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Flumazenil decreases surface expression of $\alpha 4\beta 2\delta$ GABA_A receptors by increasing the rate of receptor internalization

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Abstract

Increases in expression of $\alpha 4\beta 2\delta$ GABA_A receptors (GABARs), triggered by fluctuations in the neurosteroid THP (3 α -OH-5 α [β]-pregnan-20-one), are associated with changes in mood and cognition. We tested whether $\alpha 4\beta 2\delta$ trafficking and surface expression would be altered by *in vitro* exposure to flumazenil, a benzodiazepine ligand which reduces $\alpha 4\beta 2\delta$ expression *in vivo*. We first determined that flumazenil (100 nM – 100 μ M, IC₅₀ ~ 1 μ M) acted as a negative modulator, reducing GABA (10 μ M)-gated current in the presence of 100 nM THP (to increase receptor efficacy), assessed with whole cell patch clamp recordings of recombinant $\alpha 4\beta 2\delta$ expressed in HEK-293 cells. Surface expression of recombinant $\alpha 4\beta 2\delta$ receptors was detected using a 3XFLAG reporter at the C-terminus of $\alpha 4$ ($\alpha 4$ F) using confocal immunocytochemical techniques following 48 h exposure of cells to GABA (10 μ M) + THP (100 nM). Flumazenil (10 μ M) decreased surface expression of $\alpha 4$ F by ~60%, while increasing its intracellular accumulation, after 48 h. Reduced surface expression of $\alpha 4\beta 2\delta$ 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 $\alpha 4\beta 2\delta$ 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 $\alpha 4\beta 2\delta$ expression using flumazenil.

Keywords

flumazenil; GABA-A receptor; alpha-4; delta; receptor trafficking; pregnanolone

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1. Introduction

The $\alpha 4\beta\delta$ 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). $\alpha 4\beta\delta$ 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 (allopregnanolone or 3 α -OH-5 α [β]-pregnan-20-one), a metabolite 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 $\alpha 4\beta\delta$ 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 $\alpha 4\beta 2\delta$ 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 $\alpha 4\beta 2\delta$ and $\alpha 1\beta 2/3\delta$ GABARs are unique in that these receptors have a high sensitivity to GABA (EC₅₀=0.5 μ M) (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 ((3 α , 5 β)-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 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 $\alpha 4\beta 2\delta$ (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 $\alpha 4\beta 2\delta$ GABAR than GABA alone, as do agonists (Bianchi and Macdonald, 2003; Brown et al., 2002) with increased efficacy at $\alpha 4\beta 2\delta$ 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).

$\alpha 4\beta 2\delta$ 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 $\alpha 4\beta 2\delta$ which lack a γ subunit. In addition, an arginine at position 99 in the $\alpha 4$ (rather than histidine as found in $\alpha 1$ -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 $\alpha 4\beta 3\delta$ GABAR which can accommodate binding of other BZ ligands, including the BZ antagonist flumazenil (RO15-1788) and the BZ partial inverse agonist RO15-4513 (Hancher et al.,

2006). Binding of H^3 -RO15-4513 has been established in crude membrane fractions of recombinant $\alpha 4\beta 2\delta$ GABARs expressed in HEK-293T cells, where it produces high affinity saturable binding (Hancher 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 $\alpha 4\beta 3\delta$ GABAR. Flumazenil is well known as a BZ antagonist at GABARs of the form $\alpha [1-3,5]\beta \gamma$ 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 $\alpha 4$ subunit, such that a 10 μM concentration acutely potentiates current gated by GABA at recombinant $\alpha 4\beta 1/3\gamma 2$ 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 $\alpha 4$ or the homologous $\alpha 6$ 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 $\alpha 4$ subunit (Biggio et al., 2007), which was increased after withdrawal from 100 mM ethanol, when it coexpresses with $\gamma 2$ (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 $\alpha 6$ 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 $\alpha 4$ 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 $\alpha 4$ and δ , the present study sought to directly examine the effect of flumazenil in an isolated system, transfected HEK-293 cells, in order to determine whether flumazenil reduces $\alpha 4\beta \delta$ 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 $\alpha 4\beta \delta$ 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 $\alpha 4\beta 3\delta$ where it can reduce current gated by an EC_{20} of GABA (Dunn et al., 2003 abstract). The purpose of the present study was to confirm the effect of flumazenil at $\alpha 4\beta 2\delta$ 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 $\alpha 4\beta 2\delta$ GABARs using immunocytochemical techniques with a 3XFLAG-tagged $\alpha 4$ in HEK-293 cells following treating with GABA plus THP at concentrations we have shown produce maximal expression of the receptor (Kuver et al., 2012). This is a model of $\alpha 4\beta 2\delta$ 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 $\alpha 4\beta 2\delta$ GABARs, reducing current generated by GABA plus THP. Sustained application of the drug can decrease cell

surface expression of the $\alpha 4\beta 2\delta$ 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 (100 IU/ml) and streptomycin (100 μ g/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% CO₂, 95% O₂).

2.2. cDNA

A mouse $\alpha 4$ -3XFLAG ($\alpha 4$ F) reporter was used for all studies. This construct uses three FLAG sequences (DYKDDDDK) at the C terminus of the GABAR $\alpha 4$ 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 $\beta 2$ and δ cDNA. This construct has been described in a previous study (Kuver et al., 2012), where the GABA and gaboxadol concentration-responses of $\alpha 4\beta 2\delta$ and $\alpha 4\beta 2\delta$ were shown to be indistinguishable, suggesting that the FLAG tag does not alter functional characteristics of the receptor.

cDNA for GABAR subunits mouse $\alpha 4$ (N.L. Harrison, Columbia U., New York), rat $\beta 2$ (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 $\beta 2$ are nearly identical.)

