AMPAR and kainate receptors mediate mutually exclusive effects on GABA_A receptor expression in cultured mouse cerebellar granule neurones

Helen L. Payne,* Jane H. Ives,* Werner Sieghart† and Christopher L. Thompson*

*Centre for Integrative Neurosciences, School of Biological and Biomedical Sciences, University of Durham, Durham, UK
†Centre for Brain Research, Medical University Vienna and Section for Biochemical Psychiatry, University Clinic for Psychiatry, Vienna, Austria

Summary
Studies on animal models of epilepsy and cerebellar ataxia, e.g., stargazer mice (stg) have identified changes in the GABAergic properties of neurones associated with the affected brain loci. Whether these changes contribute to or constitute homeostatic adaptations to a state of altered neuronal excitability is as yet unknown. Using cerebellar granule neurones from control (+/+) and stg (AMPA-incompetent, KAR-competent) and stg (AMPA-incompetent, KAR-competent), we investigated whether non-NMDA receptor (NMDAR) activity regulates GABA_A receptor (GABAR) expression. Neurones were maintained in 5 mmol/L KCl-containing basal media or depolarizing media containing either 25 mmol/L KCl or the non-NMDAR agonist kainic acid (KA) (100 μmol/L). KCl- and KA-mediated depolarization down-regulated GABAR α1, α6 and β2, but up-regulated α4, β3 and δ subunits in +/+ neurones. The KCl-evoked but not KA-evoked effects were reciprocated in stg neurones compatible with AMPAR-regulation of GABAR expression. Conversely, GABA_A γ2 expression was insensitive to KCl-mediated depolarization, but was down-regulated by KA-treatment in a 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX)-reversible manner in +/+ and stg neurones compatible with a KAR-mediated response. KA-mediated up-regulation of GABAR α4, β3 and δ was inhibited by L-type voltage-gated calcium channel (L-VGCC) blockers and the Ca²⁺/calmodulin-dependent protein kinase inhibitor, 4-[(2S)-2-[(5-isouquinolinylsulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl] phenyl isoquinoline sulfonic acid ester (KN-62). Up-regulation of GABAR α4 and β3 was also prevented by calcineurin inhibitors, FK506 and cyclosporin A. Down-regulation of GABAR α1, α6 and β2 was independent of L-VGCC activity, but was prevented by inhibitors of CaN. Thus, we provide evidence that a KAR-mediated and at least three mutually exclusive AMPAR-mediated signalling mechanisms regulate neuronal GABAR expression.

Keywords: cerebellar granule neurones, depolarisation, GABA_A receptors, non-NMDA receptors, stargazer.


GABA_A receptor (GABAR) subunit genes encoding the homologous α1–6, β1–3, γ1–3, δ, ε, π and θ subunits (Barnard et al. 1998) are differentially transcribed in a cell-specific and developmentally regulated manner (Laurie et al. 1998).

Received April 16, 2007; revised manuscript received July 19, 2007; accepted August 29, 2007.

Address correspondence and reprint requests to Dr Helen L. Payne Centre for Integrative Neurosciences, School of Biological and Biomedical Sciences, Durham University, South Road, Durham, DH1 3LE, UK. E-mail: H.L.Payne@durham.ac.uk
The authors would like to dedicate this paper to the memory of Dr Christopher Thompson, an accomplished neuroscientist, an inspirational colleague and a valued friend.

Abbreviations used: 25K, 25 mmol/L KCl; 5K, 5 mmol/L KCl; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; AMPAR, AMPA receptor; BDNF, brain-derived neurotrophic factor; BZ-SR, benzodiazepine agonist-sensitive Ro 15-4513 receptor; BZ-ISR, benzodiazepine agonist-insensitive Ro 15-4513 receptor; CaM Kinase, Ca²⁺/calmodulin-dependent protein kinase; CaN, Calcineurin; CGNs, cerebellar granule cells; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DIV, days in vitro; GABAR, GABA_A receptor; Glu, glutamate receptor; KA, kainic acid; KAR, kainate receptor; KN-62, 4-[(2S)-2-[(5-isouquinolinylsulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl] phenyl isoquinoline sulfonic acid ester; L-VGCC, L-type voltage-gated calcium channel; MK-801, (5R, 10S)-(−)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; NMDAR, NMDA receptor; PBS, Phosphate-buffered solution; TARP, transmembrane AMPAR regulatory protein.
1992; Fritschy et al. 1994; Sato et al. 2005). Immature rodent cerebellar granule neurones (CGNs) for instance, express a predominance of GABARs that are pharmacologically defined as type II benzodiazepine agonist-sensitive Ro15-4513 binding receptors (BZ-SRs) (Squires et al. 1990), which are compatible with the reported repertoire of GABAR α2, α3, β2, β3 and γ2 subunits that are expressed by these neurones. From the second post-natal week onwards, CGNs establish synaptic contacts with glutamatergic mossy fibres and GABAergic golgi interneurones, coincidently the GABAR characteristics of CGNs change. Type I BZ-SRs and a benzodiazepine agonist-insensitive subtype of Ro15-4513 binding receptors (BZ-ISRs) become prominent, compatible with expression of a GABAR subunit profile comprising α1, α6, β2, β3, γ2 and δ (Laurie et al. 1992; Zheng et al. 1993; Tia et al. 1996; Wisden et al. 1996). When cultured in 5 mmol/L KCl (5K)-containing basal media, mouse CGNs were polarized, electrically active (Mellor et al. 1998) and developed a GABAR profile consistent with that of mature CGNs in vivo (Ives et al., 2002). KCl-depolarized mouse CGNs were electrically silent (Mellor et al. 1998) and developed a GABAR profile, compatible with juvenile undifferentiated CGNs (Mellor et al. 1998; Ives et al., 2002; Engblom et al. 2003). Thus, mouse CGNs are a good model system for investigating the molecular switches involved in coordinating and regulating these developmental events. It is becoming increasingly evident that the excitatory demand on a neurone influences the expression of its GABAergic properties as exemplified in many animal seizure models (Clark 1998; Leroy et al. 2004). This would seem compatible with previous data (Thompson et al. 1998; Chen et al. 1999) which indicated that the GABAergic characteristics of adult CGNs of the epileptic and ataxic mouse strains, stargazer (stg) and waggler (wg) were similar to those expected of juvenile mice. Stg and wg mice fail to express the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptor (AMPA) trafficking and targeting protein, stargazin (TARPγ2) (Chen et al. 2000). As CGNs express only TARPγ2 of the transmembrane AMPAR regulatory protein isoforms, this leads to their complete inability to surface traffic and synthetically target AMPARs and thus are devoid of glutamatergic excitatory input (Hashimoto et al. 1999). We have recently found that CGNs in stg cerebellum in vivo do develop a mature complement of GABARs, however, the abundance of synaptic and extrasynaptic α6-containing GABAR subtypes was selectively affected (Payne et al. 2007). These observations imply that activation of glutamate receptors (GluR) expressed by CGNs is integral to the establishment of adult GABAergic profiles of these neurones, but does not comprise the molecular switch responsible for transition from immature to mature GABAR complements. However, it has been reported that chronically exposing CGNs in vitro to kainic acid (KA), a non-selective non-NMDAR receptor (non-NMDAR) agonist resulted in GABAR characteristics that were also evoked by KCl-mediated depolarization and are more compatible with immature CGNs (Engblom et al. 2003). Due to the paucity of selective AMPAR and kainate receptor (KAR)-specific agonists and antagonists, it was impossible to elucidate whether these effects were KAR- and/or AMPAR-mediated or mediated by any other molecular signalling processes. To circumvent this problem and to further explore the role played by non-NMDARs in sculpting the GABAergic properties of CGNs, we have compared expression of GABARs in cultured CGNs derived from control mice (+/+), which express AMPARs and KARs, with those isolated from stg which express only KARs (Chen et al. 2003).

