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oral examination: ……

Cerebellar granule cell-specific deletion of the AMPA receptor subunit GluR-D gene

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Summary

Ionotropic glutamate receptors (iGluRs) play a major role in physiological and pathophysiological processes in the brain. The receptors are classified to three main subtypes on the basis of their pharmacological and electrophysiological properties and sequence identities. They are AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)-preferring, kainate-preferring and NMDA (N-methyl-D-aspartate)-preferring receptors. AMPA receptors mediate fast excitatory transmission at most synapses in the CNS. They form from combinations of four subunits (GluR-A to –D or GluR-1 to -4) and are variously expressed in different cell types. Cerebellar granule cells express only the GluR-D and GluR-B genes, and the resulting proteins form the heteromeric functional AMPA channels at the mossy fiber-granule cell synapses (mf-gr), by which the granule cells get excitatory inputs from distinct brain regions and send them to Purkinje cells and other inhibitory interneurons in the cerebellar cortex.

We selectively inactivated the AMPA receptor subunit GluR-D gene from adult cerebellar granule cells (Gr△GluRD) by crossing loxGluR-D mice, in which the exon11 is flanked by two loxP sites, with another line expressing Cre recombinase selectively in adult granule cells. *In situ* hybridization and immunocytochemistry studies showed that in the progeny, the GluR-D mRNA and protein are removed selectively from granule cells; GluR-B mRNA remains at wild-type levels, although the level of GluR-B protein increases. This increase in GluR-B expression, but the formation of poorly functional homomeric GluR-B channels, does not allow effective AMPA receptor function at the mossy fibre granule cell synapse: AMPA receptor responses are virtually abolished, but there was no effect on the evoked NMDA response. We expect that *in vivo* the mossy fibre to granule cell synapses are silent, as there might be no effective depolarization to allow opening of NMDA receptor channels. So in this regard the mouse phenocopies the stargazer mutation. Microarray analysis and real-time PCR showed that ablating AMPA receptor expression from cerebellar granule cells affects the expression of many genes. Despite the nearly abolished AMPA currents at the mf-gr synapse, Gr△GluRD mice have no motor impairments, likely indicating some compensatory mechanisms occurred.

Zusammenfassung

Ionotrope Glutamatrezeptoren spielen eine entscheidende Rolle bei physiologischen und pathophysiologischen Prozessen im Gehirn. Die Rezeptoren werden in drei Untergruppen eingeteilt aufgrund ihrer pharmakologischen Eigenschaften und ihrer Sequenzähnlichkeit. Diese sind die AMPA(α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid)-, und Kainat- und NMDA(N-methyl-D-aspartate)-bevorzugenden Rezeptoren. AMPA-Rezeptoren vermitteln schnelle erregende Weiterleitung an den meisten Synapsen des ZNS. Sie werden aus Kombinationen von vier Untereinheiten (GluR-A bis –D oder GluR-1 bis –4) gebildet und werden in verschiedenen Zelltypen unterschiedlich exprimiert. Cerebelläre Körnerzellen exprimieren nur die Gene GluR-D und GluR-B, die resultierenden Proteine bilden heteromere AMPA-Kanäle an den Moosfaser-Körnerzell(mf-gr)-Synapsen, durch die die Körnerzellen erregende Ströme von entfernten Hirnregionen erhalten. Die Körnerzellen ihrerseits leiten die Erregung an Purkinje-Zellen und andere inhibitorische Interneurone im cerebellären Kortex weiter.

Wir haben selektiv die AMPA-Rezeptoruntereinheit GluR-D aus adulten cerebellären Körnerzellen entfernt (GrΔGluRD) durch Kreuzen von loxGluR-D Mäusen, in denen das Exon 11 von zwei loxP-Stellen flankiert wird, mit einer Linie, die Cre-Rekombinase selektiv in adulten cerebellären Körnerzellen exprimiert. *In situ* Hybridisierung und Immunzytochemie zeigten, dass in den Nachkommen die mRNA und das Protein der GluR-D-Untereinheit selektiv aus Körnerzellen entfernt waren. Die mRNA der GluR-B-Untereinheit bleibt auf Wildtyp-Niveau, das Protein dagegen zeigt ein erhöhtes Niveau. Diese Steigerung der GluR-B Expression erlaubt wegen der Bildung von wenig funktionellen homomeren GluR-B-Kanälen keine effektive Funktion von AMPA-Kanälen an mf-gr Synapsen: AMPA-Rezeptor-Ströme sind nahezu bei Null, es gab allerdings keinen Effekt bei der evozierten NMDA-Antwort. Wir erwarten dass die mf-gr Synapsen *in vivo* stumm sind, da es vermutlich keine ausreichende Depolarisation gibt, um eine Öffnung von NMDA-Rezeptor-Kanälen zu ermöglichen. In dieser Beziehung kopiert diese Mauslinie den Phenotyp der “Stargazer“-Mutation. Mikroarray-Analyse und Real-Time-PCR zeigten, dass die Entfernung der AMPA-Rezeptorexpression aus cerebellären Körnerzellen die Expression von vielen Genen beeinflusst. Trotz der nahezu entfernten AMPA-Ströme an mf-gr Synapsen haben GrΔGluRD-Mäuse keine motorischen Defizite, was auf kompensatorische Mechanismen hindeutet.

