From synapse to behavior: rapid modulation of defined neuronal types with engineered GABA_A receptors

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In mammals, identifying the contribution of specific neurons or networks to behavior is a key challenge. Here we describe an approach that facilitates this process by enabling the rapid modulation of synaptic inhibition in defined cell populations. Binding of zolpidem, a systemically active allosteric modulator that enhances the function of the GABA_A receptor, requires a phenylalanine residue (Phe77) in the γ2 subunit. Mice in which this residue is changed to isoleucine are insensitive to zolpidem. By Cre recombinase–induced swapping of the γ2 subunit (that is, exchanging Ile77 for Phe77), zolpidem sensitivity can be restored to GABA_A receptors in chosen cell types. We demonstrate the power of this method in the cerebellum, where zolpidem rapidly induces significant motor deficits when Purkinje cells are made uniquely sensitive to its action. This combined molecular and pharmacological technique has demonstrable advantages over targeted cell ablation and will be invaluable for investigating many neuronal circuits.

A classical approach to the study of brain function is selective lesioning. Unfortunately, the interpretation of data from such studies can be confounded by compensatory changes, whereby unrelated systems are recruited to alleviate, if only partially, any deficit. A complementary method involves reversibly silencing, albeit with little or no cell-type selectivity, the activity of a pathway or nucleus through cooling or stereotaxic drug administration (for example, see refs. 1,2). Reversible approaches have advantages over permanent lesioning. First, the effects of acute regional inactivation cannot be easily overcome by compensatory changes, because the inactivated system is altered only briefly (reviewed in refs. 3,4). Second, we can learn, in principle, not only 'where', but also 'when' and for 'how long' a brain region is involved in a given function[3]. Similar considerations apply to the functional dissection of brain circuit components. Thus, to determine how specific cell types influence network properties and contribute to animal behavior, a method is needed for reversibly inactivating a single neuronal type in

a specific brain area[5]. This approach poses two challenges: first, there are many cells of each type; and second, neuronal classes are often dispersed as sparse populations in large volumes (such as subtypes of GABAergic interneurons in the hippocampus).

The effects on brain function of drugs acting on GABA_A receptors show that it is not necessary to silence a cell type to alter network properties or behavior: a modest modulation of inhibitory postsynaptic currents (IPSCs) is sufficient[6–8]. GABA_A receptors, which are present on all neurons in the mammalian brain, are ligand-gated anion-permeable channels and produce fast inhibition. They are commonly formed from two α, two β and a single γ2 subunit (encoded by *Gabrg2*)[6–8]. The α1β2γ2, α2β2γ2 and α3β2γ2 subunit combinations account for most GABA_A receptors; of these, the main subtype is α1β2γ2 (refs. 6–8). Benzodiazepines, benzodiazepine-site ligands (such as the imidazopyridine zolpidem) and anesthetics are positive allosteric modulators of GABA_A receptors[6–10]. These drugs cause a conformational alteration in the GABA_A receptor such that GABA-induced anion flux is enhanced[11]. Elegant studies of 'knock-in' mice, carrying amino acid changes at drug-binding sites in the α or β subunits, show how subtype-specific modulation of GABA_A receptors differentially influences mouse behavior[6,12–15]. These studies are 'subtractive': by changing genes encoding α or β subunits across the whole brain and observing the diminished effect of benzodiazepines, zolpidem or anesthetics on the mouse, the contributions of GABA_A receptor subtypes to specific behaviors (related to anxiety, sedation, anesthetic actions or motor control) can be deduced[6,12–15].

To develop a method for functional dissection of circuit components, we considered reversing this GABA_A receptor knock-in strategy by generating zolpidem-insensitive mice and then genetically imposing zolpidem sensitivity on a selected cell type. We chose zolpidem as the ligand because of the way in which it binds to the GABA_A receptor. One GABA_A receptor subunit, the γ2 subunit, is expressed almost universally in brain[16,17] and is incorporated into most GABA_A receptors. Zolpidem binding occurs throughout the brain[17,18] inside a pocket created between an α subunit (α1, α2 or α3) and the

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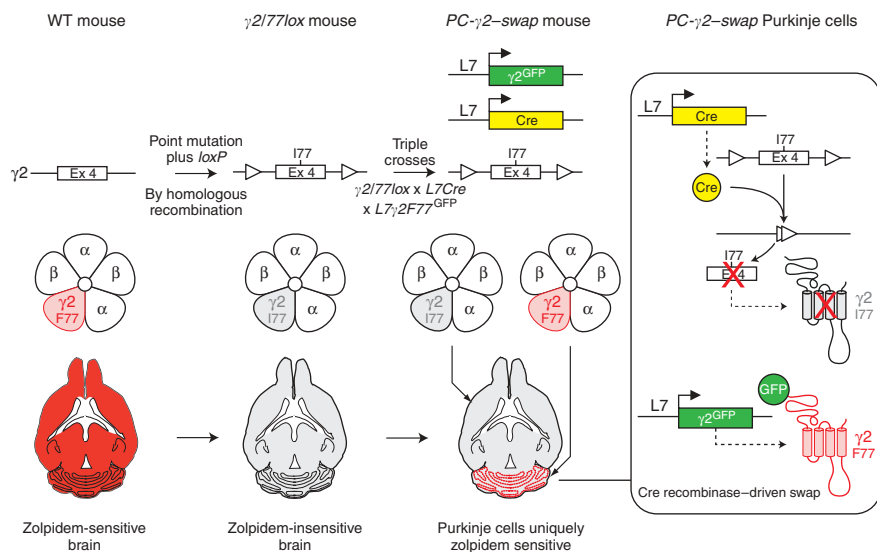
Figure 1 Strategy to restrict zolpidem sensitivity to selected neural types, using Cre recombinase to drive a γ2I77 to γ2F77 subunit swap. The genotype, the pentameric GABA_A receptors formed from α, β and γ2 (γ2F77 or γ2I77) subunits and corresponding brain sections, with zolpidem sensitivity indicated in red, are shown for three groups of mice. Homologous recombination in embryonic stem cells is used to introduce a Phe77 to isoleucine point mutation and to flank exon 4 with loxP sites. The resultant mice are crossed with mice expressing Cre recombinase and the zolpidem-sensitive γ2 subunit under the control of the Purkinje cell–selective L7 promoter to generate PC-γ2–swap mice. In the Purkinje cells of these mice, Cre catalyzes recombination between the loxP sites, resulting in excision and loss of exon 4, and thus deletion of the γ2I77 subunit (gray). In its place, the γ2F77^GFP transgene produces the wild-type zolpidem-sensitive γ2 subunit (red), tagged with GFP at its amino terminus.

