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Molecular and synaptic organization of GABA$_A$ receptors in the cerebellum: Effects of targeted subunit gene deletions

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Abstract

GABA$_A$ receptors form heteromeric GABA-gated chloride channels assembled from a large family of subunit genes. In cerebellum, distinct GABA$_A$ receptor subtypes, differing in subunit composition, are segregated between cell types and synaptic circuits. The cerebellum therefore represents a useful system to investigate the significance of GABA$_A$ receptor heterogeneity. For instance, studies of mice carrying targeted deletion of major GABA$_A$ receptor subunit genes revealed the role of α subunit variants for receptor assembly, synaptic targeting, and functional properties. In addition, these studies unraveled mandatory association between certain subunits and demonstrated distinct pharmacology of receptors mediating phasic and tonic inhibition. Although some of these mutants have a profound loss of GABA$_A$ receptors, they exhibit only minor impairment of motor function, suggesting activation of compensatory mechanisms to preserve inhibitory networks in the cerebellum. These adaptations include an altered balance between phasic and tonic inhibition, activation of voltage-independent K$^+$ conductances, and upregulation of GABA$_A$ receptors in interneurons that are not affected directly by the mutation. Deletion of the α1 subunit gene leads to complete loss of GABA$_A$ receptors in Purkinje cells. A striking alteration occurs in these mice, whereby presynaptic GABAergic terminals are preserved in the molecular layer but make heterologous synapses with spines, characterized by a glutamatergic-like postsynaptic density. During development of α1$^{0/0}$ mice, GABAergic synapses are initially formed but are replaced upon spine maturation. These findings suggest that functional GABA$_A$ receptors are required for long-term maintenance of GABAergic synapses in Purkinje cells.

Key words: *Knockout mice, benzodiazepine, Purkinje cell, granule cell, GABAergic synapse, interneurons*

Introduction

Owing to its apparent simplicity and stereotyped organization, with a clear segregation of cell types in distinct layers, the cerebellum represents an excellent structure to investigate the anatomical and functional organization of neuronal networks and major neurotransmitter systems. Most synaptic inhibition in the cerebellum is mediated by GABA$_A$ receptors, which are highly abundant despite the overwhelming presence of glutamatergic synapses formed by parallel fibers on Purkinje cell spines. GABA$_A$ receptors belong to the superfamily of ligand-gated ion channels, along with nicotinic acetylcholine receptors, glycine receptors and 5-HT$_3$ receptors (1). They form heteropentameric chloride channels gated by GABA and modulated by several clinically relevant drugs, including benzodiazepines, barbiturates, neurosteroids, and ethanol (2–4). They are assembled for a large family of subunits (α1–6, β1–3, γ1–3, δ, ε, π, and θ in mammalian brain), of which 13 are expressed in the cerebellum (5). Accordingly, the molecular organization of GABA$_A$ receptors is only partially understood and the functional significance of this diversity in cerebellar inhibitory circuits is slowly starting to emerge.

Targeted deletion of GABA$_A$ receptor subunit genes by homologous recombination has contributed much to our understanding of GABA$_A$ receptors. Focusing on the cerebellum, numerous studies have investigated molecular, pharmacological and functional properties of GABA$_A$ receptors that are less amenable for analysis in other brain regions (6). The present review gives a broad overview of recent reports on the molecular and synaptic organization of GABA$_A$ receptors in cerebellar cortex. It highlights major findings from knockout mice, with a particular focus on compensatory mechanisms allowing preservation of network organization and function despite the loss of a major constituent of synaptic transmission.

Organization of GABA$_A$ receptors in the cerebellar cortex

The heterogeneity of GABA$_A$ receptors in the cerebellar cortex stands in striking contrast to the apparent simplicity of GABAergic circuits, which are formed by four types of interneurons (stellate and basket cells in the molecular layer, Golgi cells and Lugaro cells in the granule cell layer), in addition to Purkinje cells (PC). Each type of interneuron is activated by granule cells (parallel fiber synapses) and innervates specific targets, including other interneurons (Figure 1) (7). In total, 13 GABA$_A$ receptor subunits have been detected immunochemically, including α1–α6, β1–3, γ1–3, and δ (5). The cellular distribution pattern has not yet been established for all these subunits but the major subunit combinations comprise α1βxγ2, α6βxγ2, (α1)α6βxδ, α3βxγ2, as well as α2βxγ1 (x indicates that the β subunit variants have not been determined). Despite this plethora of subunits, the majority of cell types express a limited repertoire of GABA$_A$ receptor subtypes (Figure 1). For example, PC express exclusively α1βxγ2-GABA$_A$ receptors; stellate/basket cells, α1βxγ2 and α3βxγ2; Golgi cells α3βxγ2; Bergmann glia, α2/βx/γ1 (8–14). The notable exception comes from granule cells, which express multiple receptor subtypes, despite receiving GABAergic input only from Golgi cells (15). Most
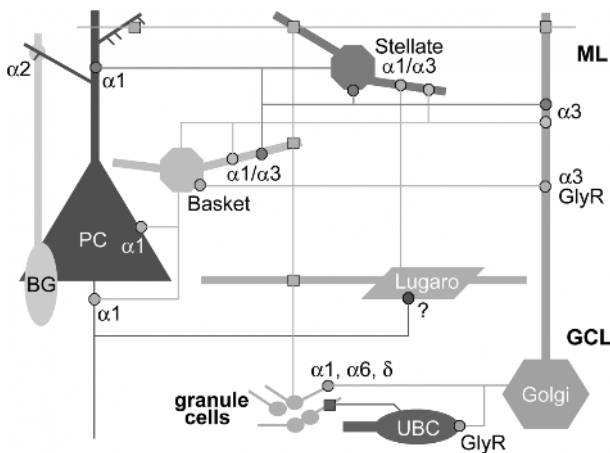
of these receptors are assembled from various combinations of α1, α6, β2, β3, γ2 and δ subunit (8,12,13,16). No information is available for Lugaro cells and unipolar brush cells (17), and conversely, several subunit expressed at low levels (α4, α5, γ3) have been identified by Western blotting only (5,18).