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1 INTRODUCTION

1.1 Cerebellum structure

The cerebellum is an important structure of the central nervous system. It is located dorsal to the brainstem and connected to the brainstem by cerebellar peduncles. It has convolutions similar to those of cerebral cortex and contains an outer cortex, an inner white matter and deep cerebellar nuclei (DCN) (Llinas and Walton, 1998; Voogd and Glickstein, 1998) below the white matter. The cortex is divided into several lobes separated by distinct fissures and is a simple three-layered structure consisting of only five types of neurons: the inhibitory GABAergic stellate, basket, Purkinje, and Golgi neurons; and the excitatory granule cells (Kandel et al., 2000). In each folium of cerebellum, the outermost layer of the cerebellar cortex is the molecular layer, occupied mostly by axons and dendrites and a few cells like basket and stellate cells. The layer below that is a monolayer of large cells, Purkinje cells, which are the central players in the circuitry of the cerebellum and the only output neurons from the cortex: they use the inhibitory neurotransmitter GABA. Below the Purkinje cells is a dense layer of tiny neurons, granule cells, which are the main population of neurons in the cerebellum. In the center of each folium is the white matter, all of the axons traveling into and out of the folia (Figure 1).

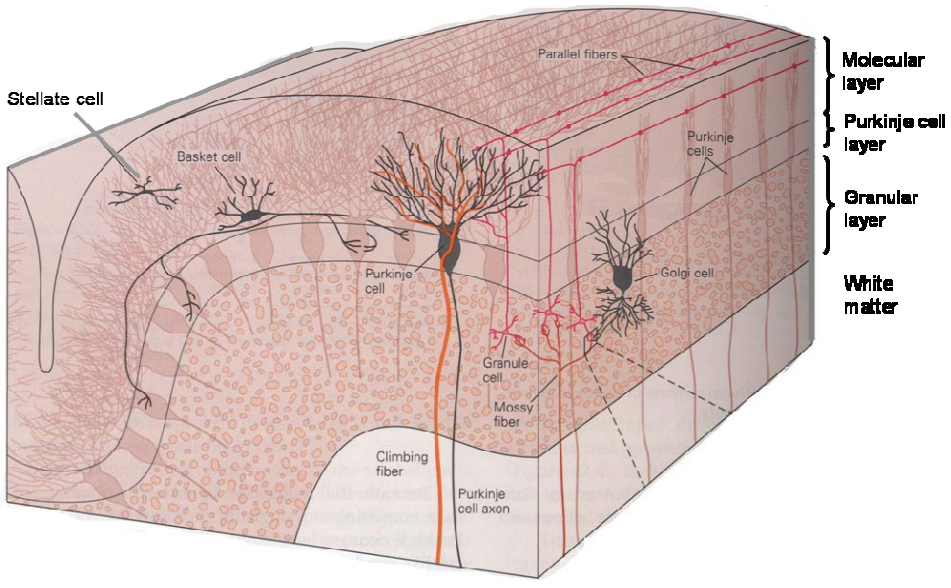


Figure 1. *Cerebellar folium & neurons in cerebellar cortex (copied from Kandel, 2000)*

The cerebellum controls movement, stores motor memories (Raymond et al., 1996), and also is involved in various cognitive processes, and emotional processing such as fear (reviewed by De Zeeuw and Yeo, 2005; Schutter and van Honk, 2005; Sacchetti et al., 2005). The cerebellum receives somatosensory input from the spinal cord, motor information from the cerebral cortex, and input about balance from the vestibular organs of the inner ear (Kandel, 2000). The functions of the cerebellar cortex are expressed solely through the modulation of the firing of cells in the DCN (Llinas and Walton, 1998). The DNC are located in the white matter beneath the cortex and project to many parts of the CNS. The only output of the cerebellar cortex is by way of Purkinje cell axons that inhibit the target neurons in the DCN and are mediated by the neurotransmitter γ-aminobutyric acid (GABA). Within the cerebellar cortex, the circuitry exists to modulate the firing of Purkinje cells (Figure 2). Cerebellar circuits consist of a main excitatory loop and an inhibitory side-loop. The Purkinje cells receive excitatory input from two afferent fiber systems, the main sources of input to the cerebellar cortex, and are inhibited by three local interneurons: basket cells, stellate and Golgi cells. The climbing fibers come from the inferior olive ascending the excitatory input into the cortex and climbing along the Purkinje cell's dendrites, whereas the granule cells, the most numerous neurons in the cerebellum and central to the cerebellar circuit, receive excitatory input from many brain areas via mossy fibers and relay this information via their ascending axon and its parallel fibers. The parallel fibres form *en passant* glutamatergic synapse in the molecular layer with the dendrites of Purkinje cells, stellate/basket interneurons and Golgi cells, all of which can be contacted by a single parallel fibre (Wisden and Farrant, 2002). These interneurons of the cerebellar cortex which are excited by mossy fiber activity through the granule cells and also by climbing fiber activity are all thought to be inhibitory to Purkinje cells or to the mossy fiber-granule cell relay. Convergence of numerous granule cells and a single climbing fibre to each Purkinje cell is a characteristic unique feature of neuronal circuitry in the cerebellum.