γ2 subunit[19]. The γ2 subunit contributes an essential residue (Phe77) to this pocket[20,21]. As shown with cloned GABA_A receptors, if Phe77 of γ2 is mutated to isoleucine, the receptors function normally with respect to activation by GABA but they are no longer modulated by zolpidem[20,21]. Pharmacological, electrophysiological and behavioral tests have shown that mice with an engineered γ2 Ile77 mutation are insensitive to zolpidem[22–24]. The Ile77 mutation does not affect the action of GABA at GABA_A receptors: in γ2 Ile77 mice, miniature IPSCs (mIPSCs) have normal rise times, amplitudes and decay kinetics[22,23].

We therefore considered that, by cell type selectively replacing ('swapping') the Ile77 γ2 subunit in zolpidem-insensitive mice with the zolpidem-sensitive (Phe77) variant, we would be able to use zolpidem to modulate reversibly the activity of any defined neuronal population. To test the feasibility of this method, we chose to use Purkinje cells, which offer straightforward genetic targeting[25] and a quantifiable behavioral readout—namely, motor performance on the rotarod. Purkinje cells provide the only output of the cerebellar cortex. Dendritic and perisomatic synaptic inhibition, provided by stellate and basket cells, respectively, alters the intrinsic activity of Purkinje cells[26] and shapes their response to excitatory synaptic input[27,28]. Purkinje cells express α1β2γ2- and α1β3γ2-type GABA_A receptors[29,30] that underlie fast GABAergic IPSCs.

Here we describe how we have made GABA_A receptors on Purkinje cells uniquely sensitive to zolpidem and then have used this drug to produce rapid and reversible behavioral effects through selective modulation of inhibitory synaptic input. To emphasize the advantages of this approach, we have also generated a static (long-term) knockout of fast GABAergic input onto Purkinje cells by producing cell type–selective deletion of their γ2 subunits, and have compared the effects of the two manipulations on the same behavioral tests under identical conditions.

RESULTS
Explanation of the strategy applied to Purkinje cells
The strategy was to produce mice devoid of zolpidem-sensitive GABA_A receptors by engineering the γ2 subunit gene (*Gabrg2*) such that the codon for Phe77 encoded isoleucine. The gene was also engineered to contain loxP sites, allowing its inactivation by Cre recombinase (**Fig. 1**). Coexpression of the zolpidem-sensitive γ2 Phe77 (hereafter γ2F77)

subunit and Cre recombinase in Purkinje cells should thus lead to a subunit swap, such that Cre-positive, γ2F77-positive Purkinje neurons would not contain γ2 Ile77 (hereafter γ2I77) subunits, which would have been deleted by Cre recombinase (**Fig. 1**). We termed these Purkinje γ2 swap mice 'PC-γ2–swap' mice: only their Purkinje cells should express zolpidem-sensitive GABA_A receptors.

Deletion of the GABA_A receptor γ2 subunit in Purkinje cells
The γ2I77lox line served as both a loxP-flanked (floxed) allele for cell type–specific ablations of GABA_A receptor γ2 subunits (γ2I77lox × cell type–specific promoter driving Cre) and the foundation line for producing cell type–selective zolpidem sensitivity (γ2I77lox × cell type–specific promoter driving Cre × cell type–specific promoter driving γ2F77 subunit 'swapping'; see **Fig. 2**, and **Supplementary Methods** and **Supplementary Fig. 1** online).

The γ2 subunit targets GABA_A receptors to postsynaptic sites and confers full (25–30 pS) single-channel conductance on the receptor[31,32]. To compare and to contrast the dynamic modulation of GABAergic input onto Purkinje cells with the long-term removal of such input, we also generated mice with a Purkinje-cell–specific deletion of γ2, and thus a deletion of synaptic GABA_A receptors. Mice with Cre recombinase expressed selectively in Purkinje cells (L7Cre mice[33]; see **Supplementary Fig. 2** online) were crossed with γ2I77lox mice, producing a Purkinje cell–selective 'static' knockout of synaptic responsiveness to GABA (these mice were termed 'PC-Δγ2' mice; **Fig. 2**).

