Diazepam-induced loss of inhibitory synapses mediated by PLCδ/ Ca\(^{2+}\)/calcineurin signalling downstream of GABAA receptors

Martin W. Nicholson\(^1\) · Aaron Sweeney\(^1\) · Eva Pekle\(^1\) · Sabina Alam\(^1\) · Afia B. Ali\(^1\) · Michael Duchen\(^2\) · Jasmina N. Jovanovic\(^1\)

Received: 4 November 2017 / Revised: 9 April 2018 / Accepted: 1 May 2018 / Published online: 14 June 2018
© The Author(s) 2018. This article is published with open access

Abstract

Benzodiazepines facilitate the inhibitory actions of GABA by binding to \(\gamma\)-aminobutyric acid type A receptors (GABA\(_A\)Rs), GABA-gated chloride/bicarbonate channels, which are the key mediators of transmission at inhibitory synapses in the brain. This activity underpins potent anxiolytic, anticonvulsant and hypnotic effects of benzodiazepines in patients. However, extended benzodiazepine treatments lead to development of tolerance, a process which, despite its important therapeutic implications, remains poorly characterised. Here we report that prolonged exposure to diazepam, the most widely used benzodiazepine in clinic, leads to a gradual disruption of neuronal inhibitory GABAergic synapses. The loss of synapses and the preceding, time- and dose-dependent decrease in surface levels of GABA\(_A\)Rs, mediated by dynamin-dependent internalisation, were blocked by Ro 15-1788, a competitive benzodiazepine antagonist, and bicuculline, a competitive GABA antagonist, indicating that prolonged enhancement of GABA\(_A\)R activity by diazepam is integral to the underlying molecular mechanism. Characterisation of this mechanism has revealed a metabotropic-type signalling downstream of GABA\(_A\)Rs, involving mobilisation of Ca\(^{2+}\) from the intracellular stores and activation of the Ca\(^{2+}\)/calmodulin-dependent phosphatase calcineurin, which, in turn, dephosphorylates GABA\(_A\)Rs and promotes their endocytosis, leading to disassembly of inhibitory synapses. Furthermore, functional coupling between GABA\(_A\)Rs and Ca\(^{2+}\) stores was sensitive to phospholipase C (PLC) inhibition by U73122, and regulated by PLCδ, a PLC isoform found in direct association with GABA\(_A\)Rs. Thus, a PLCδ/Ca\(^{2+}\)/calcineurin signalling cascade converts the initial enhancement of GABA\(_A\)R activity by benzodiazepines to a long-term downregulation of GABAergic synapses, this potentially underpinning the development of pharmacological and behavioural tolerance to these widely prescribed drugs.

Introduction

Benzodiazepines are among the most widely prescribed class of drugs worldwide. Due to their rapid anxiolytic, sedative-hypnotic, anticonvulsant and muscle relaxant effects, they are prescribed for various conditions, most prominently anxiety, insomnia and epileptic seizures [1]. Among these disorders, anxiety, often coinciding with depression, is one of the most common mental health problems in the world (World Health Organization), and is the largest cause of sickness absence in the UK [2], with ~1 in 6 adults being chronically affected according to Mental Health Foundation UK. Although benzodiazepines are very effective initially, the major limitation to their long-term use is the development of tolerance to their pharmacological effects, as well as dependence, resulting in severe withdrawal symptoms upon drug cessation [1, 3]. Nevertheless, the estimated prevalence of long-term use among patients prescribed with benzodiazepine ranges from 25 to 76%, which is equivalent to 2–7.5% of the general population [4]. Although benzodiazepines have been prescribed for over 50 years, the molecular mechanisms leading to tolerance are still poorly understood.
In the nervous system, benzodiazepines bind exclusively to the GABAARs, the main inhibitory receptors in the brain, and allosterically enhance their responsiveness to GABA [5–7]. GABAARs are GABA-gated chloride/bicarbonate channels built up as hetero-pentamers from a pool of 16 different subunits classified as α(1–6), β(1–3), γ(1–3), δ, ε, π and θ, the combination of which determines physiological and pharmacological properties, as well as the tissue and subcellular distribution of these receptors. Benzodiazepines selectively bind to γ2 subunit-containing GABAARs, which are specifically localised to GABAergic inhibitory synapses, thereby enhancing the strength of synaptic inhibition in the brain. In addition to the γ2 subunit, synaptic GABAARs incorporate two α subunits (α1, α2, α3 or α5) and two β subunits (β2 or β3), with the benzodiazepine binding site residing at the interface between the γ2 subunit and one of the α subunits [8]. In neurons, synaptic GABAARs are clustered in the vicinity of presynaptic GABA-releasing terminals, to maximise the efficacy of transmission, but they are also mobile and dynamic, being continuously trafficked between the cytoplasm and the plasma membrane and, once at the cell surface, in and out of synapses [9].

Tolerance to specific benzodiazepines develops at differing rates and degrees in patients, with sedative and hypnotic tolerance within days, and anticonvulsant and anxiolytic tolerance within weeks, as these effects are mediated by specific subtypes of synaptic GABAARs in the brain [10, 11]. Diazepam, the most widely used anxiolytic drug in the clinic, is associated with prominent side effects such as sedation, ataxia and cognitive impairments, due to its non-selective binding to and modulation of all synaptic GABAAR subtypes [1]. Believed to be an adaptation to chronic enhancement of GABAergic signalling, tolerance has been correlated to the observed uncoupling between benzodiazepines and GABA binding sites [12, 13], modifications in GABAAR subunit expression [14, 15], or changes in the level and/or signalling of other neurotransmitters [11]. Endocytosis of GABAARs has also been implicated in long-term effects of benzodiazepines, however the signalling mechanisms that regulate this process, and the effects on inhibitory synapse structure and function, remain poorly characterised [11].

We demonstrate here that prolonged treatment of neurons with diazepam leads to disruption of neuronal inhibitory GABAergic synapses as a consequence of prominent time- and dose-dependent downregulation of surface GABAARs, mediated by dynamin-dependent internalisation. In this process, prolonged activity of GABAARs triggers a metabotropic signalling pathway which involves mobilisation of intracellular Ca2+ and calcineurin-dependent dephosphorylation and endocytosis of GABAARs. This, in turn, is dependent on the activity of phospholipase C (PLC), and mediated, at least in part, by diazepam-modulated direct binding of PLCβ to GABAARs. Thus, a metabotropic PLCβ/Ca2+/calcineurin signalling cascade, activated by diazepam downstream of GABAARs, leads to a long-term downregulation of GABAARs in synapses in a negative feedback fashion, a process likely to underpin the cellular correlates of pharmacological tolerance to these drugs. As such, it provides us with a new repertoire of therapeutic drug targets that may pave the way to improving the outcomes of the long-term clinical use of benzodiazepines.

Materials and methods

Cell culture

Sprague–Dawley rats (UCL-BSU) were housed and sacrificed according to UK Home Office guidelines, following project approval by the UCL Ethics Committee. Primary cultures of cerebrocortical neurones were prepared as described previously [16]. Briefly, cortical tissue was dissected from embryonic day 16-17 (E16-17) rats, dissociated by trituration in Ca2+- and Mg2+-free Hepes-buffered saline solution (HBSS), and plated at the required density in serum-free neurobasal medium, on either 0.1 mg/ml poly-d-lysine-coated dishes or 0.1 mg/ml poly-L-lysine-coated glass coverslips or glass bottom dishes. Cultures were incubated in a humidified 37 °C/5% CO2 incubator (Heracell, Heareus, Germany), for up to 14 days prior to experimentation.