Depending on the cell type and subunit combination, GABA$_A$ receptors can be located postsynaptically or extrasynaptically, where they mediate phasic and tonic inhibition, respectively (19). Postsynaptic GABA$_A$ receptors in cerebellar neurons largely are co-localized with gephyrin, a cytoplasmic protein that is exclusively present in symmetric synapses (12). By immunofluorescence staining, they are recognized as bright clusters distributed on dendrites and somata, as well as on glomeruli of the granule cell layer (see Figure 3). Using this approach, the synaptic localization of α1- and α3-GABA$_A$ receptors was shown in the molecular layer (12); in glomeruli, α1- and in part α6-GABA$_A$ receptors can also be detected postsynaptically, confirming ultrastructural studies using postembedding immunogold electron microscopy (20–22). In whole cell patch clamp recordings of PC, spontaneous IPSCs of large amplitude predominate over EPSCs, indicating that PC are under powerful control from stellate and basket cells (23). These synaptic currents are mediated by GABA$_A$ receptors with no evidence for a contribution by strychnine-sensitive glycine receptors (24). In addition, PC also carry extrasynaptic GABA$_A$ receptors diffusely distributed on the cell surface and are modulated by tonic inhibition, notably due to GABA release from basket cells (25). Similar, high frequency and high amplitude sIPSCs have been well characterized also in stellate cells (26,27), underscoring the major role played by postsynaptic GABA$_A$ receptors in the molecular layer.

In granule cells, a major fraction of GABA$_A$ receptors is associated with the δ subunit (29,30), forming extrasynaptic receptors with a high affinity for GABA (31,32) and diffusely distributed on the soma and dendrites (12,22,28). The presence of the δ subunit, which does not co-assemble with the γ2 subunit, is determinant for the location of GABA$_A$ receptors relative to postsynaptic sites and for their functional properties. Electrophysiologically, tonic inhibition in granule cells is independent of Golgi cell action potentials (33,34). Rather, it is produced mainly by non-vesicular release of GABA and is modulated by type 1 and type 3 GABA transporters (33). So far, it is not known whether this complex regulation of tonic inhibition is related to the heterogeneity of extrasynaptic GABA$_A$ receptors in granule cells. However, it should be emphasized that the role of the δ subunit is forming receptors mediating tonic inhibition is not restricted to the cerebellum, but also occurs in thalamus and hippocampus (35,36). Furthermore, these receptors

Figure 1. Schematic organization of GABAergic circuits and GABA$_A$ receptors in the cerebellar cortex. Each cell type is indicated with a different color. Dendrites are shown in thick lines and axons in thin lines. GABAergic synapses (octogons) are shown with the color corresponding to the presynaptic cell and the main GABA$_A$ receptor subtype(s) or glycine receptors (GlyR) present postsynaptically are indicated. Glutamatergic synapses are denoted with squares. Note that Lugaro cells innervate all cell types in the molecular layer except PC. In turn, the only inhibitory input they receive comes from PC. It is not known which GABA$_A$ receptor subtype(s) they express. Basket/stellate cells express both α1- and α3-GABA$_A$ receptor, but it is not established whether they are segregated between afferents from other basket/stellate cells and from Lugaro cells. Bergman glial cells (BG) express an unusual GABA$_A$ receptor subtype, containing the α2 and γ1 subunit, which is enriched at sites of contact with PC. Abbreviations: GCL, granule cell layer; ML, molecular layer; UBC, unipolar brush cell.

are a major target for neurosteroids, which reduce neuronal excitability by enhancing tonic inhibition mediated by δ-GABA$_A$ receptors (37).

A distinct population of 'extrasynaptic', diazepam-insensitive GABA$_A$ receptors, with a probably unique subunit composition in brain ($\alpha 2\beta x\gamma 1$), is found in Bergman glia cells (10,11,14). These receptors are most abundant during early postnatal maturation and modulate K$^+$ conductances that are transiently expressed by immature Bergmann glia. As shown by immunoelectron microscopy in adult brain (10), GABA$_A$ receptors are most concentrated in glial processes wrapping PC somata and dendrites, in particular in the vicinity of symmetric synapses formed by basket cells. However, they are also concentrated in processes in contact with PC spines, in which GABA$_A$ receptors are occasionally found. This highly regulated location suggested a role for sensing GABAergic synaptic function in PC (10).

Synaptic inhibition in the cerebellum is also mediated, to a lesser extent, by glycine receptors (38,39). A majority of Golgi and Lugaro cells have a dual GABAergic/glycinergic phenotype (40,41). The specificity of transmission in their postsynaptic targets is ensured by the differential expression of receptors. Thus, spontaneous IPSCs in granule cells are mediated selectively by GABA$_A$ receptors (39), whereas Golgi cells, Lugaro cells, unipolar brush cells, and possibly other interneurons exhibit either glycinergic or mixed glycinergic/GABAergic currents (Figure 1) (42,43). At least in Golgi cells, GABA$_A$ and glycine receptors can co-exist within a given postsynaptic site (42).

