) that drastically reduce etomidate and propofol enhancement, we tested whether etomidate and propofol responses in $\alpha 4\beta 3$ receptors can be abolished with the $\beta 3N265M$ mutation that makes $\beta 3$ subunit-containing receptors (and mice) insensitive to etomidate and propofol. Figure 4 shows that the $\beta 3N265M$ mutation introduced into binary $\alpha 4\beta 3$ GABA_ARs prevents enhancement of saturating 100 μM GABA responses by 3 μM etomidate and propofol, and in fact reveals a trend towards current inhibition. In comparison, the steroid anesthetic THDOC (1 μM) retains the ability to enhance saturating GABA responses in $\alpha 4\beta 3N265M$ receptors. Original traces show that there is small amount of direct activation (in the absence of GABA) of these $\alpha 4\beta 3$ receptors by 1 μM THDOC, indicating that direct activation of receptors might have contributed to anesthetic responses at the concentrations used.

Discussion

Given that ‘binary’ GABA_ARs composed of α and β subunits are readily expressed in recombinant systems, it is important to establish their functional properties so that they can be distinguished from receptors that incorporate γ , δ or ϵ subunits into the pentamer. It is thought that most, but not all (Bencsits et al., 1999; Mortensen and Smart, 2006), native GABA_A receptors have γ , δ , or ϵ subunits in the pentameric assembly with a stoichiometry of 2 α , 2 β , and 1 γ , δ , or ϵ .

Extrasynaptic δ subunit-containing receptors are highly sensitive to GABA which allows them to sense ambient, extrasynaptic GABA concentrations and serve as a negative feedback system to prevent excessive excitation. We confirm here that $\alpha 4\beta 3\delta$ receptors expressed in HEK cells are uniquely sensitive to GABA (EC_{50} of $\sim 0.5 \mu M$) with binary $\alpha 4\beta 3$ receptors significantly less sensitive (GABA $EC_{50} \sim 1.1 \mu M$). This is consistent with the general notion that δ subunit-incorporation into functional receptors leads to an increase in GABA sensitivity. However, despite being less sensitive to GABA than $\alpha 4\beta 3\delta$ receptors, the GABA sensitivity of $\alpha 4\beta 3$ receptors remains in a range where these receptors could be activated by ambient GABA levels (300 nM GABA is $\sim EC_{20}$ on $\alpha 4\beta 3$ receptors), indicating that binary receptors, if present on the cell surface of neurons, could contribute to tonic GABA currents.

While δ subunit deletion in knock-out animals often leads to a significant reduction in tonic GABA currents in certain neurons, there are tonic GABA currents that remain in δ knock-out animals (Glykys et al., 2008; Glykys et al., 2007; Herd et al., 2008; Stell et al., 2003). Given the relatively high GABA sensitivity of binary $\alpha 4\beta 3$ receptors, and the report that $\sim 50\%$ of all $\alpha 4$ subunit-containing receptors may form binary $\alpha 4\beta$ receptor subtypes, while only 7% form $\alpha 4\beta \delta$ receptors (Bencsits et al., 1999), these binary receptors make excellent candidates for the GABA_AR subtypes responsible for mediating tonic currents and etomidate enhancement of the tonic currents that persist in δ knock-out mice (Herd et al., 2008). Behaviorally, $\delta^{-/-}$ mice show significantly reduced responsiveness to neurosteroid anesthetics, supporting the notion that δ subunit-containing GABA_ARs are important mediators of hypnotic/anesthetic actions of neuroactive steroids like alphaxolone, pregnanolone (Mihalek et al., 1999), which are close structural analogs of THDOC (THDOC is 21-hydroxyallopreganolone). However, the reduction in anesthetic efficacy (measured as duration of “loss of righting reflex”) of the GABA-enhancing anesthetics pentobarbital, propofol and etomidate were not considered statistically significant (i.e., $p < 0.02$) under the conditions and drug doses studied (Mihalek et al., 1999). This indicates that perhaps etomidate, propofol and barbiturates might be less specific for δ subunit-containing receptors and behavioral effects mediated by these drugs on δ -subunit-containing GABA_ARs might be masked by other GABA_A receptor targets, which we show here might include binary $\alpha 4\beta 3$ GABA_AR subtypes.