Experimental procedures

Materials

Hyperfilm enhanced chemiluminescence and horseradish-peroxidase-linked anti-rabbit secondary antibodies were purchased from Amersham Pharmacia (Aylesbury, Bucks, UK). Horseradish-peroxidase-linked anti-goat secondary antibody was obtained from Pierce (Chester, Cheshire, UK). [3H]muscimol and [3H]Ro15-4513 were purchased from Perkin-Elmer Lifesciences (Boston, MA, USA), Flunitrazepam, Ro15-1788 and Ro15-4513 were a gift from Hoffmann-LaRoche (Basle, Switzerland). Kainic acid (KA), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), FK506, 4-[(2S)-2-[5-isooquinolynsulfonyl]methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)phenyl isooquinoline sulfonic acid ester (KN-62) and 5,10S-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801) were obtained from Tocris (Bristol, UK).

Affinity-purified anti-GABAR α6 (1–15cys) and anti-α1 (1–15cys) subunit-specific antibodies and affinity-purified anti-TARPγ2 antibody were prepared and characterized as previously described (Ives et al., 2002; Ives et al. 2004). Anti-GABAR α4 (1–14), β2L(351–405), β3L(345–408), γ2(319–366) and δ(4–14) subunit-specific antibodies were as previously described (Jechlinger et al. 1999; Pöltl et al. 2003). Anti-GluR2 antibody was obtained from Santa Cruz Inc. (Calne, Wiltshire, UK). Anti-β-actin antibody was obtained from Sigma Chemical Company (Poole, Dorset, UK). Anti-α-tubulin antibody was obtained from Abcam (Cambridge, UK). Reagents for tissue culture were as before (Thompson and Stephenson 1994). All molecular biological consumables were obtained from Promega (Southampton, UK). PCR primers were purchased from Invitrogen Life Technologies (Paisley, UK). All other materials were obtained from commercial sources.

Animals

Wild-type strain (C3B6Fe−/−), heterozygous (C3B6Fe−/+stg) and homozygous stargazer mutant mice (C3B6Fe−/−stg) were maintained on a 12-h light/dark cycle with food and water available ad libitum. All animal procedures were conducted according to the Scientific Procedures Act 1986. No differences between +/+ and +/stg mice have been noted (Qiao et al. 1998; Hashimoto et al. 1999), therefore, we routinely combine +/+ and +/stg material for control experiments. This combined material is hereafter referred to as ‘control (+/+).’
Genotyping

In order to identify +/+ , +/stg and stg mice in a litter from P3 to P5, prior to the onset of discernible phenotypic features (from P14 to P18), we employed a genomic DNA screening system. Genomic DNA was extracted from tail tip biopsies (2–3 mm). Each biopsy was suspended in 300 μL of Nuclei Lysis solution (Promega) supplemented with 0.1 mol/L EDTA (pH 8.0) at 4°C. Proteinase K (10.5 μL, 20 mg/mL) was added and the digestion was performed at 55°C, overnight. Protein precipitation solution (Promega, 120 μL) was added and incubated for 5 min at 4°C. The supernatant isolated following centrifugation at 11 400 g for 5 min was mixed with 360 μL of isopropanol. Precipitated DNA was pelleted by centrifugation (11 400 g, 10 min) and washed with 100 μL, 70% ethanol. The dried pellet was finally resuspended in 40 μL of 0.1 mol/L H2O.

Primary cerebellar granule neurone cultures

Cerebellar granule neurone cultures (CGNs) were prepared from 6- to 7-day-old (P6/P7) mouse neonates as previously described (Ives et al., 2002). CGNs were cultured in minimal essential medium containing 10% (v/v) foetal calf serum, glutamine (2 mmol/L), gentamycin (50 μg/mL) and supplemented at 24 h, when appropriate with either 20 mmol/L KCl (25 mmol/L KCl (25K)-media) or KA (100 μmol/L). Signal transduction manipulating drugs were applied 30 min prior to KA. Fluoro-deoxyuridine (80 μmol/L) was added at 48 h to suppress the proliferation of non-neuronal cells. Cells were assayed intact at 7 days in vitro (DIV) for reversible [3H] muscimol (40 mmol/L) and [3H] Ro15-4513 (40 mmol/L) binding activity. Non-specific [3H] muscimol binding was defined as residual radioactivity following co-incubation with GABA (1 mmol/L). Benzodiazepine-insensitive-[3H]Ro15-4513 binding was determined by co-incubation with flunitrazepam (10 μmol/L), non-specific [3H]Ro15-4513 binding was defined as residual radioactivity following co-incubation with Ro15-1788 (10 μmol/L). Alternatively, cultured CGNs (7 DIV) were harvested in 0.5 mL/35 mm dish of solubilizing buffer (50 mmol/L Tris, pH 6.8, 2% w/v sodium dodecyl sulfate, 2 mmol/L EDTA) so that the expression levels of GABAR subunit proteins could be determined by immunoblotting.

