Evidence that GABA ρ subunits contribute to functional ionotropic GABA receptors in mouse cerebellar Purkinje cells

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Ionotropic γ-amino butyric acid (GABA) receptors composed of heterogeneous molecular subunits are major mediators of inhibitory responses in the adult CNS. Here, we describe a novel ionotropic GABA receptor in mouse cerebellar Purkinje cells (PCs) using agents reported to have increased affinity for ρ subunit-containing $GABA_C$ over other GABA receptors. Exogenous application of the $GABA_C$-preferring agonist *cis*-4-aminocrotonic acid (CACA) evoked whole-cell currents in PCs, whilst equimolar concentrations of GABA evoked larger currents. CACA-evoked currents had a greater sensitivity to the selective $GABA_C$ antagonist (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA) than GABA-evoked currents. Focal application of agonists produced a differential response profile; CACA-evoked currents displayed a much more pronounced attenuation with increasing distance from the PC soma, displayed a slower time-to-peak and exhibited less desensitization than GABA-evoked currents. However, CACA-evoked currents were also completely blocked by bicuculline, a selective agent for $GABA_A$ receptors. Thus, we describe a population of ionotropic GABA receptors with a mixed $GABA_A$/$GABA_C$ pharmacology. TPMPA reduced inhibitory synaptic transmission at interneurone–Purkinje cell (IN–PC) synapses, causing clear reductions in miniature inhibitory postsynaptic current (mIPSC) amplitude and frequency. Combined application of NO-711 (a selective GABA transporter subtype 1 (GAT-1) antagonist) and SNAP-5114 (a GAT-(2)/3/4 antagonist) induced a tonic GABA conductance in PCs; however, TPMPA had no effect on this current. Immunohistochemical studies suggest that ρ subunits are expressed predominantly in PC soma and proximal dendritic compartments with a lower level of expression in more distal dendrites; this selective immunoreactivity contrasted with a more uniform distribution of $GABA_A$ α1 subunits in PCs. Finally, co-immunoprecipitation studies suggest that ρ subunits can form complexes with $GABA_A$ receptor α1 subunits in the cerebellar cortex. Overall, these data suggest that ρ subunits contribute to functional ionotropic receptors that mediate a component of phasic inhibitory GABAergic transmission at IN–PC synapses in the cerebellum.

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The neurotransmitter GABA acts at ionotropic $GABA_A$ and $GABA_C$ receptors to mediate fast inhibition in the adult CNS. These receptor subtypes can be distinguished pharmacologically and also by their distinct molecular subunit composition (Johnston, 1996; Bormann, 2000); in regard to the latter, heteromeric $GABA_A$ receptors are encoded by $\alpha_{(1-6)}$, $\beta_{(1-3)}$, $\gamma_{(1-3)}$, $\delta_{(1)}$, $\varepsilon_{(1)}$, $\theta_{(1)}$ and $\pi_{(1)}$ (Whiting *et al.* 1999), whilst $GABA_C$ are composed exclusively of combinations of $\rho_{(1-3)}$ subunits (Bormann, 2000). However, ρ subunits show a high degree of amino acid sequence homology with $GABA_A$ receptor subunits and have also been suggested to represent $GABA_A$ subtypes themselves (Barnard *et al.* 1998; Didelon *et al.* 2002). In expression systems, there is evidence that ρ subunits can associate with $GABA_A$ γ_2 subunits (Qian & Ripps, 1999; Ekema *et al.* 2002; Pan & Qian, 2005). The extent to which this association occurs *in vivo* is largely unknown; however, it has been demonstrated that ρ subunits can coassemble with $GABA_A$ α_1 and γ_2 subunits in brainstem neurones (Milligan *et al.* 2004). The agonist

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CACA and the antagonist TPMPA have both been reported to have higher affinity at $GABA_C$ over other GABA receptors (Bormann, 2000). Using these agents, ionotropic GABA receptors with a mixed pharmacological profile have been reported in hippocampal (Semyanov & Kullmann, 2002; Hartmann *et al.* 2004) and brainstem (Milligan *et al.* 2004) neurones. Importantly, Milligan *et al.* (2004) correlated sensitivity to CACA with the expression of ρ_1 subunits in individual neurones, providing the first evidence that selective pharmacology may be used to probe the expression of ρ subunits in the CNS. CACA has been shown to directly activate α6-containing GABA receptors in cerebellar granule cells (Wall, 2001); however, cerebellar PCs, the focus of the present study, do not express α6 receptor mRNA or protein (Wisden *et al.* 1996).

The term $GABA_C$ was originally suggested from studies in the cerebellum in which CACA, previously demonstrated to depress firing of spinal cord neurones in a bicuculline-insensitive manner (Johnston *et al.* 1975), had no effect on the binding of the $GABA_B$ agonist baclofen to isolated membranes (Drew *et al.* 1984). Studies at a molecular level have highlighted the cerebellum as being enriched in ρ subunit transcripts; ρ_1 and ρ_2 mRNAs were shown to be selectively expressed in rat PCs and basket-like interneurones (Rozzo *et al.* 2002) and surface ρ_1 immunoreactivity has been reported in rat PC soma and dendrites (Boue-Grabot *et al.* 1998). Recently, a role for ρ subunits in the cerebellum has been further implicated with the cloning of a ρ_2 subunit from bovine tissue, the first ρ subunit to be isolated outside of the retina (Lopez-Chavez *et al.* 2005). However, despite the cloning of ρ subunits and the demonstration of their prominent expression in PCs, the *functional* contribution of this subunit to ionotropic GABA responses in the cerebellum remains to be addressed. In the present study, we provide evidence that ρ subunits contribute to a novel population of GABA receptors that play a role in phasic inhibitory transmission at cerebellar IN–PC synapses, thus adding to the molecular heterogeneity of GABA receptors in the cerebellum.

Methods

Tissue preparation and solutions

Cerebellar slices were prepared according to methods previously described in detail by Southan & Robertson (1998). Three- to five-week-old male TO mice (Harlan, UK) were humanely killed by cervical dislocation and decapitated in line with UK Home Office procedures. The brain was removed and transferred into a chilled, oxygenated sucrose-based artificial cerebrospinal fluid (aCSF) solution. The cerebellum was dissected out and parasagittal cerebellar slices (300 μm thick) were cut using a Vibratome (R. & L. Slaughter, Upminster, UK). Slices were transferred into standard aCSF solution at 37°C for 1 h and then maintained at room temperature (20–24°C). The standard aCSF contained (mM): NaCl 124, KCl 3, $NaHCO_3$ 26, NaH_2PO_4 2.5, $MgSO_4$ 2, $CaCl_2$ 2, D-glucose 10, maintained at pH 7.3 by bubbling with 95% O_2–5% CO_2. The sucrose-based solution used for dissection and slicing was identical to standard aCSF, with the exception that NaCl was replaced by isosmotic sucrose (74.5 g l^{-1}).