Stable α1β2-HEK293 cell line was maintained under selection with G418 disulphate (Neomycin; G5013-Sigma-Aldrich) and Zeocin (R25001-Invitrogen) [17], while a stable α1β2γ2-HEK293 cell line (Sanofi-Synthelabo, Paris, France) was maintained under selection with G418 disulphate [18].

Immunocytochemistry and synaptic cluster analysis

Cortical neurones (32,500/cm² plated on glass coverslips) were subjected to various treatments and processed for immunolabelling using β2γ2- and γ2-specific antibodies to label the cell surface GABAARs, and, following permeabilization, VGAT and MAP2-specific antibodies, to label GABAergic terminals and dendrites, respectively. Following incubation with the corresponding Alexa-Fluor secondary antibodies (Supplementary Table S1), samples were imaged using a laser scanning confocal microscope (Zeiss LSM 710 Meta, Zeiss, Germany) with a ×63 oil-immersion objective.

The size and number of GABAAR clusters, and their colocalization with VGAT-terminals, were analysed using Zen2.1 software, as previously described [19]. As the
imaging was done in separate channels, a threshold for each fluorophore was determined by the formula (Threshold = mean intensity + (2 × standard deviation)). GABA$_A$R clusters were defined as immuno-reactivity greater than 0.1 $\mu$m$^2$, with a mean fluorescence value greater than 2 × standard deviation of background fluorescence, which were present along the first 20 $\mu$m length of MAP2-positive primary dendrites. Colocalisation in separate channels (at least 50% overlap) was determined by overlaying the images. Synaptic elements size and numbers were analysed using Origin Pro 9.1 software, and the values were expressed as outlined in the figure legends. Normality tests were performed using the Shapiro–Wilks and Kolmogorov–Smirnov tests. After normality tests were performed on each of the groups, non-parametric statistical analysis was done using Mann–Whitney test with the confidence interval of 95%, as the groups showed non-Gaussian distribution.

**GABA$_A$R internalisation assay**

Cell-surface receptors were labelled in living cultured neurons or in α1β2-HEK293 cells transiently transfected with the myc-γ2 cDNA, as described previously [19]. Briefly, cover-slips were incubated with ice-cold Buffer A (mM: 150 NaCl; 3 KCl; 2 MgCl$_2$; 10 HEPES, pH 7.4; 5 Glucose), containing 0.35 M sucrose for 5 min, followed by incubation with mouse anti-β$_{2/3}$ antibody (MAB341-MerckMillipore) for 30 min at 4 °C in Buffer A containing 0.35 M sucrose, 1 mM EGTA and 1% BSA. Cells were further incubated at 37 °C in Buffer A containing 1 mM CaCl$_2$ and 5 µg/ml leupeptin, in the absence or presence of diazepam (1 µM, Tocris), for 1 h to allow internalisation of the labelled β$_{2/3}$ subunit-containing GABA$_A$Rs. Cells were fixed with 4% paraformaldehyde/4% sucrose/PBS (PFA/PBS) and processed for immunolabelling with anti-mouse Alexa-488 antibodies (Supplementary Table S1) for 1 h at room temperature. Samples were imaged using a Zeiss LSM 710 confocal microscope as above.

**Cell surface ELISA**

Cortical neurons (14 DIV, 100,000 cells/cm$^2$ in 24-well plates) or α1β2-HEK293 cells (111,000 cells/cm$^2$ in 24-well plates) transiently transfected with the myc-γ2 cDNA using nucleofection (Lonza, Switzerland), were subjected to treatments with vehicle (DMSO) or diazepam (1 µM), in the absence or presence of various reagents (Supplementary Table S2). All the treatments were done in duplicate. Cells were fixed with PFA/PBS, and changes in surface and total levels of GABA$_A$Rs were detected using cell surface ELISA with β$_{2/3}$ (1 µg/ml)- or myc (1 µg/ml)-specific mouse monoclonal primary antibody and HRP-conjugated anti-mouse secondary antibody [16]. Values were expressed as mean percentage of vehicle treated control (set at 100%) ± s.e.m., with the number of independent repeats of each experiment (n) indicated in the figure legends. Statistical analysis was carried out using ANOVA with Dunnett post-hoc analysis and data plotted using OriginPro 9.1.

**Electrophysiology**

Whole-cell recordings were made from cortical neurons (14 DIV), which were treated with DMSO or diazepam for 72 h, as described before above. Patch pipettes (resistance 8–10 MΩ) were pulled from borosilicate glass tubing and filled with an internal solution containing (mM): 144 K-glucosate, 3 MgCl$_2$, 0.2 EGTA, 2 Na$_2$ATP, 0.2 Na$_2$GTP, 10 HEPES pH 7.2–7.4, 300 mOsm. Spontaneous activity of the neurons was recorded in current clamp mode (SEC 05 L/H, NPI electronics, Tamm, Germany), in the presence of TTX (1 µM), D-AP5 (50 µM) and CNQX (20 µM, all from Tocris). Synaptic potentials recorded were amplified, low-pass filtered at 2 kHz, and digitised at 5 kHz using a CED 1401 interface and data acquisition programme, Signal 4.04 (Cambridge Electronic Design, Cambridge, UK), and analysed offline using Signal. Single sweep amplitudes were measured from the baseline to the peak of the IPSP, and selected for analysis if greater than 0.05 mV [19]. Statistical analysis was conducted using the Student’s t test and data plotted using OriginPro 9.1.

**Biochemical assays**

GST pull-down assays were performed, as described previously [16] using either cortical cell lysates, GFP-PLCδ- or GFP-PRIP-transfected HEK293 cell lysates, or in vitro translated PLCδ (TNT Quick Coupled Transcription/Translation kit, Promega). For communoprecipitation analysis, primary cortical neurons or α1β2γ2-HEK293 cells transfected with GFP-PLCδ, GFP-PRIP1 or both cDNAs using Calcium–phosphate procedure [20], were incubated in the absence or presence of diazepam (1 µM), or in the absence or presence of diazepam/isoguvacine, respectively, for 2 h, and lysed under non-denaturing conditions. Cell lysates were incubated with 10 µg of either nonspecific goat IgG, or GABA$_A$R α1-specific antibody [21], followed by incubation with Protein G-Sepharose. Precipitated proteins were resolved using SDS–PAGE and immunoblotting conducted as described previously [16], using antibodies described in Supplementary Table S3. Protein concentration was determined using either Bradford or BCA assays (ThermoFisher).
**Intracellular Ca\(^{2+}\) imaging**

Cortical neurones (14 DIV; 20,000 cells/cm\(^2\) in 35 mm glass bottom dishes) were incubated with 5 \(\mu\)M Fluo-4 AM/0.0025% pluronic F-127 (ThermoFisher) for 30 min at 37 \(^\circ\)C in the Recording Buffer (in mM: 10 HEPES pH 7.35, 156 NaCl, 3 KCl, 1.25 KH\(_2\)PO\(_4\), 10 D-glucose, 2 CaCl\(_2\)) containing CNQX (20 \(\mu\)M), TTX (0.5 \(\mu\)M) and D-AP5 (50 \(\mu\)M) [22]. Intracellular Ca\(^{2+}\) was recorded using Zeiss LSM 880 confocal microscope at 37 \(^\circ\)C using a \(\times40\) oil-immersion objective and recordings were analysed using Zen2.1 SP2 software (Zeiss, Germany). To quantitate changes in fluorescence intensity, the ‘Region of Interest’ tool was used to monitor regions in the somas and dendrites. The maximal fluorescence values following diazepam application were normalised to the average baseline control before the addition of diazepam (F/F\(_0\)), and analysed using ANOVA followed by Bonferroni post hoc analysis in OriginPro 9.1 software.