Cell Surface biotinylation

Cerebellar granule neurone cultures (7 DIV) were washed with ice-cold phosphate-buffered saline (PBS) + 4% sucrose, pH 8.0 and incubated for 15 min at 4°C with 1 mg/mL EZ-link Sulfo-NHS-SS-biotin (Pierce) in cold PBS + 4% sucrose. Following washing with ice-cold PBS + 4% sucrose, residual cross-linker was quenched by incubation for 10 min at 4°C with quenching buffer (50 mmol/L Tris, 0.9% NaCl, 192 mmol/L glycine, 4% sucrose). After a further wash in cold PBS + 4% sucrose, CGNs were solubilized in 166 μL lysis buffer (50 mmol/L Tris, 0.9% NaCl, 2 mmol/L EDTA, 0.1 mol/L phenylmethan sulphonyl fluoride, pH 8.0) containing 1% sodium dodecyl sulfate. Samples were made up to 1 mL total volume with 833 μL lysis buffer and 1% Triton X-100 and incubated with 100 μL of 50% slurry of streptavidin beads for 2 h at 4°C with agitation. Samples were centrifuged at 10 000 g for 1 min and the supernatant retained as unbound fraction or intracellular component. Streptavidin beads were washed with lysis buffer + 1% Triton X-100 and centrifuged for 1 min at 10 000 g, this wash procedure was repeated several times. Bound proteins were eluted by application of 50 μL of 2× sample buffer heated to 95°C for 5 min. Following centrifugation at 10 000 g for 1 min, the supernatant was retained as ‘bound fraction’ or ‘surface protein’. Samples were subjected to western blot analysis to allow for the quantification of surface versus intracellular protein.

Radioligand binding

[3H]muscimol (40 mmol/L) and [3H]Ro15-4513 (40 mmol/L) binding was conducted on intact CGNs (7 DIV) adhering to 35-mm culture dishes, cultured under 5K and KA-treated (100 μmol/L) conditions as previously described (Thompson and Stephenson 1994). Non-specific [3H]muscimol binding was determined in the presence of GABA (100 μmol/L). Non-specific [3H]Ro15-4513 binding was determined in the presence of Ro15-1788 (10 mmol/L). [3H]Ro15-4513 binding in the presence of flunitrazepam (10 μmol/L) allowed an estimation of the proportion of total-specific [3H]Ro15-4513 binding sites that were associated with either benzodiazepine agonist-sensitive GABARs (BZ-SRs) or benzodiazepine full agonist-insensitive GABARs (BZ-ISRs). Binding data were analysed using GraphPad Prism 3.0.

Immunoblotting

The procedure employed for sodium dodecyl sulfate–polyacrylamide gel electrophoresis was essentially as described (Thompson and Stephenson 1994). Polyacrylammide gel electrophoresis was performed in 10% polyacrylammide slab mini-gels. Affinity-purified subunit-specific antibodies were used at final concentrations of 1–5 μg/mL. Anti-actin and anti-tubulin antibodies were used at 1 : 2000 dilution. Immunoreactive species were detected using the enhanced chemiluminescence western blotting system with signals captured on film. Previous studies have shown that this method can be utilized to measure small changes in protein from concentrations over approximately one order of magnitude (Pollenz 1996; Heynen et al. 2000; Ives et al., 2002). In order to quantify the immunoreactive signals in our system, a dilution series of protein samples were loaded on each gel. The resulting signals were measured from films of varying exposure times using the NIH Image J software (NIH, Bethesda, MD, USA) to identify conditions, where the amount of protein applied and the image intensity of the immunoreactive signal was within a linear range. Signals lying within this range were used to compare expression between control and test samples.

All immunoreactivities were normalized to β-actin and/or α-tubulin expression and this allowed us to compare CGNs from different cultures and to compensate for minor errors incurred in processing the materials for immunoblotting and sample application. Where possible, blots were screened simultaneously for the protein of interest, and β-actin and/or α-tubulin.

Protein determination

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard protein (Lowry et al. 1951).
Statistical analyses
Data were analysed statistically by Student’s *t*-test with \( p < 0.05 \) considered statistically significant.

Results

Migration and aggregation of CGNs in vitro is influenced by AMPAR activity: bio-assay of functional AMPARs

Age-matched, littermate +/+, +/stg and stg mice were identified at P3–P5, prior to the onset of phenotypic markers, using a genomic-DNA-screening strategy. The cerebella from +/+ and +/stg neonates were pooled and used to generate control-cultured CGNs and referred as ‘+/+’ CGNs. Stg mice provided the test CGNs. Qualitative visual assessments revealed that CGNs derived from +/+ and stg cultured in basal ‘polarizing’ media (5K) were healthy and migrated on the substratum to form aggregates and extended cables of conjoint processes that contacted other aggregates. Interestingly, CGNs derived from both +/+ and stg cultured in depolarizing media (25K) failed to migrate and aggregate though they clearly developed and extended neurites. CGNs derived from +/+ mice cultured at 5K supplemented with the non-specific non-NMDAR agonist, KA (100 \( \mu \text{mol/L} \)) at 24 h post-plating, resembled CGNs cultured at 25K. In contrast, stg CGNs cultured in the presence of KA behaved similarly to +/+ CGNs cultured at 5K in the absence of KA i.e., they migrated to form cellular aggregates, implying that AMPAR-mediated depolarization inhibited CGN migration in a similar way to KCl-depolarization. Stg CGN’s inability to express the AMPAR-trafficking protein, TARP \( c_2 \) (Fig. 1) clearly impaired their ability to respond to KA in terms of their morphological characteristics and cellular dynamics, thus providing indirect evidence that under the culture conditions employed here AMPARs are functional in +/+ but not in stg CGNs, as expected (data not shown).

Developmentally regulated GABAR subunit and TARP\( \gamma_2 \)-AMPAR expression by CGNs in vivo is reciprocated in mouse CGNs in vitro

The aim of this investigation was to use cultured mouse CGNs to determine whether non-NMDAR activity influences GABAR expression. Hence it was important to evaluate, when control neurones expressed both a ‘mature’ GABAR profile and became ‘AMPAR-competent’ where they expressed AMPAR subunits and the cell surface trafficking protein, TARP\( \gamma_2 \).

Control CGNs (+/+ ) cultured at 5K were collected at 1, 3, 5, 7 and 9 DIV and screened by immunoblotting using GABAR subunit-specific antibodies, AMPAR subunit-specific antibodies and TARP\( \gamma_2 \)-directed antibodies.

