Diazepam Accelerates GABA<sub>A</sub>R Synaptic Exchange and Alters Intracellular Trafficking

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Despite 50+ years of clinical use as anxiolytics, anti-convulsants, and sedative/hypnotic agents, the mechanisms underlying benzodiazepine (BZD) tolerance are poorly understood. BZDs potentiate the actions of gamma-aminobutyric acid (GABA), the primary inhibitory neurotransmitter in the adult brain, through positive allosteric modulation of γ2 subunit containing GABA type A receptors (GABA<sub>A</sub>Rs). Here we define key molecular events impacting γ2 GABA<sub>A</sub>R and the inhibitory synapse gephyrin scaffold following initial sustained BZD exposure in vitro and in vivo. Using immunofluorescence and biochemical experiments, we found that cultured cortical neurons treated with the classical BZD, diazepam (DZP), presented no substantial change in surface or synaptic levels of γ2-GABA<sub>A</sub>Rs. In contrast, both γ2 and the postsynaptic scaffolding protein gephyrin showed diminished total protein levels following a single DZP treatment in vitro and in mouse cortical tissue. We further identified DZP treatment enhanced phosphorylation of gephyrin Ser270 and increased generation of gephyrin cleavage products. Selective immunoprecipitation of γ2 from cultured neurons revealed enhanced ubiquitination of this subunit following DZP exposure. To assess novel trafficking responses induced by DZP, we employed a γ2 subunit containing an N terminal fluorogen-activating peptide (FAP) and pH-sensitive green fluorescent protein (γ2pH<sub>FAP</sub>). Live-imaging experiments using γ2pH<sub>FAP</sub> GABA<sub>A</sub>R expressing neurons identified enhanced lysosomal targeting of surface GABA<sub>A</sub>Rs and increased overall accumulation in vesicular compartments in response to DZP. Using fluorescence resonance energy transfer (FRET) measurements between α2 and γ2 subunits within a GABA<sub>A</sub>R in neurons, we identified reductions in synaptic clusters of this subpopulation of surface BZD sensitive receptor. Additional time-series experiments revealed the gephyrin regulating kinase ERK was inactivated by DZP at multiple time points. Moreover, we found DZP simultaneously enhanced synaptic exchange of both γ2-GABA<sub>A</sub>Rs and gephyrin using fluorescence recovery after photobleaching (FRAP) techniques. Finally we provide the first proteomic analysis of the BZD sensitive GABA<sub>A</sub>R interactome in DZP vs. vehicle treated mice. Collectively, our results indicate DZP exposure elicits down-regulation of gephyrin scaffolding and BZD sensitive GABA<sub>A</sub>R synaptic availability via multiple dynamic trafficking processes.

Keywords: GABA<sub>A</sub>R, benzodiazepine, trafficking, gephyrin, mass spectrometry, diazepam, inhibitory synapse
INTRODUCTION

GABA\(_A\)Rs are ligand-gated ionotropic chloride (Cl\(^-\)) channels responsible for the majority of fast inhibitory neurotransmission in the adult CNS. The most prevalent synaptic GABA\(_A\)R subtype is composed of two \(\alpha\), two \(\beta\), and a \(\gamma\) subunit forming a heteropentamer (Usui-Oukari and Korpi, 2010). Benzodiazepines (BZDs) are a widely used clinical sedative-hypnotic drug class that selectively bind between the interface of a GABA\(_A\)R \(\gamma\) subunit and either an \(\alpha_1/2/3/5\) subunit (Vinkers and Olivier, 2012). Receptors containing these \(\alpha\) subunits are considered to be primarily synaptic, with the exception of \(\alpha_5\), which is localized both synthaptically and extrasynthaptically (Brady and Jacob, 2015). Positive allosteric modulation by BZD enhances GABA\(_A\)R inhibition by increasing the binding affinity of GABA and increasing channel opening frequency (Usui-Oukari and Korpi, 2010). Recent cryo-electron microscopy publications have provided unprecedented structural and pharmacological information about benzodiazepine-sensitive GABA\(_A\)Rs and the benzodiazepine binding site (Phulera et al., 2018; Zhu et al., 2018; Laverty et al., 2019; Masilius et al., 2019) reinvigorating interest in the molecular actions of BZD drugs. It is known that the potentiating effect of BZDs are lost after prolonged or high dose acute exposure (Tietz et al., 1989; Holt et al., 1999), characterized first by a loss of sedative/hypnotic activity followed by the anti-convulsant properties behaviorally (Lister and Nutt, 1986; Wong et al., 1986; File et al., 1988; Bateson, 2002). The induction of BZD tolerance occurs in part due to the uncoupling of allosteric actions between GABA and BZD (Galler et al., 1984; Marley and Gallager, 1989), a process that appears to rely on GABA\(_A\)R receptor internalization (Ali and Olsen, 2001; Gutierrez et al., 2014). We have previously shown that 24 h BZD treatment leads to decreased surface and total levels of the \(\alpha_2\) subunit in cultured hippocampal neurons that was dependent on lysosomal-mediated degradation (Jacob et al., 2012); however, the process by which the \(\alpha_2\) subunit is selectively targeted to lysosomes is still unknown. GABA\(_A\)R subunit ubiquitination and subsequent degradation at proteasomes or lysosomes modulates cell surface expression of receptors (Saliba et al., 2007; Arancibia-Carcamo et al., 2009; Crider et al., 2014; Jin et al., 2014; Di et al., 2016). Ubiquitination of the \(\gamma_2\) subunit is the only currently known mechanism identified to target internalized surface GABA\(_A\)R to lysosomes (Arancibia-Carcamo et al., 2009).

Another major regulator of GABA\(_A\)R efficacy is postsynaptic scaffolding. Confinement at synaptic sites maintains receptors at GABA axonal release sites for activation. Furthermore, this limits receptor diffusion into the extrasynaptic space where internalization occurs (Bogdanov et al., 2006; Gu et al., 2016). The scaffolding protein gephyrin is the main organizer of GABA\(_A\)R synaptic localization and density, as gephyrin knockdown and knock-out models show dramatic reductions in \(\gamma\)- and \(\alpha_2\)-GABA\(_A\)R clustering (Kneussel et al., 1999; Jacob et al., 2005). Evidence suggests gephyrin interacts directly with GABA\(_A\)R \(\alpha_1, \alpha_2, \alpha_3, \alpha_5, \beta_2, \) and \(\beta_3\) subunits (Tretter et al., 2008; Marie et al., 2011; Mukherjee et al., 2011; Kowalczyk et al., 2013; Tyagarajan and Fritschy, 2014; Brady and Jacob, 2015). Gephyrin recruitment is involved in inhibitory long term potentiation (Petrini et al., 2014; Flores et al., 2015), while its dispersal coincides with GABA\(_A\)R diffusion away from synapses (Jacob et al., 2005; Bannai et al., 2009). Extensive post-translational modifications influence gephyrin function (Zacchi et al., 2014; Ghosh et al., 2016). Accordingly, expression of gephyrin phosphorylation site mutants revealed complex effects on GABA\(_A\)R diffusion and gephyrin ultrastructure and scaffolding (Ghosh et al., 2016; Battaglia et al., 2018). Phosphorylation at the gephyrin serine 270 (Ser270) site has been particularly characterized to negatively modulate scaffold clustering and density, in part by enhancing calpain-1 protease-mediated degradation of gephyrin (Tyagarajan et al., 2013). Given the well-established interdependent relationship between gephyrin and the \(\gamma_2\) subunit in maintaining receptor synaptic integrity (Essrich et al., 1998; Kneussel et al., 1999; Schweizer et al., 2003; Alldred et al., 2005; Jacob et al., 2005; Li et al., 2005), impaired postsynaptic scaffolding should affect both pre-existing and newly inserted GABA\(_A\)R clustering and ultimately the efficacy of inhibitory neurotransmission. Thus a central unanswered question is if BZD exposure causes changes in gephyrin phosphorylation or protein levels.

Here we demonstrate that 12–24 h treatment with the BZD diazepam (DZP) leads to a reduction in total \(\gamma_2\) subunit and full-length gephyrin levels in vitro and in vivo. This reduction occurred coincident with enhanced \(\gamma_2\) subunit ubiquitination, but resulted in no significant change in overall \(\gamma_2\) surface levels. Using our recently published dual fluorescent BZD-sensitive GABA\(_A\)R reporter (\(\gamma_2\)P\(^{\text{H}}\)FAP), we further show that cell surface \(\gamma_2\)-GABA\(_A\)Rs are more frequently targeted to lysosomes after DZP exposure. Forester resonance energy transfer (FRET) experiments further confirmed specific loss of synaptic \(\alpha_2/\gamma_2\) GABA\(_A\)R levels following DZP treatment. The scaffolding protein gephyrin also demonstrated augmented phosphorylation at Ser270, increased cleavage and was significantly decreased in membrane and cytosolic compartments. Fluorescence recovery after photobleaching (FRAP) assays identified that DZP treatment increased the simultaneous recovery of \(\gamma_2\)-GABA\(_A\)R and gephyrin at synaptic sites, indicating reduced receptor confinement and accelerated exchange between the synaptic and extrasynaptic GABA\(_A\)R pool. This process was reversed by the BZD site antagonist Ro 15-1788. Lastly, co-immunoprecipitation, quantitative mass spectrometry and bioinformatics analysis revealed shifts in the \(\gamma_2\)-GABA\(_A\)R interactome toward trafficking pathways in vivo. Together, these data suggest that DZP exposure causes a compensatory decrease in inhibitory neurotransmission by reducing BZD-sensitive GABA\(_A\)R and gephyrin confinement at synapses and via ubiquitination and lysosomal targeting of \(\gamma_2\).