**Statistical analysis**

Statistical analyses were performed using OriginPro9.1 software. Each dataset was tested for normality using the Shapiro–Wilk test. For non-parametric data sets, the Kruskal–Wallis test followed by Mann–Whitney test was used and the results were presented using boxplots showing the median, interquartile range, standard deviation and the mean, as specified in the figure legends. For parametric data sets, either the two-tailed Student’s t-test or ANOVA followed by Bonferroni post hoc analysis was used and the results were expressed as the mean ± standard error of the mean. The exact sample size and the number of independent experiments performed, description of the samples and statistical analyses done were also specified in the figure legends. The number of timed-pregnant rats/litters was minimised by utilising the same tissue in multiple experiments, including biochemical, immunocytochemical, electrophysiological and live cell imaging experiments. Values were considered to be statistically significant for \(p<0.05\) (*).

**Fluorescent imaging of GFP-PH PLC\(_{6}\) and DsRed-PRIP1**

\(\alpha\)1\(\beta\)2y2-HEK293 cells (20,000 cells/cm\(^2\) in 35 mm glass bottom dish) were transfected with 0.2 \(\mu\)G GFP-PH\(_{PLC6}\) [23] in the absence or presence of 0.2 \(\mu\)G DsRed-PRIP1 [24] cDNA using Effectene (Qiagen) and cultured for 24 h. The cells were then incubated with Calcein Blue AM/0.0025% pluronic F-127 (ThermoFisher) for 30 min at 37 \(^\circ\)C in HBS (in mM 10 HEPES pH7.4, 150 NaCl, 3 KCl, 2 MgCl\(_2\), 5 D-glucose, 2 CaCl\(_2\)). Calcein Blue, GFP-PH\(_{PLC6}\), or DsRed-PRIP1 emission was collected at 360–450 nm, 500–560 nm or 570–600 nm, respectively. Images (12-bit resolution) were acquired every 5 s before and after the bath application of diazepam (1 \(\mu\)M) and isoguvacine (5 \(\mu\)M) for total of 15 min using Zeiss LSM 880 confocal microscope at 37 \(^\circ\)C using the \(\times40\) oil-immersion objective and analysed using Zen2.1 SP2 software (Zeiss, Germany). Alternatively, cells were treated for 1 h with diazepam (1 \(\mu\)M) and isoguvacine (5 \(\mu\)M) in HBS (in mM: 10 HEPES pH 7.4, 150 NaCl, 3 KCl, 2 MgCl\(_2\), 5 D-glucose, 2 CaCl\(_2\)) and subsequently fixed with 4% PFA/4% sucrose (w/v). Cell surface GABA\(_A\)Rs were labelled with an anti-\(\beta\)-2/3 antibody (BD17) and an Alexa Fluor 555 antibody. GFP-PH\(_{PLC6}\) emission was collected at 500–560 nm and Alexa Fluor 555 emission was collected at 570–600 nm. To quantify changes in fluorescence, the ‘Profile’ tool was used to produce fluorescence profiles for each fluorophore. The fluorescence at the membrane (F\(_m\)) and the fluorescence in the cytoplasm (F\(_c\)), measured before and after bath application of diazepam/isoguvacine, were used to produce a fluorescence ratio F\(_m\)/F\(_c\) which was analysed using Students t-test in OriginPro9.1 software.

**Results**

**Diazepam causes a time-dependent disassembly of GABAergic synapses**

Binding of diazepam to a specific allosteric site harbourabled by the GABA\(_A\)R at the interface between \(\alpha\) and \(\gamma\) subunits leads to a rapid increase in channel gating [25, 26] and results in cumulative enhancement of GABA-mediated transmission at inhibitory synapses. However, under the conditions of sustained stimulation of GABAARs by diazepam (1 \(\mu\)M; concentration analogous to measured plasma concentration of diazepam taken orally by patients [27]) over the time course of 72 h, inhibitory GABAergic synapses underwent a gradual decline in structural integrity due to a significant reduction in the size (Fig. 1a, b; *\(p<0.05\)) and number of dendritic postsynaptic GABAAR clusters (Fig. 1a, c; *\(p<0.05\)), as well as the number of colocalised GABAergic presynaptic terminals (Fig. 1a, d; *\(p<0.05\)), as observed in triple immunolabelling experiments with GABA\(_A\)R-\(\beta\)/\(\gamma\), vesicular GABA transporter (VGAT)- and microtubule-associated protein (MAP2)-specific antibodies and confocal imaging in primary cerebrocortical neurones. The extrasynaptic GABA\(_A\)R clusters monitored at the same time, which were significantly smaller in size, but larger in number than synaptic clusters, remained unaffected by diazepam (Supplementary Figure 1a and b). The total number of immunolabelled GABAergic or glutamatergic neurones remained unchanged (data not shown). Consistent with the observed structural changes, the extrasynaptic clusters (Fig. 1d, e; *\(p<0.05\)) exhibited a reduced fluorescence intensity, and the ‘Region of Interest’ tool was used to monitor regions in the somas and dendrites.
changes in synapses was a prominent reduction in the frequency and amplitude (Fig. 1e–g; *p < 0.05) of miniature inhibitory postsynaptic potentials (mIPSPs), recorded in whole-cell current clamp mode, in neurones treated with diazepam for 72 h. When Ro 15-1788 (flumazenil), a specific competitive antagonist at benzodiazepine binding site on the GABA\(\alpha\)R, was applied together with diazepam, the decrease in size (Fig. 1h, i; *p < 0.05) and number (Fig. 1h,
**Fig. 1** Diazepam causes a time-dependent breakdown of GABAergic inhibitory synapses upon direct binding to GABAARs. a Immunolabeling of postsynaptic GABAAR β2-containing clusters (red) and VGAT-positive presynaptic GABAergic terminals (cyan) along MAP2-positive primary dendrites (20 µm; blue) of cortical neurons in the absence or presence of diazepam (D; 1 µM), and the corresponding graphs showing a decrease over time in b size (median/line-IQRs; mean/dot ± s.d. whiskers; Mann–Whitney test, \( *p < 0.05 \)) and c number (mean ± s.e.m.; ANOVA/Bonferroni post-hoc test; \( *p < 0.05 \)) of synaptic β2-containing clusters, and d number of GABAergic terminals (mean ± s.e.m.; ANOVA/Bonferroni post-hoc test; \( *p < 0.05 \)) contacting n = 59, n = 70, n = 53 control (DMSO)-treated primary dendrites and n = 70, n = 67, n = 44 diazepam-treated primary dendrites for 24 h, 48 h, and 72 h, respectively. Total of n = 17, n = 18 and n = 15 control and n = 17, n = 17 and n = 17 diazepam-treated neurons, respectively, collected from two independent experiments, were analysed in each group. e Representative traces of mIPSPs recorded in cortical neurons after 72 h treatment with control (DMSO) or diazepam (D; 1 µM), before and after application of isoguvacine (+ I, 50 μM), followed by picrotoxin (+ Pic, 50 µM; scale refers to all conditions), and corresponding bar graphs (mean ± s.d.; Student’s t-test; \( *p < 0.05 \)) showing a diazepam-dependent decrease in f frequency and g amplitude of mIPSPs and the effects of isoguvacine (+ I, 50 µM; grey bars) from n = 7 control & n = 7 diazepam-treated cells collected from n = 3 independent experiments. h Immunolabelling of γ2-containing clusters (red) and VGAT-GABAergic terminals (cyan) along MAP2-positive dendrites (20 µm; blue) following 72 h treatment with control (DMSO), or diazepam (D; 1 µM), in the absence or presence of Ro 15-1788 (Ro; 25 µM), and the corresponding graphs showing a decrease in h size (median/line-IQRs; mean/dot ± s.d. whiskers; Mann–Whitney test, \( *p < 0.05 \)), and i number of synaptic γ2 clusters (mean ± s.e.m.; ANOVA/Bonferroni post-hoc test; \( *p < 0.05 \)), and k decrease in the number of GABAergic terminals (mean ± s.e.m.; ANOVA/Bonferroni post-hoc test; \( *p < 0.05 \)) contacting n = 49 control-treated & n = 45, n = 53, n = 48 diazepam-, diazepam/Ro- or Ro-treated dendrites for 72 h, respectively. The total of n = 15, n = 14, n = 13, n = 16 neurons, respectively, collected from two independent experiments, were analysed in each group. Scale bars = 20 µm (a, h-upper row) and = 5 µm (a, h-lower row)