Using recombinant $\alpha 1\beta 3\delta$ and $\alpha 6\beta 3\delta$ receptors it has been previously shown that δ subunit-containing GABA_ARs exhibit higher potency and efficacy for etomidate, propofol, as well as GABA_AR-active neurosteroids like THDOC, and barbiturates (Bianchi and Macdonald, 2003; Feng and Macdonald, 2004; Wallner et al., 2003; Wohlfarth et al., 2002). We confirm this increase in GABA efficacy for δ subunit containing receptors, but show that increases in GABA efficacy, i.e., increases in peak currents at saturating GABA concentrations, by GABA_AR-active anesthetics etomidate, propofol, and THDOC can be similar in $\alpha \beta$ and $\alpha \beta \delta$ receptors. Therefore, increases in GABA efficacy by anesthetics are not necessarily due to δ subunit incorporation. In addition, enhancement by 3 μM propofol cannot be used to distinguish binary $\alpha \beta 3$ GABA_ARs from $\alpha \beta 3\delta$ receptors as previously implied (Yamashita et al., 2006).

Anesthetic actions of etomidate, propofol and barbiturates can be blunted by mutations (e.g., $\beta 3N265M$) in GABA receptor $\beta 3$ subunits (Siegwart et al., 2002); interestingly the same point

mutation also reduces effects of high concentrations (≥ 100 mM) of EtOH on recombinant GABA_AR receptors (Mihic et al., 1997; Wallner et al., 2006b). Most importantly, $\beta 3N265M$ point mutated mice lack the immobilizing anesthetic actions of etomidate and propofol (but not alphaxalone) *in vivo* (Jurd et al., 2003). In contrast, mice with an analogous mutation in the $\beta 2$ subunit ($\beta 2N265S$) only lose the sedative effects of etomidate (Reynolds et al., 2003). Given our observation of $\beta 3$ specificity for low dose 30 mM alcohol actions, we have previously speculated that extrasynaptic δ subunit-containing GABA receptors might be preferentially associated with $\beta 3$ subunits and this might contribute to $\beta 3$ subunit-specificity of etomidate anesthetic effects as suggested by the $\beta 3N265M$ and $\beta 2N265S$ mice (Jurd et al., 2003; Reynolds et al., 2003; Wallner et al., 2003).

The action of most GABA_AR-active anesthetics are determined by putative transmembrane receptor sites (THIP/gaboxadol as a GABA mimetic is an exception), defined by mutations that eliminate action of these anesthetics (Hosie et al., 2007; Jurd et al., 2003; Mihic et al., 1997). Recent etomidate photoaffinity labeling studies have proposed that these binding sites are present at the interface between α and β subunits at transmembrane sites (Li et al., 2006). Our finding here that etomidate, propofol and THDOC enhance $\alpha 4\beta 3$ receptors, and that this enhancement is absent in $\alpha 4\beta 3N265M$ receptors is consistent with the notion that transmembrane acting GABA-anesthetics actions may be determined by binding sites formed between $\alpha\beta$ subunits. Furthermore, the loss of etomidate and propofol enhancement in $\alpha 4\beta 3N265M$ transfected cells, demonstrates that there is a negligible contribution of endogenous $\beta 2$ or $\beta 3$ subunits, previously shown to be expressed in HEK 293 cells (Davies et al., 2000; Ueno et al., 1996).

A number of studies on δ subunit-containing receptors expressed in recombinant system have suggested that GABA is only a partial agonist on these receptors as it has been demonstrated that the low efficacy on δ subunit-containing receptors can be increased by the anesthetic neurosteroid THDOC with little or no changes in GABA potency/ EC_{50} (Bianchi et al., 2002; Wallner et al., 2003; Wohlfarth et al., 2002). The amount of enhancement is dependent on the exact receptor composition with $\alpha 1\beta 3\delta$ GABA peak currents being enhanced about 10 fold, with less enhancement of peak currents seen with $\alpha 6\beta 3\delta$ receptors when expressed in HEK cells (Bianchi et al., 2002). The enhancement by 1 μM THDOC reported here with $\alpha 4\beta 3\delta$, $\alpha 4\beta 3$ and $\alpha 4\beta 3N265M$ expressed in HEK cells is similar to the enhancement previously reported with $\alpha 6\beta 3\delta$ receptors in HEK cells (Bianchi et al., 2002). The similarity of $\alpha 6\beta 3\delta$ and $\alpha 4\beta 3\delta$ in anesthetic enhancement of peak GABA responses is not surprising given that $\alpha 4$ and $\alpha 6$ subunit protein sequences are closely related. Even more dramatic increases in peak currents mediated by $\alpha 4/6\beta 3\delta$ GABA_ARs are observed in response to etomidate and THDOC (Wallner et al., 2003) and for $\alpha 1\beta 2\delta$ receptors to THDOC and tracazolate (Zheleznova et al., 2008) when expressed in oocytes. With $\alpha 1\beta 2\delta$ receptors expressed in oocytes the GABA efficacy is so low that it was concluded that these receptors are essentially silent with GABA alone and that such silent receptors can be recruited by the neurosteroid THDOC as well as tracazolate (Zheleznova et al., 2008). In contrast to what we show here with $\alpha 4\beta 3$ subunits expressed in HEK cells, Zheleznova et al. showed that currents from $\alpha 1\beta 2$ injected oocytes were not enhanced by 1 μM THDOC and tracazolate at saturating 100 μM GABA concentrations. This indicates that GABA efficacy and enhancement by anesthetics on binary $\alpha\beta$ receptors might be critically dependent on the exact subunit composition. In addition, the expression system might be important, with expression of δ subunit-containing receptors in oocyte apparently favoring lower efficacy and therefore more room for enhancement by GABA_AR-active anesthetics. Many important open questions remain, including perhaps the most pressing, whether or not native δ subunit-containing and possibly native binary $\alpha\beta$ receptors that mediate tonic currents show low GABA efficacy, and if GABA efficacy on native receptors subtypes can be modulated by pharmacological and physiological means.