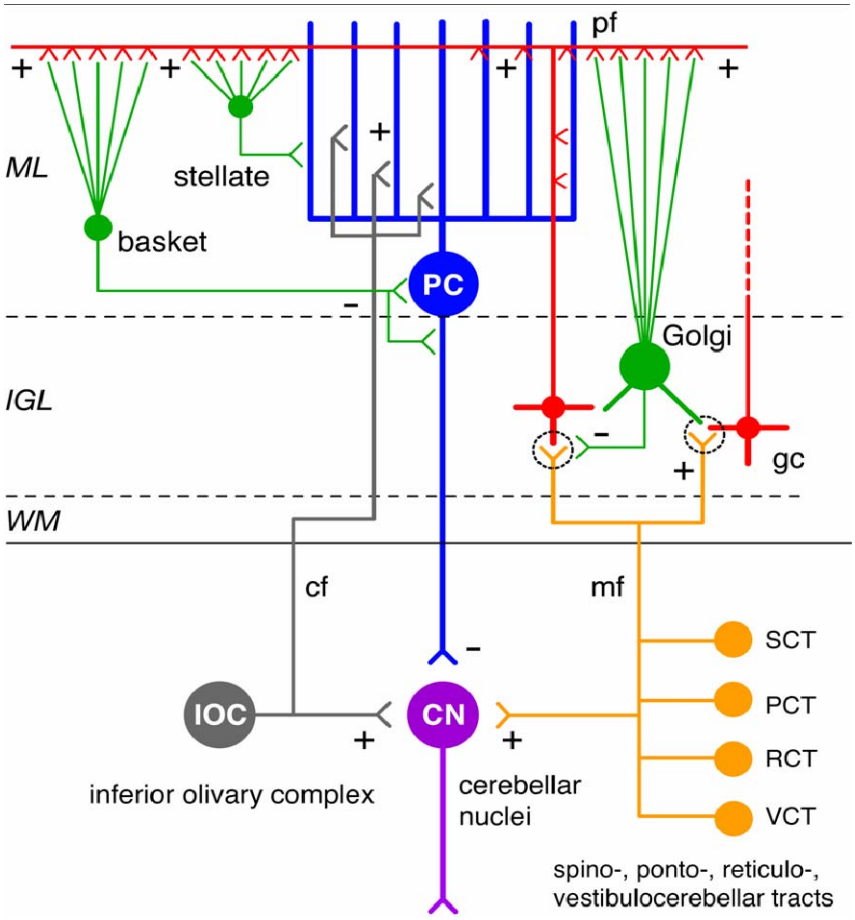


Figure 2. *Circuit diagram of the cerebellar cortex network* The two sources of afferent imputs, mossy (mf) and climbing fibers (cf) are shown; for simplicity only one of each cell type are indicated: Purkinje cell (PC); excitatory interneurons, granule cells (gc); and inhibitory interneurons, Golgi, basket and stellate cells. Golgi cells provide feed-forward and feedback inhibition to granule cells; + : excitatory input; -: inhibitory input.

Although a few granule cells may be contacted by GABA-positive mossy fibre-like terminals (Hamori and Takacs, 1989), the principal GABAergic input to these cells comes from Golgi cells (Llinas and Walton, 1998). The Golgi cell axon forms an extensive plexus contacting thousands of granule cells. Whereas the apical dendrites of Golgi cells extend into the molecular layer and receive excitatory input from parallel fibres, the basolateral dendrites and soma are contacted by mossy fibres and climbing fibres. Golgi cells thus provide both feed-forward and feed-back inhibition onto the granule cells (reviewed in Dieudonne, 1998). The importance of Golgi cells to

cerebellar function is clear: their selective ablation causes acute disruption of motor co-ordination (Watanabe et al., 1998). Similarly, reversibly and inducibly blocking synaptic transmission from granule cells to Purkinje cells produces ataxia, demonstrating the functional importance of glutamatergic transmission at this synapse (Yamamoto et al., 2003).

No synaptic connections are formed on the granule cell soma; instead both the excitatory and inhibitory inputs are confined to the distal ends of the short granule cell dendrites within a specific structure, the glomerulus. The glomeruli form around single mossy fibre terminals, and a single mossy fibre contacts dendrites from up to fifty granule cells. As each granule cell dendrite also receives input from two to three Golgi cell axon varicosities. (reviewed by Llinas and Walton,1998; Voogd et al., 1998). The Golgi cell-granule cell circuit may aid the filtering of mossy fiber sensory input during its relay to Purkinje cells (Eccles et al., 1967; Gabiani et al., 1994; Brickley et al., 1996). The feedback excitation of Golgi cells by granule cell axons could synchronize activity of both cell types, converting the spatial signal of mossy fibre input to a temporal pattern of parallel fibre activity (De Zeeuw and Yeo, 2005). Damage to the cerebellum results in ataxia, dysmetria and intention tremor.

1.2 Glutamate receptors

L-Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS), acting through both ligand-gated ion channels (ionotropic receptors, iGluRs) and G-protein coupled (metabotropic receptors, mGluRs) receptors (Hammond, 2001). Activation of these receptors is responsible for basal excitatory synaptic transmission and many forms of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD) (Bredt and Nicoll, 2003), which are thought to underlie learning and memory. They are thus also potential targets for therapies for CNS disorders such as epilepsy and Alzheimer's disease (Maren and Baudry, 1995; Asztely and Gustafsson, 1996) Overactivation of glutamate receptors produces the excitoxicity and cell death seen in stroke.

1.2.1 Ionotropic glutamate receptors

Ionotropic glutamate receptors play major roles in physiological and pathophysiological processes in the brain. The receptors are ligand-gated ion channels; ie on binding glutamate that has been released from a presynaptic neuron, the ions Na^+, K^+ and sometimes Ca^{2+} pass through a

channel in the center of the receptor complex. This flow of ions depolarizes the plasma membrane. Ionotropic receptors are divided into three main subtypes on the basis of their pharmacological and eletrophysiological characterization, and sequence identity: AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionate)-preferring, kainate-preferring and NMDA (N-methyl-D-aspartate)-preferring receptors (Figure 3; reviewed by Mayer, 2005; Chen and Wyllie, 2006; Kristensen et al., 2006). Glutamate receptors are colocalized in the postsynaptic membrane of glutamatergic synapses. They differ in several functional properties, including gating kinetics, block by extracellular magnesium, and permeablility to Ca^{2+}. Non-NMDA (AMPA and kainate) receptors primarily mediate rapid electrophysiological responses to glutamate, whereas NMDA receptors are voltage-dependent, blocked by magnesium at the resting membrane potential, mediate a slower phase of neurotransmission and have been implicated in synaptic plasticity. Some cells only express NMDA receptors without expression of functional AMPA receptors making these synapses "silent" at the resting membrane potential (Isaac et al., 1995; reviewed by Groc et al., 2006).