2.3. Transfection

Cells were transfected with $\alpha 4$ F, $\beta 2$ and δ cDNA (1:1:1; $\alpha 4$ (F): $\beta 2$: δ) using a Nucleofector (Amaxa/Lonza, Walkersville, MD) with reagents and protocols optimized for HEK-293 cells (5 μ g of cDNA was used per 100 μ l reagent). In some cases, cells were also co-transfected with 2 μ g 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.

2.4. Drug administration

Transfected HEK-293 cells were treated with GABA (10 μ M) plus THP (pregnanolone or 3 α -OH-5 β -pregnan-20-one, 100 nM) or vehicle (0.01% dimethylsulfoxide) for 48 h. In some cases, they were also treated with flumazenil (10 μ M) 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 both flumazenil (10 μ M) and/or botulinum toxin B (5 nM)

across a time-course (1, 2, 4, 6 or 24 h). In all cases, cells were harvested at the end of the treatment so that all groups would be processed in parallel (Inset, Fig. 2). Other drugs used included leupeptin, an inhibitor of lysosomal degradation which has been shown to enter cells (Mirabilla et al., 2011; Lee et al., 2010) and dynasore, a cell-permeable inhibitor of dynamin, which prevents endocytosis (Macia et al., 2006). GABA, leupeptin, dynasore and flumazenil were from Sigma Chemical Co. (St. Louis, MO), THP was from Steraloids, Inc. (Newport, RI) and botulinum toxin B was from List Biological Laboratories, Inc. (Campbell, CA).

2.5. Immunocytochemistry

2.5.1. Surface expression of $\alpha 4F$ —Initially, live cells were probed for surface expression of $\alpha 4F$ under non-permeable conditions which required that all steps be carried out on ice (Eshaq et al., 2010). Cells were first incubated with a mouse (Ms) monoclonal anti-FLAG M2 primary antibody (1:50–1:100) (Sigma, St. Louis, MO) followed by a goat (Gt) anti-Ms IgG F(ab')₂ fragment conjugated to Alexa Fluor 488 (1:500, Molecular Probes, Grand Island, NY). Cells were then fixed with 4% paraformaldehyde. In some cases, the cells were then stained with either DAPI (1:1000), a nuclear stain used as a cell marker, or permeabilized with 0.1% Triton X-100 for 5 minutes, and incubated with TO-PRO 3 (1:1000) a cell and nuclear marker (Molecular Probes) for 30 minutes. In the applicable figures, DAPI staining is in blue and TO-PRO 3 in red.

2.5.2. Intracellular expression of $\alpha 4F$ —In order to detect intracellular immunostaining, cells were probed for $\alpha 4F$ under permeabilized conditions at room temperature. Cells were initially fixed with 4% paraformaldehyde/4% sucrose and permeabilized with 0.1% Triton X-100. Cells were then blocked with 10% BSA and incubated with a Ms monoclonal anti-FLAG M2 primary antibody (1:100) (Sigma, St. Louis, MO) followed by a Gt anti-Ms IgG Alexa-488 secondary antibody (1:200) to detect $\alpha 4F$, and a Rb anti-calnexin antibody (1:500) (AbCam, Cambridge, MA) followed by Gt anti-Rb IgG Alexa-546 secondary antibody (1:100) to detect the endoplasmic reticulum (ER). All secondary antibody-Alexa fluor conjugates were from Molecular Probes (Grand Island, NY).

2.5.3. Immunofluorescence analysis—Images were visualized and captured on the Zeiss 710 or 510 inverted confocal microscope at 63X oil or 40X (Microscopy Core of NYU Langone Medical Center, NY, NY). The Image J program (NIH) (Kuver et al., 2012) was used for analysis of the immunofluorescence intensity of representative cells using the ROI (Region of Interest) manager in the Zen 2008 Light Edition program. After the threshold intensity was established, background fluorescence was subtracted from the image, and the intensity of fluorescent pixels around the circumference of the cell was calculated as the integrated density/total area (mean intensity/ μm^2). Images were captured of three cells per plate/group and the experiment repeated 3 times for each determination. Analysis of intracellular labeling was assessed using the same plane of focus that revealed ER staining.

2.6. Assessment of the rate of receptor removal from the cell surface

We assessed whether flumazenil increased the rate of removal of surface expressing $\alpha 4\beta 2\delta$ GABARs after GABA plus THP treatment of transfected HEK-293 cells. To this end, cells were treated with botulinum toxin B (5 nM) for varying lengths of time (1, 3, 5 and 24 or 48 h) to block insertion of newly formed receptors during 48 h exposure to GABA (10 μ M) plus THP (100 nM), with or without flumazenil (10 μ M). Botulinum toxin B cleaves vesicle associated membrane protein (VAMP/synaptobrevin) and prevents vesicle trafficking to the cell surface (Montecucco and Schiavo, 1995; Rummel 2013), as we have previously shown (Kuver et al., 2012). Cells were harvested at the end of the treatment so that all groups would be processed in parallel. Surface immunofluorescence was plotted as a function of time of exposure to botulinum toxin B. Analysis of the rate of receptor removal was accomplished with the least squares fit to the exponential decay function, $y = A1 \exp(-x/\tau) + y0$, where A1 is the initial amplitude minus the steady-state level, y0 is the steady-state level and τ is the decay time constant (Origin 8.5.1, Microcal, Piscataway, NJ).