While there is evidence that GABA_AR-active anesthetics are more efficacious, and apparently some of them also more potent, on extrasynaptic receptor subtypes (Bonin and Orser, 2008; Cope et al., 2005; Feng and Macdonald, 2004; Stell et al., 2003; Wallner et al., 2003; Wohlfarth et al., 2002; Zhelezнова et al., 2008), they are certainly not entirely selective for extrasynaptic receptors. Indeed, at higher concentrations they are allosteric modulators (gaboxadol/THIP as a GABA analog is an exception) on classical synaptic GABA_AR subtypes where they increase the potency of GABA (Rusch et al., 2004; Siegwart et al., 2002). The high GABA efficacy of classical γ subunit-containing synaptic receptors leaves little room for increases in the maximum GABA current in the presence of anesthetics (Rusch et al., 2004). However, similar to benzodiazepines, anesthetics slow the decay of synaptic responses, and this may contribute to sedation and anesthesia. Given that many GABA_AR-active anesthetics increase the GABA sensitivity of γ subunit-containing receptors, the presence of relevant concentrations of anesthetics might increase GABA sensitivity of certain γ subunit-containing receptor subtypes so that otherwise “silent” GABA_AR populations (possibly even regardless whether they are synaptic or extrasynaptic), could now be activated by low ambient GABA concentrations, thereby giving rise to anesthetic-induced forms of tonic GABA inhibition that could mediate behavioral aspects of anesthetic actions.

While there are currently more open questions than answers, we are confident that a better understanding of the physiology and pharmacology of native and recombinant GABA_A receptor subtypes will allow us to discriminate in the future which of the many native GABA receptors subtypes contribute to the many aspects of actions of different GABA_AR-active anesthetics.

Methods

Propofol and etomidate were obtained from Tocris. THDOC (3 α ,21-Dihydroxy-5 α -pregnan-20-one) was obtained from Sigma. Propofol, etomidate, and THDOC were dissolved in DMSO as 100 mM stock solutions. β -CCE was a gift from Ferrosan (Copenhagen). Most other standard chemicals, including EtOH, were obtained from Sigma.

Cell Transfection

GABA_AR cDNAs were as previously described (Hancher et al., 2006). To increase δ subunit incorporation into functional GABA_ARs, we transfected human embryonic kidney (HEK) cells with five-fold excess of δ subunit plasmid versus α/β plasmid DNA; the total amounts of DNA are 4 μ g α , 4 μ g β , 20 μ g δ (δ cDNA omitted for $\alpha\beta$ receptors) together with 0.4 μ g of eGFP-plasmid DNA for each 10 cm diameter plate. HEK cells were transfected using a DEAE-Dextran transfection method as previously described (Meera et al., 1997). Briefly, HEK cells were seeded at a density of $\sim 2.5 \times 10^6$ cells per 10 cm diameter culture plate with DMEM medium supplemented with 10% fetal bovine serum, and maintained at 37°C in a 5% CO₂ incubator. Cells were exposed for two hours to a transfection mixture consisting of DEAE-dextran (0.5 mg/ml in DMEM) and endotoxin-free plasmid DNA, followed by a brief shock with 5% DMSO in PBS. Electrophysiological recordings were performed starting 70 hours post transfection on cells plated on polylysine-coated cover slips.

Electrophysiological recordings in HEK cell

Whole-cell currents were recorded at room temperature using the patch clamp technique (Axopatch 200 B amplifier, Molecular Devices, USA) at a holding potential of -60 mV. The external solution is (in mM): 142 NaCl, 1 CaCl₂, 6 MgCl₂, 8 KCl, 10 glucose, and 10 HEPES, pH 7.4 (327–330 mOsm). The pipette internal solution contained in mM: 140 CsCl, 4 NaCl, 0.5 CaCl₂, 10 HEPES, 5 EGTA, 2 Mg²⁺ ATP, and 0.2 GTP. Drug solutions were applied using

a multibarrel pipette driven by a stepper motor (SF77B, Warner Instruments) with an exchange time of around 10 ms. Recording pipettes had a bath resistance of around 4 MΩ.