**Diazepam triggers internalisation of GABAAR receptors by activating calcineurin**

In correlation with diazepam-dependent changes in postsynaptic GABAARs clusters observed in primary cerebrocortical neurones, a significant overall reduction in GABAAR surface expression was detected using a GABAAR β2/3-specific antibody in cell-surface ELISA experiments (Fig. 2). However, a decrease in cell surface levels was detected earlier than the observed disassembly of GABAergic synapses, reaching statistical significance within 1 h, and steady level after 24 h, in the continuous presence of diazepam (Fig. 2a, black line; \( *p < 0.05 \)). In parallel ELISAs, in which the total levels of GABAARs were measured, a statistically significant decrease was detected after 72 h of continuous diazepam treatment (Fig. 2a, grey line; \( *p < 0.05 \)). The effects of diazepam were not only time- but also dose-dependent, with the lowest effective concentration of 1 µM (Fig. 2b; \( *p < 0.05 \)). A significant reduction in surface GABAARs was also observed in α1β2γ2mSC-HEK293 cells treated with 1 µM diazepam in the presence of 5 µM isoguvacine (Fig. 2c, \( *p < 0.05 \)). The observed decrease in surface GABAARs in neurones was abolished in the presence of Ro 15-1788 (Fig. 2d; \( *p < 0.05 \)), again confirming that this process was initiated by direct diazepam binding to GABAARs. Moreover, this decrease in surface GABAARs was also abolished by picrotoxin, a GABAAR channel blocker, in neurones and in α1β2γ2mSC-HEK293 cells (Fig. 2e, f, respectively; \( *p < 0.05 \)), and by bicuculline (Fig. 2g; \( *p < 0.05 \)), a competitive antagonist which blocked the binding of GABA to these receptors in neurones. Thus, the observed reduction in surface levels of GABAARs appears to be a consequence of prolonged diazepam-dependent stimulation of their activity evoked by endogenous GABA released from spontaneously active GABAergic neurones in culture (Fig. 1e) or by isoguvacine in α1β2γ2mSC-HEK293 cells.

A time- and dose-dependent decrease in cell surface GABAARs was also observed in the continuous presence of specific agonists of these receptors at higher concentrations, muscimol (50 μM; Supplementary Figure 2; \( *p < 0.05 \)) in primary neurones, or isoguvacine (50 μM; Fig. 2c; \( *p < 0.05 \)) in α1β2γ2mSC-HEK293 cells. Importantly, a statistically significant diazepam (1 µM)-dependent potentiation of muscimol effect at lower doses (1 or 5 µM muscimol; \( *p < 0.05 \)), but not at higher doses (10 or 50 µM muscimol; Supplementary Figure 2a; \( p > 0.05 \)) was observed, suggesting that, at higher agonist concentrations, GABAARs may have reached the maximal level of stimulation in these preparations. That GABAAR activation by muscimol was a necessary trigger for their downregulation from the cell surface was confirmed in neurones in the presence of bicuculline (Supplementary Figure 2c; \( *p < 0.05 \)) or picrotoxin (Supplementary Figure 2d; \( *p < 0.05 \)).

Crucially, the diazepam-dependent decrease in GABAAR surface levels in neurones was abolished in the presence of dynamin-inhibitory peptide (Fig. 2h; \( *p < 0.05 \)), a peptide which is able to penetrate the plasma membrane and inhibit the endocytotic machinery of the cell [28]. This indicates that the underlying cause of reduction at the cell surface was indeed dynamin-dependent endocytosis of GABAARs rather than inhibition in protein synthesis or reduced insertion into the plasma membrane.
Dynamin-inhibitory peptide also blocked a reduction in surface GABA$_A$Rs caused by high doses of muscimol (Supplementary Figure 2e; *p < 0.05). Consistent with this was the loss of cell surface receptors (Fig. 2i, red) and a concomitant intracellular accumulation of endocytosed GABA$_A$Rs (Fig. 2i, green) in the presence of diazepam (1 μM) or muscimol (50 μM; Supplementary Figure 2f), which were monitored using immunolabelling with the β$_{2/3}$ antibody and confocal imaging in neurones. A small amount of internalised β$_{2/3}$ subunits observed in neurones treated with vehicle control was likely due to constitutive internalisation of GABA$_A$Rs [19]. Likewise, a reduction of surface GABA$_A$Rs (Supplementary Figure 3b; red), with a concomitant intracellular accumulation of the endocytosed receptors (Supplementary Figure 3b; green), was also observed in α$_1$β$_{2}γ_{2}^{moc}$-HEK293 cells
GABAARs were measured by cell surface ELISA using isoguvacine (I; 5 μM) with cyclosporine A (CyA; 1 μM) in aβ2γ2 subunit-specific HEK293 cell line (n = 6). Decrease in surface GABAARs in response to low doses of isoguvacine (I; 5 μM) is potentiated by diazepam (D; 1 μM) in aβ2γ2 subunit-specific HEK293 cell line (n = 6). Diazepam (D; 1 μM) dependent decrease in surface GABAARs is inhibited by Ro 15-1788 (Ro; 25 μM; d; n = 7), picrotoxin (Pic; 50 μM; e; n = 5), or bicuculline (Bic; 50 μM; g; n = 4) in cortical neurones, and f picrotoxin (Pic; 50 μM; n = 6) in aβ2γ2 subunit-specific HEK293 cell line. Diazepam (D; 1 μM)-dependent decrease in surface GABAARs is inhibited by dynamin-inhibitory peptide (DynIP; 25 μM; n = 5) in cortical neurones following 2 h treatments. Immunolabelling of internalised (green) and surface (red) GABAARs following 2 h treatments with diazepam (D; 1 μM; n = 2; scale bar = 5 μm). Diazepam (D; 1 μM)-dependent reduction of surface GABAARs is unaffected by inhibition of PP2A or PP1 with low (0.05 μM) or high (1 μM) dose of okadaic acid, respectively (OA; n = 6), but it is prevented by inhibition of calcineurin by cyclosporine A (CyA; 1 μM; n = 6). Diazepam (D; 1 μM)/isoguvacine (I; 5 μM)-dependent reduction in surface GABAARs in aβ2γ2 subunit-specific HEK293 cell line is prevented by inhibition of calcineurin with cyclosporine A (CyA; 1 μM; n = 3). Diazepam treatments (D; 1 μM; 2 h) cause GABAAR γ2 subunit dephosphorylation at Ser327 in cortical neurones and this is prevented by cyclosporine A (CyA; 1 μM; n = 2). Immuno-blotting was done using an anti-PSer327-γ2 or anti-γ2 primary antibody, followed by alkaline phosphate-phatase-conjugated secondary antibody and a colour reaction. Quantification was done using ImageJ. Diazepam (D; 1 μM/isoguvacine (I; 5 μM)-dependent reduction in surface GABAARs in aβ2γ2 subunit-specific HEK293 cells is abolished by S327A mutation in the γ2 subunit. Changes in surface GABAARs were measured by cell surface ELISA using β2γ2 subunit-specific antibody in cortical neurones or myc-antibody in aβ2γ2 subunit-specific HEK293 cells and presented in graphs as mean ± s.e.m., with n = number of independent experiments. Statistical analysis was done using ANOVA with Bonferroni post-hoc test; *p < 0.05.