2.7. Electrophysiology

2.7.1. Whole cell patch clamp—Whole cell currents were recorded from transfected HEK-293 cells in response to GABA (10 μ M) plus THP (30 nM) or gaboxadol (100 nM) using voltage clamp techniques at a holding potential of -50 mV on a Nikon Diaphot inverted microscope. The bath perfusion solution contained (in mM): NaCl 120, CsCl 5, CaCl_2 2, MgCl_2 1, Hepes 10 and glucose 25, pH 7.4, 320 mOsm. Patch pipets (filament-capillary tubes, Sutter Instruments, Novato, CA) were fabricated from borosilicate glass using a Flaming-Brown puller to yield open tip resistances of 3 – 5 M Ω . The pipet solution contained (in mM): N-methyl-D-glucamine chloride 120, Cs_4BAPTA 5 (Calbiochem, San Diego, CA), Mg-ATP 5, and an ATP regeneration system (20 mM Tris phosphocreatine and creatine kinase). Currents were recorded at room temperature (21–22°C) using an Axopatch 1D amplifier (Axon Instruments, Union city, CA) filtered at 2 kHz (four-pole Bessel filter) and detected at 10 kHz (pClamp 8.2).

2.7.2. Pharmacology—Following drug treatments, cells were first incubated in drug-free solution for 1 h prior to voltage clamp recording, which was carried out in the presence of 1 μ M ZnCl_2 to block any binary receptors (Meera et al., 2011). Flumazenil effects were examined on current generated by GABA (10 μ M) plus THP (30 nM) to increase receptor efficacy as a model system with which to best determine potential inhibitory effects of flumazenil. This combined drug application was used to increase receptor efficacy because GABA alone generated insufficient current to reliably interpret the effect of flumazenil. The addition of THP generated robust current, and permitted consistent findings with flumazenil across a concentration range. Drugs were applied using a solenoid-controlled micropipette array 50 μ m from the cell (Smith et al., 1998) which allowed for rapid onset of drug application (20 ms). Responses to drugs were recorded for 5–10 s. Flumazenil (100 nM – 100 μ M) was pre-applied in the bath perfusion (30 s) and concomitantly with GABA plus THP when testing its effects on the current.

2.7.3. Data analysis—Analysis of peak current was accomplished with pClamp 10.1 (Axon Instruments, Union City, CA) and Origin (Microcal, Piscataway, NJ) software

packages. In all cases, 4–5 current traces were averaged for each group. Flumazenil effects on the GABA-gated current were expressed as a ratio relative to current generated by 10 μ M GABA plus 30 nM THP. A concentration-response curve was generated for flumazenil effects on GABA plus THP-generated current using the least square fit to a logistic (sigmoidal) function of the form $y = A_2 + (A_1 - A_2)(1 + (x/x_0)^p)^{-1}$, where $A_{1,2}$ are the initial and final values, respectively, x_0 is the IC_{50} and p is the Hill coefficient.

2.8. Statistics

Data are depicted as the mean $\pm$ SEM (Origin 7, Microcal, Piscataway, NJ). The analysis of variance (ANOVA) was used to evaluate significant differences between >2 groups, followed by a post-hoc Tukey's test. The Student's t -test was used to compare differences between 2 groups. In both cases, significance was established with a $P < 0.05$.

3. Results

3.1. Flumazenil reduces GABA-gated current

In contrast to its role as a BZ antagonist at $\alpha 1\beta 2\gamma 2$ GABAR, flumazenil has been shown to act as a positive GABA modulator at $\alpha 4\beta \gamma 2$ GABAR (Biggio et al., 2007; Wafford et al., 1996). Although the effect of flumazenil on δ -containing receptors has yet to be established conclusively, preliminary reports in abstract form have suggested that 100 nM of this BZ antagonist act as a negative GABA modulator at $\alpha 4\beta 2\delta$ (Dunn et al., 2003 abstract). In order to confirm these findings, we examined the effect of flumazenil on current gated by $\alpha 4\beta 2\delta$ in response to GABA plus THP. This combined treatment was used because there was insufficient current generated by GABA alone, which is a partial agonist at $\alpha 4\beta \delta$ GABARs (Brown et al., 2002), to see consistent effects of flumazenil. THP increases receptor efficacy (Bianchi and Macdonald, 2003) and generated sufficient current to observe consistent results with flumazenil.

Whole-cell patch clamp recordings of HEK-293 cells transfected with $\alpha 4\beta 2\delta$ show that flumazenil produced a concentration-dependent decrease in GABA-gated current (100 nM – 100 μ M), with an $IC_{50} \approx 1 \mu$ M (Fig. 1A, B). These findings indicate that low concentrations of flumazenil act as a negative modulator at $\alpha 4\beta 2\delta$, evaluated in the presence of THP. This newly discovered effect of flumazenil to decrease the amplitude of GABA-gated current at the extrasynaptic $\alpha 4\beta 2\delta$ GABAR raises the possibility that it could influence the surface expression of this receptor.

3.2. Flumazenil down-regulates surface expression of $\alpha 4(3XFLAG)\beta 2\delta$ surface expression

Our previous findings established the effectiveness of the positive GABA modulator, THP, in increasing cell surface expression of $\alpha 4\beta 2\delta$ GABARs (Kuver et al., 2012). Therefore, we tested whether flumazenil, as a negative modulator of $\alpha 4\beta 2\delta$ GABARs (Fig. 2), would decrease surface expression of recombinant $\alpha 4(3XFLAG)\beta 2\delta$ expressed in HEK-293 cells using our previously established model of maximal $\alpha 4\beta 2\delta$ surface expression using GABA plus THP (Kuver et al., 2012).

To this end, we performed a time-based protocol (0.5, 2, 6, 12, 24, 48 h) of flumazenil (10 μ M) treatment during a 48 h incubation of the HEK-293 cells with GABA (10 μ M) plus THP (100 nM) to determine possible effects on surface expression of $\alpha 4\beta 2\delta$. We have previously shown that 48 h exposure of HEK-293 cells with GABA plus THP at these concentrations produces peak levels of $\alpha 4\beta 2\delta$ surface expression (Kuver et al., 2012); surface expression levels are also increased by 1 μ M GABA plus THP. Thus, we used this protocol as an optimized model to increase cell surface expression of $\alpha 4\beta 2\delta$. All timed flumazenil treatments terminated at the conclusion of the 48 h incubation period (Fig. 2 timeline) so that cell processing for all groups proceeded in parallel.