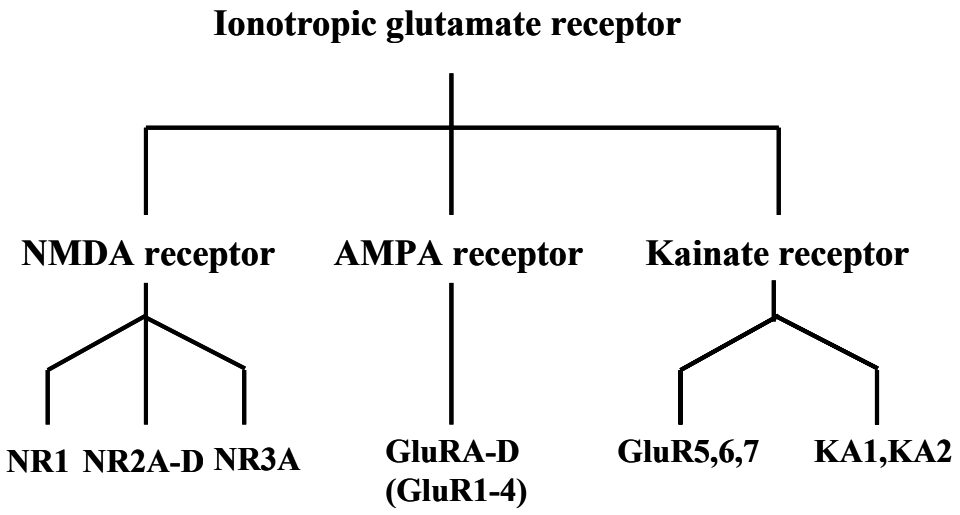


Figure 3. *Dendrogram of the mammalian members of the ionotropic glutamate receptor family*

1.2.1.1 Structure of the ionotropic glutamate receptors

NMDA, AMPA and kainate receptors are membrane-spanning glycoproteins composed of several subunits (Figure 3; reviewed by Mayer and Aemstong, 2004): within the gene family, there are four AMPA receptor subunits GluR1-4 (also called GluRA-D) (Keinaenen et al., 1990), five for kainate termed GluR5, 6, 7, KA1 and KA2 (Werner et al., 1991; Herb et al., 1992) and seven for NMDA receptor termed NR1, NR2A, 2B, 2C, 2D and NR3A,B (Moriyoshi et al., 1991, Monyer et al., 1992; Hollmann, 1994).

All ionotropic glutamate receptor subunits share a common basic structure of four hydrophobic regions within the central portion of the sequence (TM1 - 4; Figures 4 and 5; Mayer and Armstrong, 2004). Among them, there are three transmembrane domains (M1, M3, M4) plus a cytoplasm-facing re-entrant membrane loop (M2), giving these receptor subunits an extracellular N-terminus and intracellular C-terminus (Figure 4, 5 and 10). The C-terminus is often the site of splice variation and interaction sites with intracellular proteins, including binding and signaling proteins. In addition, the long loop between TM3 and TM4 is extracellular and forms part of the glutamate binding domain (S2) with the C-terminal half of the N-terminus. The other ligand-binding domain (S1) is located on the extracellular N-terminus preceded the M1 region. These two binding sites are required for the agonist selectivity. The subunits assemble heteromerically, or sometimes homomerically, as tetramers and combine with TARPS (Transmembrane AMPA Regulator Proteins, Kuner et al., 2003; Mayer and Armstrong, 2004; Nakagawa et al., 2005; Kristensen et al., 2006, see Figure 10).

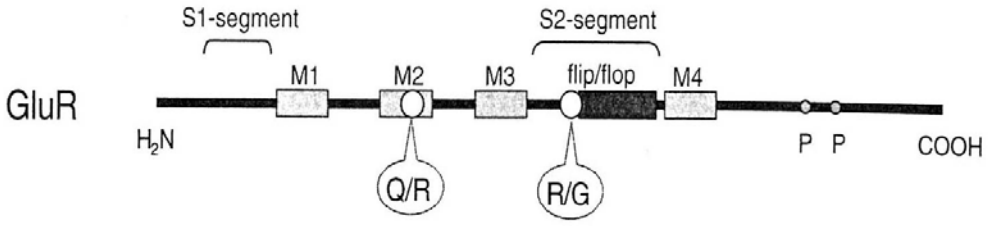


Figure 4. *Linear representation of the sequence of ionotropic glutamate receptors* The pore loop at M2 region; splicing variants: flip/flop and editing site Q/R, R/G (Kuner et al., 2003)

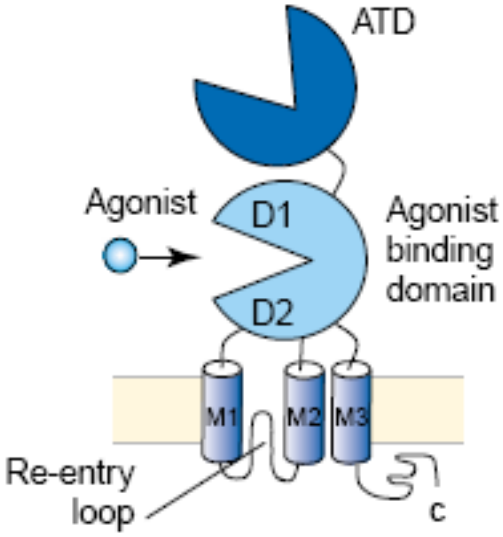


Figure 5.A *Cartoon of folding of a GluR subunit* ATD: the amino terminal domain; agonist-binding domains D1 and D2 (Kristensen et al., 2006)