The relationship between diazepam-dependent dephosphorylation and internalisation of GABAARs was further investigated in experiments in which changes in synaptic elements, the size and number of postsynaptic γ2-GABAAR clusters and the number of colocalised presynaptic GABAergic terminals, were quantitatively assessed following prolonged diazepam treatments (72 h) in the absence or presence of cyclosporine A (Fig. 3a–d). The decrease in size (Fig. 3a, b; *p < 0.05) and number of postsynaptic γ2-GABAAR clusters (Fig. 3a, c; *p < 0.05), and in the number of colocalised presynaptic, VGAT-termminals (Fig. 3a, d; *p < 0.05) detected after 72 h treatment with diazepam, were effectively abolished in the presence of cyclosporine A, further supporting the central role of calcineurin in this process. Moreover, cyclosporine A and diazepam, when applied together, caused a significant increase in the size of GABAAR clusters in comparison with the control, diazepam or cyclosporine A alone (Fig. 3a, b; *p < 0.05).

Furthermore, a functional link between prolonged diazepam-dependent stimulation of GABAARs and disassembly of GABAergic synapses, was assessed in experiments in which structural elements of synapses were analysed in the presence of bicuculline (Fig. 3e–h). Bicuculline significantly attenuated the diazepam-dependent decrease in size (Fig. 3e, f; *p < 0.05) and number of postsynaptic γ2-GABAAR clusters (Fig. 3e, g; *p < 0.05), and in the number of colocalised presynaptic VGAT-termminals (Fig. 3e, h; *p < 0.05). Diazepam and bicuculline added together also caused a significant increase in the size of GABAAR clusters in comparison with control, diazepam or bicuculline treatments alone (Fig. 3e, f; *p < 0.05).

Collectively, these data evince a cascade of signalling events triggered by prolonged stimulation of synaptic GABAARs which, in turn, leads to a calcineurin-mediated dephosphorylation and endocytosis of these receptors, and a consequent disassembly of GABAergic synapses. As GABAARs are GABA-gated chloride/bicarbonate channels which are impermeable to Ca2+, while calcineurin activation requires an increase in cytoplasmic Ca2+, the question arises as to the nature of the signalling molecules that link the activities of these seemingly unrelated pathways.

(Supplementary Figure 3a) treated with the submaximal doses of isoguvacine (5 μM), and this was further potentiated by the addition of diazepam (1 μM).

Dynamin-dependent endocytosis of GABAARs is known to be regulated by the activity of protein kinases and phosphatases which determine the state of phosphorylation of specific residues in the β and γ subunits of these receptors [29], such that dephosphorylation by protein phosphatase 1 or 2A [16], or calcineurin (Ca2+/calmodulin-dependent phosphatase 2B [30]), respectively, promotes their internalisation. To establish which of these phosphatases are involved in diazepam-triggered endocytosis of GABAARs, treatments of cultured neurones were carried out in the presence of either low (0.05 μM) or high (1 μM) doses of okadaic acid, to inhibit PP2A or PP2A/PP1, respectively, or in the presence of cyclosporine A (1 μM), to inhibit calcineurin, and surface GABAARs were monitored using cell surface ELISA. Although inhibition of PP2A alone, or both PP2A and PP1, had no effect (Fig. 2), inhibition of calcineurin significantly attenuated GABAAR endocytosis triggered by diazepam in neurones (1 μM; Fig. 2k; *p < 0.05), or diazepam/isoguvacine (1 μM/5 μM; Fig. 2l, *p < 0.05) in aβ2γ2 subunit-specific HEK293 cells, suggesting that dephosphorylation of the γ2 subunit by calcineurin is a necessary step in this process. This was corroborated in immunoblotting experiments with P-Ser277-γ2-specific antibody, in which diazepam-dependent dephosphorylation of the γ2 subunit in neurones was abolished by cyclosporine A (Fig. 2m; *p < 0.05), and further supported by cell surface ELISA experiments in which mutated S327A γ2 myc subunit [31] was expressed in the aβ2-HEK293 cell line (Fig. 2n; *p < 0.05).
Diazepam-induced loss of inhibitory synapses mediated by PLCδ/...
Diazepam triggers Ca\textsuperscript{2+} release from thapsigargin-sensitive intracellular stores by activating PLC

To investigate the functional link between GABA\textsubscript{A}Rs and calcineurin, we monitored the intracellular Ca\textsuperscript{2+} signal using the Ca\textsuperscript{2+}-indicator Fluo-4 in primary neurones by live cell imaging, which has revealed that application of diazepam (1 \mu M) evokes a prolonged increase in intracellular Ca\textsuperscript{2+} in neuronal dendrites and cell bodies (Fig. 4a, d, e, h; *p < 0.05). Importantly, this increase was completely abolished when diazepam was applied in the presence of Ro 15-1788 (Fig. 4b, d; *p < 0.05) or bicuculline (Fig. 4c, d; *p < 0.05). Furthermore, the rise in intracellular Ca\textsuperscript{2+} was also abolished when the sarcoplasmic/ endoplasmic Ca\textsuperscript{2+} stores were depleted in the presence of thapsigargin, a non-competitive inhibitor of Ca\textsuperscript{2+} ATPase [32], prior to the addition of diazepam (Fig. 4f, h; *p < 0.05). These findings indicate a critical role of the intracellular Ca\textsuperscript{2+} stores in this process and are consistent with a metabotropic-type signalling classically mediated by phospholipase C (PLC) [33]. To characterise this process further, live imaging of Fluo-4 labelled neurones was carried out in the presence of a general PLC inhibitor U73122 (10 \mu M), which effectively abolished the increase in intracellular Ca\textsuperscript{2+} in response to diazepam-dependent activation of GABA\textsubscript{A}Rs (Fig. 4g, h; *p < 0.05). In agreement with this, diazepam-dependent down-regulation of surface GABA\textsubscript{A}Rs in neurones and in \alpha\textsubscript{2}\beta\textsubscript{2}-myc-HEK293 cells was also attenuated by thapsigargin (Fig. 4i, left and right, respectively; *p < 0.05) and U73122 (Fig. 4j; left and right, respectively; *p < 0.05), but not by extracellular chelation of Ca\textsuperscript{2+} in the presence of EGTA (Fig. 4k; left and right, respectively; *p < 0.05).