A distinctive feature of postsynaptic GABA$_A$ receptors in the molecular layer is their colocalization with the dystrophin-glycoprotein complex (DCG), including β-dystroglycan, dystrophin, and α-/β-dystrobrevin (44–47). The DGC is required for proper maturation or maintenance of postsynaptic GABA$_A$ receptors in PC, as shown by a decreased number and size of clusters in mutant mice lacking either dystrophin, or both α- and β-dystrobrevin. As a consequence, synaptic inhibitory input is reduced on PC of mice lacking dystrophin (48). While the relevance of the DGC in specific GABAergic synapses is unclear, it is required for proper expression of long-term depression in PC (49), suggesting a role in synaptic plasticity. A similar situation occurs in the cerebral cortex and hippocampal formation, where large subsets of inhibitory synapses are associated with the DGC (45). In the hippocampus, absence of the DGC leads to alterations of short- and long-term synaptic plasticity (50,51). However, the DGC cannot be essential for the formation of GABAergic synapses or clustering of GABA$_A$ receptors, since it is absent in many other brain areas, such as thalamus, basal ganglia, tectum, etc. Furthermore, dystrophin expression occurs after

formation of GABAergic synapses during postnatal development (45).

The maturation of GABA$_A$ receptors during ontogeny of the cerebellum has been investigated mainly in granule cells and in PC (52,53). The subunit composition and pharmacological profile of GABA$_A$ receptor in granule cells are developmentally regulated and these changes are activity-dependent (54–57). A transient expression of the $\alpha 2$ and $\alpha 3$ subunit occurs at early postnatal stages (58–60), whereas the $\alpha 6$ and δ subunit, which are expressed selectively in granule cells (61), is delayed (11,55,60) and parallels the formation of functional GABAergic synapses from Golgi cells (62–65). This maturation pattern contrasts with that of PC, which express a prominent $\alpha 1$ subunit immunoreactivity at birth, before having formed a monolayer and being innervated by basket and stellate cells (66,67).

Effects of targeted subunit gene deletion on cerebellar GABA$_A$ receptors

Targeted deletions of eight GABA$_A$ receptor subunits ("full knockout" mice) have been reported so far in the literature ($\alpha 1$, $\alpha 3$, $\alpha 5$, $\alpha 6$, $\beta 2$, $\beta 3$, $\gamma 2$, δ). Among these only four have been analyzed for their effects on assembly of GABA$_A$ receptors and function of the GABAergic system in the cerebellum ($\gamma 2$, $\alpha 6$, δ, $\alpha 1$). The major alterations reported in these studies concern the function and pharmacology of the remaining GABA$_A$ receptors, which are partially altered due to loss of an additional subunit that has an obligatory association with the deleted subunit (for example, δ subunit in $\alpha 6^{0/0}$ mice) or due to compensatory changes in expression of remaining subunit(s) (68,69). Unexpectedly, even targeted deletions affecting major GABA$_A$ receptor subtypes, such as $\alpha 1$-GABA$_A$ receptors, have only minor behavioral consequences, suggesting that widespread adjustments take place within and outside the GABAergic system to ensure preserved function.

Most $\gamma 2^{0/0}$ mice die shortly after birth and a small percentage survive up to the third postnatal week (70). In these mice, there is an almost complete loss of $\alpha 1$ subunit and gephyrin clusters in the cerebellum, in line with the requirement of the $\gamma 2$ subunit for postsynaptic aggregation of GABA$_A$ receptors and gephyrin (71,72). No specific compensation with another $\gamma 2$ subunit variant was observed, indicating indirectly that the $\gamma 1$ and $\gamma 3$ subunits do not contribute significantly to neuronal GABA$_A$ receptors in the mouse cerebellum.

Deletion of the $\alpha 6$ subunit gene, which is exclusively expressed in cerebellar granule cells, caused a 50% overall reduction of GABA$_A$ receptors in the cerebellum (73), underscoring the relative abundance of the $\alpha 6$ subunit in these cells.

Unexpectedly, this mutation caused the disappearance of the δ subunit protein (74). However, since mRNA levels for the δ subunit were not affected, this finding suggested that the δ subunit protein requires the $\alpha6$ subunit for assembly in a receptor complex and is degraded rapidly in the absence of this mandatory partner. As a consequence, deletion of a single subunit led to the loss of multiple GABA$_A$ receptor subtypes in granule cells. This conclusion was confirmed by an autoradiographic analysis showing marked alterations of the pharmacological profile of GABA$_A$ receptors in the granule cell layer of $\alpha6^{0/0}$ mice, best explained by a concurrent loss of $\alpha6\beta x\gamma2$- and $\alpha6\beta x\delta$-GABA$_A$ receptors (69). No compensation by the $\alpha1$ subunit was detected in glomeruli or in the molecular layer of $\alpha6^{0/0}$ mice (73), indicating that the $\alpha1$ and $\alpha6$ subunit are regulated independently, although their genes are present in the same chromosomal cluster (75). Interestingly, while $\alpha6^{0/0}$ mice display no major spontaneous phenotype, a clear motor deficit could be shown upon treatment with diazepam, which could be reversed by flumazenil, indicating that it was mediated by the remaining, mainly postsynaptic, GABA$_A$ receptors in these mice (76).