GABAR subunits known to be expressed by adult CGNs in vivo were all detected in cultured +/+ CGNs by 7 DIV (Fig. 1a). GABAR \( \alpha_1 \), \( \alpha_6 \) and \( \delta \)– markers of mature CGNs were barely detectable at 1 DIV but were all up-regulated between 3 and 5 DIV (Fig. 1a). GABAR \( \beta_3 \) and \( \gamma_2 \) subunits were clearly expressed earlier in CGN maturation as is the case in vivo (Fig. 1a). TARP\( \gamma_2 \) was also barely detectable until 3 DIV and plateaued at 7 DIV (Fig. 1a). Interestingly, Glur2 was strongly expressed throughout the culture period, prior to expression of TARP\( \gamma_2 \). As expected, TARP\( \gamma_2 \) was not detected in CGNs derived from stg mice.
(Fig. 1b). It has been shown that TARPγ2 is not compensated for by any other transmembrane AMPAR regulatory protein isoforms (Chen et al. 2000). Based on this information, we decided to use CGNs at 7 DIV for our subsequent studies.

At 7 DIV, it is interesting to note that the GABAR subunit expression profile of +/+ (AMPA receptor competent) and stg CGNs (AMPA receptor incompetent) is comparable when cultured under basal conditions (Fig. 1b), indicating that the switch for expression of a repertoire of mature GABAR subunits occurs independently of AMPAR function.

Effects of KCl- and KA-mediated depolarization on GABAR expression in +/+ CGNs

We compared the abundance of the principal GABAR subunits expected to be expressed by mature control CGNs (+/+ when cultured (i) under control conditions (5K), (ii) depolarized with KCl (25K), (iii) chronically exposed to KA (100 μmol/L) to activate non-NMDARs and (iv) exposed to KA (100 μmol/L) in the presence of the non-NMDA-receptor antagonist, CNQX (20 μmol/L) to verify that any KA effects were non-NMDAR-mediated (Fig. 2).

As we had shown previously (Ives et al., 2002), depolarizing CGNs with KCl (25K), strongly down-regulated expression of GABAR α1 (6 ± 2% of 5K levels, \(p < 0.01\)), α6 (13 ± 0.5% of 5K levels, \(p = 0.016\)) and β2 (undetectable) with concomitant up-regulated expression of GABAR β3 (251 ± 10%, \(p = 0.04\)). We extended these earlier studies to cover the full complement of GABAR subunits that were expected to be prominent components of GABARs expressed by CGNs. Thus, we investigated the prevalence of γ2 and δ under these conditions. The abundance of γ2 was largely unaffected (75 ± 18%, \(p = 0.36\)) by depolarization with KCl, GABAR δ on the contrary was strongly up-regulated (245 ± 10% of 5K levels, \(p = 0.02\)). When treated with KA, the general trend of effects evoked by KCl was largely reciprocated, with GABAR α1 (28 ± 26%, \(p < 0.01\)), α6 (16 ± 20%, \(p = 0.02\)) and β2 (undetectable) expressions being down-regulated relative to controls (5K), whilst both β3 (167 ± 13%, \(p = 0.04\)) and δ (246 ± 20%, \(p = 0.04\)) were up-regulated (Fig. 2). All KA-mediated effects were reversed by CNQX. In contrast to the effects of KCl depolarization, KA caused a CNQX-reversible down-regu-

![Fig. 2](image-url) Effects of KCl- and kainic acid (KA)-mediated depolarization on GABA receptor (GABAR) subunit expression by cerebellar granule cells (CGNs) derived from control (+/+) mice. (a) CGNs from control mice (+/+) maintained in media containing 5 mmol/L KCl (5K), 25 mmol/L KCl (25K) or 5K + KA (100 μmol/L) in the absence (KA) or presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (20 μmol/L, KA + CNQX) for 7 days in vitro (DIV) were harvested, solubilized and screened for GABAR subunit expression by immunoblotting using anti-GABAR α1, α6, β2, β3, γ2 and δ subunit-specific antibodies. Immunoblots were also probed with anti-β-actin antibody as a protein loading control. Ten micrograms of CGN protein were applied per gel lane, \(n = 3–5\). (b) Immunoreactive bands were quantified by densitometry and subunit expression normalized against β-actin (Ives et al., 2002). Expression levels were estimated relative to those obtained at 5K that were assigned a value of 100%. Data shown are mean ± SEM, \(n = 3–5\).
regulation of GABAR γ2 abundance (43 ± 12%, p = 0.03) in +/- CGNs.

Effects of KCl- and KA-mediated depolarization on GABAR expression in stg CGNs

We repeated this analysis on CGNs derived from stg (Fig. 3). Depolarizing with KCl (25K) evoked almost identical effects in stg CGNs as in +/- CGNs thus, GABAR α1 (21 ± 27% of 5K levels, p = 0.05), α6 (undetectable) and β2 (undetectable) were strongly down-regulated, whilst GABAR β3 (246 ± 12% of 5K levels, p < 0.01) and GABAR δ (220 ± 17% of 5K levels, p < 0.01) were significantly up-regulated. The abundance of γ2 was largely unaffected (85 ± 1%, p = 0.19). When CGNs derived from stg were treated with KA, however, expression of GABAR α1 (75 ± 22% of 5K levels, p = 0.56), α6 (82 ± 16% of 5K levels, p = 0.2), β2 (64 ± 46% of 5K levels, p = 0.27), β3 (85 ± 26% of 5K levels, p = 0.61) and δ (93 ± 42% of 5K levels, p = 0.29) were not significantly affected. Interestingly, as with +/- CGNs, KA’s exposure caused a CNQX-reversible down-regulation of GABAR γ2 (55 ± 11%, p = 0.03) implying that this was a KAR-mediated effect.

GABAR α4 replaces α6 in depolarized CGNs

Radioligand binding assays were performed on intact CGNs (+/-, 7 DIV) to determine the GABAR pharmacological characteristics of CGNs cultured in the absence (5K) and presence of KA (5K + KA). The prevalence of [3H]muscimol binding sites, which represents the full complement of GABARs, i.e. γ2 + δ containing subtypes were not significantly different under the two culture conditions (Fig. 4a). Conversely, [3H]Ro15-4513 binding sites, which comprise the γ2-containing subtypes only were significantly less abundant in KA-treated CGNs (Fig. 4b), with total [3H]Ro15-4513 binding sites being 59 ± 9% of 5K (p = 0.02), benzodiazepine-sensitive binding sites being 74 ± 16% of 5K (p = 0.08) and benzodiazepine-insensitive binding sites being 33 ± 34% of 5K level (p = 0.003). Thus, results are consistent with KA-treatment having no effect on the total number of GABARs expressed (γ2 + δ containing receptors) but induces a down-regulation of the γ2-contain-