Electrophysiological recording

Slices were placed in a recording chamber at room temperature and perfused at 2–4 ml min^{-1} with standard aCSF bubbled with 95% O_2–5% CO_2. Individual neurones were visualized by a ×60 water immersion lens with infrared differential interference contrast optics using an upright Olympus BX50WI microscope (Olympus, Tokyo, Japan). Whole-cell recordings were performed using an EPC-9 patch-clamp amplifier (HEKA Electronik, Lambrecht, Germany), controlled by Pulse software (HEKA) with a Macintosh G4 computer. Electrodes were made from borosilicate glass (GC150-F10, Harvard Apparatus, Kent, UK) and, when filled with an intracellular solution containing (mM): CsCl 140; $MgCl_2$ 1; $CaCl_2$ 1; EGTA 10; MgATP 4; NaGTP 0.4; Hepes 10; pH 7.3, had resistances between 3 and 7 MΩ. Series resistance was typically 5–10 MΩ and was monitored and compensated by 70–90% throughout. Data were sampled at 7 kHz and filtered at one-third of the sampling frequency. Miniature inhibitory postsynaptic currents (mIPSCs), isolated by 1 μM tetrodotoxin (TTX), were identified as rapidly activating, inward currents at a holding potential of −70 mV. Agents were applied in the presence of the non-NMDA glutamate receptor antagonist 6-nitro-7-sulfamoylbenzo(*f*)quinoxaline-2-3-dione (NBQX) and the $GABA_B$ antagonist CGP 55845. Data were analysed using Pulsefit (HEKA), Axograph (Axon Instruments), Mini Analysis (Synaptosoft), Igor (Wavemetrics), Origin (Microcal), Excel (Microsoft) and Prism (GraphPad) software. Cumulative frequency plots were constructed for mIPSC inter-event intervals (bin widths 5 ms) or mIPSC amplitudes (bin widths 2 pA). Data are presented as mean value ± S.E.M., where n = number of cells. Statistical significance was determined using Student's paired or unpaired t tests, one-way ANOVA followed by Tukey's HSD test or a Mann-Whitney test, as appropriate. Cumulative frequency plots were analysed by Kolmogorov-Smirnov (KS) tests. In all cases, $P < 0.05$ was considered significant.

Immunohistochemisty

Immunohistochemistry was performed on three- to five-week-old male TO mice (Harlan, UK) using a procedure modified from Llano *et al.* (2000). Briefly, mice

were decapitated and the cerebellar vermis was extracted and placed in 4% paraformaldehyde in PBS (0.15 M) for 1 h. Parasagittal slices, 100 μm thick, were cut and left in the same PBS/fixative solution for a further 30 min at 22–25°C before sections were treated with a 1% bovine serum albumin (BSA)–PBS blocking buffer containing 0.4% Triton X-100 for 1 h at room temperature. Slices were washed in PBS and then incubated overnight (2–5°C) in an antibody mixture containing rabbit anti-$\rho_{(1\&2)}$ (1 : 600) (a kind gift from Dr Ralf Enz, Friedrich Alexander University of Erlangen-Nurnberg) or anti-$GABA_A$ α1 (1 : 300) (Alomone Laboratories, USA) and mouse anti-calbindin D_{28K} (1 : 2000) (Sigma, UK). Slices were then incubated with a secondary antibody mixture containing anti-rabbit FITC (1 : 200) and anti-mouse TRITC (1 : 200) (Stratech Scientific, UK), before being washed in blocking buffer and PBS and mounted on glass slides in Vectashield fluorescent mounting medium (Vector Laboratories, CA, USA). Images were acquired using a Zeiss LSM 510 Meta confocal microscope (488 nm and 540 nm excitation) and analysed off-line.

Immunoprecipitation

Cerebella dissected from three- to five-week-old male TO mice were homogenized in 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 150 mM NaCl, 1.5% sodium deoxycholate, 0.1% Nonidet P-40 and Complete protease inhibitor (Roche, UK) with an Ultra-Turrax blender. Pre-cleared supernatant was incubated with rabbit anti-$\rho_{(1\&2)}$ (1 : 400) overnight at 4°C. To monitor unspecific binding, the antibody was omitted from a similar sample. Following incubation, 40 μl of protein G-sepharose beads (Sigma, UK) was added and samples were incubated at 4°C for a further 4 h. The resin was collected by centrifugation and washed five times in buffer. Bound protein was eluted by boiling the resin in 40 μl of ×2 loading buffer and resolved by SDS gel electrophoresis on a 12.5% polyacrylamide gel. $GABA_A$ α1 subunits were visualized by Western blotting with a α1 antibody (1 : 200, Santa Cruz Biotechnology, Heidelberg, Germany) using enhanced chemiluminescence.

Pharmacology

The following agents were used: (+)bicuculline, bicuculline methiodide, CACA, GABA, picrotoxin, SNAP-5114, NO-711 (all Sigma, UK), and NBQX (disodium salt) and TPMPA (Tocris Cookson, Bristol, UK). CGP 55845 was a kind gift from Dr Wolfgang Froestl (Novartis, Basel, Switzerland). TTX was from Alomone (Jerusalem, Israel). All drugs were made up as ×1000 concentrated stock solutions in distilled water or DMSO ((+)bicuculline, CGP 55845 and SNAP-5114), aliquoted and stored at −20°C. Aliquots were thawed and dissolved in oxygenated aCSF immediately prior to use. Drugs were bath applied to achieve steady-state effects. In some experiments, drugs were applied focally by pressure ejection using low-resistance (~1 MΩ) borosilicate glass electrodes; electrodes were placed directly above the slice and pressure ejection of drugs also served to clean the surface of cells present in the slice.

Results

Characteristics of ionotropic GABA receptor agonist-evoked currents in Purkinje cells

Ionotropic GABA receptor-mediated responses were investigated in mouse cerebellar PCs using agents with increased affinity for $GABA_C$ (which comprise ρ subunits exclusively) over other GABA receptors. We investigated (i) the effects of exogenous application of CACA, an agonist with a > 100-fold greater potency at $GABA_C$ than at $GABA_A$ receptors (Kusama *et al.* 1993); although CACA may activate α6-containing GABA receptors (Wall, 2001), PCs do not express α6 receptors (Wisden *et al.* 1996); (ii) TPMPA, a selective $GABA_C$ antagonist with a greater than 100-fold lower dissociation constant at $GABA_C$ than at $GABA_A$ receptors (Ragozzino *et al.* 1996); and (iii) the natural agonist GABA. Whole-cell currents were recorded in the presence of 1 μM TTX (to minimize action potential-dependent transmitter release), 5 μM NBQX and 5 μM GCP 55845 to reduce any contribution from non-NMDA and $GABA_B$ receptors, respectively.

Bath application of GABA (10–500 μM) evoked concentration-dependent, inward currents at hyperpolarized holding potentials that exhibited desensitization at higher concentrations (Fig. 1*A* and *B*; see also Fig. 1 in online Supplemental material). CACA (50–500 μM) also evoked inward currents in PCs which were of smaller amplitude than those evoked by equimolar concentrations of GABA (Fig. 1*A*, *B* and *C*; see also online Supplemental material Fig. 1). GABA- and CACA-evoked inward currents in PCs were both significantly reduced by application of 100 μM TPMPA (Fig. 1*B*). The effect of TPMPA was measured at the approximated submaximal EC_{20} point of the GABA or CACA dose–response relationship (~50 μM and ~250 μM, respectively, see Supplemental material Fig. 1). Under these conditions, 100 μM TPMPA blocked a significantly larger proportion of current evoked by 250 μM CACA (53.4 ± 7.2%, $n = 5$; Fig. 1*Bb* and *c*) than that evoked by 50 μM GABA (23.9 ± 8.7%, $n = 5$, $P < 0.05$; Fig. 1*Ba* and *c*). These results suggest that CACA-evoked currents had an increased sensitivity to TPMPA in comparison to currents evoked by GABA. $GABA_C$ receptors are characterized by their insensitivity to bicuculline (Johnston, 1996; Bormann, 2000); however, CACA-evoked currents in PCs

were abolished completely by 50 μM bicuculline (Fig. 1*C*; $n = 6$), as were GABA-evoked currents ($n = 5$, data not shown). In addition, CACA-evoked currents were also abolished by 10 μM picrotoxin ($n = 3$, data not shown). Taken together, our data suggest that PCs express a novel subtype of ionotropic GABA receptor with a mixed $GABA_A/GABA_C$ pharmacology.