Deletion of the δ subunit gene did not result in a loss of GABA$_A$ receptors in the cerebellum, as shown by autoradiography and Western blot analysis. An increase in receptors containing the $\gamma2$ subunit was noted, along with $\alpha6$ and βx, suggesting that $\gamma2$ and δ compete for assembly with these subunits in granule cells from wildtype mice (77). Functionally, a loss of modulation of sIPSCs by the neurosteroid THDOC selectively occurs in granule cells, but not stellate cells of $\delta^{0/0}$ mice (78), underscoring the importance of δ-GABA$_A$ receptors for the in vivo action of neurosteroids (79). Behaviorally, deletion of the δ subunit gene produces no overt impairment in motor function or motor learning, similarly to the $\alpha6$ subunit gene (76). This observation should not lead to the conclusion that tonic inhibition mediated by extrasynaptic GABA$_A$ receptors in granule cells is irrelevant. Rather, the lack of phenotype is due to compensatory upregulation of voltage-independent K$^+$ channels (two-pore-domain K$^+$ channels TASK-1 and TASK-3), which increases the membrane conductance to the same extent as the tonic inhibition mediated by $\alpha6$-GABA$_A$ receptors in wildtype mice (80).

Like for the $\alpha6$ subunit, targeted deletion of the $\alpha1$ subunit gene has major consequences for GABA$_A$ receptors in the cerebellum. A profound loss of binding sites for benzodiazepine ligands occurs (81). This decrease is mirrored in the 50% reduction of $\beta2,3$ and $\gamma2$ subunit proteins in crude membrane extracts from the cerebellum (18,81), pointing to a decrease in the assembly of GABA$_A$ receptors in the absence of the $\alpha1$ subunit. This phenotype is not unique to the cerebellum but has been observed in other brain regions, as well (82). Despite the major reduction in the number of GABA$_A$ receptors, $\alpha1^{0/0}$ mice display no anatomical anomaly and no gross behavioral impairment (83–85). The most obvious deficits include a mild impairment of motor coordination and a kinetic and postural tremor that is reminiscent of essential tremor in human; this tremor was responsive to drug therapies that alleviate symptoms of essential tremor in patients and is likely related to the loss of $\alpha1$-GABA$_A$ receptors in multiple stations of the motor system, including the cerebellum (86).

Compensatory changes in GABA$_A$ receptor subunit expression in $\alpha1^{0/0}$ mice

A major feature of $\alpha1^{0/0}$ mice, despite the 50% overall loss of GABA$_A$ receptors, is the compensatory up-regulation of subunits that are normally expressed at low levels, which might partially replace the missing $\alpha1$-GABA$_A$ receptors. In particular, expression of the $\alpha3$, $\alpha4$ and $\alpha6$ subunit proteins is increased, as shown by two independent *in vivo* studies (18,81). *In vitro*, the loss of $\alpha1$-GABA$_A$ receptors induced an increase in $\alpha6\beta x\delta$-receptors (and possibly $\alpha4\beta x\delta$), suggesting that tonic inhibition partially compensates for the loss of synaptic receptors (87). Functionally, these adaptations are reflected in the maintenance of postsynaptic currents in granule cells, but with altered kinetic properties. In particular, the decay time constants of remaining receptors are prolonged, reflecting the substitution of $\alpha1$-GABA$_A$ receptors by another subtype. Since $\alpha1$-GABA$_A$ receptors have faster kinetics than those containing the $\alpha2$ or $\alpha3$ subunits (88,89), this factor was taken as an index for the sustained presence of these subunits in mature granule cells of mutant mice (87,90).

