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Research report

Flumazenil decreases surface expression of α4β2δ $GABA_A$ receptors by increasing the rate of receptor internalization

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ABSTRACT

Increases in expression of α4βδ $GABA_A$ receptors (GABARs), triggered by fluctuations in the neurosteroid THP (3α-OH-5α[β]-pregnan-20-one), are associated with changes in mood and cognition. We tested whether α4βδ trafficking and surface expression would be altered by in vitro exposure to flumazenil, a benzodiazepine ligand which reduces α4βδ expression in vivo. We first determined that flumazenil (100 nM–100 μM, IC_{50} = ~1 μM) acted as a negative modulator, reducing GABA (10 μM)-gated current in the presence of 100 nM THP (to increase receptor efficacy), assessed with whole cell patch clamp recordings of recombinant α4β2δ expressed in HEK-293 cells. Surface expression of recombinant α4β2δ receptors was detected using a 3XFLAG reporter at the C-terminus of α4 (α4F) using confocal immunocytochemical techniques following 48 h exposure of cells to GABA (10 μM) + THP (100 nM). Flumazenil (10 μM) decreased surface expression of α4F by ~60%, while increasing its intracellular accumulation, after 48 h. Reduced surface expression of α4β2δ after flumazenil treatment was confirmed by decreases in the current responses to 100 nM of the GABA agonist gaboxadol. Flumazenil-induced decreases in surface expression of α4β2δ were prevented by the dynamin blocker, dynasore, and by leupeptin, which blocks lysosomal enzymes, suggesting that flumazenil is acting to increase endocytosis and lysosomal degradation of the receptor. Flumazenil increased the rate of receptor removal from the cell surface by 2-fold, assessed using botulinum toxin B to block insertion of new receptors. These findings may suggest new therapeutic strategies for regulation of α4β2δ expression using flumazenil.

1. Introduction

The α4βδ $GABA_A$ receptor (GABAR) is a pentameric membrane protein which gates a Cl^- conductance and is one of many possible subtypes which mediate inhibition in the brain (Olsen and Sieghart, 2009). This receptor expresses extrasynaptically (Wei et al., 2003) where it underlies a tonic inhibitory current (Smith et al., 2009). α4βδ GABARs normally have low expression in the CNS (Pirker et al., 2000; Wisden et al., 1992), but are capable of a high degree of plasticity. *In vivo* studies have shown that naturally occurring fluctuations in neuroactive steroids such as THP ([allo]pregnanolone or 3α-OH-5α[β]-pregnan-20-one), metabolites of the ovarian steroid progesterone (Compagnone and Mellon, 2000), can increase surface expression of this receptor at puberty (Shen et al., 2007), across the estrous cycle (Lovick et al., 2005; Maguire et al., 2005) and post-partum (Maguire and Mody, 2009; Sanna et al., 2009), in areas such as CA1 hippocampus, dentate gyrus and the midbrain central grey, as can direct administration of exogenous steroid to female rodents (Smith et al., 2006). Increased surface expression of α4βδ GABARs increases tonic inhibition (Shen et al., 2010), which has been shown to generate greater inhibitory current than phasic inhibition (Bai et al., 2000). This receptor is also a sensitive target for low dose alcohol (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003) in cells which have high intracellular levels of protein kinase C-δ (Messing et al., 2007). Increased expression of α4β2δ GABARs produced by hormone fluctuations in vivo can in many cases be correlated with alterations in anxiety, seizure susceptibility as well as learning deficits, suggesting that these receptors may play an important role in pathophysiological conditions (Smith et al., 2007).