Our results show that 10 μ M flumazenil significantly ($P < 0.05$) decreased cell surface expression produced by GABA plus THP exposure (Fig. 2), an effect first seen after a 2 h flumazenil exposure (initiated after 46 h exposure of cells to GABA plus THP). Maximal decreases in FLAG surface immunofluorescence were noted at 24 h (49.9%) and 48 h (62%) of flumazenil exposure with levels of surface expression not significantly different from cells treated with vehicle or flumazenil alone (Fig. 2A, B). Conversely, 30 min of flumazenil treatment had an insignificant effect on surface expression of the receptor compared to GABA plus THP alone, which increased surface expression 3-fold compared to vehicle ($P < 0.05$). This timecourse of flumazenil effects is similar to our earlier studies, where flumazenil was administered following 48 h GABA plus THP exposure (data not shown). In that case, significant decreases were observed after 2 h flumazenil exposure, with maximal effects at 24–48 h flumazenil exposure.

Exposure of cells to flumazenil alone had no effect on surface expression compared to vehicle (Fig. 2B). Thus, our findings suggest that a negative modulator reduces expression of the $\alpha 4(3XFLAG)\beta 2\delta$ receptor on the cell surface when expression levels of this receptor are increased by GABA plus THP.

3.3. Gaboxadol-gated current of $\alpha 4\beta 2\delta$ GABARs decreases after 24–48 h of flumazenil exposure

To determine if the decrease in observed cell surface expression of $\alpha 4\beta 2\delta$ receptors produced by flumazenil reflected a decrease in functional receptors, we recorded responses of recombinant $\alpha 4\beta 2\delta$ GABARs to 100 nM gaboxadol, a GABA agonist, which, at this concentration is selective for δ -containing GABAR (Brown et al., 2002; Meera et al., 2011). To this end, whole cell voltage clamp recordings were carried out after transfected HEK-293 cells were exposed to 48 h GABA plus THP with various time-based flumazenil treatments (0.5, 2, 6, 24 and 48 h) similar to the protocol used in Figure 2. Gaboxadol-induced currents decreased with increasing exposure to flumazenil beginning with 2 h of exposure (30% decrease, $P < 0.05$), with maximal decreases occurring with 24–48 h of exposure (70% decrease, $P < 0.05$, Fig. 3). The observed decreases in peak response to gaboxadol support our immunocytochemical results indicating that surface expression of $\alpha 4\beta 2\delta$ decreases following flumazenil administration in the presence of GABA plus THP.

3.4. Flumazenil increases the intracellular localization of $\alpha 4\beta 2\delta$ after GABA plus THP treatment

We measured intracellular $\alpha 4$ levels to identify the mechanism for down-regulation of its surface expression by flumazenil. After 48 h of treatment with vehicle or GABA plus THP, with or without flumazenil (48 h), the $\alpha 4\beta 2\delta$ -transfected HEK cells were probed with an anti-FLAG antibody under permeabilized conditions. Intensity of labeling was determined within the intracellular compartment by using the same plane of focus which revealed ER staining in the merged image. Intracellular expression of the recombinant $\alpha 4(3XFLAG)\beta 2\delta$ was highest in the control (vehicle) group ($P < 0.05$, Fig. 4). 48 h treatment with GABA plus THP reduced this intracellular labeling by 38.1% ($P < 0.05$) compared to vehicle-treated control. However, concomitant 48 h treatment with flumazenil partially reversed the effect of GABA plus THP treatment, increasing intracellular expression by ~50% compared to GABA plus THP ($P < 0.05$), suggesting that it may have an effect on receptor trafficking or receptor re-cycling as detailed in the discussion.

3.5. Flumazenil-induced reduction in surface levels of $\alpha 4\beta 2\delta$ after 48 h GABA plus THP are reversed by inhibiting dynamin

Internalization via the clathrin-coated pit has been considered the primary mechanism for reducing GABA_AR surface expression at synaptic (Kittler et al., 2000) and extrasynaptic sites (Joshi and Kapur, 2009), but this mechanism has not yet been investigated in $\alpha 4\beta 2\delta$ receptor down-regulation. To test if this same mechanism is responsible for down-regulation of extrasynaptic $\alpha 4\beta 2\delta$ membrane receptors by flumazenil we used dynasore (80 μ M) to block internalization through dynamin inhibition in HEK-293 cells. Dynasore acts immediately to block the dynamin protein (Macia et al., 2006), so an exposure time of 30 min has been used in internalization studies of membrane receptors (Oh et al., 2012). Cells were treated with dynasore during the final 30 min of a 48 h exposure of transfected HEK-293 cells to GABA plus THP with or without flumazenil, because GABA plus THP can increase $\alpha 4\beta 2\delta$ surface expression within 20 min (Kuver et al., 2012). In fact, 30 min of dynasore treatment prevented the decrease in cell surface expression of $\alpha 4\beta 2\delta$ produced by flumazenil on GABA plus THP-treated cells (Fig. 5). Dynasore treatment resulted in more than a 10-fold increase in surface FLAG labeling compared to flumazenil-treated GABA plus THP cells ($P < 0.05$). Surface labeling at this time-point was not significantly different than that observed for the GABA plus THP group not treated with flumazenil.