Fig. 3 Effects of KCI- and kainic acid (KA)-mediated depolarization on GABA<sub>a</sub> receptor (GABAR) subunit expression by cerebellar granule cells (CGNs) derived from stargazer (stg) mice. (a) CGNs from stargazer mice (stg) mice maintained in media containing 5 mmol/L KCl (5K), 25 mmol/L KCl (25K) or 5K + KA (100 μm/L) in the absence (KA) or presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (20 μmol/L, KA + CNQX) for 7 days in vitro (DIV) were harvested, solubilized and screened for GABA<sub>a</sub> receptor by immunoblotting using anti-GABA<sub>a</sub> α1, α6, β2, β3, γ2 and δ subunit-specific antibodies. Immunoblots were also probed with anti-β-actin antibody as a protein loading control. Ten micrograms of CGN protein were applied per gel lane, n = 3–5. (b) Immunoreactive bands were quantified by densitometry and subunit expression normalized against β-actin (Ives et al., 2002). Expression levels were estimated relative to those obtained at 5K that were assigned a value of 100%. Data shown are mean ± SEM, n = 3–5.

© 2007 The Authors
ing subtypes. This indicated that δ-containing receptors were up-regulated, despite the apparent reduced availability of the α6 subunit (Fig. 2), the preferred δ-subunit assembly partner in CGNs. We investigated whether the alternative δ assembly partner α4, normally the preferred assembly partner of δ in forebrain structures, such as the thalamus was induced by KA-mediated depolarization as we have previously shown for KCl-depolarized CGNs (Payne et al. 2006).

Figure 5 shows that α4 expression was indeed up-regulated in +/- CGNs following KCl- and KA-mediated depolarization, the latter in a CNQX-reversible manner. Interestingly, α6 was barely detectable in +/- CGNs cultured under KCl-depolarizing conditions (25K; Fig. 2) and thus appears to be replaced entirely by α4.

Activating AMPARs increases the cell surface expression of α4δ-containing GABARs

We next determined whether AMPAR-mediated up-regulation of α4 and δ subunits resulted in their increased expression at the CGN surface. We conducted cell surface biotinylation assays to evaluate the proportion of subunits located both intracellularly and at the cell surface. Figure 6 shows that α6 was expressed at the cell surface of CGNs cultured at 5K but was undetectable at the cell surface of KA-treated CGNs. GABAR α6 detected in KA-treated CGNs was almost exclusively restricted to intracellular domain(s). GABAR α4 expression was undetectable at the cell surface of CGNs cultured at 5K, but was prominent at the surface of KA-treated CGNs. GABAR δ was also up-regulated at the cell surface of KA-treated CGNs, compared to control neurones (5K; Fig. 6). The results are compatible with a switch from α6βδ to α4βδ receptors at the surface of KA-treated CGNs. The intracellular protein β-actin being detected entirely in the ‘intracellular’ fraction was not biotinylated, thus verifying the validity of this approach to discriminate between cell surface and intracellular proteins (Fig. 6). These results were confirmed using an alternative approach that utilized the membrane impermeable cross-linker, BS3 (Ives et al. 2002; data not shown).
down-regulation of CaN inhibitors, FK506 (10 μmol/L) or in the presence of kainic acid (KA, 100 μmol/L), KA and nifedipine were added to the culture media at 1 day in vitro (DIV). CGNs were collected in solubilizing buffer at 7 DIV and screened by immunoblotting using anti-GABAR α6, β and γ subunit-specific antibodies as probes. Immunoblots were also probed with anti-β-actin antibody as a protein loading control. Ten micrograms of CGN protein were applied per gel lane, n = 3.

Fig. 6 Qualitative evaluation of the relative levels of cell surface expressed GABA_A receptor (GABAR) α6, δ and γ4 subunits by +/- cerebellar granule cells (CGNs) maintained in a media containing 5 mmol/L KCl (5K) or 5K + kainic acid (KA) (5K + KA). CGNs from control mice (+/+), maintained in media containing 5K or 5K + KA (100 μmol/L) for 7 days in vitro (DIV) were subjected to cell surface protein biotinylation assays as described in the methods. Proteins that were residents in the CGN plasma-membrane (‘cell surface’) versus those that were located in intracellular domains (‘intracellular’) were identified by immunoblotting using GABAR α6, δ and γ4 subunit-specific antibodies, n = 3. β-actin was used as a control to validate this approach as a means to discriminate proteins found at the cell surface as opposed to those restricted to the cell interior. Due to the differential avidity of the antibodies for their respective antigens, it is not possible to extrapolate any information regarding the abundance of the respective proteins that were probed.

Signalling pathways involved in AMPAR-mediated up-regulation of GABAR α4, β3 and δ and down-regulation of GABAR α1, α6 and β2

The KA-mediated up-regulation of GABAR α4, β3 and δ expression in control CGNs was blocked by L-type voltage-gated calcium channel (L-VGCC) blockers, nifedipine (KA + Nifedipine, 10 μmol/L, Fig. 7) and verapamil (10 μmol/L; data not shown). However, KA-mediated down-regulation of α1, α6 and β2 was insensitive to blockade by L-VGCC blockers (Fig. 7). We then investigated whether NMDAR activity (Ca2+-flux) or down-stream Ca2+-activated enzymes; Ca2+/calmodulin-dependent protein kinase (CaM kinase) and calcineurin (CaN) were involved. CaN inhibitors, FK506 (1 μmol/L) and cyclosporin A (500 nmol/L; data not shown) inhibited AMPAR-mediated down-regulation of α1, α6 and β2 expression by +/- CGNs. We established that KA evoked an inhibition of expression of α1 (by 54 ± 19%), α6 (66 ± 9%) and β2 (72 ± 6%) subunits compared to expression under basal culture conditions (5K, Fig. 8). FK506 restored α1 (81 ± 11%) and β2 (62 ± 14%) expression (Fig. 8). Neither the CaM kinase inhibitor, KN-62 (10 μmol/L) nor the application of the NMDAR open-channel blocker, MK-801 (10 μmol/L) had any effect on α1, α6 or β2 (Fig. 8) expression (data not shown). Somewhat surprisingly, AMPAR-mediated up-regulation of α4 and β3, but not δ was also reversed by CaN inhibitors (99 ± 16%, 95 ± 9%, and 279 ± 10%, respectively), whilst up-regulation of all three was reversed, following inhibition of CaM kinase (72 ± 11%, 58 ± 3% and 41 ± 20%, respectively). Indeed KN-62 inhibited expression was lower than that expressed under basal conditions. The amounts of GABAR α6 and δ expressed in the presence of KA (KA, depolarized) and FK506 (KA + FK506, CaN inhibited) were uniquely much higher than that detected under basal conditions (5K, Fig. 8) and were not paralleled by effects on α1, α4, α6, β2 or β3 expression. KN-62 caused a significant reduction of β3 and δ below basal levels implying that basal expression of β3 and δ are under the influence of ‘constitutive’ CaM kinase activity of the cultured neurones. This was unlikely due to spontaneous AMPAR activity of the cultures as CNQX was without effect on basal expression of any GABAR subunits (data not shown).