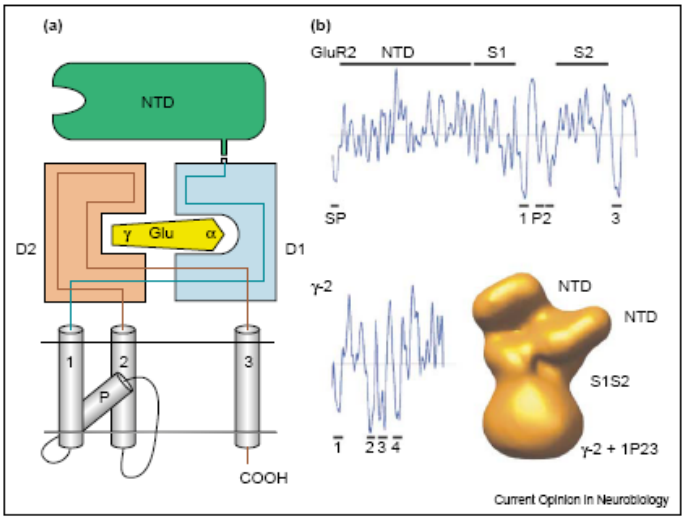


Figure 5.B *Domain structure in glutamate receptor ion channels* (a) Each subunit consists of a bilobed amino-terminal domain (NTD), the two-domain ligand-binding core (D1 D2), an ion channel with three membrane-spanning segments (1–3) and a pore loop (P), and a cytoplasmic domain of variable length. (b) Low-resolution structures of iGluRs and accessory subunits, illustrated by hydropathy plots for GluR2 (top) and the γ-2 subunit of the TARP family (lower left), and by a single-particle image of an AMPA receptor complex with TARPs. (reproduced from Mayer, 2005).

1.2.2 Diversity of the AMPA-type glutamate receptors

AMPA receptors mediate fast excitatory transmission at most synapses in the CNS and are composed of subunits GluRA-D (or GluR1-4), products from separate genes. The receptor properties allow high tempral precision, short latency of action potential initiation, and EPSP (excitatory post synaptic potential) coincidence detection.

Alternative splicing of AMPA receptor subunit genes: flip and flop

Each AMPA receptor subunit gene can produce two alternatively spliced versions, named flip (i) and flop (o), which determine the desensitization rate of channels (Sommer et al 1990; Quirk et al., 2004). The flip/flop region, a cassette of 38 amino acids encoded by exons 14 and 15 (in GluR-B), is located extracellularly between TM3 and TM4 (see Figure 4 and 6). In response to fast application of glutamate (1mM) at rat and human recombinant AMPA receptors, the time constants of desensitization between GluRAi and GluRAo receptors are the same, whereas the flip isoforms for GluRB-D receptors have significantly slower desensitization rates compared with the flop isoforms (Quirk et al., 2004). Three amino acid residues in the flip–flop region (Thr765, Pro766, and Ser775 in flip and Asn765, Ala766, and Asn775 in flop) contribute to splice variant differences in the desensitization rate (Quirk et al., 2004). These residues may confer differences in flip and flop receptor desensitization rates by directly and/or indirectly influencing the stability of the interface between adjacent subunits in the receptor tetramer. Cerebellar granule cells switch their expression of AMPA receptor subunit GluR-D splice variants from mostly flip forms in early stages to predominantly flop forms in the adult rat brain (Monyer et al., 1991). These findings suggest that rapid desensitization of AMPA receptors can be regulated by the expression and alternative splicing of GluR-D gene transcripts (Monyer et al., 1991).

The C-terminus of the AMPA receptors is also alternatively spliced. A small percentage of GluR-B protein exhibits a long C terminus (Köhler et al., 1994; Kolleker et al., 2003). The cerebellum expresses GluR-Dc (Gallo et al., 1992), which has a C terminus that is shorter than that of GluR-D and is homologous to the tail of GluR-B short (Figure 6). This could have important functional consequences as the C-terminus is also the site for multiple protein-protein interactions. For example, association of glutamate receptors with identified proteins containing PDZ domains (**P**SD-95/SAP-90, **D**iscs-large, **Z**O-1 homologous domain) is dependent on the C-terminal amino

acids. The COOH terminus of GluR-B binds to a pair of multi-PDZ proteins—GRIP/APB (Dong et al., 1997; Srivastava et al., 1998) and also to a single PDZ protein, PICK1 (Xia et al., 1999). Thus different splice variants may interact differently with the same set of proteins leading to, for instance, differential subunit localization.

RNA editing

A further modification leading to AMPA receptor subunit diversification is RNA editing, which following conversion (editing) of nucleotides in the primary transcript leads to single amino acid exchanges in the resulting protein (Sommer et al., 1991; Seeburg, 2002).