Data analysis

Whole-cell currents were analyzed using Clampfit 9 (Molecular Devices). The normalized concentration-response data were least-square fitted (using the “Solver” function in Microsoft EXCEL) using the Hill equation: $I/I_{\max} = (1/(1 + (EC_{50}/[A])^n))$ where EC_{50} represents the concentration of the agonist ($[A]$) inducing 50% of the maximal current evoked by a saturating concentration of the agonist and n is the Hill coefficient. I is the peak current evoked by a given concentration of GABA. $I_{\max}$ is the maximal current at saturating GABA concentrations.

Glossary

GABA, γ -aminobutyric acid; GABA_AR, type A GABA receptor; HEK, human embryonic kidney; β -CCE, β -carboline-3-carboxyethyl ester; EtOH, ethanol; THDOC, tetrahydrodeoxycorticosterone or 3 α ,21-dihydroxy-5 α -pregnan-20-one.

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Fig. 1. Expression of the δ subunit with $\alpha 4$ and $\beta 3$ leads to increased GABA-sensitivity

A,B) Representative recordings showing GABA dose response data from cells transfected with (A) $\alpha 4 \beta 3$ and (B) $\alpha 4 \beta 3 \delta$ receptors. GABA concentrations of 0.1, 0.3, 1, 3, 10 and 30 μ M were applied. The 0.3 μ M GABA responses are marked below the traces; C) Average GABA dose response curve of $\alpha 4 \beta 3 \delta$ and $\alpha 4 \beta 3$ receptors. GABA EC_{50} values $\pm$ SD are, $\alpha 4 \beta 3 = 1.3 \pm 0.1$ μ M (n=8), for $\alpha 4 \beta 3 \delta$ $EC_{50} = 0.53 \pm 0.02$ μ M (n=6). The GABA sensitivities of receptors made from $\alpha 4 \beta 3$ and $\alpha 4 \beta 3 \delta$ subunits are significantly different ($p < 0.01$).

Fig. 2. Expression of the δ subunit with $\alpha 4 \beta 3$ confers sensitivity to 30 mM EtOH, 1 μ M β -CCE and insensitivity to 1 μ M Zn^{2+}

Shown are representative recordings from three ($\alpha 4 \beta 3$) or four ($\alpha 4 \beta 3 \delta$) similar recordings. To evoke currents, 300 nM GABA was applied, either alone (black trace), with 30 mM EtOH (green trace), with 1 μ M β -CCE (pink), or with 1 μ M Zn^{2+} (light blue).

Fig. 3. Both $\alpha 4\beta 3$ and $\alpha 4\beta 3\delta$ receptors are enhanced by the anesthetics THDOC, etomidate and propofol at saturating (100 μ M) GABA concentrations

A bar graph summarizing the mean enhancement relative to control for 3 μ M etomidate, 3 μ M propofol and 1 μ M THDOC. Open bars show effects on $\alpha 4\beta 3$ (n=9 for etomidate and propofol, n=8 for THDOC), shaded bars on $\alpha 4\beta 3\delta$ (n=3). Enhancements of 100 μ M GABA evoked peak current responses by 3 μ M etomidate and propofol, and 1 μ M THDOC are statistically significant ($p < 0.01$). A comparison of $\alpha 4\beta 3\delta$ vs. $\alpha 4\beta 3$ receptors responses to THDOC, etomidate and propofol shows that differences are not statistically significant ($p=0.32$ for etomidate, $p=0.76$ for propofol, $p=0.69$ for THDOC).

Fig. 4. In mutant $\alpha 4\beta 3N265M$ binary receptors the enhancing effects of 3 μM etomidate and 3 μM propofol but not 1 μM THDOC are abolished

The responses shown in A, B and C are from the same recording from a single $\alpha 4\beta 3N265M$ transfected cell (out of four similar recordings). (A) Etomidate (3 μM), (B) propofol (3 μM) and (C) THDOC (1 μM) were applied either alone or together with a saturating (100 μM) GABA concentration and compared to a saturating 100 μM GABA response. (D) Summary data (n=4, error bars are standard error of mean). Voltage clamp errors occurring with larger GABA currents may lead to underestimates in the amounts of current enhancement by anesthetics and overestimates of the amount of variability, particularly in the case of THDOC.