3.6. $\alpha 4\beta 2\delta$ surface levels increase after inhibition of lysosomal degradation following 48 h treatment of HEK-293 cells with flumazenil and gaboxadol

Other studies have implicated the endosomal pathway in the down-regulation of synaptic surface receptors via adaptin protein 2 (AP2) and $\beta 2$ subunit interaction (Kittler et al., 2000). Therefore, we measured $\alpha 4\beta 2\delta$ cell surface expression after disabling lysosomal enzymes to determine if the observed effects on down-regulation of the $\alpha 4/\delta$ -containing receptor were linked to endocytic sorting. This was accomplished with leupeptin (1 μ M), an inhibitor of proteases found mainly in lysosomes responsible for protein degradation (Arancibia-Carcamo et al., 2009; Eriksen et al., 2010). In this study, we used gaboxadol, the high efficacy agonist of $\alpha 4\beta 2\delta$, to increase levels of its surface expression (Kuver et al., 2012) in

order to compare flumazenil-induced effects on surface $\alpha 4\beta 2\delta$ expression with those from the studies using GABA plus THP.

Similar to its effect on GABA plus THP-treated cells, 48 h flumazenil exposure reduced surface expression of $\alpha 4\beta 2\delta$ by ~90% after gaboxadol treatment. However, leupeptin administration completely prevented this flumazenil-induced down-regulation of receptor surface expression following gaboxadol treatment ($P < 0.05$, Fig. 6A, B). These data suggest that flumazenil increases degradation through endocytic sorting of $\alpha 4\beta 2\delta$.

3.7. Flumazenil accelerates the rate of receptor removal from the cell surface

Although our data suggest that endocytosis plays a role in flumazenil-induced decreases in surface expression of $\alpha 4\beta 2\delta$ GABARs, it does not directly address whether flumazenil alters the rate of removal of the receptor from the cell surface. Therefore, we examined this possibility in transfected HEK-293 cells treated with GABA plus THP, with or without flumazenil, for 48 h. To this end, we blocked receptor insertion for varying lengths of time (1, 3, 5 and 24 or 48 h) with application of botulinum toxin B (5 nM), known to cleave vesicle associated membrane protein-2 (VAMP2/synaptobrevin) and prevent vesicle trafficking to the cell surface (Montecucco and Schiavo, 1995; Rummel 2013). Endogenous VAMP2 is found in HEK293 cells (Singh et al., 2004) where it regulates membrane insertion of proteins, as we have shown in a previous study (Kuver et al., 2012). We then assessed surface labeling of $\alpha 4F$ using the immunofluorescence techniques described above. Results were plotted as a function of surface labeling versus time of exposure to botulinum toxin B, and the curve fitted with a single exponential decay function.

The time constant for the decrease in surface labeling of $\alpha 4F$ after blockade of receptor insertion was decreased by more than 50% by flumazenil (τ , 2.6 ± 0.23 h, flumazenil with GABA plus THP, versus τ , 5.71 ± 0.89 h, GABA plus THP; $t(8) = 3.38$, $P < 0.01$, Fig. 7) in cells treated with GABA plus THP. These results suggest that the rate of receptor removal is significantly accelerated by 10 μ M flumazenil. In contrast, GABA plus THP treatment does not alter the rate of receptor removal which is similar to vehicle (τ , 5.7 ± 0.64 h) as we have previously shown (Kuver et al., 2012.) This time-course is consistent with findings from recent studies (Joshi and Kapur, 2009) demonstrating that the surface half-life of δ -containing GABAR is on the order of hours, in contrast to γ -containing GABAR, where it is on the order of minutes.

4. Discussion

Our findings suggest that when applied acutely, flumazenil can decrease current gated by $\alpha 4\beta 2\delta$ GABARs, recorded in the presence of THP. Further, we show here that chronic 2 – 24 h exposure to flumazenil decreases cell surface expression of this receptor in transfected HEK-293 cells which had been increased by previous exposure to GABA plus THP. These studies used both immunocytochemical and electrophysiological/pharmacological techniques, suggesting a loss of functional receptors. Flumazenil-induced decreases in receptor surface expression were mediated via an acceleration in the rate of receptor removal from the cell surface through endocytotic and lysosomal degradation pathways. These findings may suggest novel therapeutic approaches for pathologies associated with hormonal

states where $\alpha 4\beta 2\delta$ GABARs are increased by fluctuations in endogenous THP (Maguire et al., 2007; Sabaliauskas et al., 2014).

4.1. BZ ligands: differences in binding to $\alpha 4\beta 2\delta$ GABARs

The binding of BZ inverse agonists and antagonists, such as flumazenil, to $\alpha 4\beta 2\delta$ GABARs is distinct from BZ agonists, which do not bind to these receptors due to an arginine to histidine substitution at residue 99 (Kleingoor et al., 1993) and the absence of a γ subunit which forms part of the binding pocket for BZ agonists (Sigel, 2002). However, reports suggest that flumazenil shares a binding site with ethanol (ETOH) (Hancher et al. 2006). ETOH itself can be a positive modulator of $\alpha 4\beta 2\delta$ GABARs at low concentrations (3 – 30 mM) (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003), an effect dependent upon protein kinase C- δ , although conflicting reports (Baur et al., 2009) have appeared. These low concentrations of ETOH act through a binding site which is also shared by the BZ partial inverse agonist RO15-4513; ETOH can prevent binding of [H3]-RO15-4513 to crude membranes of HEK-293T cells transfected with $\alpha 4\beta 3\delta$ ($K_i \sim 8$ mM) (Hancher et al. 2006). Binding of [H3]-RO15-4513 is also prevented by flumazenil ($K_i = 8.3$ nM), suggesting that the BZ partial inverse agonist and antagonist share a similar binding site. However, binding of [H3]-RO15-4513 is not displaced by classic BZ agonists, including diazepam, midazolam and flunitrazepam; thus, these agonists do not share this binding site, as expected for a receptor containing an $\alpha 4$ subunit and lacking a γ subunit. Surprisingly, only RO15-4513, but not flumazenil, prevents the modulatory actions of ETOH at $\alpha 4\beta 3\delta$ GABARs (Wallner et al., 2006). Although RO-15-4513 and flumazenil are structurally similar, an azido group at the C7 position of the BZ ring on RO15-4513 which is not present on flumazenil may be the moiety which overlaps with the ETOH-binding site. Earlier studies showed that mutations of histidine 101 in $\alpha 1$ of recombinant $\alpha 1\beta 2\gamma 2$ to residues (K or E) which prevent binding of a BZ agonist result in agonist-like effects of flumazenil (Dunn et al., 1999). These results are consistent with recent findings suggesting that flumazenil binds to different regions of the binding pocket occupied by BZ agonists (Kucken et al., 2003; Sigel and Luscher, 2011).