We next compared the action of CACA and GABA at distinct sites on the PC dendritic arbor. Agonists were focally applied by pressure ejection from a glass pipette (see

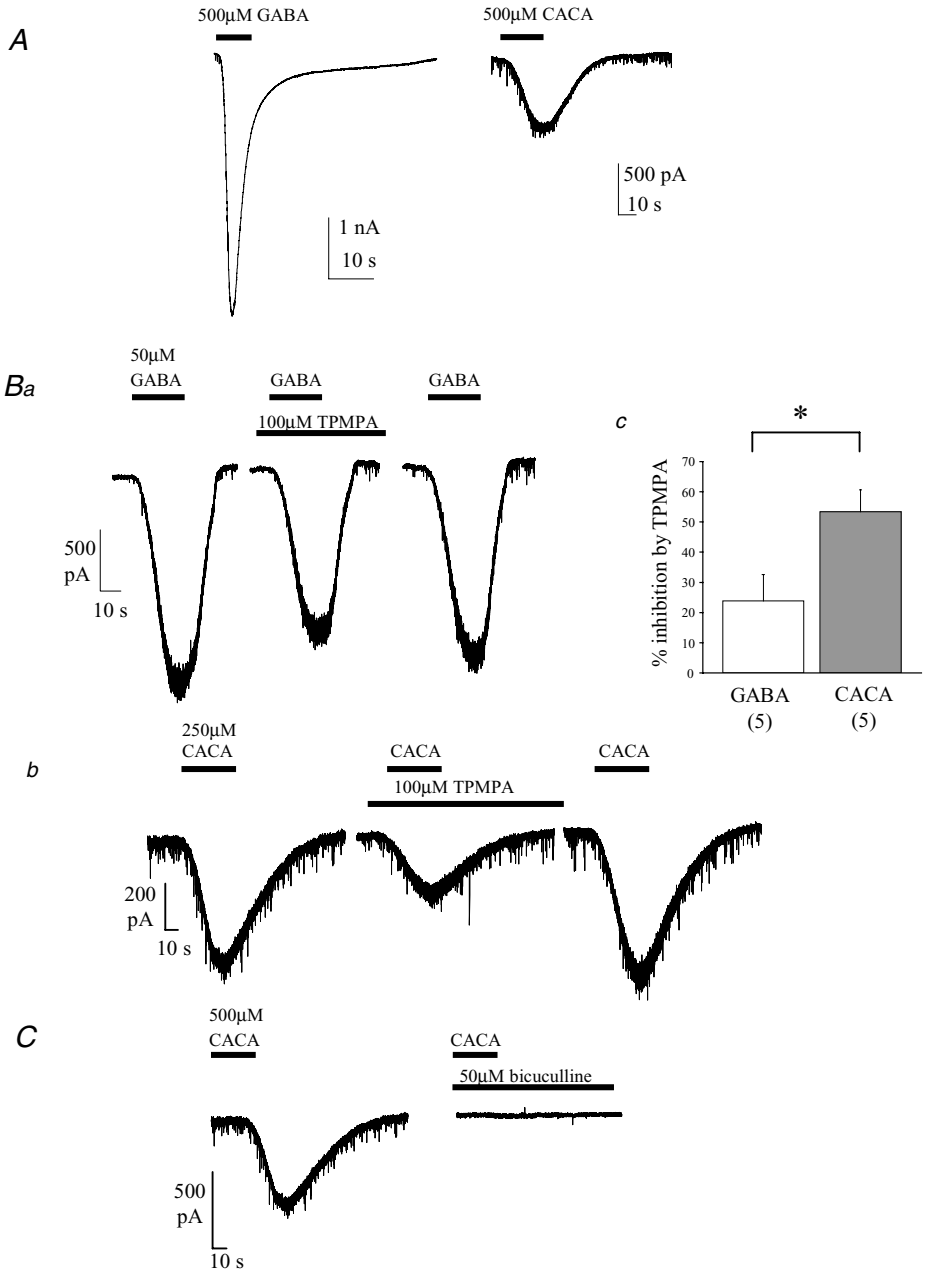

Figure 1. Ionotropic GABA receptors display an unusual pharmacological profile
A, example raw data traces of GABA- and CACA-evoked inward currents. Holding potential ($V_H = -70$ mV). Inward mIPSCs, due to spontaneous, action potential-independent release of endogenous GABA, are also evident. Note also current desensitization induced by GABA. *B*, example raw data traces showing reversible TPMPA-induced reduction of GABA- (*a*) and CACA- (*b*) evoked current, and summary data (*c*; mean ± S.E.M. with number of replicants (*n*) in parentheses). TPMPA produced a larger block of CACA- than GABA-evoked currents (*$P < 0.01$); $V_H = -70$ mV. *C*, example raw data traces showing bicuculline-induced abolition of CACA-evoked current; $V_H = -70$ mV.

Methods) at three distinct sites: the PC soma, proximal dendrites (~30–60 μm away from the soma) and distal dendrites (outer edge of the molecular layer) (marked as 'a', 'b' and 'c', respectively, in Fig. 2). CACA-evoked currents typically displayed a sharp attenuation in amplitude with increasing distance from the soma (Fig. 2*A* and *B*); for example, when CACA was applied to the outer third of the molecular layer (position 'c') there was a significant $90 \pm 2\%$ ($n = 5$, $P < 0.05$) decrease in current amplitude compared to that observed when CACA was applied directly to the soma (position 'a') (Fig. 2*B*). In contrast, GABA (applied in the presence of 100 μM TPMPA to block any ρ subunit-containing GABA receptors) evoked larger, desensitizing currents that displayed far less attenuation; for example, at distal dendritic sites (c) the current attenuation was only $11 \pm 5\%$ ($n = 7$) (Fig. 2*B*). CACA-evoked currents also displayed differences in activation (quantified as time-to-peak values) than observed for GABA: for example, when agonists were applied directly to the cell soma (position 'a'), the mean time-to-peak for CACA (2.84 ± 0.56 s, $n = 5$) was significantly slower than for GABA (0.45 ± 0.03 s, $n = 7$; $P < 0.05$).

Overall, the different response profiles between agonists were consistent with CACA activating a subpopulation of receptors, predominantly expressed at somatodendritic locations, whilst GABA acted non-selectively at receptor sites throughout the PC.