MATERIALS AND METHODS

Cell Culture, Transfection, Expression Constructs and Mice

Cortical neurons were prepared from embryonic day 18 rats and nucleofected with constructs at plating (Amaxa). The \(\gamma_2\)P\(^{\text{H}}\)FAP construct was characterized in Lorenz-Guertin et al. (2017) and
RFP-gephyrin was described in Brady and Jacob (2015). The γ2RFP construct was generated by PCR cloning and fully sequenced: the red fluorescent protein mCherry replaced pHluorin in the previously published γ2PHFP construct (Jacob et al., 2005). GFP-ubiquitin was a gift from Nico Dantuma (Addgene plasmid # 11928) (Dantuma et al., 2006). 8–10 weeks old male C57BL/6J mice (Jackson Laboratory) were maintained on a reverse 12 h dark/light schedule. Mouse cortical brain tissue was collected and flash frozen 12 h after I.P. injection with either vehicle or diazepam [in 40% PEG, 10% EtOH, 5% Na Benzoate, 1.5% Benzyl alcohol (Hospira)]. All procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

**Reagents, Antibodies, and MG Dye**

Diazepam (cell culture, Sigma; injections, Hospira); Ro 15-1788 (Tocris Bioscience); calpain-1 inhibitor MDL-28170 (Santa Cruz); 1788 (Tocris Bioscience); guinea pig anti-Cruz); γ; (Tocris Bioscience); Primary antibodies: rabbit GAPDH (WB) (14C10, Cell Signaling); rabbit (P)GSK3β (WB) (9322, Cell Signaling); rabbit (P)ERK (WB) (4370, Cell Signaling); mouse ERK (WB) (9315, Synaptic Systems); chicken GFP (WB) (GFP-1020, Aves); mouse gephyrin mAb7a (IF, phospho) (147011, Synaptic Systems) (adjusted to pH 7.4 with NaOH). Images were acquired using a Nikon A1 confocal microscope with a 60× oil objective (N.A., 1.49) at 3× zoom. Data were analyzed in NIS Elements software (Nikon, N.Y.). Measurements were taken from whole cell or averaged from three dendritic 10 μm regions of interest (ROI) per cell. For fixed imaging, media was quickly removed and coverslips were washed twice with Dulbecco’s Phosphate Buffered Saline (DPBS) and immediately fixed with 4% paraformaldehyde and then blocked in PBS containing 10% fetal bovine serum and 0.5% bovine serum albumin. Surface antibody staining was performed under non-permeabilized conditions overnight at 4°C. Intracellular staining was performed overnight at 4°C following 0.2% Triton-X permeabilization for 10 min in blocking solution. Synaptic sites were determined during analysis by binary thresholds and colocalization with GAD-65. Extrasynaptic intensity was measured by taking the total dendrite ROI sum intensity minus background and synaptic fluorescence intensity. Dendritic fluorescence was measured using binary thresholds. Experimental conditions were blinded during image acquisition and analysis. The ROUT test (Q = 1%) or Grubbs’ Test (alpha = 0.05) was used to remove a single outlier from a data set.

**Fixed and Live-Imaging**

Measurements were made on days in vitro (DIV) 15–19 cortical neurons. Live-imaging performed in Hepses-buffered saline (HBS), containing the following (in mM): 135 NaCl, 4.7 KCl, 10 Heps, 11 glucose, 1.2 MgCl₂, and 2.5 CaCl₂ (adjusted to pH 7.4 with NaOH). Images were acquired using a Nikon A1 confocal microscope with a 60× oil objective (N.A., 1.49) at 3× zoom. Data were analyzed in NIS Elements software (Nikon, N.Y.). Measurements were taken from whole cell or averaged from three dendritic 10 μm regions of interest (ROI) per cell. For fixed imaging, media was quickly removed and coverslips were washed twice with Dulbecco’s Phosphate Buffered Saline (DPBS) and immediately fixed with 4% paraformaldehyde and then blocked in PBS containing 10% fetal bovine serum and 0.5% bovine serum albumin. Surface antibody staining was performed under non-permeabilized conditions overnight at 4°C. Intracellular staining was performed overnight at 4°C following 0.2% Triton-X permeabilization for 10 min in blocking solution. Synaptic sites were determined during analysis by binary thresholds and colocalization with GAD-65. Extrasynaptic intensity was measured by taking the total dendrite ROI sum intensity minus background and synaptic fluorescence intensity. Dendritic fluorescence was measured using binary thresholds. Experimental conditions were blinded during image acquisition and analysis. The ROUT test (Q = 1%) or Grubbs’ Test (alpha = 0.05) was used to remove a single outlier from a data set.

**Lysosomal Targeting Assay**

Neuron surface and lysosomal-association assays utilized MG-BTau dye for surface receptor pulse-labeling. DIV 15–16 neurons were treated with vehicle or DZP for 8–12 h, then pulse labeled with 100 nM MG-B Tau for 2 min at room temperature in HBS. Neurons were then washed 5× times with HBS and returned to conditioned media ± DZP for 1 h. To identify lysosomal targeting, 50 nM LysoTracker Blue DND-22 (Life Technologies) and the lysosomal inhibitor, Leupeptin (200 μM Amresco), was added 30 min prior to imaging. Following incubation, neurons were washed and imaged in 4°C HBS. Two–three neurons were immediately imaged per culture dish within 10 min of washing. For image analysis, independent ROIs were drawn to capture the soma, three 10 μm sections of dendrite and the whole cell. Binary thresholds and colocalization measurements were performed to identify MG-BTau, pHGFP synaptic GABAAR clusters and lysosomes. Total surface pHGFP expression was determined by taking the entire cell surface signal following background subtraction.

**NH4Cl Intracellular Imaging**

DIV 15–16 neurons were washed and continuously perfused with HBS + treatment at room temperature. Multiposition acquisition was used to image 2–3 neurons per dish. An initial image was taken to identify surface γ2PHFAP GABAARs. Neurons were then perfused with NH4Cl solution to collapse the cellular pH gradient and were reimaged. NH4Cl solution (in mM): 50 NH4Cl, 85 NaCl, 4.7 KCl, 10 Heps, 11 glucose, 1.2 MgCl₂, and 2.5 CaCl₂ (adjusted to pH 7.4 with NaOH). pHGFP intensity was measured following background subtraction and smoothing. Surface/total levels were determined by dividing the first image (surface only) from the second image (total). The spot detection tool in Nikon Elements was used to select count larger intracellular vesicles positive for γ2PHFAP. A stringent threshold was set to identify brightly fluorescent circular objects with a circumference of approximately 0.75 μm. Values reflect new vesicle objects that were only seen after NH4Cl perfusion (second image – first image).

**Intermolecular FRET Imaging, Characterization and Analysis**

The α2 pHGFP (α2PH) construct was previously published (Tretter et al., 2008) and the γ2RFP construct was generated by PCR cloning and fully sequenced. DIV 15–16 neurons were treated with Veh or DZP for 20–28 h, then washed and continuously perfused with HBS at room temperature. Images were acquired with a 60× objective at 2× zoom. For each cell, an initial image was acquired containing two channels to identify surface α2PH (excited by 488 laser, emission band pass filter 500–550) and γ2RFP participating in FRET (excited 488 FRET, emission band pass filter 575–625 nm, FRET channel). A second, single channel image was taken immediately following with only 561 nm excitation to reveal total γ2RFP levels (excited by 561 laser, emission band pass filter 575–625 nm). For synaptic quantifications, binary thresholding based on intensity was applied with smoothing and size exclusion (0–3 μm) factors.
FRET and 561 channel binaries shared identical minimum and maximum binary threshold ranges. Individual synaptic ROIs were created to precisely target and measure synaptic clusters containing both α2PH and γ2RFP. Manual trimming and single pixel removal were used to remove signal not meeting the criteria of a receptor cluster. Restriction criteria were applied in the following order: (1) at least 15 synapses measured per cell, (2) FRET γ2RFP: raw γ2RFP sum intensity ratio must be less than one, (3) synaptic α2PH mean intensity of at least 500, and (4) α2PH sum intensity limit of 300% of average sum intensity. ROI data was then normalized to vehicle control as percent change. The percentage of RFP participating in FRET was also calculated using FRET RFP:Total RFP ratio.

Fluorescence resonance energy transfer activity was directly assessed by acceptor (γ2RFP) photobleaching. Photobleaching ROIs were implemented on 2 synapses per cell. Pre-bleaching images were acquired every 5 s, followed by a γ2RFP photobleaching event using 80% 561 nm laser power. After photobleaching, image capturing resumed without delay using pre-bleach laser power settings for 2 min. Image analysis incorporated background subtraction and the measurement of percent change in α2PH/FRET γ2RFP ratio over the time course. FRET efficacy measurements compared directly adjacent α2PH and γ2RFP subunits in a GABAAR complex. Live-imaging with perfusion of pH 6.0 extracellular imaging saline solution (MES) was used to quench the pH-dependent GFP fluorescence from the α2PH donor fluorophores and show the dependence of FRET on surface α2PH fluorescence. Acridic extracellular saline solution, MES solution pH 6.0 (in mM): 10 MES, 135 NaCl, 4.7 KCl, 11 glucose, 1.2 MgCl2, and 2.5 CaCl2 (adjusted to pH 7.4 with NaOH). Images were collected under HBS conditions for 1 min at 20 s intervals, and then followed by a 2 min MES wash with the same imaging interval to quench donor emissions. FRET RFP mean intensity was measured under both conditions and normalized to HBS. Percent or fold change in FRET RFP emissions were reported as indicated.