KAR-mediated down-regulation of γ2 expression was insensitive to FK506, cyclosporin A, KN-62 and MK-801 (data not shown).

Discussion

The ataxic mutant mouse, stargazer (stg) fails to express TARPγ2, a transmembrane AMPAR-trafficking and synaptic-targeting protein. As a consequence, the cerebellar mossy
fibre-granule cell synapses are devoid of AMPARs and hence electrically silent (Hashimoto et al. 1999; Chen et al. 2000) leading to aberrant expression of GABAergic characteristics of CGNs (Thompson et al. 1998; Chen et al. 1999). This raised the question ‘Do glutamatergic afferents influence GABAR expression by a non-NMDAR mediated signalling pathway(s)?’ We utilized cultured CGNs from control and stg mice to address this issue for two reasons.

First, CGNs derived from neonatal murine cerebella can be maintained long-term in vitro under basal, non-depolarizing culture conditions. Secondly, CGNs derived from control mice express functional AMPARs and KARs, (Chen et al. 2000, 2003) whilst CGNs derived from stg express only functional KARs (Chen et al. 2000, 2003) enabling us to dissect out the functional role(s) played by AMPARs and KARs in the expression of inhibitory GABARs. Here we Fig. 8 Dissecting out the signalling pathways involved in kainic acid (KA)-mediated changes in GABA<sub>A</sub> receptor (GABAR) expression by cerebellar granule cells (CGNs). CGNs (+/+) were cultured under basal conditions [5 mmol/L KCl (SK), lane 1] in the presence of KA (100 μmol/L KA, lane 2) and in the presence of KA (100 μmol/L) with calcineurin (CaN) inhibitors, FK506 (1 μmol/L, KA + FK506, lane 3), or with Ca<sup>2+</sup>/calmodulin-dependent protein kinase (CaM kinase) inhibitor, 4-[(2S)-2-[[5-isoquinolinylsulfonyl]methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl] phenyl isoquinoline sulfonic acid ester (KN-62) (10 μmol/L, KA + KN-62, lane 4). KA and drugs were applied to the culture media at 1 day in vitro (DIV). CGNs were collected in solubilizing buffer at 7 DIV and screened by immunoblotting using anti-GABAR subunit-specific antibodies. Immunoblots were also probed with anti-α-tubulin antibody as a protein loading control. Ten micrograms of CGN protein were applied per gel lane. Data shown are mean ± SEM, asterisk represents "p < 0.05."
show that functional AMPARs are not required for the transition from immature to mature GABAR expression profiles – GABAR \( \alpha_6 \) and \( \delta \) subunits, markers of mature CGNs are expressed at comparable levels in neurones derived from \(+/+\) and \( \text{stg} \) cultured at 5K (Fig. 1b). The transition to a mature GABAR profile is also maintained in \( \text{stg} \) CGNs \textit{in vivo} (Payne et al. 2007). It is the amount of \( \alpha_6, \beta_3 \) and \( \delta \) subunits expressed that is compromised \textit{in vivo}. GABAR \( \alpha_6 \) gene expression by CGNs has already been described to be a cell autonomous, pre-programmed event that is independent of environmental influences such as specific neurotransmitter innervation (Bahn et al. 1999; Wisden et al. 2002) which our data supports. Indeed, we failed to observe any overt differences in GABAR expression between \(+/+\) and \( \text{stg} \) CGNs when cultured under basal conditions (5K, Fig. 1b), despite the fact that we had previously reported that the expression of GCN maturation markers GABAR \( \alpha_6 \) and \( \beta_3 \) subunits and BZ-ISR were severely compromised, while \( \alpha_1 \) and \( \beta_2 \) subunits and BZ-ISR were largely unaffected (Thompson et al. 1998), and that GABAR functional characteristics of adult CGNs of the allelic mutant wagger \textit{in vivo} (Chen et al. 1999) mirrored those of juvenile GABARs expressed by wild-type mice. This implied that either the abnormalities in GABAR expression reported in \( \text{stg} \) CGNs \textit{in vivo} were a consequence of aberrant signalling through cerebellar circuits that were not maintained in dissociated cultures \textit{in vitro} and/or that non-NMDARs expressed by 7 DIV CGNs were not active in 5K media. To test the latter possibility, we applied the non-NMDAR agonist KA to the culture media to act as exogenous agonist. Following artificial activation of non-NMDARs in this way, we did observe discernible differences in the GABAR expression profiles of \(+/+\) and \( \text{stg} \) CGNs in culture. GABAR \( \alpha_1, \alpha_6 \) and \( \beta_2 \) subunit expression was down-regulated (Fig. 2), \( \alpha_4 \) (Fig. 5), \( \beta_3 \) and \( \delta \) expression was up-regulated (Fig. 2) relative to \(+/+\) neurones cultured at 5K. The KA-mediated effects were inhibited by the non-NMDAR antagonist CNQX, but not by the GABAR antagonist picrotoxin (data not shown). The latter suggests that these effects are not driven by KA-mediated GABA-release from synaptically coupled Golgi interneurones, that are known to contaminate this culture (Mellor et al. 1998) and the subsequent GABAR activity of CGNs. KA had no effect on GABAR \( \alpha_1, \alpha_4, \alpha_6, \beta_2, \beta_3 \) or \( \delta \) expression by CGNs derived from \( \text{stg} \) as expected, as these neurones fail to express functional AMPARs. The effects evoked by KA on GABAR \( \alpha_1, \alpha_4, \alpha_6, \beta_2, \beta_3 \) and \( \delta \) expression in \(+/+\) CGNs (but not \( \text{stg} \) CGNs) were paralleled by a K+-depolarizing stimulus (25K, Figs 2 and 3) on both \(+/+\) and \( \text{stg} \)-derived neurones indicating that the signalling pathway(s) activated by AMPAR-mediated depolarization were mimicked by K+-mediated membrane-depolarization and that the membrane-depolarization-activated signalling pathway(s) were intact in \( \text{stg} \) neurones (Figs 2 and 3).