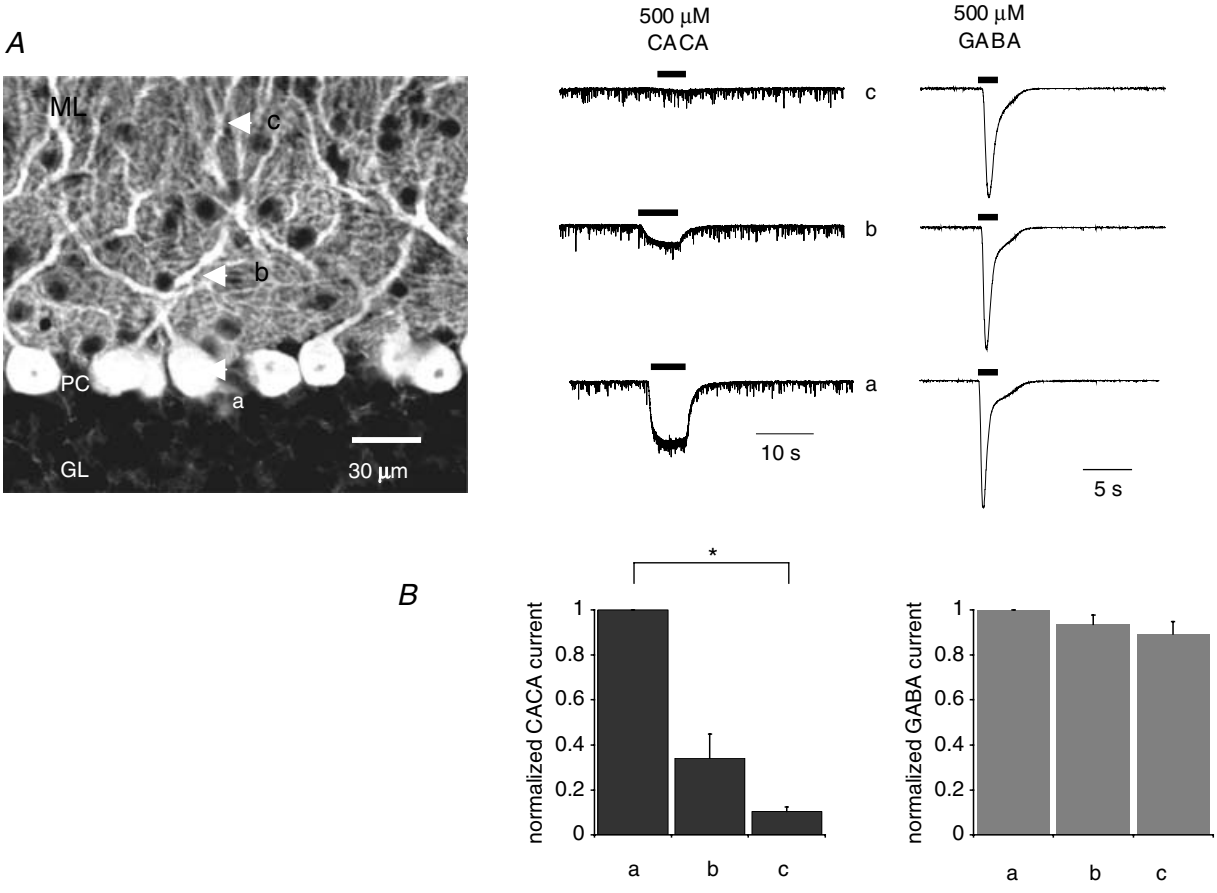

Figure 2. Focal application of ionotropic GABA receptor agonists evoke membrane currents with distinct profiles

A, agonists were focally applied to distinct sites, as approximately indicated by arrows on the left-hand schematic panel, namely directly onto the Purkinje cell (PC) soma (a), ~30–60 μm away from the soma (b), and at the edge of the inner-third of the molecular layer (ML) (c); the granular layer (GL) is also shown for reference. Representative raw data traces for 500 μM CACA and 500 μM GABA are shown for corresponding positions (a–c); responses were normalized to peak agonist effect at the cell soma. CACA responses typically showed a sharp attenuation in amplitude with increasing distance from the soma, whilst GABA responses showed a far less pronounced attenuation. Note also that CACA-evoked currents had a slower onset and much less desensitization in comparison to those evoked by equimolar GABA. *B*, summary data for effects of 500 μM CACA ($n = 5$) and 500 μM GABA ($n = 7$) showing differences in degree of attenuation of current amplitude with increasing distance from the soma, $^*P < 0.05$, unpaired *t* test assuming unequal variance.

Effects of TPMPA on synaptic transmission

We next examined the contribution of TPMPA-sensitive receptors to inhibitory synaptic transmission at cerebellar IN–PC synapses. Bath application of 50 μM TPMPA caused a clear reduction in mean mIPSC amplitude from -58.5 ± 2.8 to -44.1 ± 2.7 pA ($P < 0.001$, $n = 7$), equivalent to a reduction of $24.6 \pm 2.8\%$ (Fig. 3*A* and *Bb*); this decrease was fully reversible (to -56.5 ± 3.1 pA, $n = 7$; $P < 0.01$). These data are reflected by a pooled cumulative frequency plot showing a reversible reduction in mean mIPSC amplitude induced by TPMPA (Fig. 3*Ba*; $n = 7$, each replicant $P < 0.05$, Kolmogorov-Smirnov (KS) test). Interestingly, 50 μM TPMPA also caused a decrease in mean mIPSC frequency, from 11.5 ± 1.6 to 9.0 ± 1.5 Hz ($n = 7$, $P < 0.001$), equivalent to a reduction of $22.6 \pm 3.1\%$ (Fig. 3*A* and *Cb*), with recovery to 10.7 ± 1.4 Hz ($n = 7$, $P < 0.01$). These data are reflected by a reversible increase in the mIPSC inter-event interval induced by TPMPA (Fig. 3*Ca*; $n = 7$, each replicant $P < 0.05$, KS test). Higher concentrations of TPMPA (100 μM) caused no further reduction in mean mIPSC amplitude ($24.6 \pm 1.7\%$) or frequency ($15.2 \pm 3.4\%$).

The reduction in mean mIPSC frequency by TPMPA may be due to smaller amplitude events falling below the detection threshold. However, examination of mIPSC amplitude distribution histograms (Fig. 4*A*) revealed that there was no clear leftward shift in the peak of the distribution (at 18 pA for both control (Fig. 4*Aa*)

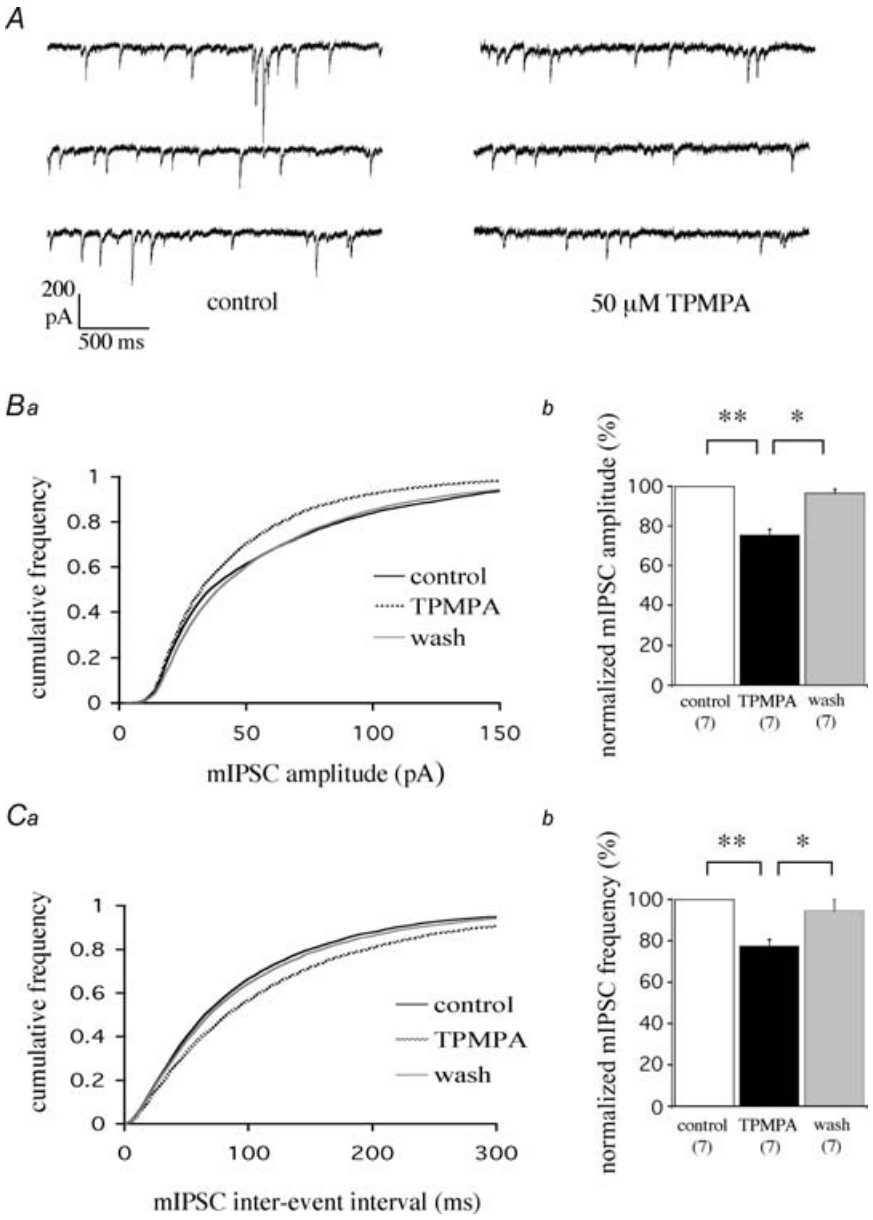

Figure 3. TPMPA reduces synaptic transmission in Purkinje cells
A, example raw data traces showing the inhibition of mean mIPSC amplitude and frequency by TPMPA; $V_H = -70$ mV. *B*, TPMPA-induced reduction in mIPSC amplitude as shown by a pooled cumulative frequency plot ($n = 7$) for mIPSC amplitude (*a*; each replicant $P < 0.05$, KS test), and summary of normalized data (*b*; $^*P < 0.01$, $^{**}P < 0.001$, unpaired *t* test assuming unequal variance). *C*, TPMPA-induced reduction in mIPSC frequency as shown by a pooled cumulative frequency plot ($n = 7$ cells) for mIPSC inter-event intervals (*a*; each replicant $P < 0.05$, KS test), and summary of normalized data (*b*; $^*P < 0.01$; $^{**}P < 0.001$, unpaired *t* test assuming unequal variance).