Purkinje cell–restricted γ2F77^GFP expression
For the zolpidem-sensitive γ2F77 subunit swap, we tagged the γ2F77 subunit at its amino terminus with enhanced green fluorescent protein, eGFP (γ2F77^GFP; see **Supplementary Methods** and ref. 34) to distinguish it from the endogenous γ2I77 subunit. By using the L7 Purkinje cell–specific promoter[25], mice were produced with expression of γ2F77^GFP restricted to Purkinje cells. One of the L7γ2F77^GFP lines (of six screened) gave strong expression of the mRNA specifically in Purkinje cells, as detected by in situ hybridization with a GFP-specific probe. We then made triple crosses (γ2I77lox × L7Cre × L7γ2F77^GFP (see Methods)) to generate PC-γ2–swap mice (**Fig. 2**). At the cellular level, we first established that the γ2F77^GFP subunit in these mice was targeted to GABAergic synapses, and that IPSCs onto Purkinje cells were uniquely sensitive to zolpidem.

Figure 2 Summary of mouse genotypes. *In situ* hybridization autoradiographs show the expression of various GABA$_A$ receptor γ2 subunit mRNA in brain sections from *γ2I77lox*, *PC-Δγ2* and *PC-γ2–swap* adult mice. The corresponding transgene content (genotype) of the mice is indicated above the images (arrowheads, *lox*P sites; Ex 4, exon 4). The *γ2I77lox* and *PC-Δγ2* autoradiographs were obtained with a γ2-specific (exon 4) oligonucleotide. To visualize selectively the *γ2F77*^{GFP} mRNA, the *PC-γ2–swap* image was obtained with a GFP-specific oligonucleotide. Magnifications of the boxed areas are shown at the bottom. In *γ2I77lox* cerebellum, exon 4–containing *γ2I77* mRNA is present in all layers of the cerebellum, but is most evident in the granule cell (Gr) and Purkinje cell (PC) layers. In *PC-Δγ2* cerebellum, *γ2I77* mRNA is absent from the Purkinje cell layer. In *PC-γ2–swap* brains, *γ2F77*^{GFP} mRNA is present only in Purkinje cells. Scale bars, 2 mm and 300 μm.

γ2F77^{GFP} is enriched at GABAergic synapses

Primary GFP fluorescence and GFP immunoreactivity in *PC-γ2–swap* mice was selectively localized to Purkinje cells throughout the cerebellum (**Supplementary Fig. 3** online). The rest of the brain in *PC-γ2–swap* mice (or littermate control mice) had no GFP immunoreactivity. Purkinje cells of *PC-γ2–swap* mice contained granular GFP immunoreactivity in both their cell body (excluding the nucleus) and their proximal dendrites, presumably corresponding to γ2F77^{GFP} associated with endo-membranes (**Fig. 3**). Strongly immunopositive puncta and 0.5–1-μm segments of membrane were present in the soma and the main dendrites (**Fig. 3**). Scattered GFP-positive puncta were also present throughout the molecular layer and presumably corresponded to smaller dendrites of Purkinje cells (**Fig. 3**).

To confirm that the γ2F77^{GFP} subunits were located in GABAergic synapses, we performed triple immunolabeling (**Fig. 3**). The GFP-positive segments always colocalized with the endogenous α1 subunit of the GABA$_A$ receptor. Immunoreactivity for the α1 subunit was also present as short segments and puncta in the molecular layer outside the GFP patches, corresponding to GABAergic synapses on other cerebellar neurons. Most GFP patches, both on Purkinje cells and in the neuropil,
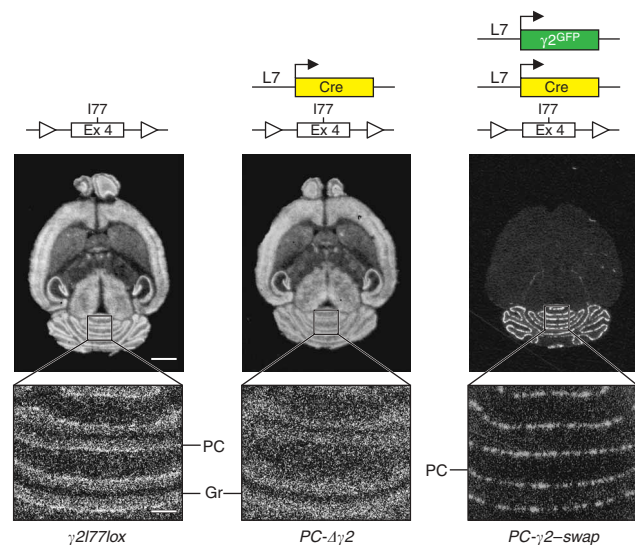
were apposed to glutamic acid decarboxylase (GAD)-immunoreactive structures, representing GABAergic nerve terminals (**Fig. 3**).

Restriction of zolpidem sensitivity to Purkinje cells

To determine the zolpidem sensitivity of GABAergic synaptic transmission in the different mouse lines, we recorded mIPSCs from Purkinje cells and molecular layer interneurons (presumptive stellate cells) in slices prepared from adult mice. As reported previously[22], the properties (amplitude, rise time and decay) of mIPSCs in *γ2I77* mice were similar to those of mIPSCs in *γ2F77/F77* and C57BL/6 mice, confirming that the *γ2F77I* point mutation was a neutral substitution. The *γ2I77lox* mice generated here differ from previously reported *γ2F77I* mice[22] only by the inclusion of a *lox*P site located 5′ to exon 4 (**Supplementary Fig. 1**). Accordingly, mIPSCs recorded in both Purkinje cells (amplitude, 57.5 ± 5.2 pA; 10–90% rise time, 296 ± 11 μs; decay ($τ_w$; see **Supplementary Methods**), 2.7 ± 0.2 ms; charge transfer, 165.4 ± 13.8 fC; $n = 11$ cells) and stellate cells (61.3 ± 10.4 pA; 328 ± 16 μs; 2.6 ± 0.1 ms; 194.4 ± 33.3 fC; $n = 5$) had properties similar to those of the corresponding mIPSCs reported previously[22].