4.2. Flumazenil as a negative modulator and regulator of $\alpha 4\beta 2\delta$ surface expression

In this study we establish the role of flumazenil as a negative modulator of $\alpha 4\beta 2\delta$ GABARs, which was demonstrated robustly when GABA-gated current was recorded in the presence of THP. THP increases the efficacy of $\alpha 4\beta 2\delta$ GABARs, at which GABA is only a partial agonist (Bianchi and Macdonald, 2003).

We have previously shown that high efficacy agonists and positive modulators of $\alpha 4\beta 2\delta$ GABARs can increase cell surface expression of the receptor when applied for 24–48 h (Kuver et al., 2012). This is consistent with results from earlier studies showing that when GABA is a full agonist at GABARs such as $\alpha 1\beta 2\gamma 2$, GABA alone can increase surface expression of the receptor (Eshaq et al., 2010). Although the initial trigger for this effect is not established, it is noteworthy that flumazenil as a negative modulator of the receptor can exert the opposite effect, decreasing cell surface expression of $\alpha 4\beta 2\delta$ GABARs, which are increased by either GABA plus THP or by the high efficacy agonist gaboxadol. Although the mechanism of its effect is not known, because HEK-293 cells were used in the present

study, we can rule out potential effects of flumazenil on other GABARs which contain BZ binding sites. The concentration of flumazenil producing optimal effects in reducing surface expression was 10 μ M, higher than the 1 μ M concentration used for complete blockade of BZ-gated current in transfected cells (Kucken et al., 2000).

The present results are also consistent with a previous study (Zhou and Smith, 2009) suggesting that regulation of native α 4-containing GABAR by GABAR modulators is dependent upon the effect of the drug, where positive modulators such as pentobarbital and THP increase receptor expression, while negative modulators or antagonists decrease receptor expression. The physiological mechanism for the observed flumazenil effects in the present study is not known and could be a result of potential allosteric effects of the drug on the receptor.

Flumazenil did not, however, decrease surface levels of α 4 expression under control conditions. This finding is similar to those reported in an earlier study (Zhou and Smith, 2009) which demonstrated that α 4 expression, assessed by Western blot, is correlated with changes in GABA-gated current, rather than simply through ligand-receptor interactions. In this study, α 4 expression was increased by drugs which increase GABA-gated current, while α 4 expression was decreased by drugs which decrease GABA-gated current. Reducing the driving force with bumetanide reduced the effect of the GABA-modulators to alter α 4 expression. Under unstimulated conditions, flumazenil has no effect on the current and did not alter α 4 surface expression in either the previous study or the current findings. Alternatively, the binding of GABA plus THP may allosterically change the receptor to permit flumazenil to reduce receptor surface expression.

4.3. Flumazenil effects on α 4 surface expression

Flumazenil has previously been shown to decrease expression of native α 4-containing GABARs without changing δ expression (Biggio et al., 2007) and the homologous α 6 (Zheng et al., 1996 in cultured cerebellar granule cells, although in the latter study, it also increased α 1 and δ expression, assessed using Western blot and immunocytochemical techniques. Our findings, however, suggest that flumazenil decreases in α 4 surface expression are accompanied by reductions in δ surface expression, based on the electrophysiology results. The data from the present study are consistent with findings from the α 4 $^{-/-}$ mouse demonstrating that δ surface expression is significantly reduced in CA1 hippocampus and thalamus in the absence of α 4 surface expression (Sabaliauskas et al., 2012; Peng et al., 2014), suggesting that the effects of flumazenil on δ surface expression may be both subunit and site-specific.

Our recent study also suggests that *in vivo* treatment of adult, male rats with flumazenil for 48 h reduces elevated hippocampal levels of α 4 β 2 δ expression, assessed by Western blot, produced by chronic methamphetamine (Shen et al., 2013). This decrease in α 4 β 2 δ expression prevented stress-triggered anxiety, which is one outcome of methamphetamine withdrawal (Shen et al., 2013). Interestingly, in one study, flumazenil was shown to be effective in reducing relapse in methamphetamine-dependent individuals (Urschel et al., 2009), suggesting a possible clinical application for the drug.

Early studies reported an effect of flumazenil in reversing the effects of chronic BZs on BZ binding and tolerance (Roca et al., 1990; Gonsalves and Gallager, 1988). Although the mechanism is not known, the insensitivity to BZs reported in these studies may reflect an increase in the BZ-insensitive $\alpha 4$ subunit. Thus the fact that flumazenil could reverse this insensitivity is consistent with the idea that flumazenil can decrease $\alpha 4$ surface expression.