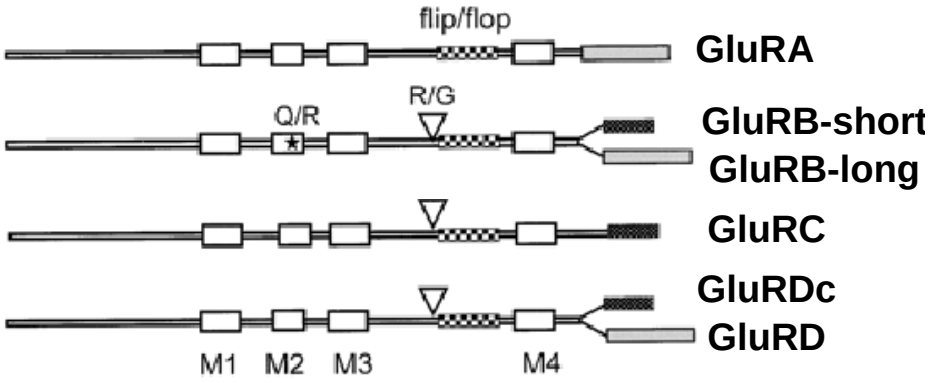


Figure 6. *Schematic diagram of the alternative splicing and editing of AMPA receptor subunits* (Dingledine et al., 1999)

1.3 Structure and functional properties of AMPA receptor

Like all the ionotropic glutamate receptors subunits, AMPA GluR subunits have an extracellular N-terminus and an intracellular C-terminus (Figure 5; Mayer, 2005). Many native AMPA receptor channels are impermeable to calcium, a function controlled by the GluR-B subunit (Seeburg 2002; Kittler, 2006). The calcium permeability of receptors with the GluR-B subunit is determined by the post-transcriptional editing of the GluR-B mRNA by the ADAR editing enzymes (Adenosine Deaminase), which change a single amino-acid in the TM2 region from glutamine (Q) to arginine (R) (Figure 6). This is the so called "Q/R editing site", which determines the single channel properties. Receptors with GluR-B(Q) are calcium permeable whereas

GluR-B(R) containing receptors are not (Sommer et al., 1991; Seeburg, 2002; Kittler, 2006). It was also found that the single-channel conductance is relatively high for calcium-permeable AMPA receptors but lower for calcium-impermeable channels containing edited subunits (Swanson et al., 1997). Under normal circumstances, almost all the GluR-B protein expressed in the CNS is in the GluR-B(R) form, giving rise to calcium-impermeable AMPA receptors. This, along with the interactions with other intracellular proteins, makes GluR-B perhaps the most important AMPA receptor subunit. Mice in which the GluR-B editing can no longer function die at very young age possibly because of Ca2+ toxicity to neurons (Higuchi et al., 2000; Wang et al., 2000). The further importance of the need to fully edit the GluR-B subunit comes from studies showing that pathological alterations in ADAR activity might down-regulate editing activity, so producing more Ca^{2+} permeable AMPA receptors and neuronal death (reviewed by Kittler, 2006).

AMPA receptors mediate most excitatory (depolarizing) currents in conditions of basal neuronal activity: they have a major influence in the strength of the synaptic response, whereas NMDA receptors remain silent at the resting membrane potential (Nowak et al., 1984), they are crucial for the induction of specific forms of synaptic plasticity, such as long-term potentiation (LTP) and long-term depression potentiation (LTD). The phosphorylation/dephosphorylation of AMPA receptors (or associated proteins) could modify channel function by altering various parameters, including single-channel conductance, the number of active channels, receptor desensitization and the probability that an agonist-bound receptor will open at the peak of a response (Cull-Candy, 2002). The effects of phosphorylation on the GluR-A AMPA subunit are probably involved in long-term changes in transmission (Sodeling et al., 1994). AMPA receptors are highly mobile/dynamic (Borgdoff and Choquet, 2002). Regulated insertion and removal of AMPA receptors at the synapse might provide a mechanism for altering synaptic efficacy, and for storing information in the brain (Clem and Barth, 2006).

1.4 Pharmacological characteristics of AMPA receptors

All AMPA receptors can be activated by AMPA, kainate and other agonists (see Table1, Chen and Wyllie, 2006). In response to glutamate AMPA receptors rapidly desensitize. Certain drugs are able to distinguish between flip and flop isoforms of AMPA receptor subunits. Cyclothiazide

(CTZ) suppresses desensitization only of the flip isoform, whereas the molecule PEPA (4-［2-(phenylsulfonylamino) ehtylthio］-2,6–difluorophenoxyacetamide), preferentially potentiates flop isoforms (Sekiguchi et al., 1997).

The excellent broad-spectrum blockers of non-NMDA receptors (AMPA and kainate receptors), CNQX, NBQX, and DNQX have been proved (see Table 1). But these have not usually been effective at distinguishing between AMPA and kainate receptors. The 2,3 benzodiazepines (GYKI compounds: GYKI-52466 and GYKI-53655) are non-competitive antagonists at non-NMDARs, with more selectivity towards AMPARs (Donevan and Rogawski, 1993; Wilding and Huettner, 1995; Paternain et al., 1995).

Recently, the crystal structure study of ionotropic glutamate receptors has provided the possible mechanisms of partial agonism, agonist selectivity and desensitization. The binding site of AMPA receptors for glutamate is located in two extracellular domains S1 and S2 (see Figure 5) which plays the role in forming the glutamate-binding pocket and in determining agonist selectivity (Stern-Bach et al., 1994). Three residues from the S1 domain (Pro 478, Thr 655, and Glu 705) make direct hydrogen bonds with glutamate (Chen et al., 2006). The competitive antagonist DNQX has a slight different pharmacophoric pattern from glutamate. The two carbonyl groups of DNQX mimic the α-carboxyl group of glutamate interacting with both Thr480 and Arg485 residues (Chen et al., 2006). The individual ligand-binding cores form dimmers and the stability of these interdimer interactions determines the extent of densensitization in AMPA receptors. Like Cyclothiazide within the interdimer region that abolish desensitization has been shown to increase the stability of the dimmer complex (Stern-Bach et al., 1998; Sun et al., 2002).