Synaptic Exchange Rate FRAP Imaging

Neurons were washed and media was replaced with HBS + treatment. Imaging was performed in an enclosed chamber at 37°C. An initial image was taken for baseline standardization. Photobleaching was performed by creating a stimulation ROI box encompassing two or more dendrites. This stimulation region was photobleached using the 488 and 561 lasers at 25% power for 1 min. The same stimulation ROI was used for every cell in an experiment. Immediately following photobleaching, 10 nM MG-Tau dye was added to the cell culture dish to re-identify surface synaptic GABAAR clusters. Time-lapse imaging was then started every 2 min for 60 min. During image analysis, objects were only considered synaptic if they demonstrated colocalization with γ2PHFAP pHGFP signal, RFP-gephyrin signal and had obvious surface MG-BTau fluorescence. ROIs were drawn measuring the rate of fluorescence recovery at 4–8 synaptic sites and one extrasynaptic site (10 μm long region; Bezier tool) per cell. For data analysis, synapse post-bleach fluorescence intensity time point data was first normalized to pre-bleach fluorescence intensity (post-bleach/pre-bleach).

Normalized synapse post-bleach data was then calculated as percent change from t0 [(tx/t0)−100, where x = min]. Individual synapses were then averaged to calculate fluorescence recovery and statistically significant changes across time points.

Western Blot and Immunoprecipitation

Protein concentration was determined by BCA protein assay for all biochemistry. Neurons were lysed in denaturing buffer for immunoprecipitation: 50 mM Tris HCl, 1 mM EDTA, 1% SDS, 2 mM Na3VO4, 10 mM NaF, 50 mM N-ethylmaleimide, protease inhibitor cocktail (Sigma). Lysates were sonicated and heated at 50°C for 20 min, then diluted 1:5 in RIPA buffer (50 mM Tris HCl pH 7.6, 150 mM NaCl, 1% Igepal, 0.5% Sodium deoxycholate, 1 mM EDTA, 2 mM Na3VO4, 10 mM NaF, 50 mM N-ethylmaleimide, protease inhibitor cocktail). Standard immunoprecipitation procedures were performed using overnight incubation with γ2 subunit antibody or rabbit IgG (sci2027; Sigma), 1 h incubation with Protein A Sepharose 4B beads (Invitrogen), three RIPA buffer washes, and loading for SDS-PAGE. After electrophoresis and transfer to nitrocellulose membrane, samples were probed with primary antibody overnight followed by the appropriate horseradish peroxide (HRP)-coupled secondary antibody.

Membrane and Subcellular Fractionation

Cultured neurons were lysed using fractionation buffer: 50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 2 mM Na3VO4, 10 mM NaF, 320 mM sucrose, 0.25% Igepal, and protease inhibitor cocktail. Lysates were spun at 88,881 g for 30 min at 4°C to separate pellet (membrane) from supernatant (cytosol). Fraction integrity was tested by localization specific markers in all experiments (Supplementary Figure S2 and data not shown).

Co-immunoprecipitation

Mice were intraperitoneally (I.P.) injected with vehicle control or 10 mg/kg DZP and sacrificed 12 h post-injection (n = 4 mice per treatment). Mouse cortical tissue was homogenized in co-IP buffer (50 mM Tris HCl pH 7.6, 50 mM NaCl, 0.25% Igepal, 1 mM EDTA, 2 mM Na3VO4, 10 mM NaF, 50 mM N-ethylmaleimide, and Sigma protease inhibitor cocktail) using a Dounce homogenizer. Tissue was solubilized with end-over-end mixing at 4°C for 15 min, and then spun at 1,000 g to remove non-solubilized fractions. Each immunoprecipitation tube contained 375 μg of tissue lysate brought up to 1 ml volume using co-IP buffer. Lysates were precleared using Protein A Sepharose 4B beads (Invitrogen) for 1 h at 4°C. Lysate was then immunoprecipitated overnight with 2.5 μg rabbit γ2 subunit antibody (224003, Synaptic Systems) or 2.5 μg rabbit IgG (2027, Santa Cruz). The next day, 40 μl Protein A Sepharose slurry was added and mixed for 2 h at 4°C on a rotator. Beads were then washed 3 x at 4°C on a rotator in 1 ml co-IP buffer. Beads were denatured with SDS-PAGE loading buffer [Laemmli Sample buffer (Bio-Rad) + β-mercaptoethanol] with heat at 70°C for 10 min and intermittent vortexing. Two immunoprecipitation reactions were performed per animal and were pooled into a single tube without beads to be used for downstream in-gel digestion.
Mass Spectrometry and Data Processing
Immunoprecipitated proteins were separated by electrophoresis in Criterion XT MOPS 12% SDS-PAGE reducing gels (Bio-Rad), with subsequent protein visualization by staining with Coomassie blue. Each gel lane was divided into six slices. After de-staining, proteins in the gel slices were reduced with TCEP [tris(2-carboxyethyl)phosphine hydrochloride] and then alkylated with iodoacetamide before digestion with trypsin (Promega). HPLC-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) was accomplished by data-dependent acquisition on a Thermo Fisher Orbitrap Fusion Lumos Tribirid mass spectrometer. Mascot (Matrix Science; London, United Kingdom) was used to search the MS files against the mouse subset of the UniProt database combined with a database of common contaminants. Subset searching with a database of common contaminants. Subset searching against the mouse subset of the UniProt database combined with a database of common contaminants. Subset searching of the Mascot data, determination of probabilities of peptide assignments and protein identifications, were accomplished with the MudPIT option. Identification criteria were: minimum of two peptides; 96% peptide threshold; 1% FDR; 99% protein threshold. One each entire gel lane were combined via the “MudPIT” option. Assignments and protein identifications, were accomplished with a database of common contaminants. Subset searching against the mouse subset of the UniProt database combined with a database of common contaminants. Subset searching of the Mascot data, determination of probabilities of peptide assignments and protein identifications, were accomplished with the MudPIT option. Identification criteria were: minimum of two peptides; 96% peptide threshold; 1% FDR; 99% protein threshold. One each entire gel lane were combined via the “MudPIT” option.

Statistics
Relevant statistical test information is described in the figure legends or within the individual methods sections. p-values are reported in the results section if significance is between 0.01 and <0.05 or if the data is approaching significance.

RESULTS

DZP Exposure Modifies γ2-GABA_R and Gephyrin Levels
We first examined if DZP exposure reduced surface levels of γ2-GABA_Rs and altered gephyrin Ser270 phosphorylation in cortical neurons by immunofluorescence (Figure 1A). Cortical neurons were treated for 24 h with vehicle or 1 μM DZP, then immunostained for surface γ2, followed by permeabilization and immunostaining with GAD65 (glutamic acid decarboxylase 65, a marker for presynaptic GABAergic terminals) and the phospho-Ser270 specific gephyrin mAb7a antibody (Kuhse et al., 2012; Kalbouneh et al., 2014). Image analysis identified no sizable change in surface synaptic (91.6 ± 5.3%) or extrasynaptic (93.3 ± 3.8%) γ2 intensity in DZP treated neurons relative to control, but DZP induced a significant 18.9 ± 7.4% (p = 0.033) increase in synaptic phospho-gephyrin (Figure 1B). No change in extrasynaptic phosphorylated Ser270 gephyrin was measured. We repeated this DZP treatment and examined total and phospho-gephyrin levels in dendrites (Figure 1C). Again DZP significantly enhanced phospho-Ser270 gephyrin compared to vehicle (132 ± 12%; p = 0.013), while a decrease in overall gephyrin levels was found (69.7 ± 5.4%) (Figure 1D). Accordingly, the mean ratio of phospho/total gephyrin was 78.1 ± 21% higher following DZP (Figure 1D). Complimentary biochemical studies using membrane fractionation were used to compare cytosolic, membrane, and total protein pools in cortical neurons. In agreement with immunofluorescence data, membrane levels of γ2 (0.929 ± 0.06) were not reduced after 1 μM DZP, although the total pool of γ2 was diminished (0.793 ± 0.07) (Figures 2A,B) compared to vehicle. Cytosolic levels of γ2 (1.03 ± 0.06) were also unchanged. Comparatively, DZP reduced full-length gephyrin in every compartment measured relative to control (cytosol: 0.871 ± 0.03; membrane: 0.722 ± 0.06; total: 0.695 ± 0.05). We confirmed the integrity of our fractions using cytosolic and membrane specific markers (Supplementary Figure S2).

Next we assessed if the decrease in gephyrin and γ2 total levels at 24 h was a result of altered gene expression. qRT-PCR experiments revealed no difference in gephyrin (p = 0.206), γ2, or control GABA_R β3 subunit mRNA levels between vehicle and DZP treated neurons (Figure 2C). To determine if post-translational modification of γ2 also occurs coincident with decreased γ2 protein levels, we examined ubiquitination of γ2 in response to DZP exposure. We reasoned that changes in

Bioinformatics Analysis
Ingenuity Pathways Analysis (IPA) (Ingenuity Systems) was used for cellular pathway analysis. Relative fold levels of DZP proteins compared to vehicle were used for analysis. To be suitable for IPA analysis, proteins NF-DZP were assigned a value of −1E+99, while proteins NF-V were assigned a value of 1E+99. Significant enrichment in protein networks were calculated by right tailed Fisher's exact test. Z-score analysis is a statistical measure of an expected relationship direction and observed protein/gene expression to predict pathway activation or inhibition. IPA core analysis was searched to determine direct and indirect relationships within 35 molecules per network and 25 networks per analysis. All data repositories available through IPA were used to determine experimentally observed and highly predicted interactions occurring in mammalian tissue and cell lines. Ratio data were converted to fold change values in IPA, where the negative inverse (−1/x) was taken for values between 0 and 1, while ratio values greater than 1 were not affected. Proteins found to be enhanced in their agreement with immunofluorescence data, membrane levels of γ2 (0.929 ± 0.06) were not reduced after 1 μM DZP, although the total pool of γ2 was diminished (0.793 ± 0.07) (Figures 2A,B) compared to vehicle. Cytosolic levels of γ2 (1.03 ± 0.06) were also unchanged. Comparatively, DZP reduced full-length gephyrin in every compartment measured relative to control (cytosol: 0.871 ± 0.03; membrane: 0.722 ± 0.06; total: 0.695 ± 0.05). We confirmed the integrity of our fractions using cytosolic and membrane specific markers (Supplementary Figure S2).