4.4. Time-course of flumazenil's effect on $\alpha 4\beta 2\delta$ surface expression

Flumazenil effectively decreased receptor surface expression as early as 2–6 h after the onset of drug exposure, by accelerating the rate of receptor removal and decreasing the surface half-life from 5 to 1.3 h. In contrast, the mechanism for GABA plus THP's effect to increase surface receptor expression was by increasing the rate of membrane insertion (Kuver et al., 2012). Thus, flumazenil is not acting to merely block the effect of GABA plus THP, which is consistent with the concept that the binding sites for GABA, steroids and flumazenil are distinct (Olsen and Sieghart, 2009).

This is further substantiated by the fact that flumazenil's effect to decrease surface expression of $\alpha 4\beta 2\delta$ was observed following elevated expression of the receptor (2–24 h flumazenil exposure occurring after 24–46 h GABA plus THP exposure) as well as during concomitant 48 h application of GABA plus THP with flumazenil. Our earlier studies, in fact, demonstrated that flumazenil rapidly reduces surface expression of $\alpha 4\beta 2\delta$ GABARs following 48 h exposure to GABA plus THP (data not shown) across a similar time-course, with return to control levels by 2 h, again considerably faster than the 5 h surface half-life of untreated cells.

4.5. Flumazenil effects on $\alpha 4\beta 2\delta$ internalization

In the present study, disabling dynamin and thereby inhibiting clathrin-dependent endocytosis with dynasore blocked the effect of flumazenil to reduce surface expression of $\alpha 4\beta 2\delta$. This finding substantiates the conclusion that internalization is the putative mechanism underlying down-regulation by flumazenil. The fact that 30 min exposure to dynasore reversed flumazenil-induced reductions in cell surface expression of $\alpha 4\beta 2\delta$ GABARs suggests that, unlike membrane insertion, regulation of endocytic mechanisms are rapid. This time-course is also sufficient to permit THP to increase membrane insertion of $\alpha 4\beta 2\delta$, which is significant by 30 min (Kuver et al., 2012). These findings suggest that flumazenil is causing the activation of proteins in the initial steps of the endocytic pathway and that this may be the rate-limiting step in its mechanism to down-regulate surface expression of $\alpha 4\beta 2\delta$ receptors. Recent studies (Gonzalez et al., 2012) have shown that endocytosis of δ -containing GABARs induced by ethanol is produced by binding of the GABAR δ subunit to clathrin adaptor protein 2 (AP2). Similar processes mediate endocytosis of $\gamma 2$ -containing GABARs, which also require serine phosphorylation, the first step in the endocytic pathway (Kanematsu et al., 2007).

4.6. $\alpha 4\beta 2\delta$ GABARs and neuropsychological disorders

$\alpha 4\beta 2\delta$ GABARs may play an important role in neuropsychological disorders associated with hormone fluctuations (Smith et al., 2007). These receptors are a sensitive target for neurosteroids such as THP (Belelli et al., 2002; Brown et al., 2002; Wohlfarth et al., 2002), a

metabolite of the ovarian hormone progesterone, which is also released in response to sustained stress (Droogleever Fortuyn et al., 2004; Mukai et al., 2008; Purdy et al., 1991). Increases in $\alpha 4\beta 8$ GABAR expression at puberty, across the ovarian cycle (Lovick et al., 2005; Lovick, 2007; Maguire et al., 2005; Maguire and Mody, 2007) and pregnancy (Maguire et al., 2009; Maguire and Mody, 2009; Sanna et al., 2009) are associated with anxiety, panic responses and seizure susceptibility, suggesting a role for this receptor in pathologies associated with hormonal fluctuations.

4.7. $\alpha 4\beta 8$ GABARs and Premenstrual Dysphoric Disorder

Expression of $\alpha 4\beta 8$ GABARs is also increased in CA1 hippocampus following withdrawal from the neuroactive steroid THP in a rodent model of premenstrual dysphoric disorder (PMDD) (Smith et al., 2006). This syndrome typically occurs in the luteal phase of the menstrual cycle (Cunningham et al., 2009), when fluctuations in circulating levels of THP and its parent compound progesterone occur. Similar to the rodent model, women with PMDD have abnormal, dysphoric responses to ovarian steroids such as THP or progesterone (Schmidt et al., 1998), respectively. Interestingly, flumazenil can trigger panic responses in PMDD (Le Melledo et al., 2000), despite a relative insensitivity to benzodiazepine agonists (Sundstrom et al., 1997), consistent with its role as a negative modulator of $\alpha 4\beta 8$, as reported in both human studies and in the rodent model of PMDD (Smith et al., 2006).

4.8. Conclusions

In conclusion, our findings suggest that flumazenil can reduce cell surface expression of $\alpha 4\beta 28$ GABARs that are up-regulated by steroid treatment. Flumazenil may thus have important therapeutic value in neuropsychological (Damgaard et al., 2011; Feng et al., 2010) and addictive disorders (Shen et al., 2013) which may involve this receptor.

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Highlights

- Flumazenil is a negative modulator of $\alpha 4\beta\delta$ GABARs in the presence of THP
- Flumazenil reduces THP-induced $\alpha 4\beta\delta$ GABAR surface expression
- Flumazenil increases $\alpha 4\beta\delta$ receptor endocytosis
- Flumazenil effects on $\alpha 4\beta\delta$ internalization require dynamin

Figure 1. Flumazenil reduces current gated by $\alpha 4\beta 2\delta$ GABA_A receptors

A, Effect of FLU on GABA (10 μ M) current gated by $\alpha 4\beta 2\delta$ GABARs in the presence of 30 nM THP, included to increase receptor efficacy. Arrow, start of ligand application. FLU produced a concentration-dependent (0.01 – 100 μ M) decrease in GABA current (IC_{50} =1 μ M). B, Representative traces. n=4–5 cells/group.