In contrast to granule cells, no compensation occurs in PC which lose all functional GABA$_A$ receptors in mutant mice. Patch clamp recording analyses showed a complete loss of spontaneous and evoked IPSCs and no compensation by strychnine-sensitive glycine receptors (9,86). No evidence for tonic inhibition could be found either in PC of $\alpha1^{0/0}$ mice. A detailed immunohistochemical analysis was therefore undertaken to understand how this loss of GABA$_A$ receptors affects the synaptic organization of the GABAergic system in the molecular layer (18). On the cellular level, this study confirmed the reduction of $\beta2,3$ and $\gamma2$ subunit expression, as well as the upregulation of the $\alpha3$ subunit, mainly in interneurons of the molecular layer (Figure 2). Subcellularly, the loss of $\alpha1$ subunit causes a strong, but only partial decrease of gephyrin clusters in the molecular layer (Figure 3). Staining for the $\alpha3$ subunit, which is normally present in a minor fraction of gephyrin clusters, revealed a prominent
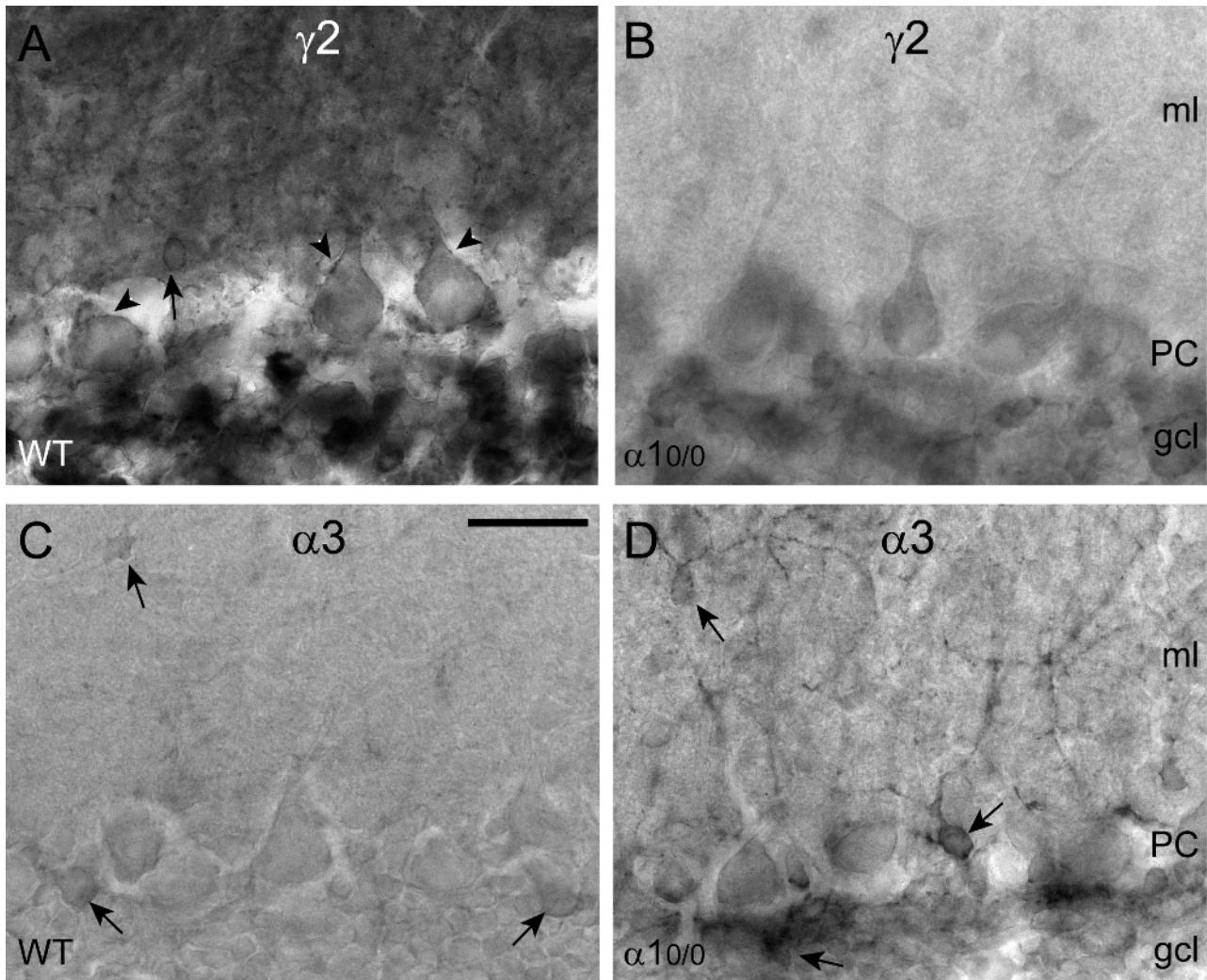
Figure 2. Differential regulation of GABA$_A$ receptor subtypes in the cerebellar cortex of $\alpha1^{0/0}$ mice, as shown by immunoperoxidase staining for the $\gamma2$ and $\alpha3$ subunit. (A) Strong and widespread expression of the $\gamma2$ subunit in the molecular layer (ml), in PC and in the granule cell layer (gcl) of wildtype (WT) mice. The arrow points to a strongly labeled interneuron and arrowheads indicate $\gamma2$ subunit immunoreactivity on PC, outlining the cell surface. (B) Marked reduction of $\gamma2$ subunit staining in a section from a mutant mouse. Note that PC are unlabeled. The granule cell layer (gcl) also appears weakly labeled, and interneurons in the ml are almost undetectable. (C–D) Striking up-regulation of the $\alpha3$ subunit, which in wildtype is weakly expressed in Golgi cells and some ml interneurons (arrows). In $\alpha1^{0/0}$ mice, the $\alpha3$ subunit staining outlines individual interneurons in the ml and in the gcl (arrows), whereas PC remain unlabeled. Scale bar, 50 μm.

increase in the number of $\alpha3$ subunit clusters in the molecular layer, most likely representing postsynaptic receptors in stellate/basket cells and Golgi cell dendrites (Figure 3). These observations are in line with a study in which miniature IPSPs recorded from stellate cells in whole-cell patch clamp experiments had slower kinetics in $\alpha1^{0/0}$ mice than in wildtype, compatible with the expression of $\alpha3$-GABA$_A$ receptors (88). The changes in $\alpha3$ subunit expression were specific for this subunit, since no alteration of the $\alpha2$ subunit (present in Bergman glia) was observed, whereas the $\alpha4$ and $\alpha5$ subunits were not detected immunohistochemically in the cerebellum of either wildtype or $\alpha1^{0/0}$ mice (18).