The biophysical and pharmacological properties of α4β2δ and α1β2/3δ GABARs are unique in that these receptors have a high sensitivity to GABA (EC_{50} = 0.5 μM) (Brown et al., 2002; Sundstrom-Poromaa et al., 2002; Zheleznova et al., 2008), which is, however, a partial agonist at these receptors. Thus, modulators such as THP and the related THDOC ((3α,5β)-3,21-dihydroxypregnan-20-one) increase receptor efficacy when acutely applied (Bianchi and Macdonald, 2003; Zheleznova et al., 2008), due to increases in the mean open time of the channel by the addition of a third

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longer open state (Bianchi and Macdonald, 2003). Our previous work suggests that prolonged exposure to drugs which increase receptor efficacy are also associated with increases in cell surface expression of α4β2δ (Kuver et al., 2012). Hence, a 48 h exposure of HEK-293 cells to THP in combination with GABA results in higher surface expression of α4β2δ GABAR than GABA alone, as do agonists (Bianchi and Macdonald, 2003; Brown et al., 2002) with increased efficacy at α4β2δ GABAR compared to GABA, gaboxadol (THIP or 4,5,6,7-tetrahydroisoxazolo(5,4-c) pyridin-3-ol) and β-alanine (Kuver et al., 2012).

α4β2δ GABARs are insensitive to modulation by benzodiazepine (BZ) agonists (Knoflach et al., 1996 Wafford et al., 1996). BZ agonists bind between the α and γ subunits (Sigel, 2002); thus binding of these agonists would be prevented in receptors such as α4β2δ which lack a γ subunit. In addition, an arginine at position 99 in the α4 (rather than histidine as found in α1-3, 5) also precludes binding of BZ agonists (Knoflach et al., 1996; Wieland et al., 1992). However, recent studies suggest that there is a modified BZ binding site on α4β3δ GABAR which can accommodate binding of other BZ ligands, including the BZ antagonist flumazenil (RO15-1788) and the BZ partial inverse agonist RO15-4513 (Hanchar et al., 2006). Binding of H^3- RO15-4513 has been established in crude membrane fractions of recombinant α4β2δ GABARs expressed in HEK-293T cells, where it produces high affinity saturable binding (Hanchar et al., 2006). Flumazenil is effective as a competitive inhibitor of this binding, suggesting that in contrast to BZ agonists, flumazenil is able to bind to α4β3δ GABARs. Flumazenil is well known as a BZ antagonist at GABARs of the form α[1-3,5]βγ where it has no direct effect on its own, but when applied acutely blocks the effects of other BZ ligands on GABA-gated current and reduces sedation produced by BZ overdose (Olsen and Sieghart, 2009). Conversely, this drug has atypical effects at receptors containing the α4 subunit, such that a 10 μM concentration acutely potentiates current gated by GABA at recombinant α4β1/3γ2 GABARs (Wafford et al., 1996) recorded in the absence of a benzodiazepine agonist.

Recent in vitro studies have suggested that in addition to its acute effects on GABA-gated current, prolonged exposure to flumazenil can also regulate surface expression of GABARs containing α4 or the homologous α6 subunit (Biggio et al., 2007; Zheng et al., 1996), but there are conflicting reports on the direction of the effect of flumazenil on δ subunit expression. Flumazenil has been shown to decrease expression of the α4 subunit (Biggio et al., 2007), which was increased after withdrawal from 100 mM ethanol, when it coexpresses with γ2 (Biggio et al., 2007; Cagetti et al., 2003), without altering δ expression. However, another study showed that in vitro application of 10 μM flumazenil for 4–6 h to cultured cerebellar granule cells increases expression of the δ subunit in association with decreased expression of the homologous α6 subunit (Zheng et al., 1996). In contrast, a recent study from our lab showed that 48 h in vivo treatment with flumazenil reduces hippocampal expression of both α4 and δ subunits, which are increased by chronic treatment of rats with methamphetamine (Shen et al., 2013). Considering these diverse reports of flumazenil's effects on α4 and δ, the present study sought to examine the effect of flumazenil in an isolated system, transfected HEK-293 cells, in order to determine whether flumazenil reduces α4βδ surface expression in vitro as a direct effect by altering receptor trafficking as a result of membrane insertion or endocytosis of the receptor. Although the in vivo approach has physiological relevance, it does not permit determination of the mechanism of flumazenil's effect on α4βδ expression.