In situ hybridization data suggested that our Cre-*lox*P strategy eliminated the γ2 subunit from Purkinje cells, a condition necessary to facilitate the Ile77 to Phe77 swap (**Fig. 2**). To confirm this elimination, we also recorded from Purkinje cells of *PC-Δγ2* mice and their *γ2I77lox/γ2I77lox* littermates. Whereas mIPSCs were present in every control Purkinje cell ($n = 21$), they were absent from all Purkinje cells of *PC-Δγ2* mice ($n = 10$; **Fig. 4a**), verifying the effective deletion of the γ2 subunit and the elimination of fast GABAergic synaptic input onto Purkinje cells.
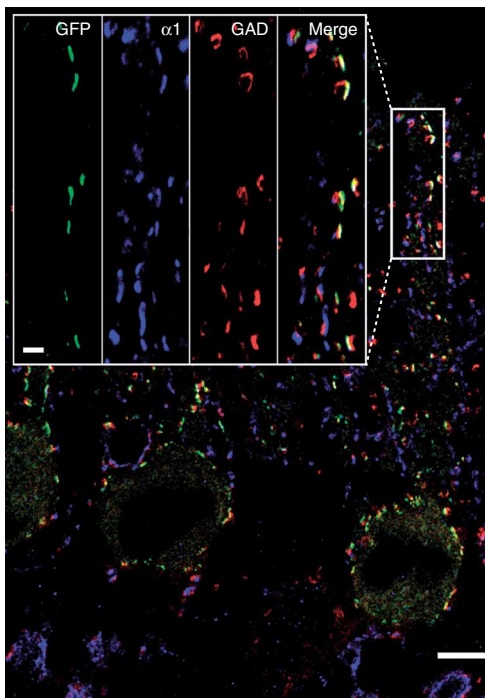
Figure 3 Synaptic expression of the γ2F77^{GFP} subunit in Purkinje cells of adult *PC-γ2–swap* mice. A confocal microscopic image shows immunoreactivity for GFP (green), the α1 subunit of the GABA$_A$ receptor (blue) and GAD (red) in the cerebellum. Note the short segments of GFP immunoreactivity surrounding the somata and dendrites of Purkinje cells. Glomeruli in the granule cell layer are immunonegative for GFP. Inset shows magnified images of the boxed area, indicating that individual GFP patches also contain α1 subunit immunoreactivity and are apposed to GAD-immunoreactive structures corresponding to GABAergic nerve terminals. Other GAD-positive structures apposed to α1 subunit immunoreactive segments represent GABAergic terminals that innervate interneuron (stellate/basket cell) dendrites. Scale bars, 15 μm and 2 μm (inset).
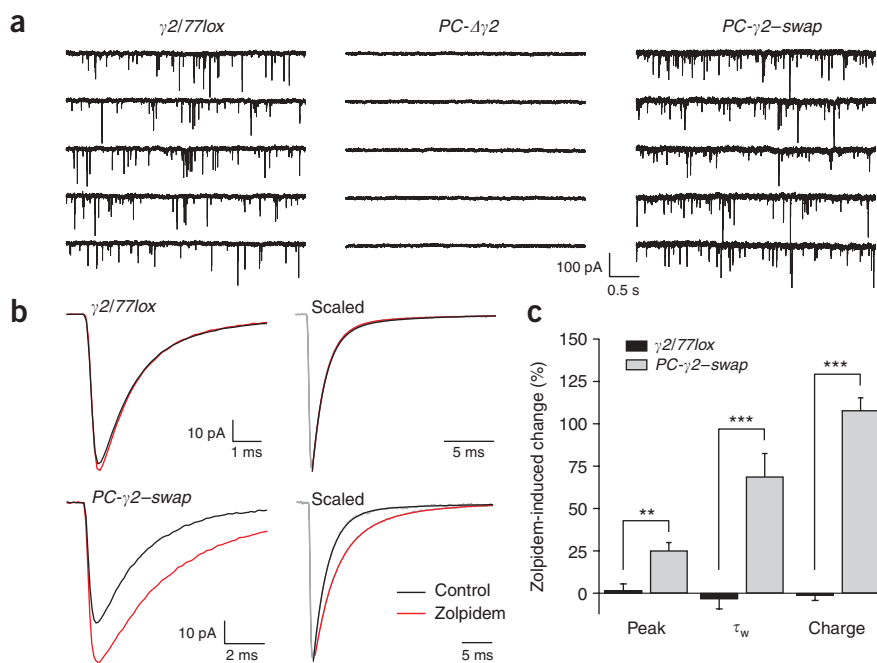
Figure 4 Potentiation of GABA$_A$ receptor–mediated mIPSCs by zolpidem in Purkinje cells of *PC-γ2–swap* mice. (**a**) Continuous 20-s segments of whole-cell recordings (−70 mV) from Purkinje cells of *γ2I77lox* (postnatal day 140), *PC-Δγ2* (P109) and *PC-γ2–swap* (P134) mice. mIPSCs (downward current deflections) are absent from the *PC-Δγ2* recording, but appear normal in the *PC-γ2–swap* recording. (**b**) Superimposed averaged mIPSCs in the absence (black) and presence (red) of zolpidem in *γ2I77lox* (top) and *PC-γ2–swap* (bottom) mice, before (left) and after (right) peak scaling (same cells as in **a**). Smooth red or black lines on the decaying phase of the scaled traces (gray) are fits of double-exponential functions. Zolpidem (1 μM) increased the amplitude and decay of the mIPSCs recorded from the *PC-γ2–swap* Purkinje cell, but had no effect on those recorded from the *γ2I77lox* cell. (**c**) Pooled data showing the effect of zolpidem on peak amplitude, $τ_w$ and charge transfer. Columns indicate means and vertical error bars indicate s.e.m. (*******P* < 0.01, ***P < 0.001, Mann Whitney *U*-test; *n* = 8 and 5 for *γ2I77lox* and *PC-γ2–swap* mice, respectively).