The comparison of compensatory changes in GABA$_A$ receptor expression between the granule cell layer and the molecular layer in $\alpha1^{0/0}$ mice highlights major features of the regulation of these receptors: (i) In granule cells expressing multiple GABA$_A$ receptor subtypes, an increase of the remaining subunit(s) might replace the missing subunit. However, this compensation is only partial, since postsynaptic GABA$_A$ receptors colocalized with gephyrin and mediating mainly phasic inhibition appear to be replaced by extrasynaptic receptors mediating tonic inhibition. This finding is supported by the decreased $\gamma2$ subunit staining in the granule cell layer. A similar situation has been observed in neurons of the thalamic ventrobasal complex, in which postsynaptically clustered $\alpha1$-GABA$_A$ receptors are replaced by extrasynaptic $\alpha4$-GABA$_A$ receptors lacking the $\gamma2$ subunit and gephyrin (18). Taken together, these observations suggest that GABA$_A$ receptor subtypes are not interchangeable within a given type of neuron and that the lack of interaction of $\alpha4$ or δ-GABA$_A$ receptors with
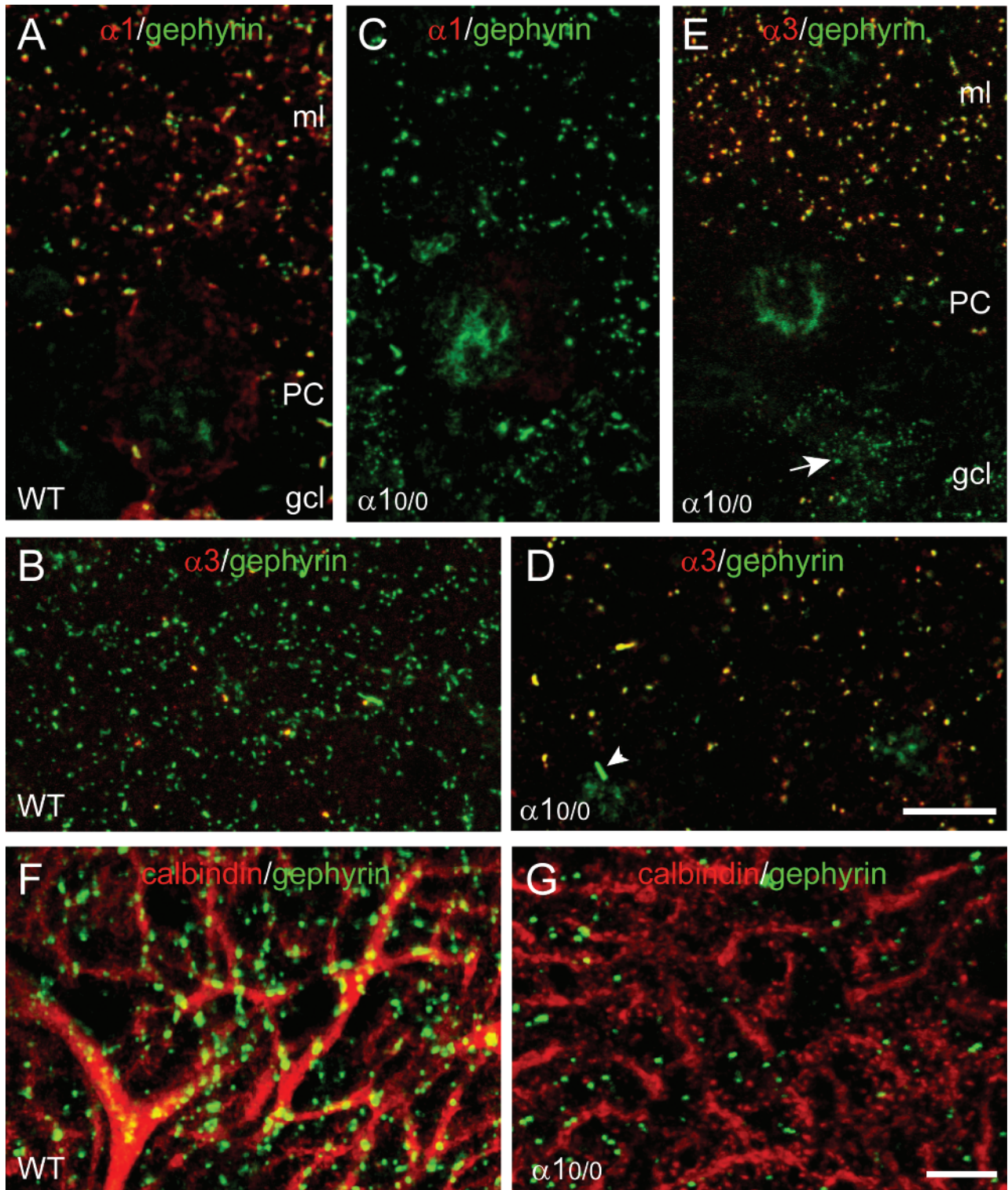
Figure 3. Partial disruption of gephyrin clustering and up-regulation of the $\alpha 3$ subunit in the molecular layer (ml) of $\alpha 1^{0/0}$ mice. Double immunofluorescence staining with the markers indicated in each panel. (A) Colocalization of the $\alpha 1$ subunit (red) and gephyrin (green) in discrete clusters (yellow) in the ml. Note that the soma of the PC is not labeled for gephyrin, whereas a few gephyrin clusters lacking the $\alpha 1$ subunit are evident in the ml. (B) Staining for the $\alpha 3$ subunit (red) in the ml reveals very few clusters, which are colocalized (yellow) with gephyrin (green). (C) Complete loss of $\alpha 1$ subunit staining and decreased density of gephyrin clusters (green) in the molecular layer of an $\alpha 1^{0/0}$ mouse. The weak labeling of the nucleus in non-specific and unrelated to the genotype. (D–E) Upregulation of $\alpha 3$ subunit-positive clusters (red) in the ml of $\alpha 1^{0/0}$ mice, which are associated with gephyrin (green) in virtually all remaining clusters. In panel D, the nuclei of two interneurons appear weakly stained in green. The arrowhead points to a large gephyrin aggregate, presumably located intracellularly in the interneuron. In panel E, note that no $\alpha 3$ subunit staining becomes apparent in the numerous small gephyrin clusters in the granule cell layer (gcl; arrow). (F–G) Loss of gephyrin clusters (green) on the dendrites of PC (labeled with calbindin, red) in $\alpha 1^{0/0}$ mice; the remaining gephyrin-positive clusters are mainly distributed on non-labeled structures between PC dendrites. Scale bars, A–D: 20 µm; F–G: 10 µm. See (18) for details.

gephyrin is not due to a competitive disadvantage with α1-GABA$_A$ receptors. (ii) In PC expressing only the α1-GABA$_A$ receptors, no compensatory expression of another α subunit variant occurs. However, overexpression of other GABA$_A$ receptor subtypes takes place in neighboring cells, such as Golgi and stellate cells, presumably to preserve the function of the cerebellar network. It is unclear how this up-regulation of α3-GABA$_A$ receptors is triggered, but stellate and Golgi cells are linked synaptically with PC via Lugaro cells (Figure 1). This observation strongly suggests that expression of GABA$_A$ receptors is regulated by synaptic activity in the network. (iii) The upregulation of 'minor' GABA$_A$ receptor subunits in the cerebellum of α1$^{0/0}$ mice occurs at the posttranslational level, as it is not accompanied by a change in mRNA transcription (81). Therefore, it appears that the turnover of GABA$_A$ receptor subunits can be regulated dynamically, allowing neurons to adjust the use of specific GABA$_A$ receptor subtypes for proper function.