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ubiquitination of γ2 would likely precede the loss of total γ2 seen at 24 h (Figures 2A,B). GFP-ubiquitin transfected cortical neurons were treated with vehicle or 1 μM DZP for 12 h. Neurons were lysed under denaturing conditions to isolate the γ2 subunit from the receptor complex (Supplementary Figure S3). Immunoprecipitation of the γ2 subunit revealed a 2.13-fold increase (p = 0.015) in ubiquitination in DZP treated neurons relative to vehicle (Figures 2D,E). Furthermore, just as observed with 24 h DZP treatment, a reduced total pool of γ2 was also found at 12 h (p = 0.020) (Figures 2D,E). Notably, this is the first demonstration of endogenous γ2 ubiquitination occurring in neurons (previous findings were of recombinant receptors in HEK cells) (Arancibia-Carcamo et al., 2009; Jin et al., 2014). To investigate mechanisms underlying reduced full-length gephyrin levels, we examined gephyrin cleavage. Gephyrin is degraded post-translationally by the protease calpain-1 (Tyagarajan et al., 2013; Costa et al., 2015; Kumar et al., 2017), and gephyrin Ser270 phosphorylation promotes cleavage by calpain-1 (Tyagarajan et al., 2013). Consistent with the enhanced gephyrin Ser270 phosphorylation (Figure 1) and reduced full-length levels (Figures 1, 2) we found a significant increase in the ratio of cleaved/full-length gephyrin after 24 h DZP in vitro (Figures 2F,G). We confirmed the identity of the gephyrin cleavage product using a well-characterized glutamate stimulation protocol that induces gephyrin cleavage in cultured neurons (Costa et al., 2015; Kumar et al., 2017), a process blocked by calpain-1 inhibition (Supplementary Figure S4).

Finally, we wanted to determine if similar mechanisms occur in vivo following DZP treatment. Prior publications show that BZDs and metabolites are not present 24 h post-injection due to rapid drug metabolism in rodents (Yoong et al., 1986; Xie and Tietz, 1992; Van Sickle et al., 2004; Markowitz et al., 2010). Furthermore, BZD uncoupling does not persist 24 h after a single dose (15 mg/kg) or 2 weeks daily DZP treatment, whereas uncoupling can be seen 12 h after a single injection, indicating this is the appropriate time point for measuring in vivo loss of γ2-GABA₉R function (Holt et al., 1999). Accordingly, mice were given a single intraperitoneal (iP) injection of 10 mg/kg DZP or vehicle control, and cortex tissues were harvested 12 h later. We found DZP significantly reduced the total pool of γ2 (87.3 ± 3.0%) and full-length gephyrin (73.9 ± 9.1%; p = 0.046) relative to vehicle treated mice at 12 h post injection (Figures 2H,I). These findings indicate both BZD-sensitive GABA₉Rs and full-length gephyrin are downregulated by post-translational mechanisms after initial DZP treatment in vitro and in vivo to temper potentiation of GABA₉R function.

**DZP Enhances Intracellular Accumulation and Lysosomal Targeting of γ2-GABA₉Rs**

We then investigated if surface γ2-containing GABA₉Rs are more frequently targeted to lysosomes after DZP exposure by live-imaging. For these experiments we used our recently characterized optical sensor for synaptic GABA₉R (γ2DH-FAP). This dual reporter is composed of a γ2 subunit tagged with an N-terminal pH-sensitive GFP, myc, and the fluorogen-activating peptide DL5 (Lorenz-Guerin et al., 2017). The pH-sensitive GFP tag selectively identifies cell surface GABA₉Rs and the DL5 FAP binds malachite green (MG) dye derivatives including MG-BTau (Szent-Gyorgyi et al., 2008, 2013; Pratt et al., 2017). MG-BTau is cell impermeable and non-fluorescent until bound to DL5. Upon binding, MG-BTau fluoresces in the far red spectral region (~670 nM). This FAP-dye system allows for selective labeling of surface γ2-containing GABA₉Rs which can then be tracked through various phases of trafficking (Lorenz-Guerin et al., 2017). As previously shown, γ2DH-FAP GABA₉Rs are expressed on the neuronal surface, form synaptic clusters, do not perturb neuronal development and show equivalent functional responsiveness to GABA and DZP both in the...
absence and presence of MG dyes (Lorenz-Guertin et al., 2017). We transfected neurons with γ2pH-FAP and treated them with DZP for 8–16 h. Neurons were then pulse-labeled with 100 nM MG-BTau dye and returned to conditioned media at 37°C ± DZP for 1 h. The lysosomal inhibitor leupeptin (200 µM) and the lysosomal specific dye, Lysotracker (50 nM), were added after 30 min. At the end of the incubation, neurons were washed in 4°C saline to inhibit trafficking and immediately used for live-imaging experiments. Representative images demonstrate MG-BTau labeled γ2pH-FAP-GABA<sub>A</sub>Rs localized on the cell surface (Figure 3A) and at synaptic clusters on dendrites (Figure 3B) based on colocalization with surface specific pHGFP signal. MG-BTau further reveals internalized receptors at lysosomes (Figure 3C). Image quantification showed synaptic γ2-GABA<sub>A</sub>R intensity remained largely unchanged (Figure 3D). Importantly, we found a significant 8.0 ± 2.5% (p = 0.015) enhancement in the mean intensity of GABA<sub>A</sub>Rs labeled with MG-B Tau at lysosomes following DZP (Figure 3E). The area of GABA<sub>A</sub>Rs colocalized at lysosomes trended toward an increase in DZP treated cells (140.2 ± 23.6%; p = 0.144) but did not reach significance.

We complemented these lysosomal targeting studies with an NH<sub>4</sub>Cl live-imaging approach that allows us to compare the ratio of cell surface vs. intracellular GABA<sub>A</sub>Rs in living neurons. γ2pH-FAP expressing neurons were treated with vehicle or DZP for 24 h. Additional control groups included the BZD antagonist Ro 15-1788 (1–2 h) to reverse the effects of DZP. Neurons were actively perfused with HEPES buffered saline (HBS) treatment and an initial image was taken of surface pHGFP receptor signal (Figure 3F). Neurons were then exposed to pH 7.4 NH<sub>4</sub>Cl solution to neutralize the pH gradient of all intracellular membrane compartments, revealing internal pools of γ2 containing GABA<sub>A</sub>Rs. Analysis revealed no change in surface γ2 levels between treatments (Figure 3G) consistent with Figures 1, 2. However, the number of large intracellular vesicles (circular area ∼0.75 µm) containing receptors was significantly enhanced (p = 0.047) (Figure 3H).
consistent with increased localization in intracellular vesicles. Ro 15-1788 and DZP + Ro 15-1788 treated neurons were not significantly different from vehicle. Overall, these findings suggest γ2-GABA_A_R ubiquitination, intracellular accumulation, lysosomal targeting and degradation are part of the adaptive response to DZP.
Surface Levels of Synaptic α2/γ2 GABA<sub>R</sub> Are Decreased Following DZP

Despite the increase in ubiquitination and lysosomal targeting of γ2-GABA<sub>R</sub>s after DZP, we did not detect decreased overall surface or synaptically localized γ2 levels. This suggested two possibilities, one being that a slight decrease in surface γ2-GABA<sub>R</sub>s could be challenging to detect with current methods (DZP treated cells total γ2 levels 80% of control in cultured cortical neurons; 85% in vivo). Alternatively, there could be an increase in γ2 subunit assembly with BDZ-insensitive α subunits (γ2α4β) (Wafford et al., 1996) with a concomitant reduction in surface levels of BDZ-sensitive receptors (γ2α1/2/3/5β). Our previous work showed 24 h BDZ exposure in hippocampal neurons causes decreased total and surface levels of the α2 GABA<sub>R</sub> subunit via lysosomal mediated degradation, without any changes in receptor insertion or removal rate (Jacob et al., 2012). To determine if α2/γ2 GABA<sub>R</sub>s are specifically decreased by DZP treatment, we developed an intermolecular FRET assay, using pH-sensitive GFP tagged α2<sup>PH</sup> (Tretter et al., 2008) as a donor fluorophore and a red fluorescent protein (RFP) tagged γ2 subunit (γ2<sup>RFP</sup>) as an acceptor. FRET is an accurate measurement of molecular proximity at distances of 10–100 Å and is highly efficient if donor and acceptor are within the Förster radius, typically 30–60 Å (3–6 nm), with the efficiency of FRET being dependent on the inverse sixth power of intermolecular separation ( Förster, 1965). Synaptic GABA<sub>R</sub>s exist as five subunits assembled in γ2-α-β-α-β order forming a heteropentameric ion channel (Figure 4A). We first expressed α2<sup>PH</sup> and γ2<sup>RFP</sup> in neurons and examined their ability to participate in intermolecular FRET. Photobleaching of the acceptor γ2<sup>RFP</sup> channel enhanced donor α2<sup>PH</sup> signal (Supplementary Figure S5), confirming energy transfer from α2<sup>PH</sup> to γ2<sup>RFP</sup>. Next, we confirmed measurable FRET only occurs between α2<sup>PH</sup>/γ2<sup>RFP</sup> in surface GABA<sub>R</sub> at synaptic sites; FRET was blocked with quenching of donor α2<sup>PH</sup> when the extracellular pH was reduced from 7.4 to 6.0 (Figures 4A,B). Following FRET assay validation, α2<sup>PH</sup>/γ2<sup>RFP</sup> GABA<sub>R</sub> expressing neurons were treated for 24 h with vehicle or DZP and examined for total synaptic α2<sup>PH</sup> and γ2<sup>RFP</sup> fluorescence as well as the γ2 FRET signal (Figure 4C). These studies identified a DZP-induced reduction in synaptic α2 (−12.6%), synaptic γ2 (−14.3%) and diminished association of α2 with γ2 in synaptic GABA<sub>R</sub>s as measured by decreased FRET γ2 signal (−10.6%; p = 0.024) (Figure 4D). In summary, this sensitive FRET method indicates that cortical neurons show a similar susceptibility for α2 subunit downregulation by BDZ treatment as seen in hippocampal neurons (Jacob et al., 2012). Furthermore it identifies a DZP-induced decrease in a specific pool of surface synaptic BDZ-sensitive γ2-GABA<sub>R</sub>.