The new ampakines drugs, act as active positive allosteric modulators of AMPARs, which can enhance fast excitatory transmission throughout the brain; promote the induction of LTP resulting in memory enhancement (Staubli et al., 1994a; 1994b); they can increase production of neurotrophin leading to selective behavioral effects (Lauterborn et al., 2000).

AMPA receptor ion channels display rapid gating, and their deactivation and desensitization determine the timing of synaptic transmission. AMPAR potentiators can promote AMPAR signaling by blunting desensitization and slowing deactivation (Staubli et al., 1994a). It has been found that the AMPAR auxillary subunit stargazin changes the pharmacology of AMPAR

potentiators by showing additive effects on it and also increases the affinity of AMPAR potentiators for glutamate receptor subunits. Furthermore, stargazin modulates the subunit specificity of AMPAR potentiators to make flop receptors sensitive to cyclothiazide and flip receptors sensitive to PEPA (Tomita et al., 2006).

Table 1. Pharmacological and functional properties of AMPA receptors

Agonists	Antagonists
•Activated by glutamate (EC_{50}～ 500μM)[1]	•Selectively blocked by GYKI-53655[8] and ATPO[9]
•All activated by AMPA[3]	•Unaffected or slightly potentiated by La^{2+}[10]
•Activated by kainate (EC_{50}～ 100μM)[4]	•Blocked by CNQX, NBQX and related compounds[11,12]
•Activated by domoate[4]	
•Activated by SYM 2081 (4-methyglutamate) EC_{50}=200 ～ 300μM[5]	•Not affected by NS-102 or LY294486; weakly antagonized by LY293558[12]
•Rapid desensitization with AMPA or glutamate *much less* rapid with kainate[6]	
•Desensitization suppressed by diazoxide, cyclothiazide[9] and PEPA[7]	
Permeability	
•High- or low Ca2+ permeability (depending on editing of subunits)[13]	•Channel conductances ～ 200fs-30pf (depending on editing)[14]

Notes: modified from reference of Cull-Candy, 2002
1. Lomeli et al., 1994; Partin et al., 1996; 2. Schiffer et al., 1997; 3. Hollmann et al., 1989; Nakanishi et al., 1990; 4. Hollmann et al., 1989; Nakanishi et al., 1990; Patneau et al., 1994; 5. Wilding and Huettner, 1997; Zhou et al., 1997; Donevan et al., 1998; 6. Partin et al., 1993; Wong and Mayer, 1993; 7. Sekaguchi et al., 1997; 8. Donevan and Rogawski, 1993; Paternain et al., 1995; Wilding and Huettner, 1995; Bleakman et al., 1996; 9. Wahl et al., 1998; 10. Reichling and MacDermott, 1991; Huettner et al., 1998; 11. Honore et al., 1988; Nakanishi et al., 1990; Sommer et al., 1992; 12. Reviewed by Bleakman and Lodge, 1998; 13. Egebjerg and Heinemann, 1993; Jonas and Burnashev, 1995; 14. Swanson et al., 1997; Wyllie et al., 1993.

1.5 AMPA receptor subunit expression in the brain

Native AMPA receptors are tetramers composed of heteromeric assemblies of different subunits (Nakagawa et al., 2005). But the complexes are not stable, their localizations and trafficking change during synaptic plasticity (Malinow et al., 2000; Anotonova et al., 2001; Daw et al., 2000; Zamanillo et al., 1999). The AMPA receptor subunit genes are differentially expressed in depending on brain region and cell types (reviewed Wisden et al., 2000; Figure 7). GluR-A mRNA is most abundant in the hippocampus, amygdala and cerebellar Bergmann glia. GluR-B is nearly universally expressed, but is particularly highly expressed in cerebellar granule cells,

neocortex and the hippocampus. GluR-B is absent or expressed at lower levels in most GABAergic interneurons (Monyer et al., 1999). GluR-C expression is highest in neocortex and hippocampus. GluR-D is highly expressed in the cerebellum with comparatively light expression in the forebrain (Keinaenen et al., 1990). In the forebrain it is expressed in the reticular thalamic nuclei and subtypes of GABAergic interneuron, where it is often found with the GluR-A subunit and forms Ca-permeable AMPA receptors with fast kinetics (Geiger et al., 1995).

1.5.1 AMPA receptor subunit gene expression in the cerebellum

In the cerebellum, the expression levels of AMPA receptor subunits mRNAs in different cells are also distinct (see Wisden et al., 2000, see Figure 8). For example, Purkinje cells express GluR-A flop, GluR-B flip and flop, and GluR-C flip mRNAs; GluR-A expression is the weakest (Keinaenen et al., 1990; Sommer et al., 1990; Monyer et al., 1991; Sato et al., 1993). Bergmann glial cells express the GluR-A flip and –D flip mRNAs (Keinaenen et al., 1990; Sommer et al., 1990; Monyer et al.,1991; Sato et al., 1993; Burnashev et al.,1996; Gallo et al., 1992; Kondo et al., 1997). Granule cells contain only GluR-B flip and GluR-D flop mRNAs. Stellate/basket cells contain GluR-B and GluR-C mRNAs; Golgi cells possibly have GluR-C mRNA (Keinänen et al., 1990). And GluR-B, GluR-C and GluR-D express in the cerebellar nuclei (Wisden et al., 2000), see Figure 8. In the cerebellar granule cells only the GluR-B flip and GluR-D flop transcripts are found, forming heteromeric functional AMPA receptors.