and TPMPA (Fig. 4*Ab*)). Interneuronal synapses which impinge on the PC soma display large amplitudes and fast rise times (see Llano *et al.* 2000) and are thought to arise from adjacent basket cell inputs. TPMPA caused a clear reduction in large amplitude events (for example, events > 150 pA were largely abolished in 50 μM TPMPA, Fig. 4*Ab*). These data suggest that postsynaptic TPMPA-sensitive receptors are present at somatodendritic IN–PC synapses, consistent with our results after exogenous CACA application (see Fig. 2*A*).

In order to investigate the potential contribution from atypical GABA receptors expressed at presynaptic sites, we determined the effects of TPMPA on mIPSC 10–90% rise times. The distribution of rise times was best described by the sum of two Gaussian components, with a population of fast events (peak $= 0.49 \pm 0.03$ ms, $n = 7$) accounting

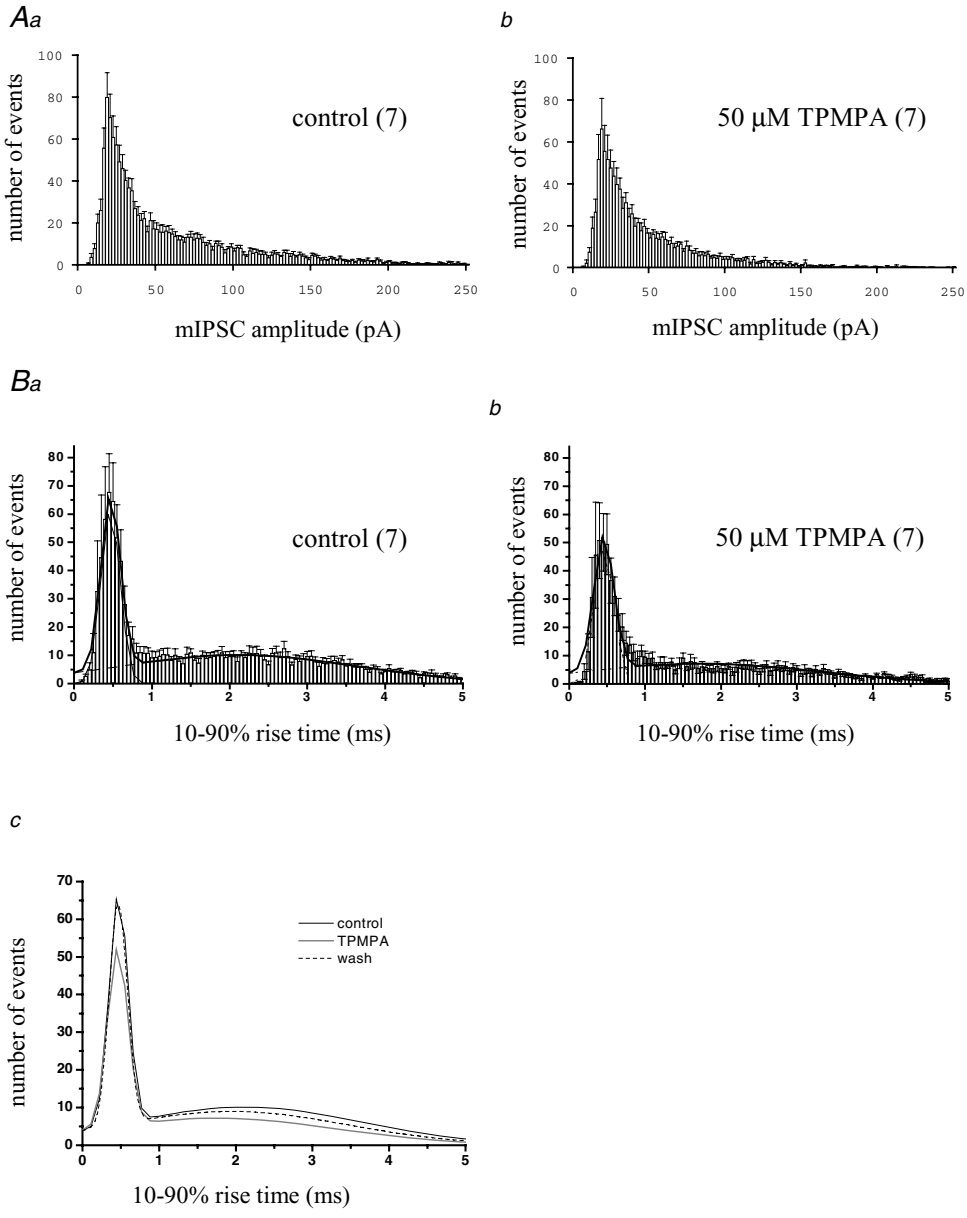

Figure 4. TPMPA has both pre- and postsynaptic actions
A, mIPSC amplitude distribution histograms in control (*a*) and TPMPA-treated cells (*b*). Values are mean ± S.E.M. with number of replicants (*n*) in parentheses. Bin width = 2 pA. TPMPA caused a reduction in larger events, but no shift in the peak response. *B*, 10–90% rise time distribution histograms in control (*a*) and TPMPA-treated cells (*b*). Bin width = 0.05 ms. Data are best described by the sum of two Gaussian components fitted to grouped data, shown for clarity in c: sum of two Gaussians fitted to control, TPMPA and wash data. TPMPA reduced the number of events in both fast and slow rise-time populations.

for 32 ± 4% of the total, and a population of slower events with rise times centred between 1 and 5 ms (= slow events: peak = 2.46 ± 0.27 ms, $n = 7$) (Fig. 4*Ba*). Fast events are likely to originate from synapses on or near the PC soma, arising predominantly from inhibitory basket cell inputs; in contrast, slower events represent a more heterogeneous population, arising from proximal and more distal dendritic sites, with the slower rise time reflecting dendritic filtering of these events (King *et al.* 1993) and reduced fidelity of voltage clamp. Application of 50 μM TPMPA caused a reduction in the number of both fast and slow events (Fig. 4*Bb* and *c*) without any clear shift in either the fast (0.48 ± 0.04 ms, $n = 7$; $P = 0.63$) or slow (2.29 ± 0.33 ms, $n = 7$; $P = 0.25$) peak. This reduction in frequency was consistent with TPMPA acting on presynaptic receptors expressed throughout the PC dendritic arbor.

Overall, these data suggest that TPMPA reduced inhibitory synaptic transmission at IN–PC synapses by acting on a population of ionotropic GABA receptors present at both presynaptic and postsynaptic sites.