Selective ERK Inactivation After DZP Treatment

To gain additional mechanistic insight into the molecular mechanisms controlling phosphorylation and degradation of gephyrin observed in Figures 1, 2, we performed a DZP time series experiment to measure changes in expression or activation of the gephyrin regulating kinases ERK, GSK3β, and CDK5. CDK5 and GSK3β phosphorylate gephyrin at the Ser270 site (Tyagarajan et al., 2011; Kalbouneh et al., 2014), while ERK phosphorylates a neighboring Ser268 residue (Tyagarajan et al., 2013). We first measured ERK activation by examining ERK phosphorylation across time points. DZP treatment caused a significant decrease in ERK phosphorylation at 45 min (−50.2%), 3 h (−44.5%) and 6 h (−51.2%), with a recovery in phosphorylation to vehicle levels occurring around 12 and 24 h (Figures 5A,B). Total ERK levels were unchanged after DZP, except for a significant enhancement in expression at the 12 h time point, coinciding with recovery of ERK phosphorylation. We did not detect a change in the

![Figure 4](attachment:figure4.png)

**FIGURE 4** | Intermolecular FRET reveals decreased dendritic synaptic α2/γ2 surface GABA<sub>R</sub>s after DZP. (A) Diagram and time-series images of cortical neurons expressing donor α2<sup>PH</sup> (green) and acceptor γ2<sup>RFP</sup> during imaging at pH 7.4 and pH 6.0. Surface α2<sup>PH</sup> (green) signal and intermolecular FRET (teal) between α2/γ2 subunits occurs at pH 7.4, but is eliminated by brief wash with pH 6.0 extracellular saline and quenching of the α2<sup>PH</sup> donor pH-GFP fluorescence. (B) Quantification of relative FRET at pH 7.4 and pH 6.0 (n = 20 synapses). (C) Neurons α2<sup>PH</sup> (green) and γ2<sup>RFP</sup> (red) were treated with vehicle or DZP for 20–28 h ± and then subjected to live-imaging. For each cell, an initial image used 488 nm laser excitation to identify surface α2<sup>PH</sup> and FRET γ2<sup>RFP</sup>. A second image was taken immediately afterwards to acquire γ2<sup>RFP</sup> total levels (561 nm laser excitation). Dendritic lengths show multiple synaptic clusters with α2<sup>PH</sup>/γ2<sup>RFP</sup> surface GABA<sub>R</sub>s. (D) Synaptic cluster intensity quantification of α2<sup>PH</sup>, γ2<sup>RFP</sup>, and FRET γ2<sup>RFP</sup> (at least 15 synapses per cell; n = 335–483 synapses; 6 independent cultures). Int., fluorescence intensity. Image scale bars = 2 μm. [p < 0.05, **p < 0.01, ****p < 0.0001, paired t-test (B); Student’s t-test (D); error bars ± s.e.m].
phosphorylation or total levels of GSK3β (Figures 5C,D) or expression of CDK5 (Figures 5E,F). This data indicates that kinases involved in gephyrin phosphorylation at Ser270 do not demonstrate global changes after DZP, suggesting that the kinases may be recruited to gephyrin, or that an unknown phosphatase responsible for dephosphorylating Ser270 is inhibited after DZP exposure. Conversely, ERK inactivation by DZP is predicted to decrease phosphorylation of the functionally relevant Ser268 site of gephyrin, which has also been implicated in gephyrin synaptic remodeling (Tyagarajan et al., 2013). Gephyrin point mutant studies suggest reduced phosphorylation at Ser268 coupled with enhanced Ser270 phosphorylation, or the inverse, promotes calpain-1 degradation and scaffold remodeling (Tyagarajan et al., 2013). This data provides evidence that a known kinase pathway responsible for fine-tuning GABA<sub>A</sub>R synapse dynamics (Brady et al., 2017) and scaffold (Tyagarajan et al., 2013) is robustly inactivated by DZP.

**Synaptic Exchange of γ2-GABA<sub>A</sub>Rs and Gephyrin Are Accelerated After Prolonged DZP Treatment**

We previously found 24 h BZD exposure reduces the amplitude of miniature inhibitory postsynaptic currents (mIPSCs) (Jacob et al., 2012), suggesting changes in synaptic GABA<sub>A</sub>R function. Having identified both reductions in full-length gephyrin (Figures 1, 2) and BZD sensitive GABA<sub>A</sub>Rs (Figures 2, 4), we next tested if DZP treatment altered the synaptic retention properties of gephyrin and/or GABA<sub>A</sub>Rs. Neurons expressing γ2<sup>HA</sup>FAP and RFP-gephyrin were used for live-imaging fluorescence recovery after photobleaching experiments (FRAP) to measure synaptic and extrasynaptic exchange following exposure to vehicle, 1 µm DZP, 5 µm Ro 15-1788, or DZP + Ro 15-1788. After an initial image was taken, dendrites were photobleached, and signal recovery was measured every 2 min over 30 min at synaptic sites and extrasynaptic regions (Figure 6A synapses panel; Figure 6B larger dendritic region with white arrows denoting extrasynaptic region). MG-BTau dye was added directly after the photobleaching step to immediately re-identify the photobleached surface synaptic GABA<sub>A</sub>Rs, and improve spatial measurements (Figure 6B). These experiments revealed synaptic γ2 turnover rates were nearly doubled in DZP treated neurons, a process reversed by Ro 15-1788 co-treatment (Figure 6C). DZP also accelerated gephyrin synaptic exchange rates compared to vehicle, with Ro 15-1788 co-treatment restoring exchange to control levels. No significant correlation was found between cluster area measured and fluorescence recovery rates of γ2 and gephyrin across all conditions, suggesting synaptic exchange rate is independent of cluster size (Supplementary Figure S6). Moreover, no statistical difference was found in γ2 or gephyrin extrasynaptic exchange rates (Figure 6D). These findings suggest concurrent reduction of gephyrin and GABA<sub>A</sub>R synaptic confinement is a compensatory response to mitigate prolonged DZP potentiation of GABA<sub>A</sub>Rs.

**Co-immunoprecipitation and Quantitative Proteomics of γ2 GABA<sub>A</sub>R Following DZP Injection**

We sought to observe DZP-induced changes in receptor trafficking in vivo. As an orthogonal approach, we utilized label-free quantitative proteomics to measure changes in the quantities
of proteins associated with γ2-GABA_ARs in the cortex of mice after DZP. Cortical tissue was collected from DZP- or vehicle-treated mice 12 h post injection, lysed, and immunoprecipitated with anti-γ2 subunit antibody or IgG control. Following label-free mass spectrometry analysis, spectrum counts were used to assess relative abundance of γ2-associated proteins.

A total of 395 proteins were identified using our inclusion criteria: minimum of two peptides; identified in at least three samples overall or in two of three samples in a specific treatment group; demonstrated at least 3:1 enrichment over IgG control across at least three samples overall (Supplementary Dataset 1). The relative abundance of γ2-GABA_ARs associated proteins in the DZP group compared to vehicle was used to determine which proteins were increased (Table 1) or decreased (Table 2). As a result we identified 46 proteins with elevated levels of interaction with γ2-GABA_ARs, including 10 proteins that were only found in the DZP treated group (Table 1, not found in vehicle samples, NF-V). Notably, we found a significant (p < 0.05) increase in γ2 association with 14-3-3 protein family members tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein gamma (also known as 14-3-3γ) and tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein epsilon (also known as 14-3-3ε), the phosphatase protein phosphatase 3 catalytic subunit alpha (also known as calcineurin/PPP3CA) and a near significant increase in the GABA_A Rs α5 subunit (p = 0.057), suggesting DZP induced changes in GABA_A Rs surface trafficking (Qian et al., 2012; Nakamura T. et al., 2016), synaptic retention (Bannai et al., 2009, 2015; Muir et al., 2010; Niwa et al., 2012; Eckel et al., 2015), and receptor composition (van Rijnsoever et al., 2004). In contrast, 23 proteins were found to co-immunoprecipitate with γ2 less in DZP animals relative to control, seven of which were only present in the vehicle treatment group (Table 2, not found in DZP, NF-DZP). Interestingly, the calcium-sensitive kinase CaMKIIα, which can regulate GABA_A R membrane insertion, synaptic retention and drug binding properties (26, 70–72), was found to be significantly decreased in interaction with γ2-GABA_A R following DZP injection in vivo.

Bioinformatics Analysis of the γ2 GABA_A R Interactome

To better understand the consequences of the DZP-induced shift in the γ2-GABA_A R protein interaction network, protein fold change data was subjected to core Ingenuity Pathway Analysis (IPA). Top enriched canonical pathways with −log(p-value) > 6.2 are shown in Figure 7. Notably, GABA receptor signaling pathways were highly enriched, as expected, although IPA was unable to determine pathway activation status by z-score analysis. γ2-GABA_A R association with proteins involved in 14-3-3 mediated signaling and RhoA signaling pathways were greatly increased after DZP (Figure 7A, orange), while interaction with proteins involved in ERK5 signaling and sirtuin signaling pathways were reduced (Figure 7A, blue) relative to vehicle.