In Purkinje cells of *PC-γ2–swap* mice, mIPSC amplitude (46.4 ± 7.2 pA), 10–90% rise time (307 ± 8 μs), $τ_w$ (3.1 ± 0.2 ms) and charge transfer (158.6 ± 21.7 fC) of mIPSCs (*n* = 8) were not significantly different from their corresponding measures in *γ2I77lox* mice (see above; all *P* > 0.05). These data verify effective deletion of the γ2I77 subunit and its replacement with the γ2F77^{GFP} subunit. Although basic mIPSC properties in the two lines were indistinguishable, modulation of mIPSCs by zolpidem was, as predicted, markedly different. Zolpidem had no effect on the amplitude or kinetics of mIPSCs in Purkinje cells of *γ2I77lox* mice; in the *PC-γ2–swap* mice, by contrast, it significantly raised the amplitude (49.7 ± 9.9 to 62.0 ± 12.1 pA), prolonged $τ_w$ (3.2 ± 0.2 to 5.3 ± 0.2 ms) and increased the charge transfer (178.9 ± 29.0 to 377.5 ± 13.6 fC) of Purkinje cell mIPSCs (*n* = 5, all *P* < 0.05; **Fig. 4b,c**). The restoration of zolpidem sensitivity was restricted to Purkinje cells: zolpidem had no effect on the properties of mIPSCs recorded from stellate cells in slices from the same mice (**Supplementary Fig. 4** online).

In female *PC-γ2–swap* mice, transgene expression was mosaic and about 50% of Purkinje cells expressed the transgene. Most probably, the *L7γ2F77*^{GFP} transgene integrated on an X chromosome. Accordingly, some Purkinje cells from females showed a complete lack of mIPSCs (equivalent to that seen in the *PC-Δγ2* mice). In cells where the γ2F77^{GFP} transgene was expressed, however, mIPSCs were normal and showed zolpidem sensitivity comparable to that of mIPSCs in γ2F77/F77 or C57BL/6 mice[22]. In male mice, at least 90% of Purkinje cells swapped their γ2 subunits, and only male mice were used for behavioral tests involving zolpidem.

Dynamic modulation of inhibition produces motor deficits

What role does GABAergic inhibition of Purkinje cells have in motor coordination? Unexpectedly, mice with no fast synaptic inhibition onto Purkinje cells (*PC-Δγ2* mice) had no motor disabilities and showed neither ataxia nor tremor. They performed the rotarod task to the same standard as their littermate controls (**Fig. 5a**). Given this observation, we considered what would be the behavioral effect of acute modulation of fast GABAergic input onto Purkinje cells when *PC-γ2–swap* mice

were given zolpidem. Without drugs, *PC-γ2–swap* mice showed no motor disabilities: they learned the rotarod task to the same standard as their littermate controls. On the first day of training, a small difference in latency to fall was detected, but this difference disappeared during subsequent training days (**Fig. 5a**).

Intraperitoneal administration of zolpidem to *PC-γ2–swap* mice produced no sedative or overt ataxic effect. Within 1–5 min of drug injection, mice were tested on the rotarod. Zolpidem significantly reduced the ability of trained *PC-γ2–swap* mice to stay on the rotating rod (**Fig. 5b**; repeated measures analysis of variance (ANOVA), $F_{1,84}$ = 10.4, *P* < 0.001; zolpidem treatment × mouse line interaction, $F_{1,84}$ = 47.6, *P* < 0.001; Newman-Keuls post hoc test, *P* < 0.001). With the initial dose of 3 mg/kg (body weight) of zolpidem, the effect was highly significant (**Fig. 5b**) and almost saturated. No greater effect was produced by a cumulative dose of 9 mg/kg of zolpidem. In an additional experiment, a single dose of 12 mg/kg of zolpidem shortened the latency to fall in the *PC-γ2–swap* mice from 144 ± 30 s to 96 ± 48 s (*P* < 0.05), indicating that the lowest doses used produced the maximal motor-impairing effect. These effects of zolpidem were produced without sedation. This observation was expected, because the γ2I77 subunit, retained in all cells other than Purkinje cells, prevents the normal effects of zolpidem on, for example, sleep regulating centers[13,22]. The *γ2I77lox* littermates were all unaffected.

To further test fine motor coordination and balance, we used the 'walking beam' test, whereby mice have to cross a suspended rod. Without zolpidem, there were no differences between *PC-γ2–swap* mice and their *γ2I77lox* littermates (repeated measures ANOVA, $F_{1,148}$ = 2.01, *P* > 0.05, data not shown). After administration of zolpidem (12 mg/kg), however, *PC-γ2–swap* mice needed significantly more time to traverse the beam (treatment × mouse line interaction, repeated measures ANOVA, $F_{1,26}$ = 9.84, *P* < 0.01; Newman-Keuls post hoc test, *P* < 0.001 for mouse line). By contrast, their *γ2I77lox* littermates were not affected (**Fig. 5c**).