Effects of TPMPA on tonic GABA conductances

Homomeric $GABA_C$ receptors in recombinant expression systems are characterized by their lack of desensitization, and also by their high agonist affinity (reviewed by Zhang *et al.* 2001); as such, they represent ideal candidates to mediate tonic inhibitory currents (Semyanov *et al.* 2004; Kullmann *et al.* 2005). Tonic conductances are activated by increases in ambient GABA concentrations; in contrast, phasic currents are activated by vesicular transmitter release (see Farrant & Nusser, 2005). Therefore, we investigated the potential contribution of atypical ionotropic GABA receptors to inhibitory tonic currents. Under basal conditions, application of TPMPA or bicuculline had no clear effect on the holding current required to voltage clamp PCs (data not shown). A tonic conductance could be induced in PCs by the combined application of NO-711 (a selective GABA transporter subtype 1 (GAT-1) antagonist) and SNAP-5114 (a GAT-2/3/4 antagonist). Application of 40 μM NO-711 and 50 μM SNAP-5114 (in the continued presence of the $GABA_B$ antagonist CGP 55845) induced a significant increase in the PC holding current (+48 ± 10 pA, $n = 6$ cells) (Fig. 5*A* and *B*). Baseline root mean square (RMS) noise (4.4 ± 0.2 pA, $n = 6$) was also significantly increased by NO-711/SNAP-5114 application (7.9 ± 0.6 pA, $n = 6$; $P < 0.001$), as also illustrated in Fig. 5*A*. All-point histograms, constructed from 500 ms of event-free recordings, revealed a broader distribution of baseline noise in the presence of NO-711/SNAP-5114 than seen in control (Fig. 5*C*). When NO-711 or SNAP-5114 (both up to 100 μM) was applied individually they were without effect (data not shown). The increase in holding current and baseline noise are characteristic of a tonic conductance and these results suggest that, under conditions of high ambient GABA concentrations, different GAT protein isoforms synergistically modulate extrasynaptic levels of GABA at PC synapses. Application of 50 μM TPMPA had no clear effect on the amplitude of the NO-711/SNAP-5114-induced current (+71 ± 22 pA, $n = 6$, $P = 0.17$; Fig. 5*A* and *B*) or baseline RMS noise levels (9.2 ± 0.9 pA, $n = 6$), as reflected in all-point histograms (Fig. 5*C*). In contrast, subsequent application of 10 μM bicuculline methiodide (BMI) to the same cell reduced the NO-711/SNAP-5114-induced current (−2 ± 17 pA in comparison to control levels, $n = 6$, $P < 0.01$; Fig. 5*A* and *B*), reduced RMS noise (4.4 ± 0.5 pA, $n = 6$, $P < 0.01$; see also Fig. 5*A* and *C*), and also blocked all mIPSCs.

Taken together, our data suggest that atypical GABA receptors do not significantly contribute to tonic GABA conductances in PCs. A further finding here is that the novel receptor identified in PCs can be distinguished from the tonic $GABA_A$ conductance on the basis of sensitivity to TPMPA (but not to bicuculline). Thus, whilst TPMPA caused a clear reduction in inhibitory synaptic transmission mediated by phasic GABA release (Figs 3 and 4), we saw no clear effect of TPMPA on the GAT antagonist-induced increase in holding current or membrane noise.

Immunohistochemical localization and co-immunoprecipitation of GABA ρ and α1 subunits

The electrophysiological actions of CACA and TPMPA may be consistent with the presence of ρ subunit-containing ionotropic GABA receptors in PCs; therefore, we investigated the expression of ρ subunits in mouse cerebellum. Double immunolabelling for ρ subunits and calbindin D_{28K}, a specific marker for PCs, revealed an abundant expression of ρ subunits in the somatodendritic and proximal dendritic compartments of PCs and low-level expression in the distal dendrites (Fig. 6*A*). Moreover, punctate staining was observed throughout the molecular layer and pinceau regions of PCs, indicating the possible expression of ρ subunits on axon terminals of cerebellar basket and stellate cells. The ρ subunit distribution in PCs was consistent with the profile of CACA/TPMPA responses in electrophysiological experiments, supporting the hypothesis that ρ subunit-containing GABA receptors mediate the atypical pharmacological responses reported in PCs. We also compared ρ subunit immunoreactivity with that of the $GABA_A$ α1 subunit, a prominent subunit in PCs (Fritschy & Mohler, 1995; Wisden *et al.* 1996). As illustrated in Fig. 6*B*, in contrast to the more restricted distribution of ρ subunits, α1 subunits were expressed

uniformly throughout the PC soma, proximal and distal dendrites. The α1 staining also correlated well with electrophysiological responses to GABA (Fig. 2), suggesting that α1-containing receptors mediate a major component of GABA action (Fritschy *et al.* 2006).

An attractive possibility, suggested by the mixed $GABA_A/GABA_C$ pharmacological properties and the subunit distribution, is that the atypical receptors represent novel heteromers comprising conventional $GABA_A$ subunits together with ρ subunits. Therefore, we performed co-immunoprecipitation studies to investigate if ρ subunits could coassemble in the cerebellum. Protein complexes were precipitated from cerebellum lysates using ρ antibody and these complexes were found also to contain α1 subunits (Fig. 7); thus, it appears that ρ subunits are expressed in the same neuronal compartment as α1 subunits. Taken together our data suggest that ρ subunits may coassemble with α1 subunits to form functional GABA receptor heteromultimers which contribute to inhibitory synaptic transmission at IN–PC synapses in the cerebellum.

Discussion

Identification of a novel ionotropic GABA receptor in Purkinje cells

The present study identifies an ionotropic GABA receptor subtype in mouse cerebellar PCs with a number of features that distinguish it from other receptors previously described in the cerebellum (Wisden *et al.* 1996). We show that the $GABA_C$-preferring agonist CACA activates a current with sensitivity both to the selective $GABA_C$ antagonist TPMPA and the $GABA_A$ antagonist bicuculline. Focal application of CACA produced effects that differed from those seen with the natural agonist GABA; thus, CACA-evoked currents exhibited a far sharper attenuation with increasing distance from the PC soma, had a slower