We further examined alterations in functional network association relevant to receptor trafficking by checking the predicted activation status of select pathways when only using proteins which were found to be increased or decreased (Tables 1, 2). Figure 7B lists γ2-GABA_A R major functional pathways found to be altered by DZP, contributing to processes such as endocytosis (z-score = 2.626), organization of cytoskeleton (z-score = 0.672), and development of neurons (z-score = -0.293). Significant protein changes (p < 0.05)...
### TABLE 1 | Proteins demonstrating increased association with γ2-GABArRs after DZP in vivo by mass spectrometry.

| Ratio (DZP/V) | P-value | UniProtKB | Gene ID | Entrez gene name | Location | Type(s) |
|---------------|---------|-----------|---------|------------------|----------|---------|
| 9.6           | 8.9E-02 | Q14B2     | GRM2    | Glutamate metabotropic receptor 2 | Plasma Membrane | G-protein coupled receptor |
| 9.5           | 4.3E-02 | P12960    | CNTN1   | Contactin 1      | Plasma Membrane | Enzyme |
| 7.0           | 5.9E-02 | P11276    | FN1     | Fibronectin 1    | Extracellular Space | Enzyme |
| 5.4           | 2.7E-02 | E9Q4P0    | KXD1    | KXD motif containing 1 | Cytoplasm | Other |
| 5.4           | 3.1E-02 | Q62277    | SYP     | Synaptophysin    | Cytoplasm | Transporter |
| 5.0           | 5.0E-03 | Q0QXY6    | EHD3    | EH domain containing 3 | Cytoplasm | Other |
| 5.0           | 7.8E-02 | P48774    | GSTM3   | Glutathione S-transferase mu 3 | Cytoplasm | Enzyme |
| 4.9           | 1.9E-05 | P38647    | HSRA9   | Heat shock protein family A (Hsp70) member 9 | Cytoplasm | Other |
| 4.7           | 6.4E-02 | Q91V41    | RAB14   | RAB14, member RAS oncogene family | Cytoplasm | Enzyme |
| 4.2           | 6.4E-02 | P48758    | CBR1    | Carbonyl reductase 1 | Cytoplasm | Enzyme |
| 4.2           | 7.2E-02 | Q8K3F6    | KCNQ3   | Potassium voltage-gated channel subfamily Q member 3 | Plasma Membrane | Ion channel |
| 4.2           | 3.3E-02 | AA0DRJ036 | Nefm    | Neurofilament, medium polypeptide | Plasma Membrane | Other |
| 4.0           | 7.9E-02 | Q92111    | TF      | Transferin       | Extracellular Space | Transporter |
| 3.8           | 8.6E-02 | Q9CY2Z    | TPD52L2 | Tumor protein D52 like 2 | Cytoplasm | Other |
| 3.3           | 4.2E-02 | Q99K00    | ACO2    | Aconitase 2      | Cytoplasm | Enzyme |
| 2.6           | 8.0E-02 | Q9EKF6    | DPYS5L  | Dihydropyrimidinase like 5 | Cytoplasm | Enzyme |
| 2.4           | 2.2E-02 | P56480    | ATP5FB1 | ATP synthase F1 subunit beta | Cytoplasm | Transporter |
| 2.4           | 9.2E-02 | P46006    | SYT1    | Synaptotagmin 1  | Cytoplasm | Transporter |
| 2.4           | 3.7E-02 | Q6P1J1    | CRMP1   | Collapsin response mediator protein 1 | Cytoplasm | Enzyme |
| 2.2           | 3.8E-02 | Q9DB20    | ATP5PO  | ATP synthase peripheral stalk subunit OSCP | Cytoplasm | Transporter |
| 1.9           | N.A.    | P61027    | RAB10   | RAB10, member RAS oncogene family | Cytoplasm | Enzyme |
| 1.8           | 9.6E-02 | P63017    | HSRA8   | Heat shock protein family A (Hsp70) member 8 | Cytoplasm | Enzyme |
| 1.8           | 1.8E-02 | P63011    | RAB3A   | RAB3A, member RAS oncogene family | Cytoplasm | Enzyme |
| 1.3           | 1.3E-02 | P17426-2  | AP2A1   | Adaptor related protein complex 2 subunit alpha 1 | Cytoplasm | Transporter |
| 1.7           | 9.1E-02 | P18760    | CFL1    | Collin 1         | Nuclear | Other |
| 1.7           | 8.2E-02 | Q9Z929    | SUCLA2  | Succinate-CoA ligase ADP-forming beta subunit | Cytoplasm | Enzyme |
| 1.7           | 4.0E-02 | P63328    | PPP3CA  | Protein phosphatase 3 catalytic subunit alpha | Cytoplasm | Phosphatase |
| 1.7           | 7.1E-02 | Q8R191    | SYNGR3  | Synaptogyrin 3   | Plasma Membrane | Other |
| 1.6           | 5.7E-02 | Q8BHJ7    | GABAR5  | Gamma-aminobutyric acid type A receptor alpha5 subunit | Plasma Membrane | Ion channel |
| 1.6           | 1.8E-02 | O35129    | PHE2    | Prohibitin 2      | Cytoplasm | Transporter |
| 1.6           | 1.6E-02 | P61982    | YWHAG   | Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein gamma (14-3-3 gamma) | Cytoplasm | Other |
| 1.6           | 5.7E-02 | P60710    | ACTB    | Actin beta       | Cytoplasm | Other |
| 1.6           | 5.3E-02 | P07901    | HSPI0AA1 | Heat shock protein 90 alpha family class A member 1 | Cytoplasm | Enzyme |
| 1.5           | 7.8E-03 | P67778    | PHB     | Prohibitin       | Nuclear | Transcription regulator |
| 1.5           | 8.1E-02 | Q3UGC7    | EIF3J   | Eukaryotic translation initiation factor 3 subunit J | Cytoplasm | Translation regulator |
| 1.5           | 7.5E-02 | Q8VEM8    | SLCC5A3 | Solute carrier family 25 member 3 | Cytoplasm | Transporter |
| 1.3           | 3.3E-02 | P60710    | ACTB    | Actin beta       | Cytoplasm | Other |
| NF-V          | 7.9E-04 | P62259    | YWHAE   | Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein epsilon (14-3-3 epsilon) | Cytoplasm | Other |
| NF-V          | 1.0E-02 | P63044    | VAMP2   | Vesicle associated membrane protein 2 | Plasma Membrane | Other |
| NF-V          | 1.6E-02 | P16660    | INA     | Internexin neuronal intermediate filament protein alpha | Cytoplasm | Other |
| NF-V          | 3.7E-02 | Q9QYM9    | TMEFF2  | Transmembrane protein with EGF like and two follistatin like domains 2 | Cytoplasm | Other |
| NF-V          | 4.1E-02 | Q6PHN9    | RAB35   | RAB35, member RAS oncogene family | Cytoplasm | Enzyme |
| NF-V          | 6.0E-02 | P19246    | NEFH    | Neurofilament heavy | Cytoplasm | Other |
| NF-V          | 6.3E-02 | Q9C213    | UQORC1  | Ubiquinol-cytochrome c reductase core protein 1 | Cytoplasm | Enzyme |
| NF-V          | 1.2E-06 | Q9CQ07    | ATP5PB  | ATP synthase peripheral stalk-membrane subunit b | Cytoplasm | Transporter |
| NF-V          | 2.8E-06 | P80317    | CCT6A   | Chaperonin containing TCP1 subunit 6A | Cytoplasm | Other |
| NF-V          | 2.8E-06 | Q9CWSO    | DDAH1   | Dimethylarginine dimethylaminohydrolase 1 | Cytoplasm | Enzyme |

**Ratio (DZP/V)** is fold change in DZP animals’ peptide spectral counts (SC) relative to control vehicle treated animals. **NF-V**, not found in vehicle samples. **N = 3** animals per treatment condition, t-test. Significant results (**p** < 0.05) and near significant results (**p** < 0.1) are shown.
TABLE 2 | Proteins demonstrating decreased association with γ2-GABA<sub>A</sub>Rs after DZP in vivo by mass spectrometry.

| Ratio DZP/V | P-value | UniProtKB | Gene ID | Entrez gene name | Location | Type(s) |
|------------|---------|-----------|---------|-----------------|----------|---------|
| 0.2        | 3.4E-02 | P62717    | RPL18A  | Ribosomal protein L18a | Cytoplasm | Other   |
| 0.2        | 3.9E-02 | P62874    | GNB1    | G protein subunit beta 1 | Plasma Membrane | Enzyme |
| 0.2        | 4.2E-02 | Q60900-2 | ELAVL3  | ELAV like RNA binding protein 3 | Nucleus | Other   |
| 0.2        | 5.8E-02 | Q92099    | WDR7    | WD repeat domain 7 | Cytoplasm | Other   |
| 0.3        | 2.0E-02 | P53026    | RPL10A  | Ribosomal protein L10a | Nucleus | Other   |
| 0.3        | 3.4E-02 | Q91VM5    | Rbmol1  | RNA binding motif protein, X-linked like-1 | Nucleus | Other   |
| 0.4        | 6.9E-02 | P49132    | Hrp1a   | Heterogeneous nuclear ribonucleoprotein A1 | Nucleus | Other   |
| 0.4        | 8.5E-02 | Q8BG05    | Hrp3a   | Heterogeneous nuclear ribonucleoprotein A3 | Nucleus | Transporter |
| 0.4        | 7.9E-02 | Q922F4    | TUBB6   | Tubulin beta class V | Cytoplasm | Other   |
| 0.5        | 5.2E-02 | P62334    | PSMC6   | Proteasome 26S subunit, ATPase 6 | Nucleus | Peptidase |
| 0.5        | 2.9E-04 | P11798    | CAMK2A  | Calcium/calmodulin dependent protein kinase II alpha | Cytoplasm | Kinase |
| 0.5        | 3.6E-02 | E9PV14    | EPB41L1 | Erythrocyte membrane protein band 4.1 like 1 | Plasma Membrane | Other   |
| 0.7        | 6.8E-02 | P16330    | CNP     | 2',3'-cyclic nucleotide 3' phosphodiesterase | Cytoplasm | Enzyme |
| 0.7        | 7.6E-02 | C56643    | AP1B1   | Adaptor related protein complex 1 subunit beta 1 | Cytoplasm | Transporter |
| 0.8        | 1.4E-02 | P63869    | TUBA1A  | Tubulin alpha 1a | Cytoplasm | Other   |
| 0.8        | 8.0E-02 | P52480    | PKM     | Pyruvate kinase M1/2 | Cytoplasm | Kinase |
| NF-DZP     | 1.5E-02 | P61358    | RPL27   | Ribosomal protein L27 | Cytoplasm | Other   |
| NF-DZP     | 3.5E-02 | Q821X4-3  | ILF3    | Interleukin enhancer binding factor 3 | Nucleus | Transcription regulator |
| NF-DZP     | 5.5E-02 | Q80UJO    | ELAVL2  | ELAV like RNA binding protein 2 | Cytoplasm | Other   |
| NF-DZP     | 2.8E-06 | Q3UH88    | CCDC177 | Coiled-coil domain containing 177 | Other | Other   |
| NF-DZP     | 2.8E-06 | P49615    | CSDK5   | Cyclic dependent kinase 5 | Nucleus | Kinase |
| NF-DZP     | 2.8E-06 | Q6ZW3     | RPL10   | Ribosomal protein L10 | Cytoplasm | Translation regulator |
| NF-DZP     | 2.8E-06 | Q9OCQ69   | UQCRQ0  | Ubiquinol-cytochrome c reductase complex III subunit VII | Cytoplasm | Enzyme |