The behavioral effects produced by zolpidem in *PC-γ2–swap* mice could be blocked by flumazenil (Ro 15-1788), which antagonizes

Figure 5 Motor performance on a rotarod and horizontal beam after systemic administration of zolpidem. (**a**) The rotarod was accelerated from 5 to 30 r.p.m. over 180 s. Data for $\gamma2I77lox$ littermate control ($n = 20$), $PC\text{-}\gamma2\text{–}swap$ ($n = 20$) and $PC\text{-}\Delta\gamma2$ ($n = 5$) mice are presented as the daily means ± s.e.m. of six trials for males and females combined (*$P < 0.05$ for $PC\text{-}\gamma2\text{–}swap$ versus $\gamma2I77lox$ control mice on training day 1, repeated measures ANOVA and Newman-Keuls post hoc tests). (**b**) Effects of zolpidem on motor performance. Shown is the effect of cumulative doses (3+3+3 mg/kg i.p., total 9 mg/kg) of zolpidem on the rotarod performance of $\gamma2I77lox$ control ($n = 9$) and $PC\text{-}\gamma2\text{–}swap$ ($n = 5$) male mice. The rotarod was accelerated from 5 to 30 r.p.m. over 180 s (***$P < 0.001$ between mouse lines and $P < 0.001$ versus saline injection within the $PC\text{-}\gamma2\text{–}swap$ line, one-way ANOVA and Newman-Keuls post hoc tests). Data are presented as means ± s.e.m. (**c**) Effect of 12 mg/kg of i.p. zolpidem on the walking beam performance of $\gamma2I77lox$ control ($n = 9$) and $PC\text{-}\gamma2\text{–}swap$ ($n = 5$) male mice. Mice were pre-trained for 4 d to traverse the 100-cm long, 0.8-cm diameter wooden beam. Columns represent means and vertical error bars s.e.m. (***$P < 0.001$ between mouse lines and $P < 0.001$ versus saline injection within the $PC\text{-}\gamma2\text{–}swap$ line, two-way ANOVA and Newman-Keuls post hoc tests).
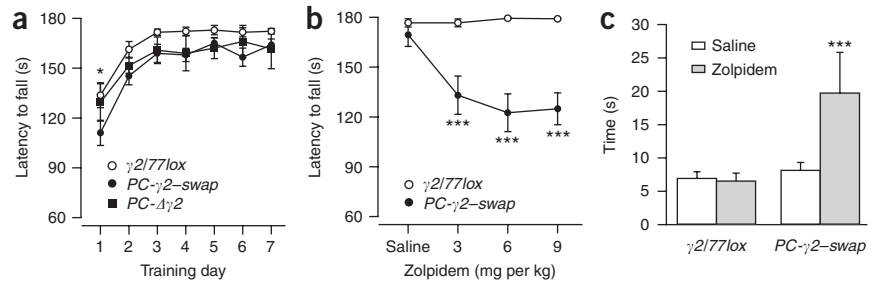
the binding of zolpidem to GABA$_A$ receptors containing the γ2F77 subunit[24]. $PC\text{-}\gamma2\text{–}swap$ mice were pre-treated with vehicle or 15 mg/kg of flumazenil and then given a single dose of 6 mg/kg of zolpidem. Immediately afterwards they were tested on the rotarod. The latency to fall in the vehicle pre-treated group ($n = 6$) shortened from 173 ± 13 s to 137 ± 13 s (one-way ANOVA and Newman-Keuls post hoc test, $P < 0.05$), but stayed the same in the group pre-treated with flumazenil (170 ± 13 s; one-way ANOVA, $P > 0.05$ $n = 4$).

DISCUSSION
Rapid modulation avoids adaptational change
Motor coordination on the accelerating rotarod is a complex task, involving many brain areas, cell types and synapses. Here, to demonstrate the feasibility of a reversible method for manipulating defined synapses, we have isolated the contribution of GABA$_A$ receptor–mediated synaptic input onto Purkinje cells to this task. Dynamically modifying GABAergic input, through acute systemic administration of zolpidem to $PC\text{-}\gamma2\text{–}swap$ mice, produced a result different from that of static long-term removal of the input (through knockout of synaptic GABA$_A$ receptors in $PC\text{-}\Delta\gamma2$ mice). Under the same test conditions, static deletion did not reduce performance on the rotarod, whereas fast and reversible enhancement of the same GABAergic input produced acute debilitating effects. These contrasting results reflect, in the chronic intervention, the adaptive abilities of neurons and networks to engage alternative strategies to achieve similar ends (for example, see refs. 4,35,36). Nevertheless, chronic interventions can be informative. When no compensation occurs, ablating a component can demonstrate its necessity for a particular process. Indeed, $PC\text{-}\Delta\gamma2$ mice do have profound deficits in the consolidation of cerebellar motor memories, indicating that fast inhibition onto Purkinje cells provides essential functions that cannot be compensated for after chronic ablation (P. Wulff et al., unpublished data). Thus, pursuing both types of study (chronic ablation and reversible intervention) gives a fuller picture of how a 'component' (here, a defined synaptic input to a target cell) contributes to a 'system' (here, cerebellar motor coordination).