To monitor the activation of PLC by diazepam/iso- guvacine, an indirect method was employed in which GFP-tagged PH domain of PLC\textsubscript{6} (GFP-PH\textsubscript{PLC\textsubscript{6}} [23]) was expressed in \alpha\textsubscript{2}\beta\textsubscript{2}-HEK293 cell line [18]. In confocal live cell imaging experiments, due to activation of endogenous PLC and depletion of PI\textsubscript{2} in response to diazepam/iso- guvacine, the GFP-PH\textsubscript{PLC\textsubscript{6}}, initially predominantly plasma membrane bound (F\textsubscript{m}), showed partial translocation to the cytoplasm (F\textsubscript{c}) within 5 min (green traces; Fig. 5a), resulting in a significant decrease in F\textsubscript{m}/F\textsubscript{c} ratio in comparison with controls (Fig. 5b; *p < 0.05). That this activation was long-lasting was shown by fluorescent imaging of GFP-PH\textsubscript{PLC\textsubscript{6}} in \alpha\textsubscript{2}\beta\textsubscript{2}-HEK293 cells (green traces; Fig. 5c), which were fixed after 1 h in continuous presence of diazepam/iso- guvacine and immunolabelled with GABA\textsubscript{A}R-\beta\textsubscript{2}-specific antibody at the cell surface (pink traces; Fig. 5c), thus yielding not only a significant decrease in F\textsubscript{m}/F\textsubscript{c} ratio (Fig. 5d; *p < 0.05), but also a decrease in surface GABA\textsubscript{A}Rs.

Collectively, these data indicate that sustained diazepam-dependent activation of GABA\textsubscript{A}Rs in neurones, beyond its ionotropic effects, also has a long-lasting metabotropic effect mediated by a PLC/Ca\textsuperscript{2+}/calcineurin signalling cascade which facilitates receptor internalisation from the cell surface.

Regulation of GABA\textsubscript{A}R interaction with PLC\textsubscript{6} or PRIP1 by diazepam

Diazepam/iso- guvacine-dependent activation of PLC and its requirement in diazepam-dependent mobilisation of intracellular Ca\textsuperscript{2+} suggests that GABA\textsubscript{A}Rs may be in association with some of the 23 known isoforms of PLC [33], via an interaction that might directly impinge on the activity of these enzymes. To test this hypothesis, the PLC\textsubscript{6} isofrom was initially investigated given its structural similarity to PRIP1 (PLC-related but catalytically inactive protein 1 [34]), which has been previously shown to directly associate with GABA\textsubscript{A}Rs [35]. Coimmunoprecipitation experiments from control and diazepam-treated neurones demonstrated that PLC\textsubscript{6} binds to GABA\textsubscript{A}Rs in controls, but disassociates from them when diazepam is applied (Fig. 6a), while
PRIP1 shows the opposite translocation (Fig. 6b). To investigate these interactions further, in vitro binding assays were carried out in which purified GST-fusion proteins incorporating the intracellular TM3-4 loop of different GABAAR subunits were incubated with lysates of PLCδ-GFP expressing HEK293 cells, revealing that PLCδ binds to the β2 and β3 subunits of GABAARs specifically (Fig. 5c). Using truncation mutants of the β3 subunit TM3-4 loop, two distinct binding regions, between 303–333 (Q1) and 366–396 (Q3) residues, were identified (Fig. 5d) and, in subsequent experiments, demonstrated to mediate the direct binding of PLCδ (Fig. 5e). Interestingly, PRIP1-GFP was also found to interact with the same Q1 and Q3 regions of the GABAAR β3 subunit as PLCδ (Fig. 5f), suggesting that, in situ, these two proteins may be in competition for their binding to GABAARs. Based on the sequence similarity in the Q1 and Q3 regions between the β2 and β3 subunits, a potential binding sequence for both PLCδ and PRIP1 was identified (FXXXGXQXXK; Fig. 5g).

Dissociation of PLCδ from GABAARs upon their activation by diazepam/isoguvacine and the concurrent increase in PRIP1 binding were confirmed by coimmunoprecipitation.
from lysates of α1β2γ2-HEK293 cells transfected with GFP-PLCδ and GFP-PRIP1 cDNA (Fig. 5h), further supporting the observation that the binding of these proteins is regulated by the level of GABAAR activation, but in a mutually exclusive manner.

Altogether, the data suggest that a switch in association between GABAARs and catalytically-active PLCδ versus catalytically inactive PRIP1, may be a critical regulatory step in the signalling pathway that leads to diazepam-dependent internalisation of GABAARs. To test this hypothesis, the intracellular localisation of GFP-PHPLCδ and dsRed-PRIP1 was monitored simultaneously by live cell imaging in transfected α1β2γ2-HEK293 cells before and 5 min after the bath application of diazepam/isoguvacine (Fig. 6i). GFP-PHPLCδ showed no apparent translocation from the membrane to the cytoplasm (green traces, Fig. 6i) and no change in F_m/F_c ratio (Fig. 6j, left) when dsRed-PRIP1 was also expressed, suggesting that activation of endogenous PLCδ in response to diazepam/isoguvacine was blocked. In contrast, dsRed-PRIP1 showed further accumulation in the plasma membrane (red traces, Fig. 6i) resulting in an increase in F_m/F_c ratio (Fig. 6j, right). Moreover, a decrease in surface GABAARs in response to diazepam/isoguvacine in α1β2γ2-myc-HEK293 cells detected by cell surface ELISA, was completely abolished by over-expression of PRIP1 (Fig. 5k), suggesting that PRIP1 may serve as an inhibitor of this signalling pathway, thereby preventing the process of GABAARs internalisation and alleviating the consequent loss of inhibitory GABAergic synapses (Fig. 5l).

**Discussion**

The prevalence of stress-related psychiatric disorders, particularly anxiety mixed with depression, panic attacks or insomnia, leads to an estimated 12 million prescriptions of benzodiazepines every year in the UK (UK Addiction Treatment Centres). However, our current understanding of the long-term effects of benzodiazepines on cellular and molecular processes in the brain remains limited.

In this study we have revealed that prolonged exposure of neurons to diazepam activates a novel Ca^{2+} signalling cascade downstream of GABAARs, which in a negative feedback fashion, leads to a gradual removal of these
receptors from the postsynaptic membrane and disassembly of inhibitory synapses, thus rendering the system unresponsive to any further diazepam treatments. Although studied in vitro, these processes are closely correlated in time to in vivo downregulation of GABA_ARs and the onset of tolerance to benzodiazepines in rodents [36]. These processes are however in sharp contrast with the initial diazepam-dependent facilitation of GABA_ARs channel gating activity [25], increased mobilisation of GABA_ARs to synapses [37, 38], and enhanced inhibitory synaptic transmission [39], possibly representing a form of neuronal adaptation in order to maintain a critical balance between the excitation and inhibition in the brain.