Ratio DZP/V is fold change in DZP animals’ peptide SC relative to control vehicle treated animals. NF-DZP, not found in DZP samples. N = 3 animals per treatment condition, t-test. Significant results (p < 0.05) and near significant results (p < 0.1) are shown.

conserved between two or more pathways include decreased γ2-GABA<sub>A</sub>R association with CAMKIIα and CDK5 and enhanced association with calcineurin/PPP3CA, the intracellular trafficking protein RAB35 and the cytoskeletal protein NEFH (also known as heavy neurofilament protein). As an additional measurement, we performed gene ontology (GO) database analysis of proteins which were found to be increased in DZP treated mice relative to vehicle control (Table 3). GO analysis identified enrichment in γ2 association with proteins involved in intracellular trafficking and cellular localization biological pathways after DZP, consistent with IPA analysis findings. Taken together, these results suggest DZP modifies intracellular and surface trafficking of γ2-GABA<sub>A</sub>Rs both in vitro and in vivo.

**DISCUSSION**

This work identifies key trafficking pathways involved in GABA<sub>A</sub>R neuroplasticity in response to initial DZP exposure. Using a combination of biochemical and imaging techniques, we identified total γ2 subunit levels are diminished in response to 12–24 h of DZP exposure in vitro and in vivo. Concurrent with the decrease in the overall γ2 pool, we found DZP treatment enhanced ubiquitination of this subunit. Use of an innovative optical sensor for BZD sensitive GABA<sub>A</sub>Rs both in vitro and in vivo suggests alterations in trafficking at the cell surface and intracellularly. Collectively, this work defines a DZP-induced reduction of gephyrin scaffolding coupled with increased synaptic exchange of gephyrin and GABA<sub>A</sub>Rs.

7.4 NH<sub>4</sub>Cl revealed increased intracellular receptor pools, providing further evidence that DZP enhances γ2-GABA<sub>A</sub>R lysosomal accumulation, a response reversed by BZD antagonist Ro 15-1788 treatment. We used novel intersubunit FRET based live-imaging to identify that surface synaptic α2/γ2 GABA<sub>A</sub>Rs were specifically decreased after DZP, suggesting these receptor complexes were subjected to ubiquitination, lysosomal targeting, and degradation. In addition to DZP modulation of receptor trafficking, the postsynaptic scaffolding protein gephyrin demonstrated significant plasticity including increased Ser270 phosphorylation and production of gephyrin proteolytic fragments, concurrent with a decrease in total and membrane full-length gephyrin levels and ERK inactivation. Given the fundamental role of gephyrin in scaffolding GABA<sub>A</sub>Rs and regulating synaptic confinement, we used simultaneous FRAP live-imaging of receptors and scaffold in neurons to monitor inhibitory synaptic dynamics. We found ~24 h DZP exposure accelerates both the rate of gephyrin and GABA<sub>A</sub>R exchange at synapses as shown by enhanced fluorescence recovery rates. Control experiments using the BZD antagonist Ro 15-1788 were able to reverse the DZP induced loss of synaptic confinement, reducing gephyrin and GABA<sub>A</sub>R mobility back to vehicle levels. Finally, we used label-free quantitative mass spectrometry and bioinformatics to identify key changes in γ2-GABA<sub>A</sub>R protein association in vivo suggesting alterations in trafficking at the cell surface and intracellularly. Collectively, this work defines a DZP-induced reduction of gephyrin scaffolding coupled with increased synaptic exchange of gephyrin and GABA<sub>A</sub>Rs.
FIGURE 7 | Continued
This dynamic flux of GABA<sub>A</sub>Rs between synapses and the extrasynaptic space was associated with enhanced γ<sub>2</sub>-GABA<sub>A</sub>R accumulation in intracellular vesicles and γ<sub>2</sub>-GABA<sub>A</sub>R subtype specific lysosomal degradation. We propose DZP treatment alters these key intracellular and surface trafficking pathways ultimately diminishing responsiveness to DZP.

Numerous classical studies have examined gene and protein expression adaptations in GABA<sub>A</sub>R subunits after BDZ exposure with minimal agreement that a specific change occurs (Bateson, 2002; Uusi-Oukari and Korpi, 2010; Vinkers and Olivier, 2012). Here molecular mechanistic insight is provided, through direct measurements of enhanced ubiquitination of the γ<sub>2</sub> subunit (Figure 2), lysosomal targeting (Figure 3), reduced surface synaptic α<sub>2</sub>γ<sub>2</sub> GABA<sub>A</sub>R levels (Figure 4), and reduced synaptic confinement (Figure 6) of DZP-sensitive GABA<sub>A</sub>Rs. Together this suggests BDZ exposure primarily decreases synaptic retention of γ<sub>2</sub> containing GABA<sub>A</sub>R while downregulating surface levels of the α<sub>2</sub> subunit. Ubiquitination of the γ<sub>2</sub> subunit by the E3 ligase Ring Finger Protein 34 (RUNF 34) (Jin et al., 2014) is the only currently known mechanism targeting internalized synaptic GABA<sub>A</sub>Rs to lysosomes (Arancibia-Carcamo et al., 2009). Due to the requirement of the γ<sub>2</sub> subunit in all BDZ-sensitive GABA<sub>A</sub>Rs, it is likely that ubiquitination of the γ<sub>2</sub> subunit is a contributing factor for increased lysosomal-mediated degradation in response to DZP. Despite a small decrease in the γ<sub>2</sub> total protein, changes in surface levels were not significant by biochemical approaches, consistent with evidence that γ<sub>2</sub>-GABA<sub>A</sub>R surface levels are tightly regulated to maintain baseline inhibition and prevent excitoxicity. For example, in heterozygous γ<sub>2</sub> knockout mice a 50% reduction in γ<sub>2</sub> levels appears to be compensated by increased cell surface trafficking, resulting in only an approximately 25% reduction in BDZ binding sites in the cortex and a limited reduction in synaptic GABA<sub>A</sub>R clusters (Crestani et al., 1999; Ren et al., 2015). In contrast, homozygous γ<sub>2</sub> knockout mice show a complete loss of behavioral drug response to BDZ and over 94% of the BDZ sites in the brain (GABA binding sites unchanged) and early lethality (Gunther et al., 1995). Similarly, studies have shown that prolonged GABA<sub>A</sub>R agonist or BDZ application increases γ<sub>2</sub> GABA<sub>A</sub>R internalization in cultured neurons, while surface GABA<sub>A</sub>R levels are variably affected (Chaumont et al., 2013; Nicholson et al., 2018). Importantly, by using high sensitivity surface GABA<sub>A</sub>R intersubunit FRET measurements we were able to detect a decrease in BDZ sensitive α<sub>2</sub>/γ<sub>2</sub> GABA<sub>A</sub>Rs (Figure 4).