Several methods to study network components, using either fast (millisecond) or relatively slow (hours to days) interventions, are available. Light-activated ion channels, receptors or pumps enable ultrafast pinpoint activation or inhibition of neurons[37,38]. Although excellent for use in vitro or in vivo for transparent species, these approaches are likely to be less applicable to the study of behavior and distributed populations of cells in vivo in mammals. The Drosophila allatostatin receptor system, which couples to mammalian potassium GIRK channels, allows reversible silencing of neurons within seconds and, like light-activated channels, is not limited to a particular vertebrate species[39,40]. The agonist peptide allatostatin, however, requires intracerebral injection and its uneven diffusion in the brain may cause difficulties for in vivo studies. Methods that block synaptic transmission have very slow kinetics[41,42], but these approaches seem to be useful for long-term (days) synaptic silencing[41,42]. By contrast, our strategy does not silence or activate cells. Instead, it confers the ability to modulate reversibly the action of an endogenous neurotransmitter and thus it more subtly interferes with network function. These various methods are complementary, potentially enabling a full range of physiological processes to be investigated.

We have shown that zolpidem, by promoting a restricted and specific increase in fast GABAergic inhibition from molecular layer interneurons onto Purkinje cells, decreases the ability of mice to carry out complex motor tasks. To gauge the success of the strategy, it is important to know the extent of motor deficit that one might expect to see after such Purkinje cell–specific modulation. Zolpidem is a potent hypnotic, acting on GABA$_A$ receptors in brain areas that regulate sleep[13]. We have shown previously that in wild-type mice zolpidem produces severe sedation and deficits in rotarod performance[22]; given this sedation, we cannot estimate what fraction of the performance deficit originates from modulation of the cerebellum and motor centers. However, comparable data from studies on benzodiazepine-insensitive mice, notably α1H101R mice, are available. In cerebellar circuits α1βγ2 is the predominant GABA$_A$ receptor type[29,30], and in a wild-type cerebellum zolpidem will modulate IPSCs onto most cell types. From studies of mice (α1H101R) with α1βγ2 receptors made insensitive to diazepam[12,14,15], the net 'contribution' of modulating α1βγ2-type GABA$_A$ receptors to rotarod behaviors (as judged by decreased latencies to fall) is about 25% (this reflects modulation of α1 receptors throughout the brain, not just in the cerebellum[14,15]). We found here that zolpidem, acting on α1βγ2 receptors in Purkinje cells alone, induced a 33% decrease in latency to fall, broadly fitting with results from α1H101R mice[14,15]. The effects seem to be greater than those obtained after blockade of GABA release from Purkinje cell terminals in the deep cerebellar nuclei (15% decrease in latency to fall with the rod rotating at 80 r.p.m.; ref. 41).

Methodological considerations
Our approach is designed to enable zolpidem, through the cell type–selective modulation of GABA$_A$ receptor–mediated synaptic currents,

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to be used in behaving animals to alter the activity of defined neuronal populations. For this strategy, active GABAergic synapses are needed. The method requires that targeted neurons express $\alpha 1$, $\alpha 2$ or $\alpha 3$ subunits together with any β subunit and the $\gamma 2$ subunit. Many cell types do indeed express predominantly $\alpha 1\beta\gamma 2$- or $\alpha 2\beta\gamma 2$-type receptors; for example, various subtypes of hippocampal GABAergic interneuron have zolpidem-sensitive GABA$_A$ receptors[10,23]. In Purkinje cells, zolpidem modulation primarily affects synaptic inhibition[22]; these cells do not show a tonic GABA$_A$ receptor–mediated current[30,43]. Other cell types, however, may express zolpidem-sensitive extrasynaptic GABA$_A$ receptors (containing $\alpha 1$, $\alpha 2$ or $\alpha 3$, β and $\gamma 2$ subunits) with GABA-dependent or GABA-independent tonic activity[7,44]. If such cells were targeted by our strategy, the activity of these receptors would also be enhanced by zolpidem.

Zolpidem can be used both in slices and in behaving animals; it can be administered by intraperitoneal (i.p.) injection or by stereotaxic injection into the brain. Zolpidem binds preferentially to $\alpha 1$-containing GABA$_A$ receptors ($\alpha 1\beta 2\gamma 2$ or $\alpha 1\beta 3\gamma 2$; inhibition constant, $K_i = 20$ nM), but at higher concentrations it will modulate receptors containing $\alpha 2$ or $\alpha 3$ subunits ($\alpha 2\beta\gamma 2$ or $\alpha 3\beta\gamma 2$; $K_i = 400$ nM). Because zolpidem can be given at doses of up to 30 mg/kg in $\gamma 2I77$ mice with no effects[22], it can be used to modulate cell types that express only $\alpha 2$- and $\alpha 3$-containing GABA$_A$ receptors. Zolpidem tartrate is the preferred preparation because of its maximal water solubility. An advantage of our method is that zolpidem has a rapid and short-acting effect: zolpidem maximally occupies its binding sites in mouse brain within minutes of i.p. injection and has a half-life of 20 min (ref. 45).

In addition to zolpidem, various other GABA$_A$ receptor modulatory drugs, such as the β-carbolines DMCM and β-CCM, the imidazobenzodiazepine bretazenil, the cyclopyrrolone zopiclone, and the quinolines PK 8165 and PK 9084, depend on the Phe77 residue of the $\gamma 2$ subunit for binding[20,21,24]. On neuronal membranes isolated from $\gamma 2I77$ mouse brains, the binding affinities of these drugs are greatly reduced[24]. Some of these drugs, for example DMCM and β-CCM, are inverse agonists at the benzodiazepine binding site of GABA$_A$ receptors and decrease receptor function[11,20,21]. The usual effect of DMCM administration (seizures) is abolished in $\gamma 2I77$ mice[46]. Thus, in principle, both zolpidem and an inverse agonist can be used to bidirectionally modulate fast inhibition onto neurons that have undergone a $\gamma 2$ subunit swap.