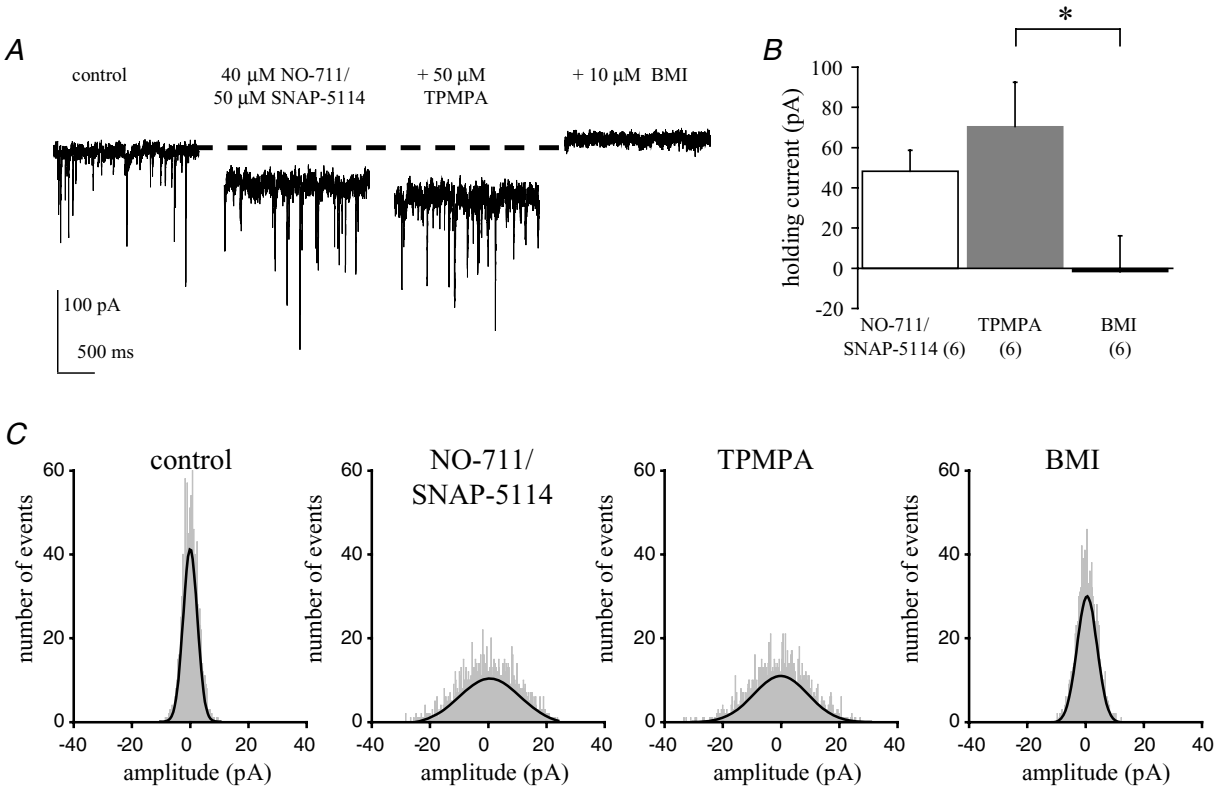

Figure 5. TPMPA has no effect on a NO-711/SNAP-5114-induced tonic GABA current in Purkinje cells
A, example raw data trace of NO-711/SNAP-5114-induced increase in membrane holding current, lack of effect of TPMPA and subsequent block of NO-711/SNAP-5114-induced current by bicuculline methiodide (BMI). Note also the increase in baseline noise induced by NO-711/SNAP-5114 and subsequent reduction in noise by BMI (but not TPMPA); $V_H = -70$ mV. *B*, summary data for effects on holding current. $V_H = -70$ mV, $^*P < 0.01$. *C*, representative all-point histograms of 500 ms event-free recording under different conditions. NO-711/SNAP-5114 caused a broadening in the distribution of baseline noise (shaded region) compared to control cells; whilst TPMPA was without effect, BMI narrowed the noise distribution. Mean values of single Gaussian fits to distributions from 6 cells were 4.2 ± 0.3 pA (control); 7.8 ± 0.6 pA (NO-711/SNAP-5114; $P < 0.01$ *versus* control); 8.7 ± 0.7 pA (TPMPA; $P = 0.10$ *versus* NO-711/SNAP-5114) and 4.5 ± 0.5 pA (BMI; $P < 0.01$ *versus* TPMPA).

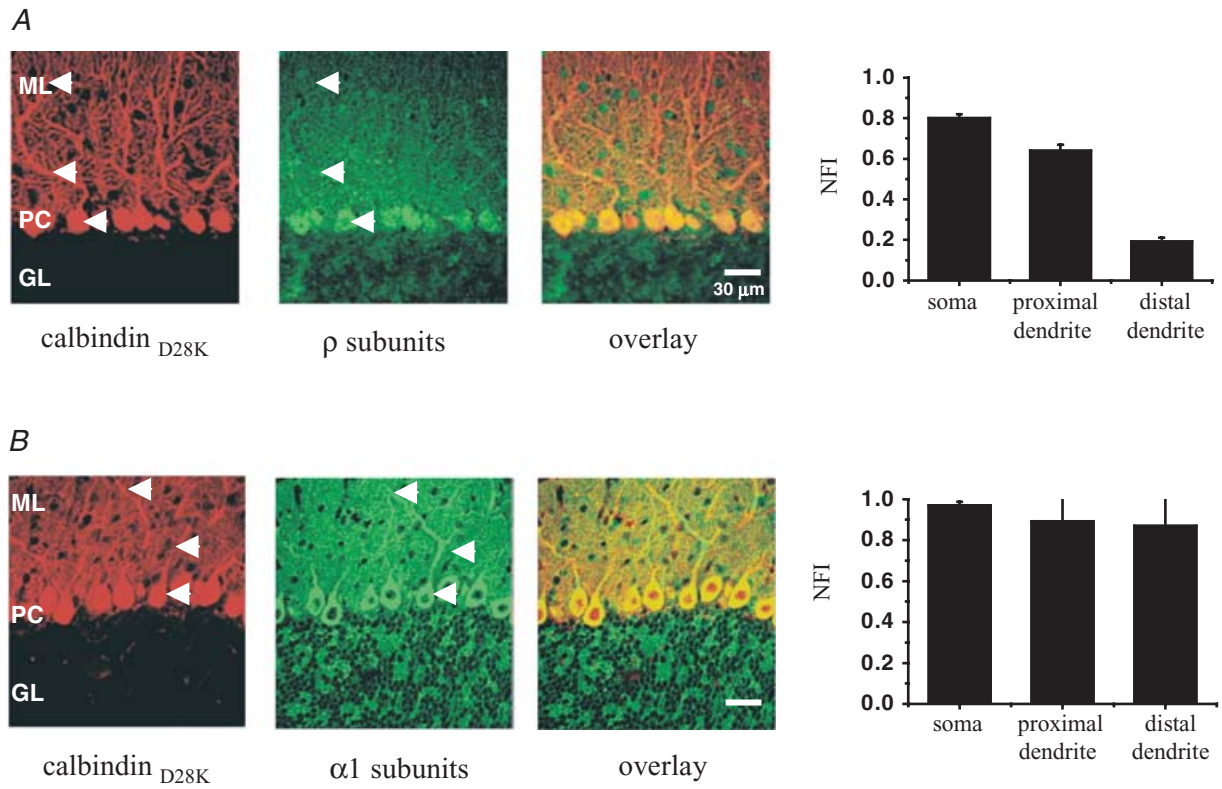

Figure 6. ρ subunit expression in mouse cerebellum, and comparison with α1 subunit expression
Confocal images showing $\rho_{(1\&2)}$ subunit (green; *A*) and α1 subunit (green) together with calbindin D_{28K} (red) immunoreactivity (*B*) in the mouse cerebellar cortex (> 3 weeks). *A*, strong $\rho_{(1\&2)}$ subunit immunostaining can be observed in the somatic and somatodendritic regions of Purkinje cells (PC) while moderate staining is observed in the molecular layer (ML). *B*, strong, uniform α1 subunit immunostaining can be observed in the somatic, proximal and distal dendritic regions of PCs and in the ML. The mean normalized fluorescence intensities (NFI) of $\rho_{(1\&2)}$ and α1 subunit immunoreactivity in somatic, proximal dendritic and distal dendritic locations (see arrows) of cerebellar PCs are also shown ($n = 4$ separate slices each).

time-to-peak and displayed little or no desensitization. We also demonstrate that TPMPA-sensitive receptors mediate a component of GABAergic transmission at IN–PC synapses. TPMPA-induced reductions in mean mIPSC amplitude suggest a postsynaptic localization of receptors, consistent with responses to exogenous CACA. TPMPA also caused a reduction in frequency of events with both 'fast' and 'slow' rise times, consistent with these receptors also being expressed at presynaptic sites.

Ionotropic GABA receptors with a pharmacological profile similar to that reported here have also been described in other neurones in the CNS; thus, CACA-activated currents with sensitivity to both bicuculline and TPMPA have been reported in neurones from the hippocampus (Semyanov & Kullmann, 2002; Hartmann *et al.* 2004) and brainstem (Milligan *et al.* 2004). In this regard, could it be that CACA and TPMPA are less selective for $GABA_C$ over $GABA_A$ receptors than suggested in the literature? CACA has been reported to activate α6-containing GABA receptors (Wall, 2001); however, α6 receptor mRNA or protein is not expressed in PCs (Wisden *et al.* 1996). Moreover, currents mediated by α6 receptors are not blocked by TPMPA (Wall, 2001) and also have a somewhat low sensitivity to bicuculline (Thompson *et al.* 1996). In the present study, CACA-evoked currents had a greater sensitivity to TPMPA than GABA-evoked currents, similar to that observed in hippocampal neurones (Hartmann *et al.* 2004). In addition, the clear differences in response profile between CACA and the natural agonist GABA (see above) are consistent with CACA activating a subpopulation of receptors. We demonstrate that 50 μM TPMPA reduced synaptic transmission at IN–PC synapses, with no further effect at higher (100 μM) concentrations; these levels are well below the reported dissociation constant (~320 μM) for TPMPA at $GABA_A$ receptors in *Xenopus* oocytes expressing cerebral cortex poly(A) RNA (Ragozzino *et al.* 1996). At 100 μM, TPMPA has no action on GABA-evoked currents in recombinant $\alpha1\beta2\gamma2$ receptors (P. Thomas, UCL, personal communication) and is reported to be without effect on $GABA_A$ receptors in native neurones (Wall, 2001; Fischer *et al.* 2005).