The most likely implication of these changes is a reorganization of inhibitory circuits in the molecular layer of α1$^{0/0}$ mice to compensate for the loss of phasic and tonic inhibition in PCs. This conclusion, if confirmed by morphological analysis, implies that GABA$_A$ receptors control, at least indirectly, the number of inhibitory synapses formed in a given circuit to set its overall level of activity. As shown in Figure 1, there are two major sources of inhibitory input on Golgi cells and molecular layer interneurons: Lugaro cells, which provide a dual glycinergic/GABAergic input onto Golgi cells and stellate/basket cells, which innervate each other and Golgi cells. This synaptic reorganization suggests growth of interneuron axonal arbors in the molecular layer of mutant mice and/or change in synaptic target specificity. A possible mechanism underlying this change in connectivity is discussed in the next section.

Requirement of GABA$_A$ receptors for long-term maintenance of GABAergic synapses

The observation that PC in α1$^{0/0}$ mice are completely devoid of functional GABA$_A$ receptors provided a unique incentive to investigate the effect of such a deletion on GABAergic synapses (9,86). At the light microscopy level, the morphology of PCs, as revealed by staining for calbindin or parvalbumin is unaffected in α1$^{0/0}$ mice and they are present in normal numbers (86). Likewise, the distribution of climbing fibers is undistinguishable from that seen in wildtype mice (9). GABAergic terminals in the molecular are readily detectable, but are larger and less regularly distributed than in wildtype. However, striking alterations in the organization of GABAergic synapses were evident at the ultrastructural level (9),

as summarized in Figure 4. In sections processed for GABA immunogold labeling, the vast majority of GABAergic presynaptic profiles formed synapses with PC spines, characterized by an asymmetric postsynaptic specialization indistinguishable from that formed in glutamatergic synapses (Figure 4A). These aberrant presynaptic terminals nevertheless were GABAergic, as shown by the presence of vesicular GABA transporter and the absence of vesicular glutamate transporters type 1 and type 2, which were readily detected in parallel (Figure 4C) and climbing fibers, respectively. Furthermore, most aberrant GABAergic terminals originated from stellate or basket cells, as seen by labeling for parvalbumin. On the dendrites of PC, the number of symmetric, type II synapses was reduced by about 75%, indicating a failure to form or to maintain GABAergic synapses in the absence of postsynaptic GABA$_A$ receptors (9).

Unexpectedly, GABAergic synapses on PC somata, which are formed selectively by basket cell terminals, were not affected in α1$^{0/0}$ mice and retained their morphology, neurochemical phenotype, and content of presynaptic vesicles (Figure 4B). Therefore, the synaptic deficit observed in the molecular layer is selective for axo-dendritic synapses. Morphological and functional examination of the cerebellum during postnatal development revealed no transient expression of GABA$_A$ receptors in PC of mutant mice. GABAergic axons contacting PC dendrites and somata were seen from P7 onwards. Ultrastructurally, the first synapses formed by terminals immunolabeled for GABA were symmetric on dendritic shafts. However, aberrant terminals, forming heterologous synapses with spines appeared first at P10 and their number increased rapidly thereafter (Figure 4D). These results were taken as evidence that initial steps of GABAergic synapse formation are not affected in α1$^{0/0}$ mice (9). However, a clear deficit for the maintenance of GABAergic synapses on PC dendrites occurs in the absence of functional GABAergic transmission.