Our experiments demonstrate that sustained activation of GABA_ARs by diazepam, in the presence of ambient GABA (Fig. 1e [39]), is a key trigger of this signalling cascade which involves PLC_δ activation, mobilisation of intracellular Ca^{2+} and activation of calcineurin. A key role of calcineurin in diazepam-dependent endocytosis of GABA_ARs is in agreement with the previously reported effects on GABA_AR migration out of the synaptic contacts [31, 40, 41], and internalisation from the cell surface [42].
Another important step in this cascade is diazepam-dependent rise in intracellular Ca\textsuperscript{2+} originating from the thapsigargin-sensitive intracellular stores and mediated by the activation of PLC\textgreek{d}, which is consistent with a metabotropic-type signalling by GABA\textsubscript{AR}s in mature neurons operating in addition to their ‘canonical’ ionotropic signalling as chloride/bicarbonate channels. In contrast to immature neurons, where GABA\textsubscript{AR} activation generally leads to depolarisation and influx of Ca\textsuperscript{2+} through voltage-gated Ca\textsuperscript{2+} channels [50, 51], this signalling pathway is insensitive to the extracellular chelation of Ca\textsuperscript{2+} with EGTA. Furthermore, the requirement for PLC activity in this process is in agreement with the biochemical evidence for a direct association between GABA\textsubscript{AR} \( \beta \) subunits and PLC\textgreek{d}, which was also detected in recent proteomic analyses of GABA\textsubscript{AR} binding proteins [52, 53]. Although PLC\textgreek{d} may not be the only isoform of PLC able to interact with GABA\textsubscript{AR}s, its extensive structural similarity with PRIP1, a well characterised GABA\textsubscript{AR} receptor interacting protein [54], is of significance. Interestingly, our experiments demonstrate that PLC\textgreek{d} disassociates from GABA\textsubscript{AR}s with diazepam treatments, suggesting that this displacement may be required for its activation, particularly if the PLC\textgreek{d} binding to GABA\textsubscript{AR}s resembles its binding to calmodulin via an auto-inhibitory region which renders it inactive [55]. Exactly how dissociation of PLC\textgreek{d} from GABA\textsubscript{AR}s occurs remains to be elucidated, although we hypothesise that this could be caused by a conformational change in the receptor upon activation, or by accumulation of negatively charged chloride and depletion of bicarbonate, possibly changing the pH or osmolality [56] in the vicinity of the receptor. At the same time, however, these conditions facilitate the binding of PRIP1 to GABA\textsubscript{AR}s, which, together with identified common binding sites in the \( \beta \) subunits, suggests that the two proteins may be in competition with each other. The interplay between PLC\textgreek{d} and PRIP1 in binding to GABA\textsubscript{AR}s may represent an important on/off switching mechanism regulating this signalling pathway and downstream endocytosis of GABA\textsubscript{AR}s. This is because PRIP proteins are not only catalytically inactive variants of PLC\textgreek{d} unable to generate IP\textsubscript{3} and DAG [34], but they also inhibit PLC signalling due to high affinity binding of IP\textsubscript{3}, thereby sequestering it away from the IP\textsubscript{3} receptor on the intracellular Ca\textsuperscript{2+} stores [57]. That overexpression of PRIP1 inhibited diazepam-dependent activation of PLC\textgreek{d} and diazepam-dependent down-regulation of surface GABA\textsubscript{AR}s in heterologous systems, suggests that this protein can act as an inhibitor by outcompeting the PLC\textgreek{d} binding to GABA\textsubscript{AR}s. In neurons, PRIP1 is likely to be a temporary block of this pathway, due to its removal together with GABA\textsubscript{AR}s undergoing endocytosis [58]. This is in agreement with
the effects of PRIP gene deletion in mice, which show increased intracellular Ca\(^{2+}\) and calcineurin activity [57], decreased levels of synaptic \(\gamma_2\)-containing GABA\(_A\)Rs receptors, reduced sensitivity to diazepam and increased anxiety-like behaviour [59]. We therefore hypothesise that, when the ‘off’ switch (PRIP) is no longer present, PLC\(\delta\) recruitment to GABA\(_A\)Rs allows continuous activation of this signalling pathway and depletion of synaptic GABA\(_A\) receptors, leading to their structural and functional deficits in inhibitory synapses. This diazepam-induced breakdown of inhibitory GABAergic synapses not only correlates well in time with the development of tolerance but also provides a likely explanation for the severe withdrawal symptoms, increased anxiety and even seizures, observed in animal models and patients following sudden termination of chronic benzodiazepine treatment [60], possibly due to uncontrolled excitatory drive in the absence of functional inhibition.

This signalling mechanism offers a new spectrum of possible molecular interventions that could be tailored towards extending the initial highly beneficial clinical outcomes of benzodiazepines, while preventing the subsequent disruption of GABAergic synapses and development of pharmacological and behavioural tolerance to these widely prescribed drugs.

Acknowledgements This work was supported by the Biotechnology and Biological Sciences Research Council UK New Investigator Grant BB/C507237/1 (to J.N.J), Medical Research Council UK project grant (G0800498), Wellcome Trust and UCL School of Pharmacy funds. We thank Professor Jean-Marc Fritschy (University of Zurich) and Professor Anne Stephenson for providing us with the GABA\(_A\) \(\gamma_2\) subunit- and \(\alpha_1\)-specific antibodies, respectively, Professor Masato Hirata and Dr Akiko Mizokami (Kyushu University) for GFP-PRIP1 and dsRed-PRIP1 cDNAs, and PRIP-specific antibodies, Dr Steve Marsh (UCL) for GFP-PRIP1 cDNA, Dr Lorena Arancibia-Carcamo and Professor Josef Kittler (UCL) for mutant S327A \(\gamma_2\) subunit cDNA, and Gianandrea Broglia and Ljubi Sihra-Jovanovic for technical assistance. We thank Professor Talvinder Sihra (UCL Department of Neuroscience, Physiology and Pharmacology) for critical reading of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