Is a loss of GABAR autonomous AMPAR activity responsible for the aberrant expression of GABAR in \( \text{stg} \)?

Clearly, we have shown that AMPAR activity in CGNs does influence the GABAR profile which these neurones subsequently express. However, a loss of AMPAR function at the CGN-mossy fibre synapse in \( \text{stg} \) would not appear to be the sole cause of aberrant GABAR expression. From our \textit{in vivo} observations (Payne et al. 2007) on \( \text{stg} \), we would have expected the principal effects of competent AMPAR signalling (kainic-acid-treated \(+/+\) neurones) to show an elevated steady-state level of expression of \( \alpha_6, \beta_3 \) and \( \delta \) subunits in \(+/+\) CGNs, compared to electrically active \(+/+\) CGNs (5K CGNs) and AMPAR incompetent \( \text{stg} \) CGNs, as these subunits were strongly down-regulated in \( \text{stg} \) cerebellum \textit{in vivo}, where AMPAR activity is abrogated. The \( \beta_3 \) and \( \delta \) subunits were up-regulated as predicted (Fig. 2), however, unexpectedly, the \( \alpha_6 \) subunit expression level was down-regulated by KA treatment of \(+/+\) CGNs. Nonetheless, GABAR \( \alpha_6 \) subunit expression was unaffected by KA treatment in CGNs derived from \( \text{stg} \), as expected. Despite the fact that the effect of stimulating AMPARs on GABAR \( \alpha_6 \) subunit expression is entirely compatible with GABAR \( \alpha_6 \) subunit being a depolarization/CaN ‘off’ gene (Sato et al. 2005), our data with cultured CGNs would predict that the stargazer mutation would result in the up-regulation of the \( \alpha_6 \) subunit \textit{in vivo} which we have shown is not the case (Thompson et al. 1998; Payne et al. 2007). Clearly, regulation of the \( \alpha_6 \) subunit must be subjected to other regulatory mechanisms which are disrupted by the inability of \( \text{stg} \) CGNs to express TARPy2 e.g. compromised expression of brain-derived neurotrophic factor (BDNF, 24). BDNF has been shown to play a role in up-regulating \( \alpha_6 \) subunit expression in CGNs \textit{in vitro} (Bulleit and Hsieh 2000). Expression of the NMDAR NR2C subunit also a marker of CGN maturation has recently been shown to be up-regulated by membrane depolarization (elevated KCl) as long as CaN was simultaneously inhibited – this enabled BDNF release from CGNs which acts as an autocrine messenger, activating CGN tyrosine kinase type B receptors, the extracellular signal regulated kinase 1/2 pathway and NR2C transcription (Suzuki et al. 2005). We report here that depolarization (AMPAR-mediated) evokes up-regulation of GABAR \( \alpha_6 \) and \( \delta \) expression relative to basal conditions if CaN is inhibited with FK506 (Fig. 8), which parallels the sequence of events associated with NR2C expression in CGNs – we propose the same mechanism dictates the unique regulation of GABAR \( \alpha_6 \) expression in CGNs. We are currently investigating this further.

From our \textit{in vivo} studies on GABAR expression in adult \( \text{stg} \) cerebellum, we would have predicted that \( \alpha_1, \beta_2 \) and \( \gamma_2 \) would be either unaffected by KA treatment or weakly up-regulated, as these subunits are either not affected by the stargazer mutation \textit{in vivo} (Thompson et al. 1998) or weakly down-regulated \textit{in vivo} (Payne et al. 2007). Indeed, \( \gamma_2 \)
expression was not affected by AMPAR activation in CGNs from +/+ mice, as predicted. However, contrary to predictions, both α1 and β2 subunits were down-regulated in CGNs from +/+, following KA treatment. Thus, although our data clearly shows that the GABAR expression profiles of CGNs are subject to regulation by AMPAR activity, the loss of functional AMPARs at the stg mossy fibre-CGN synapse in vivo offers only a partial explanation as to why stg CGNs develop an abnormal GABAR profile. Discrepancies between in vitro and in vivo results may also be because of a non-physiological chronic activation of AMPARs mimicked in culture. Further investigations of the down-stream consequences of the stargazer mutation need to be conducted to fully understand the mechanisms underpinning aberrant GABAR expression in stg.

Nonetheless, we have shown here that KA-mediated effects on GABAR α1, α4, α6, β2, β3 and δ expression are AMPAR-mediated. GABAR γ2 expression was not affected by K⁺-depolarization, but was down-regulated by KA in a CNQX-reversible manner in both +/+ and stg CGNs, implying a role for KARs that was independent of membrane depolarization and Ca²⁺-signalling (Figs 2, 3 and 9). Our radioligand binding data suggested that the KA-treated CGNs expressed a comparable number of GABARs to their untreated counterparts, but a switch from γ2-containing (synaptic) to δ-containing (extrasynaptic) GABARs occurred. However, it cannot be excluded that no change in [³H]muscimol binding could result from a loss of δ-subunit containing GABAR, which have a five-fold higher affinity for the ligand (Quirk et al. 1995). Commensurate with a switch from δ-containing to γ2-containing GABAR, we found that expression of GABAR γ2 protein was reduced and that δ protein was increased, as was the amount of δ located at the CGN membrane surface (Fig. 6) where one would expect to find δ assembled into pentameric GABARs. This was paralleled by an increase in cell surface expression of one of the δ subunit’s two preferred assembly partners, the GABAR α4βδ subtype. Whilst the expected δ partner in CGNs, α6 was barely detectable at the cell surface (Fig. 6). Our data suggest that GABAR α4 must have been predominantly recruited into the α4βδ subtype, a predicted extrasynaptic GABAR conferring tonic inhibition. The CGNs would appear to have made an adaptive response to increased excitatory input (KA treatment of CGNs from +/+) by posting a greater number of GABARs to extrasynaptic domains at the expense of synaptic receptors, a situation that would seem to be contrary to what we observed in the dentate granules of stg (Payne et al. 2006). Interestingly, in stg CGNs in vivo, where excitatory drive of CGNs is reduced relative to controls, we found a strong down-regulation of extrasynaptic GABARs (Payne et al. 2007), the extrasynaptic tonic inhibition conferring α6βδ receptors (Brickley et al. 1996, 1999; Nusser et al. 1998) which are responsible for eliciting > 97% of GABAR-mediated inhibition in CGNs and are thus pivotal for information transfer in the cerebellum (Hamann et al. 2002).