For its full potential to be realized, the method that we describe will require mouse Cre lines with suitable cell type–specific expression. The brain region– and cell type–specific gene expression patterns identified in, for example, the Allen Brain Atlas project will eventually aid this aim[47]. We used triple crosses of mouse lines, but the procedure could be streamlined by using Cre-mediated switching of an Ile77 to a Phe77 exon or viral delivery of a $\gamma 2F77$-*ires*-*Cre* transgene into $\gamma 2I77lox$ brains. We anticipate that the unique features of our method will be invaluable for investigating the contribution of specific neurons to brain function and behavior.

METHODS

Transgenic mouse production and breeding. All procedures were done in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986, had ethical approval from the Regierungspräsidium Karlsruhe or were approved by the Laboratory Animal Committee of the University of Helsinki.

Production of the $\gamma 2I77lox$ and $L7\gamma 2F77^{GFP}$ lines and mouse genotyping are described in the **Supplementary Methods**. Mice homozygous for the $\gamma 2I77lox$ gene and heterozygous for the $L7\gamma 2F77^{GFP}$ transgene were crossed with mice homozygous for $\gamma 2I77lox$ and heterozygous for an *L7Cre* transgene[33]. Littermates of the following genotypes were used for the experiments: $\gamma 2I77lox$/ $\gamma 2I77lox$/$L7\gamma 2^{GFP}$/$L7Cre$ (*PC-$\gamma 2$–swap*), $\gamma 2I77lox$/$\gamma 2I77lox$/$L7Cre$ (*PC-$\Delta\gamma 2$*) and $\gamma 2I77lox$/$\gamma 2I77lox$ (littermate controls).

In situ hybridization. We carried out *in situ* hybridization as described[16]. The probe sequences to exon 4 of the $\gamma 2$ subunit and *GFP* are given in the **Supplementary Methods**.

Triple labeling immunohistochemistry. We used the following primary antibodies: affinity-purified rabbit antibody to the $\alpha 1$ subunit[48] (0.5 μg/ml), guinea-pig antiserum to EGFP (1:100; a gift from R. Tomioka, RIKEN, Japan) and sheep antibody to GAD (1:1,000; ref. 49). See **Supplementary Methods** for detailed protocol and image acquisition.

Electrophysiology. Whole-cell recordings were made from neurons in parasagittal slices of cerebellar vermis prepared from adult male and female mice. A bicarbonate-buffered 'external' solution and a CsCl-based 'internal' solution (near symmetrical Cl$^-$ concentration) were used. mIPSCs were recorded at -70 mV in the presence of 5 μM CNQX (6 cyano-7-nitroquinoxaline-2,3-dione disodium), 10 μM D-AP5 and 0.5–1.0 μM tetrodotoxin. In all tests, synaptic currents recorded under such conditions were completely blocked by the GABA$_A$ receptor antagonist SR 95531 (gabazine, 10–20 μM; data not shown). All recordings were made at near physiological temperature (34–38 °C), except those from *PC-$\Delta\gamma 2$* mice and their littermates, which were made at room temperature (22–25 °C). For full details of the recording and analysis, see **Supplementary Methods**.

Zolpidem (Tocris Bioscience) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mM and was used at 1 μM (final DMSO 0.002%). Zolpidem was applied to each slice only once, and its effect on mIPSCs was determined only after equilibration for at least 2 min.

Statistical tests were done with GraphPad Prism software (Prism 3.0, GraphPad Software Inc.). Except where noted, treatment groups and mouse lines were compared with two-tailed paired or unpaired Student's t-tests, as appropriate. Where data were non-normally distributed (Shapiro-Wilk test), non-parametric tests were used (Wilcoxon Matched pairs or Mann-Whitney U-test). The level of significance was set at $P < 0.05$.

Mouse behavioral experiments. Male and female $\gamma 2I77lox$ ($n = 20$; all bred as littermates from the compound crosses, see above), *PC-$\gamma 2$–swap* ($n = 20$) and *PC-$\Delta\gamma 2$* ($n = 5$) mice aged 4–10 months (20–45 g) were used in tests. Basic behavioral characterization was done as described in the **Supplementary Methods**.

Rotarod and horizontal beam tests were done as described[50]. The mice were given an i.p. injection of zolpidem (Sanofi-Synthelabo AB) 1–5 min before the rotarod and walking beam tests (1–4 trials). Zolpidem tartrate was crushed from a tablet, suspended in physiological saline and injected at 10 ml/kg (body weight). Drug dosing was either acute (one bolus injection) or cumulative (3 plus 3 plus 3 mg/kg, 5 min between injections). We used cumulative dosing of zolpidem with repeated rotarod testing in male mice. There was a minimum washout period of 3 d between pharmacological tests. Full details are given in the **Supplementary Methods**.

Statistical analyses of motor behavior were performed with SPSS 12.0.1 (SPSS Inc.) or GraphPad Prism software. Treatment groups and mouse lines were compared with either repeated measures ANOVA or one-way ANOVA, followed by Newman-Keuls post hoc test or Dunnett's test. In all statistical tests, the significance level was set at $P < 0.05$.

Note: Supplementary information is available on the Nature Neuroscience website.

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