Figure 2. Flumazenil reduces surface expression of $\alpha 4\text{F}\beta 2\delta$ after 48 h GABA plus THP exposure
A, B, Cell surface expression of $\alpha 4\text{F}\beta 2\delta$ expressed in HEK-293 cells. Cells were treated with vehicle (0.01% DMSO) or GABA(10 μM)+THP(100 nM) and harvested after 48 h. Inset, Flumazenil (FLU, F, 10 μM) was added at various times after GABA+THP treatment, so that all cells were harvested at the same time. FLAG expression was assessed with immunofluorescence (488 λ , F(ab')₂ fragment IgG). A, Representative images. Surface fluorescence intensity was greatest after 48 h GABA+THP treatment and reduced by FLU exposure. Cell stain, TO-PRO-3; scale bar, 10 μm . B, Averaged data. (n=9; *F(8,63)=13.23, P<0.0001, ANOVA; P<0.05 vs. 48 h GABA + THP, pos-hoc Tukey's test). Scale, 10 μm .

Figure 3. Flumazenil exposure reduces responses of recombinant $\alpha 4\beta 2\delta$ GABARs to gaboxadol Whole cell patch clamp recordings of peak current responses (in pA) to acutely applied gaboxadol (GBX, 100 nM) in cells treated for 48 h with GABA plus THP or vehicle as well as FLU for the indicated times. A, Summary data and B, representative current traces show that long-term FLU treatment (6, 24, 48 h) reduces GBX-gated current. Arrow, start of ~500 ms ligand application. (n=5 cells/group, *F(6,28)=36.7, $P<0.0001$, ANOVA; $P<0.05$ vs. 48 h GABA+THP with 48 h FLU, post-hoc Tukey's test).

Figure 4. Retention of $\alpha 4\text{F}\beta 2\delta$ in the intracellular compartment after flumazenil administration to GABA plus THP-treated cells

A, Representative images (40 X) showing (left to right) immunofluorescence for intracellular $\alpha 4\text{F}$ (488 λ) and endoplasmic reticulum (ER, 546 λ) after 48 h treatment of transfected HEK-293 cells with GABA + THP with or without flumazenil (FLU, 10 μM). (Control, 0.01% DMSO) 48 h FLU (lower panel) increased intracellular $\alpha 4\text{F}$. B, Averaged data of the mean intracellular $\alpha 4\text{F}$ intensity. (n=9, *F(2,6)=162.5, P<0.0001, ANOVA; P<0.05 vs. other groups, post-hoc Tukey's test). Scale, 10 μm .

Figure 5. Dynasore effects on $\alpha 4\text{F}\beta 2\delta$ surface expression after flumazenil administration to HEK-293 cells treated with GABA plus THP for 48 h

Surface labeling of $\alpha 4\text{F}\beta 2\delta$ expressed in HEK-293 cells after blockade of endocytosis with dynasore (DYN, 80 μM). Cells were treated for 48 h with vehicle (0.01% DMSO) or GABA (10 μM) + THP (100 nM) with or without flumazenil (FLU, 10 μM). DYN was added for 0.5 h at the end of the 48 h incubation. A, Representative (40 X) confocal images. Upper panel (left to right), Cells treated with GABA + THP alone, GABA + THP plus DYN, GABA+THP plus FLU; GABA+THP plus DYN and FLU. DYN prevented the decrease in surface FLAG immunofluorescence produced by FLU after 48 h GABA + THP treatment. Representative images were enhanced for visualization purposes (brightness (40%) and contrast (60%)). Cell stain, DAPI (546 λ), scale bar, 10 μm . B, Averaged data. (n=9, *F(3,32)=45.6, P<0.0001, ANOVA; P<0.05 vs. other groups, post-hoc Tukey's test)

Figure 6. Leupeptin effects on $\alpha 4\text{F}\beta 28$ surface expression after flumazenil administration to gaboxadol - treated HEK-293 cells

Surface labeling of $\alpha 4\text{F}\beta 28$ expressed in HEK-293 cells. Cells were treated with gaboxadol (GBX, 10 μM) for 48 h in addition to either vehicle, flumazenil (FLU, 10 μM), leupeptin (LEU, 1 μM) or FLU plus LEU. A, Representative (40 X) confocal images. Surface FLAG immunofluorescence (488 λ) was reduced by FLU, but this effect was prevented by LEU. Cell stain, DAPI (350 λ), scale bar, 10 μM . B, Averaged data. (n=9, $F(3,32)=614.4$, $P<0.0001$, ANOVA; * $P<0.05$ vs. FLU, ** $P<0.05$ vs. GBX+LEU, *** $P<0.05$ vs. GBX, post-hoc Tukey's test.) Scale, 10 μm .

Figure 7. Flumazenil accelerates the rate of $\alpha 4\text{F}\beta 2\delta$ receptor removal from the cell surface
 Surface labeling of $\alpha 4\text{F}\beta 2\delta$ expressed in HEK-293 cells was assessed after 48 h exposure to GABA (10 μM) + THP (100 nM) with or without flumazenil (FLU, 10 μM). Botulinum toxin B (BX, 5 nM) was added to block receptor insertion at various times prior to staining (0, 1, 3, 5 and 24 or 48 h). A, Representative confocal images (40 X) of surface $\alpha 4\text{F}$ staining (488 λ , green) after treatment with BX for the indicated time periods, administered without (upper panel) or following 48 h treatment with FLU (lower panel). Scale, 10 μm . Representative images were enhanced for visualization purposes (brightness (40%) and contrast (60%)). B, Averaged data. The rate of disappearance of surface $\alpha 4\text{F}$ labeling is plotted as a function of time (n=5 cells per time point). The plot was best fit to a single exponential decay function, which was accelerated by FLU (τ , 2.6 $\pm$ 0.23 h) compared to GABA+THP alone (τ , 5.71 $\pm$ 0.89 h; t(8)=2.76, P=0.0247).