We then evaluated three independent pathways that were involved in these AMPAR-mediated bi-directional changes in GABAR expression (Fig. 9). Up-regulation of GABAR α4 and β3 was influenced by two L-VGCC-dependent (nifedipine and verapamil inhabitable) routes, one involving CaN (FK506 and cyclosporin A inhibitable) and a second involving CaM Kinase (inhibition by KN-62), the latter appearing to be solely responsible for the up-regulation of δ (Fig. 9) verifying the observations made by Gault and Siegel (1997) using rat CGNs. These effects on α4, β3 and δ expression were reproduced by KCl depolarization (Figs 2 and 3) implying that AMPAR activation evoked sufficient sustained membrane depolarization (Δψf, Fig. 9) to activate...
L-VGCCs and subsequent Ca\(^{2+}\)-stimulation of both CaN and CaM kinase. Whether CaN and CaM kinase are the final steps in pathways or constitute intermediates in enzyme cascades still has to be elucidated. For example, CaN may activate or inhibit adenylyl cyclases (Chan et al. 2005) and inhibit the mitogen activated protein kinase pathway (Suzuki et al. 2005), indeed we have already shown that adenylyl cyclase(s) play a role in GABAR expression in CGNs (Thompson et al. 2000). Whether they influence transcription or post-translational phosphorylation status of all or some subunits remains to be resolved.

AMPAR-mediated down-regulation of GABAR \(\alpha_1\), \(\gamma_6\) and \(\beta_2\) subunits was found to be an L-VGCC-independent process that nonetheless was dependent upon activation of CaN. This was mimicked by KCl-depolarization implying that a depolarization-induced elevation of intracellular Ca\(^{2+}\) concentration was involved. Whether the Ca\(^{2+}\) originated from non-L-VGCCs, intracellular stores or Ca\(^{2+}\)-permeable AMPARs which are abundant in cultured CGNs (Jones et al. 2000) remains to be resolved. NMDAR blockade with MK-801 did not abrogate these responses, eliminating NMDARs as the Ca\(^{2+}\) source. CaN constitutes a component of the signalling pathway that transforms activation of AMPARs to a down-regulation of \(\alpha_1\), \(\gamma_6\) and \(\beta_2\), yet is capable of doing so without activating the CaN-dependent pathway that up-regulates \(\alpha_4\) and \(\beta_3\) (Figs 7–9). We can only assume, this must be because of spatial compartmentalization of pathways and/or calcium sources such that \(\alpha_1\), \(\gamma_6\) and \(\beta_2\) subunits can be regulated independent of \(\alpha_4\) and \(\beta_3\) by signal transduction pathways that both involve CaN. It has recently been reported that immature, relatively depolarized, migratory CGNs in vivo express depolarization/CaN ‘off’ genes (genes that are switched on when CaN is switched on and/or vice versa) that have roles in CGN proliferation, migration and early post-mitotic differentiation. Interestingly, GABAR \(\alpha_1\), \(\gamma_6\) and \(\delta\) are all reported to be CaN ‘off’ genes (i.e. genes that are switched on when CaN is switched off and/or vice versa, (Sato et al. 2005)) whose transcription would be expected to be up-regulated when CaN activity is curtailed e.g. by hyperpolarization of the resting membrane potential (Sato et al. 2005). As CGNs mature in vivo, the resting membrane potential has been reported to become hyperpolarized (Zanzouri et al. 2006) favouring transcription of depolarization/CaN ‘off’ genes – the protein products of many, which have roles in synaptic neurotransmission. Thus our data are largely compatible with these observations, identifying AMPARs as a source of the depolarizing input that can activate/stimulate CaN and down-regulate expression of GABAR \(\alpha_1\) and \(\gamma_6\) in (+/+ or −/−) CGNs. GABAR \(\delta\) is also considered to be a CaN ‘off’ gene but refractory to KCl-mediated CaN activation (Sato et al. 2005) implying that the source of [Ca\(^{2+}\)]\(_{i}\) that activates CaN to regulate \(\delta\) gene expression is different from that required to regulate GABAR \(\alpha_1\) and \(\gamma_6\) genes. Indeed, we found an anomaly in terms of \(\delta\) expression as AMPAR-activation of CaN would be expected (Sato et al. 2005) to evoke down-regulation of \(\delta\) mRNA, whilst CaN inhibitors would be expected to induce up-regulation. In our hands, AMPAR-activation evoked up-regulation of \(\delta\) protein that was refractory to inhibitors of CaN but reversed by inhibition of CaM kinase. Our results are in agreement with those of Gault and Siegel 1997; Martikainen et al. 2004; \(\delta\) gene expression, (44) and Salonen et al. 2006 but incompatible with those predicted by Sato et al. 2005. Further studies are required to resolve this discrepancy, which ultimately requires a concerted effort to study transcription, translation and post-translational receptor aspects of all subunits to fully understand the mechanisms involved.

Finally, GABAR \(\gamma_2\) expression, insensitive to membrane depolarization was down-regulated by a KAR-dependent mechanism that was independent of L-VGCCs, CaM kinase, CaN and NMDAR activity.

We have reported here the consequences of non-NMDAR activation on GABAR protein expression, assembly and trafficking in CGNs and have begun to dissect out the signal transduction pathways involved. Whether these effects of AMPAR activity on GABAR expression are because of transcriptional and/or protein phosphorylation regulated processes remains to be elucidated, though we have previously shown that KCl-depolarization caused down-regulation of GABAR \(\alpha_1\), \(\gamma_6\), \(\beta_2\) and \(\beta_3\) mRNAs (Ives et al., 2002) – GABAR \(\alpha_1\), \(\gamma_6\) and \(\beta_2\) proteins were down-regulated in parallel however, \(\beta_3\) protein was significantly up-regulated. These previous studies clearly need to be extended to include the full complement of GABAR subunits as studied here and ultimately to dissect the complete signalling pathways involved, as the anomaly with the effects of membrane depolarization on \(\beta_3\) mRNA and protein indicate that the complete spectrum of regulatory mechanisms on gene/protein expression need to be considered. Nonetheless, it is clear that these studies may have bearing on our understanding and design of treatment regimes of hyperexcitability syndromes that result as a consequence of excessive GluR activation.

Acknowledgements

This study was possible through awards of research grants to CLT from the Wellcome Trust (Ref No. 066204), the Biotechnology and Biological Sciences Research Council (BBSRC, Ref No. 12/0014803) and Merck Sharp and Dohme (PhD studentship award to HLP) for which we are very grateful.

References