The role of inhibitory scaffolding changes in responsiveness to BDZ has been largely under investigated. Phosphorylation of gephyrin at Ser270 is mediated by CDK5 and GSK3β, while a partnering and functionally relevant Ser268 site is regulated by ERK (Tyagarajan et al., 2013). DZP time series experiments revealed a global decrease in ERK phosphorylation but not GSK3β, without a change in total kinase levels of ERK, GSK3β, or CDK5 over the course of the assay (except 12 h ERK) (Figure 5). A previous model by Tyagarajan et al. (2013) using gephyrin point mutants at Ser268 and Ser270 suggested that enhanced Ser270 phosphorylation coupled with decreased Ser268 phosphorylation by ERK promotes gephyrin remodeling and calpain-1 degradation. This is consistent with the ERK inactivation measured in our data and the increase in gephyrin Ser270 phosphorylation demonstrated by immunofluorescence after DZP (Figure 1), enhanced gephyrin degradation and decreased full-length gephyrin levels (Figures 1, 2). Calpain-1 mediated gephyrin cleavage can occur within 1 min in hippocampal membranes (Kawasaki et al., 1997), and cleavage products are increased following in vitro ischemia at 30 min and up to 48 h following ischemic events in vivo (Costa et al., 2015). Gephyrin cleavage may be occurring at earlier time points than the DZP 24 h mark measured here (Figures 2F,G), coinciding with ERK dephosphorylation as early as 45 min (Figures 5A,B). One limitation of our results is that measuring total and phospho levels of these kinases does not directly address changes in association or

| GO biological process | GO term ID | Fold enrichment | P-value |
|-----------------------|------------|-----------------|---------|
| Transport             | GO:0006810 | 4.2             | 9.8E-09 |
| Establishment of localization | GO:0051234 | 4.0 | 2.5E-08 |
| Establishment of localization in cell | GO:0051649 | 6.8 | 2.0E-07 |
| Localization | GO:0051179 | 3.3 | 2.5E-07 |
| Intracellular transport | GO:0046907 | 7.2 | 1.6E-06 |
| Regulation of localization | GO:0032879 | 4.3 | 3.7E-06 |
| Intracellular protein transport | GO:0006886 | 9.1 | 7.8E-06 |
| Regulation of transport | GO:0051049 | 5.1 | 2.2E-05 |
| Cellular localization | GO:0051641 | 4.8 | 4.8E-05 |
| Protein transport | GO:0015031 | 6.1 | 2.2E-04 |

Proteins found to have enhanced association with γ<sub>2</sub>-GABA<sub>A</sub>Rs during Scaffold analysis were searched in the GO Ontology database for biological process pathway enrichments. PANTHER Overrepresentation test and Fisher's Exact test analysis were searched in the GO Ontology database for biological process enrichment.
regulation of gephyrin, although it does provide an additional piece of evidence supporting gephyrin cleavage by calpain-1 and scaffold remodeling. Accordingly, our previous work found 30 min treatment with the GABA$_A$R agonist muscimol in immature neurons (depolarizing) leads to ERK/BDNF signaling and decreased Ser270 phosphorylated gephyrin levels at synapses and overall (Brady et al., 2017). Thus, ERK activation status negatively correlates with the level of phosphorylation at gephyrin Ser270.

Recent work has demonstrated 12 h DZP treatment of organotypic hippocampal slices expressing eGFP-gephyrin causes enhanced gephyrin mobility at synapses and reduced gephyrin cluster size (Vlachos et al., 2013). Here we found the synaptic exchange rate of γ2 GABA$_A$Rs and gephyrin to be nearly doubled at synapses in cortical neurons after ~24 h DZP exposure (Figure 6). γ2 extrasynaptic recovery in DZP treated neurons was variable but also trended toward an increase relative to controls (Figure 6), which could be a result of increased diffusion of receptors out of the synaptic space. This effect occurred coincident with the formation of truncated gephyrin cleavage products (Figure 2), which has previously been shown to decrease γ2 synaptic levels (Costa et al., 2015). These findings are also consistent with our previous work showing RNAi gephyrin knockdown doubles the rate of γ2-GABA$_A$R turnover at synaptic sites (Jacob et al., 2005). Later quantum dot single particle tracking studies confirmed γ2 synaptic residency time is linked to gephyrin scaffolding levels (Renner et al., 2012). Importantly, GABA$_A$R diffusion dynamics also reciprocally regulate gephyrin scaffolding levels (Niwa et al., 2012), suggesting gephyrin and GABA$_A$Rs synaptic residency are often functionally coupled. Accordingly, γ2 subunit and gephyrin levels both decrease in responses to other stimuli including status epilepticus (Gonzalez et al., 2013) or prolonged inhibition of IP$_3$ receptor-dependent signaling (Bannai et al., 2015). Additionally, chemically induced inhibitory long-term potentiation (iLTP) protocols demonstrate gephyrin accumulation occurs concurrent with the synaptic recruitment of GABA$_A$Rs within 20 min (Petriti et al., 2014). Collectively, these proteins display a high degree of interdependence across different experimental paradigms of inhibitory synapse plasticity occurring over minutes to days.

Increasing receptor synaptic retention enhances synaptic currents, while enhanced receptor diffusion via decreased scaffold interactions reduces synaptic currents. For example, reduction of gephyrin binding by replacement of the α1 GABA$_A$R subunit gephyrin binding domain with non-gephyrin binding homologous region of the α6 subunit results in faster receptor diffusion rates and a direct reduction in mIPSC amplitude (Mukherjee et al., 2011). Similarly, enhanced diffusion of GABA$_A$Rs following estradiol treatment also reduces mIPSCs in cultured neurons and in hippocampal slices (Mukherjee et al., 2017). In contrast, brief DZP exposure (<1 h) reduces GABA$_A$R synaptic mobility (Levi et al., 2015) without a change in surface levels (Gouzer et al., 2014), consistent with initial synaptic potentiation of GABA$_A$R neurotransmission by DZP. Together with our current findings, this suggests post-translational modifications on GABA$_A$R subunits or gephyrin that enhance receptor diffusion are a likely key step leading to functional tolerance to BZD drugs.

It is a significant technical challenge to examine dynamic alterations in receptor trafficking occurring in vivo. To overcome this we examined changes in γ2-GABA$_A$R protein association following DZP injection in mice using quantitative proteomics and bioinformatics analysis. This work revealed shifts toward γ2-GABA$_A$R association with protein pathway networks associated with endocytosis and organization of cytoskeleton (Figure 7B and Table 3), confirming similar fluctuations in membrane and intracellular trafficking occur in vivo and in vitro after DZP. We also found that shifts in association of proteins involved in the development of neurons (CAMKIIα, CDK5, NEFH, and calcineurin/PPP3CA) suggested an inhibition in this pathway after DZP (Figure 7B). When considering all protein hits between vehicle and DZP, γ2-GABA$_A$R association with proteins involved in 14-3-3 mediated signaling and RhoA signaling pathways were greatly increased after DZP (Figure 7A, orange), while interaction with proteins involved in EIF2 signaling and sirtuin signaling pathways were reduced (Figure 7A, blue). 14-3-3 proteins are heavily linked in GABA$_A$R intracellular to surface trafficking (Qian et al., 2012; Nakamura T. et al., 2016), and the RhoA signaling pathway is directly involved in actin cytoskeleton organization (Negishi and Kato, 2002) and α5-GABA$_A$R anchoring (Hausrat et al., 2015), providing further evidence of GABA$_A$R shifts in membrane and cytosolic trafficking after DZP exposure.

Recent inhibitory synapse proteomics studies have identified a number of new protein synaptic constituents or modulators of GABA$_A$R function (Butko et al., 2013; Kang et al., 2014; Nakamura Y. et al., 2016; Uezu et al., 2016; Ge et al., 2018). We show here that proteins known to have roles in synaptic function and trafficking of membrane receptors show changes in their association with γ2-receptors. For example, the calcium-sensitive kinase CaMKIIα was found to be significantly decreased in interaction with γ2-GABA$_A$R following DZP, which can regulate GABA$_A$R membrane insertion, synaptic retention and drug binding properties (Churn et al., 2002; Marsden et al., 2010; Saliba et al., 2012; Petriti et al., 2014) (Table 2). Calcineurin/PPP3CA has been recognized as a key regulator of GABA$_A$R synaptic retention and plasticity (Bannai et al., 2009; Muir et al., 2010; Niwa et al., 2012; Bannai et al., 2015; Eckel et al., 2015) and has been linked to the response to DZP in vitro (Nicholson et al., 2018). Here we provide the first evidence that DZP exposure enhances the association of calcineurin with γ2-GABA$_A$Rs in vivo. Furthermore, DZP was found to enhance γ2 association with 14-3-3 protein family members (Table 1), which are known mediators of GABA$_A$R surface and intracellular trafficking (Qian et al., 2012; Nakamura T. et al., 2016). γ2 coassembly with the GABA$_A$R α5 subunit was also elevated post DZP exposure (Table 1). Interestingly, the α5 subunit is required for the development of BZD sedative tolerance in mice (van Rijnsoever et al., 2004). It is notable that our proteomic studies are in part limited by the specificity of our antibody used and general downstream effects of reduced neuronal activity.
Future follow up studies using the DZP site antagonist R015-1788 will be needed to dissect the individual roles of proteins found to be significantly altered in their association with GABA<sub>A</sub>R, and their physiological and pharmacological importance to BZD tolerance and inhibitory neurotransmission.

Through application of novel and highly sensitive fluorescence imaging approaches combined with in vivo proteomics, we provide unprecedented resolution of GABA<sub>A</sub>R synapse plasticity induced by BZDs at both the level of the single neuron and cortex. Our study reveals that sustained initial DZP treatment diminishes synaptic BZD sensitive GABA<sub>A</sub>R availability through multiple fundamental cellular mechanisms: through reduction of the post-synaptic scaffolding protein gephyrin; shifts toward intracellular trafficking pathways and targeting of receptors for lysosomal degradation; and enhanced synaptic exchange of both gephyrin and GABA<sub>A</sub>Rs. Proteomic and bioinformatics studies using DZP-treated mouse brain tissue provide further evidence that altered γ<sub>2</sub>-GABA<sub>A</sub>R surface and intracellular trafficking mechanisms play a critical role to the response to DZP in vivo. These results define key events leading to BZD irresponsiveness in initial sustained drug exposure. Future studies utilizing this dual approach will address the neuroadaptations produced by long term BZD use to systematically identify the effects of a critical drug class that has seen a tripling in prescription numbers over the last two decades (Bachhuber et al., 2016).

**AUTHOR CONTRIBUTIONS**

JL-G and TJ designed the research. JL-G, TJ, and SW wrote and revised the manuscript. JL-G performed the biochemistry, immunoprecipitation, bioinformatics analysis and fixed and live imaging acquisition and analysis in Figures 1–6. MB performed the FRET imaging and analysis. JL-G and SD performed the tissue collection. TJ, JL-G, and SW designed the Mass spectrometry experiments. SW and the Weintraub lab performed mass spectrometry and data was analyzed by JL-G, TJ, and SW.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2019.00163/full#supplementary-material
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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