Although not yet tested rigorously, preliminary findings have appeared in abstract form suggesting that 100 nM flumazenil can act as a negative modulator at α4β3δ where it can reduce current gated by an EC_{20} of GABA. The purpose of the present study was to confirm the effect of flumazenil at α4β2δ GABARs under conditions where their surface expression was increased, in the presence of THP. It was also our goal to test the effect of flumazenil on cell surface expression of α4β2δ GABARs using immunocytochemical techniques with a 3XFLAG-tagged α4 in HEK-293 cells following treatment with GABA plus THP at concentrations we have shown produce maximal expression of the receptor (Kuver et al., 2012). This is a model of α4β2δ surface expression that produces consistent results in studies of receptor regulation (Kuver et al., 2012). Our findings suggest that flumazenil is a negative modulator at α4β2δ GABARs, reducing current generated by GABA plus THP. Sustained application of the drug can decrease cell surface expression of the α4β2δ GABARs which are increased by 48 h treatment of the cells with GABA plus THP.

2. Materials and methods

2.1. Cell culture

This study used human embryonic kidney (HEK) 293 cells (ATCC, Manassas, VA), maintained in Dulbecco's Modified Eagle's Medium (DMEM/F-12, Invitrogen, Carlsbad, CA) which was supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO), penicillin (100IU/ml) and streptomycin (100 μg/ml) (Invitrogen, Carlsbad, CA). Cells were grown on MatTek glass bottom dishes (MatTek Corp, Ashland, MA) at 37 °C in a humidified incubation chamber (5% CO_2, 95% O_2).

2.1.1. cDNA

A mouse α4-3XFLAG (α4F) reporter was used for all studies. This construct uses three FLAG sequences (DYKDDDDK) at the C terminus of the GABAR α4 subunit for immunocytochemical detection with a high signal:noise ratio. It is expressed in a CMV-14 expression vector (Sigma, St. Louis, MO) and yields functional expression of the full receptor when transfected with β2 and δ cDNA. This construct has been described in a previous study (Kuver et al., 2012), where the GABA and gaboxadol concentration-responses of α4Fβ2δ and α4β2δ were shown to be indistinguishable, suggesting that the FLAG tag does not alter functional characteristics of the receptor.

cDNA for GABAR subunits mouse α4 (N.L. Harrison, Columbia U., New York), rat β2 (J. Bracamontes, Washington U, St. Louis) and human δ (K. Wafford, Merck, Sharp and Dohme, UK) were used for all studies, expressed in pcDNA3.1. (Mouse, rat and human cDNA sequences for β2 are nearly identical.)

2.1.2. Transfection

Cells were transfected with α4F, β2 and δ cDNA (1:1:1; α4(F):β2:δ) using a Nucleofector (Amaxa/Lonza, Walkersville, MD) with reagents and protocols optimized for HEK-293 cells (5 μg of cDNA was used per 100 μl reagent). In some cases, cells were also co-transfected with 2 μg eGFP cDNA (Amaxa/Lonza) for visualization of transfected cells under fluorescence microscopy where the transfection efficiency was consistently 70–80%. The final surface density of plated HEK-293 cells was 10,000 cells/plate.

2.1.3. Drug administration

Transfected HEK-293 cells were treated with GABA (10 μM) plus THP (pregnanolone or 3α-OH-5β-pregnan-20-one, 100 nM) or vehicle (0.01% dimethylsulfoxide) for 48 h. In some cases, they were also treated with flumazenil (10 μM) for varying lengths of time (0.5, 6, 24 or 48 h) culminating at the end of the 48 h GABA plus THP exposure period (a 2 d experiment). In initial studies, the 48 h GABA plus THP exposure period preceded flumazenil administration (a 4 d experiment). However, the 2 d experiment presented in this paper was chosen over the 4 d experiment because both produced similar results. In other cases they were treated with