Altogether, these observations confirm that neurotransmitter-mediated activity is not essential for synapse formation (91–94) and indicate that differential mechanisms ensure long-term stability of synapses in various cell compartments. While the normal morphology and content of presynaptic vesicles of basket cell terminals suggest that these terminals remain competent for GABA synthesis and release, activation of postsynaptic GABA$_A$ receptors is not required for maintenance of this synapse. A different situation prevails in dendrites, where most GABAergic synapses are lost in adult animals. It is possible that spines exert a synaptogenic action of GABAergic terminals, which is sufficient to attract them when they form 'silent' contacts with their normal postsynaptic targets. It is
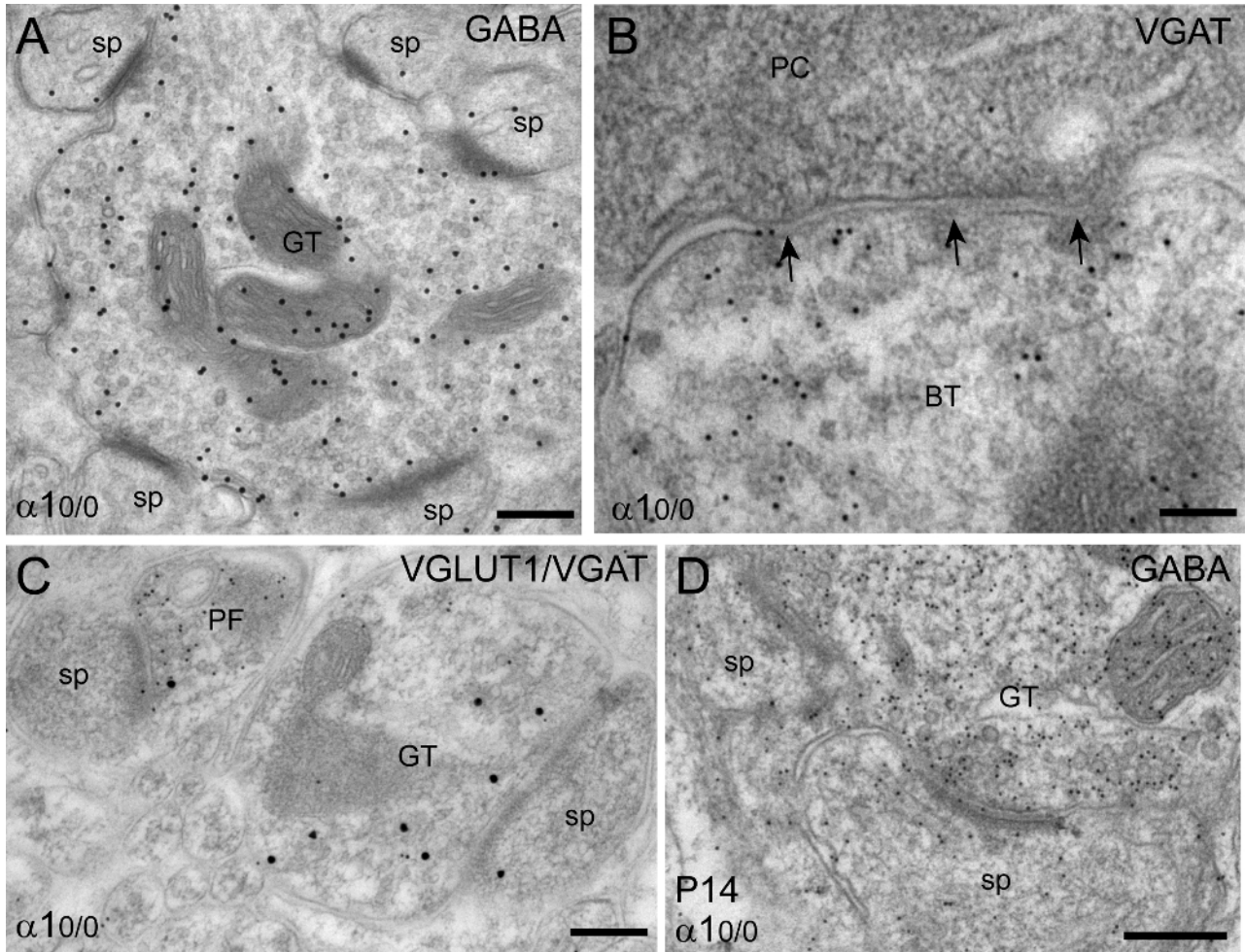
Figure 4. Formation of heterologous synapses in the molecular layer of $\alpha1^{0/0}$ mice, as visualized by immunoelectron microscopy. Each panel depicts a section from a mutant mouse (A–C) adult, (D) P14. Immunogold labeling of mitochondria is non-specific and should be disregarded. (A) Example of a GABAergic terminal (GT) labeled for GABA using a secondary antibody coupled to 20 nm gold particles, which forms asymmetric synapses with 6 spine profiles (sp) within a single ultrathin section. (B) A basket cell terminal (BT) labeled for vesicular GABA transporter (VGAT; 10 nm immunogold particles) makes symmetric synapses (arrows) with the soma of a PC. Such terminals were indistinguishable from those seen in wildtype mice. (C) Segregation of type 1 vesicular glutamate transporter (VGLUT1; 10 nm gold particles) and VGAT (20 nm gold particles) between parallel fiber terminals (PF) and GABAergic terminals, even when both make asymmetric synapses with spines. (D) Example of heterologous synapses formed during postnatal development between a GABAergic terminal and two spines (GABA labeling with 10 nm gold particles). Scale bars, A, D: 200 nm; B, C: 100 nm. For details, see (9).

also conceivable that the presence of GABA$_A$ receptors and/or gephyrin is required for synaptic anchoring of a molecule mediating transsynaptic homo- or heterophilic interaction with a presynaptic partner, thereby explaining the profound reduction of symmetric synapses on dendritic shafts. It is of note that the formation of basket cell synapses on the axon initial segment of PC depends on specific interactions with extracellular molecules (95) and that gephyrin clusters are not detectable on PC somata, despite the prominent clustering of the $\alpha1$ subunit (Figure 3), indicating a distinct mechanism for GABA$_A$ receptor clustering compared to dendrites. In any case, the lack of long-term stability of stellate cell synapses onto PC dendrites might contribute to the formation of supernumerary synapses between interneurons, as discussed in the previous section.

Conclusions

The analysis of targeted deletion of GABA$_A$ receptor subunit genes expressed in the cerebellum has unraveled seemingly contradictory findings: GABA$_A$ receptor subtypes are functionally not interchangeable and do not replace a missing subunit, as seen best by the loss of δ subunit protein in $\alpha6^{0/0}$ mice and by the increase in extrasynaptic receptors in granule cells of $\alpha1^{0/0}$ mice. However, wide ranging compensations contribute to the maintenance of function, in particular when the mutation concerns a major GABA$_A$ receptor subtype. Adaptations can even occur in cell types that are not affected by the mutation, as witnessed by the increase in $\alpha3$-GABA$_A$ receptors in Golgi cells of $\alpha1^{0/0}$ mice. As a consequence, behavioral deficits are minimal compared to the strong impairment produced by acute pharmacological blockade or

stimulation of GABA$_A$ receptors in vivo. While the stereotyped organization of the cerebellum facilitates these investigations, additional studies will be required to determine to which extent these findings can be generalized to other brain regions and other GABA$_A$ receptor subtypes.

Acknowledgements

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