In summary, the pharmacological responses to $GABA_C$-preferring agents in cerebellar PCs are consistent

with a role for a novel ionotropic GABA receptor subtype. Overall, these atypical receptors display some properties of $GABA_C$ receptors, but their sensitivity to bicuculline places them formally in the $GABA_A$ receptor family.

Potential contribution of ρ subunits to atypical GABA receptors in Purkinje cells

Having identified a novel ionotropic GABA receptor subtype in PCs with a mixed $GABA_C$/$GABA_A$ pharmacology, we can speculate on its potential molecular composition. An attractive possibility is that responses to $GABA_C$-preferring agents are mediated by ionotropic GABA receptors containing ρ subunits (see Zhang *et al.* 2001). There is extensive evidence for the presence of transcripts for ρ_1 and ρ_2 subunits within the mammalian cerebellum (Boue-Grabot *et al.* 1998; Rozzo *et al.* 2002; Lopez-Chavez *et al.* 2005). More specifically, ρ_1 and ρ_2 mRNAs were shown to be selectively expressed in rat PCs and basket-like interneurones (Rozzo *et al.* 2002). Although, there is not necessarily a linear relationship between mRNA levels and protein expression, Boue-Grabot *et al.* (1998) reported ρ_1 immunoreactivity in the soma and dendrites of PCs. Consistent with the latter study, we demonstrate prominent ρ subunit staining in somatodendritic regions, but with reduced expression at more distal sites. Importantly, the ρ subunit distribution in PCs exhibited a clear correlation with the profile of CACA responses (Fig. 2) and TPMPA action on inhibitory synaptic transmission at IN–PC synapses (Figs 3 and 4), thus indicating that ρ subunit-containing receptors mediate the atypical pharmacological responses in PCs.

Recently, a ρ_2 subunit has been cloned from bovine cerebellum and functionally expressed in *Xenopus* oocytes (Lopez-Chavez *et al.* 2005); homomeric ρ_2 receptors supported bicuculline-insensitive, TPMPA-sensitive currents, as reported for other ρ_2 subunits (Pan *et al.* 2005; Alakuijala *et al.* 2005). The present study suggests that similar homomeric ρ subunit receptors do not contribute to functional responses in cerebellar PCs. In particular, the sensitivity of CACA-evoked currents to bicuculline means that the GABA receptor subtype identified here cannot be ascribed exclusively to the ρ subunit-containing $GABA_C$ class of receptors (as discussed above). Overall, a consensus view of our data may be that atypical ionotropic GABA receptors represent novel heteromers comprising conventional $GABA_A$ subunits together with ρ subunits. In support of this hypothesis, we show that ρ subunits can coassemble with $GABA_A$ receptor α1 subunits in the mouse cerebellum, similar to findings in rat brainstem (Milligan *et al.* 2004). The demonstration that ρ and α1 subunits are present in the same neuronal compartment suggests that these subunits may coassemble in the cerebellum and that heteromer formation is likely to be obligatory for the functionality of ρ subunit-containing receptors in PCs. Moreover, the immunohistochemistry data argue that the presence of ρ subunits would restrict expression of such a complex principally to somatic and proximal dendritic regions of PCs.

Functional relevance of atypical GABA receptors in Purkinje cells

The present study characterizes a population of novel ionotropic GABA receptors which functionally mediate a component of inhibitory synaptic transmission at IN–PC synapses. Moreover, our findings are consistent with a contribution of ρ subunits to the molecular composition of such receptors. The actions of exogenous CACA, together with clear reduction in mIPSC amplitude by TPMPA, suggest a postsynaptic localization. These data are supported by ρ subunit immunoreactivity and identify a new substrate for GABAergic inhibition in the cerebellum.

The potential role of presynaptic ionotropic receptors in transmitter release has been highlighted (Engelman & MacDermott, 2004). As discussed above, the TPMPA-induced reduction in mIPSC frequency suggest that atypical receptors may also have a presynaptic localization; these data are supported by punctate ρ subunit staining consistent with expression on axon terminals of basket and stellate cells. However, the corresponding TPMPA-induced reduction in mIPSC amplitude makes it difficult to assess accurately the relative contribution of any pre- *versus* postsynaptic actions (see also Hartmann *et al.* 2004). The antagonist data suggest that receptors expressed at IN–PC synapses would normally act to increase transmitter release. Anionic presynaptic receptors have been reported to mediate depolarizing responses leading to increased

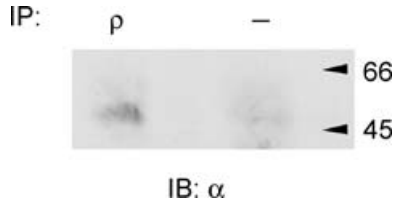

Figure 7. ρ subunits co-immunoprecipitate with α1 subunits in cerebellum lysates
Protein complexes precipitated from cerebellum lysates using anti-$\rho_{(1\&2)}$ and probed with the α1 antibody resulted in a band at the molecular weight appropriate to the α1 subunit. No bands were detected when the $\rho_{(1\&2)}$ antibody was omitted. The data are representative of $n = 3$ separate experiments. IP, immunoprecipitation antibody; IB, immunoblot antibody.

transmitter release (Turecek & Trussell, 2001, 2002), and such actions are likely to be dependent on the reversal potential of permeant Cl^- ions (see Engelman & MacDermott, 2004). Interestingly, although the intracellular Cl^- concentrations in presynaptic terminals are unknown here, the effects of presynaptic $GABA_A$ receptor activation may be either facilitatory or inhibitory amongst individual cerebellar interneurone synapses (Chavas & Marty, 2003), making the overall impact on transmitter release difficult to assess. However, such effects are likely to impinge on presynaptic inhibition mediated by other classes of GABA receptors at these synapses (Harvey & Stephens, 2004).

Homomeric $GABA_C$ receptors have high agonist affinity and are associated with persistently open channels, suggesting a possible contribution of ρ subunit-containing receptors to tonic GABA conductances. We demonstrate that combined application of NO-711 (GAT-1 antagonist) and SNAP-5114 (GAT-2/3/4 antagonist) induced a tonic current in PCs. These data suggest for the first time that different GAT protein isoforms, in addition to GAT-1 (Chiu *et al.* 2005), are involved in modulating extrasynaptic levels of GABA at PC synapses. In this regard, we show that GAT-1 acts synergistically with GAT-2/3/4 isoforms to regulate GABA levels, as also reported for tonic conductances in neocortical neurones (Keros & Hablitz, 2005). The NO-711/SNAP-5114-induced tonic current in PCs was not affected by TPMPA, but was blocked by bicuculline. These data suggest that atypical GABA receptors do not contribute to tonic GABA conductances in PCs, and that distinct GABA receptor subtypes, composed of different combinations of subunits, may underlie tonic and phasic signalling (Farrant & Nusser, 2005). This study also provides further evidence that TPMPA is not acting as a non-selective GABA antagonist. Taken together, our data suggest that atypical ionotropic GABA receptors act selectively to mediate a component of phasic inhibitory synaptic transmission at cerebellar IN–PC synapses.

In summary, we describe a novel population of GABA receptors with a mixed $GABA_A$/$GABA_C$ pharmacology that contributes to functional responses in PCs. We present evidence that GABA ρ subunits contribute to these atypical receptors and may form heteromeric complexes with $GABA_A$ $\alpha 1$ subunits. This study increases our understanding of the diversity of ionotropic GABA receptors within the cerebellum and suggests distinct substrates for inhibition in the CNS.

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Supplemental material

The online version of this paper can be accessed at:
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and contains supplemental material consisting of a figure and legend entitled: Figure 1. Ionotropic GABA receptor agonists evoke membrane currents in Purkinje cells.

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