References

1. Rudolph U, Knollach F. Beyond classical benzodiazepines: novel therapeutic potential of GABAA receptor subtypes. Nat Rev Drug Discov. 2011;10:685–97.
2. Spiers N, Qassem T, Bebington P, McManus S, King M, Jenkins R, et al. Prevalence and treatment of common mental disorders in the English national population, 1993–2007. Br J Psychiatry. 2016;209:150–6.
3. Lader M. Benzodiazepine harm: how can it be reduced? Br J Clin Pharmacol. 2014;77:295–301.
4. Lugoboni F, Quaglio G. Exploring the dark side of the moon: the treatment of benzodiazepine tolerance. Br J Clin Pharmacol. 2014;77:239–41.
5. Downing SS, Lee YT, Farb DH, Gibbs TT. Benzodiazepine modulation of partial agonist efficacy and spontaneously active GABA(A) receptors supports an allosteric model of modulation. Br J Pharmacol. 2005;145:894–906.
6. Gielen MC, Lumb MJ, Smart TG. Benzodiazepines modulate GABA\(_A\) receptors by regulating the preactivation step after GABA binding. J Neurosci. 2012;32:5707–15.
7. Mohler H. The legacy of the benzodiazepine receptor: from flumazenil to enhancing cognition in Down syndrome and social interaction in autism. Adv Pharmacol. 2015;72:1–36.
8. Sieghart W. Structure, pharmacology, and function of GABA\(_A\) receptor subtypes. Adv Pharmacol. 2006;54:231–63.
9. Luscher B, Fuchs T, Kilpatrick CL. GABA receptor trafficking-mediated plasticity of inhibitory synapses. Neuron. 2011; 70:385–409.
10. Bateson AN. Basic pharmacologic mechanisms involved in benzodiazepine tolerance and withdrawal. Curr Pharm Des. 2002;8:5–21.
11. Vinkers CH, Olivier B. Mechanisms underlying tolerance after long-term benzodiazepine use: a future for subtype-selective GABA(A) receptor modulators? Adv Pharmacol Sci. 2012;2012:416864.
12. Roca DJ, Rozenberg I, Farrant M, Farb DH. Chronic agonist exposure induces down-regulation and allosteric uncoupling of the gamma-aminobutyric acid/benzodiazepine receptor complex. Mol Pharmacol. 1990;37:37–43.
13. Graville MC. Activation-induced regulation of GABAA receptors: is there a link with the molecular basis of benzodiazepine tolerance? Pharmacol Res. 2016;109:92–100.
14. van Rijnsoever C, Tauber M, Chouli MK, Keist R, Rudolph U, Mohler H, et al. Requirement of alpha5-GABAA receptors for the development of tolerance to the sedative action of diazepam in mice. J Neurosci. 2004;24:6785–90.
15. Uusi-Oukari M, Korpi ER. Regulation of GABA(A) receptor subunit expression by pharmacological agents. Pharmacol Rev. 2010;62:97–135.
16. Jovanovic JN, Thomas P, Kittler JT, Smart TG, Moss SJ. Brain-derived neurotrophic factor modulates fast synaptic inhibition by regulating GABA(A) receptor phosphorylation, activity, and cell-surface stability. J Neurosci. 2004;24:522–30.
17. Brown LE, Nicholson MW, Arama JE, Mercer A, Thomson AM, Jovanovic JN, et al. gamma-Aminobutyric Acid Type A (GABAA) receptor subunits play a direct structural role in synaptic contact formation via their N-terminal extracellular domains. J Biol Chem. 2016;291:13926–42.
18. Fuchs C, Abibol K, Burden JJ, Mercer A, Brown L, Iball J, et al. GABA(A) receptors can initiate the formation of functional inhibitory GABAergic synapses. Eur J Neurosci. 2013;38:3146–58.
19. Oakley D, Ali AB, Rampersaud N, Harkavyi A, Fuchs C, Whitton PS, et al. Dopamine-dependent tuning of striatal inhibitory synaptogenesis. J Neurosci. 2010;30:2935–50.
20. Jordan M, Wurm F. Transfection of adherent and suspended cells by calcium phosphate. Methods. 2004;33:136–43.

21. Pollard S, Duggan MJ, Stephenson FA. Further evidence for the existence of alpha subunit heterogeneity within discrete gamma-aminobutyric acidA receptor subpopulations. J Biol Chem. 1993;268:3753–7.

22. McKenzie M, Duchen MR. Impaired cellular bioenergetics causes mitochondrial calcium handling defects in MT-ND5 mutant cybrids. PLoS ONE. 2016;11:e0154371.

23. Stauffer TP, Ahn S, Meyer T. Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P2 concentration monitored in living cells. Curr Biol. 1998;8:343–6.

24. Kanematsu T, Yasunaga A, Mizoguchi Y, Kuratani A, Kittler JT, Jovanovic JN, et al. Modulation of GABA(A) receptor phosphorylation and membrane trafficking by phospholipase C-related inactive protein/protein phosphatase 1 and 2A signaling complex underlying brain-derived neurotrophic factor-dependent regulation of GABAAergic inhibition. J Biol Chem. 2006;281:22180–9.

25. Rogers CJ, Twyman RE, Macdonald RL. Benzodiazepine and beta-carbol ine regulation of single GABA(A) receptor channels of mouse spinal neurones in culture. J Physiol. 1994;475:69–82.

26. Hanson SM, Czajkowski C. Structural mechanisms underlying benzodiazepine modulation of the GABA(A) receptor. J Neurosci. 2008;28:3490–9.

27. Friedman H, Greenblatt DJ, Peters GR, Metzler CM, Charlton MD, Harmatz JS, et al. Pharmacokinetics and pharmacodynamics of oral diazepam: effect of dose, plasma concentration, and time. Clin Pharmacol Ther. 1992;52:139–50.

28. Kittler JT, Delmas P, Jovanovic JN, Brown DA, Smart TG, Moss SJ, et al. Constitutive endocytosis of GABA receptors by an association with the adaptin AP2 complex modulates inhibitory synaptic currents in hippocampal neurones. J Neurosci. 2000;20:7972–7.

29. Nakamura Y, Darnieder LM, Deeb TZ, Moss SJ. Regulation of GABA(A)Rs by phosphorylation. Adv Pharmacol. 2015;72:97–146.

30. Wang J, Liu S, Haditsch U, Tu W, Cochrane K, Ahmadian G, et al. Interaction of calcineurin and type-A GABA receptor gamma 2 subunits produces long-term depression at CA1 inhibitory synapses. J Neurosci. 2003;23:826–36.

31. Muir J, Arancibia-Carcamo IL, MacAskill AF, Smith KR, Griffin LD, Kittler JT, et al. NMDA receptors regulate GABA(A) receptor lateral mobility and clustering at inhibitory synapses through serine 327 on the gamma2 subunit. Proc Natl Acad Sci USA. 2010;107:16679–84.

32. Treiman M, Caspersen C, Christensen SB. A tool coming of age: a novel pHluorin-tagged gamma-aminobutyric acid receptor, Type A (GABAA), alpha2 subunit knock-in mouse. J Biol Chem. 2011;286:21667–77.

33. Terunuma M, Jang IS, Ha SH, Kittler JT, Kanematsu T, Jovanovic JN, et al. GABA(A) receptor phospho-dependent modulation is regulated by phospholipase C-related inactive protein type 1, a novel protein phosphatase 1 anchoring protein. J Neurosci: Off J Soc Neurosci. 2004;24:7074–84.

34. Miller LG, Greenblatt DJ, Bamhill JG, Shader RI. Chronic benzodiazepine administration. I. Tolerance is associated with benzodiazepine receptor downregulation and decreased gamma-aminobutyric acid A receptor function. J Pharmacol Exp Ther. 1988;246:170–6.
54. Kanematsu T, Takeuchi H, Terunuma M, Hirata M. PRIP, a novel Ins(1,4,5)P3 binding protein, functional significance in Ca2+ signaling and extension to neuroscience and beyond. Mol Cells. 2005;20:305–14.

55. Sidhu RS, Clough RR, Bhullar RP. Regulation of phospholipase C delta1 through direct interactions with the small GTPase Ral and calmodulin. J Biol Chem. 2005;280:21933–41.

56. Chavas J, Forero ME, Collin T, Llano I, Marty A. Osmotic tension as a possible link between GABA(A) receptor activation and intracellular calcium elevation. Neuron. 2004;44:701–13.

57. Toyoda H, Saito M, Sato H, Tanaka T, Ogawa T, Yatani H, et al. Enhanced desensitization followed by unusual resensitization in GABAA receptors in phospholipase C-related catalytically inactive protein-1/2 double-knockout mice. Pflug Arch. 2015;467:267–84.

58. Kanematsu T, Fujii M, Mizokami A, Kittler JT, Nabekura J, Moss SJ, et al. Phospholipase C-related inactive protein is implicated in the constitutive internalization of GABAA receptors mediated by clathrin and AP2 adaptor complex. J Neurochem. 2007;101:898–905.

59. Kanematsu T, Jang IS, Yamaguchi T, Nagahama H, Yoshimura K, Hidaka K, et al. Role of the PLC-related, catalytically inactive protein p130 in GABAA receptor function. EMBO J. 2002;21:1004–11.

60. Schoch P, Moreau JL, Martin JR, Haefely WE. Aspects of benzodiazepine receptor structure and function with relevance to drug tolerance and dependence. Biochem Soc Symp. 1993;59:121–34.