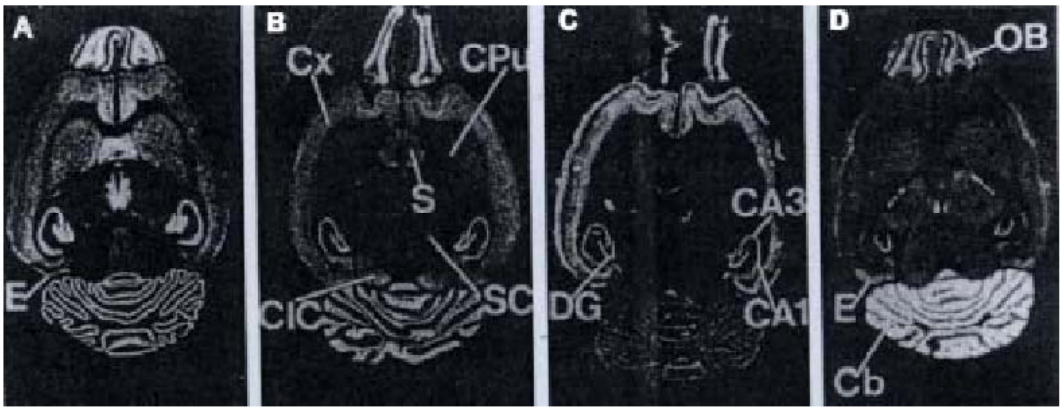


Figure 7. *AMPA receptor subunit mRNA distribution in horizontal sections of adult rat brain* A: GluRA expression; B:GluRB; C:GluRC; D:GluRD. Cx, neocortex; Cpu, caudate putamen; Cb, cerebellum; CIC, central nucleus of the inferior colliculus; S, septal nuclei; SC, superior colliculus; E, entorhinal cortex; DG,dentate gyrus; OB,olfactory bulb; CA1&CA3,Hippocampus region (Keinänen et al., 1990)

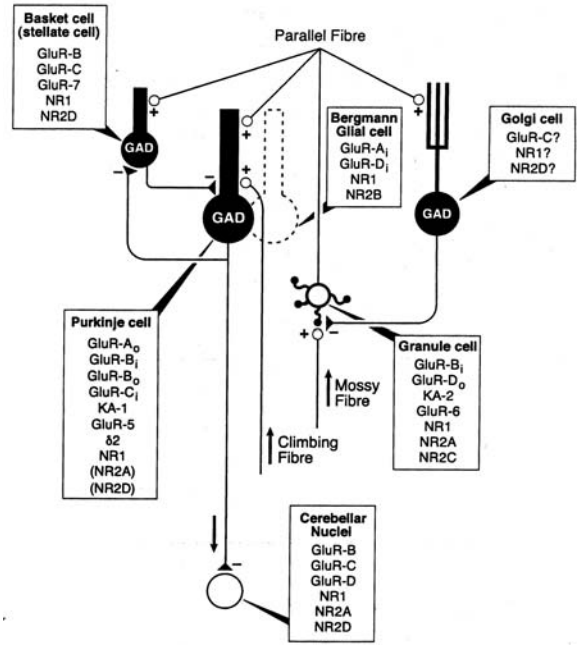


Figure 8. *The cell types in the adult rat cerebellum and their expression of the AMPA, NMDA and kainate receptor subunit mRNAs* (Wisden et al., 2000)

1.6 PDZ domains and AMPA receptors

On the postsynaptic plasma membrane of excitatory synapses there is an electron-dense region referred to as the postsynaptic density (PSD). This membrane specialization is thought to be important for the clustering of postsynaptic glutamatergic receptors and for the assembly of the postsynaptic signaling machinery (Hall et al., 1993; Garner et al., 1996); part of the assembly of the PSD involves a super-family of proteins with PDZ domains. The PDZ domain was identified initially as a common element present in three structurally related proteins: **P**SD-95/SAP90, **D**LG and **Z**O1 (Garner et al., 2000; Hata et al., 1998). The PDZ domain, consisting of about 90 amino acid residues, binds to short peptide sequences with 10-100nM affinity (Saras et al., 1996). Most of these short peptide sequences are located on the C-terminal tails of the interacting proteins. In the CNS of vertebrates the PDZ-domain-containing proteins, which interact with the cytoplasmic C-terminus of glutamate receptor subunits, play a fundamental role in their synaptic localization and function. The length and sequence divergence in the C-terminus of the subunits may influence the binding with different PDZ domains to affect the assembly receptors, trafficking and signal

transduction. There is an example described in the diversity of ionotropic glutamate receptors. In the AMPA receptors the subunit GluR-A has a type-I PDZ-binding site (-ATGL) at its extreme C-terminus and has been shown to interact with synapse-associated protein-97 (SAP97), whereas Glu-B and GluR-C have a type-II PDZ-binding site (-SVKI) at their extreme C-terminus and interact via this motif to numerous PDZ proteins such as GRIP (glutamate receptor-interacting protein), ABP (AMPA-binding protein), and PICK-1 (PKC-interacting protein) (Henley, 2003). These proteins have been implicated in the clustering of AMPA receptors or their turnover, whereas SAP97 may be a critical molecule involved in the synaptic delivery of GluR-A-containing receptors. In addition, GluR-B specifically binds to NSF (N-ethylmaleimide-sensitive factor), a chaperone protein implicated in SNARE (soluble N-ethylmaleimide-sensitive factor attached protein receptor) complex disassembly (Henley, 2003).

The molecular heterogeneity of PSDs between different brain regions has been elicited by relative and absolute quantification of Postsynaptic Density Proteome (PSP) (Cheng et al., 2006).Some 620 proteins have been identified in purified PSDs (Collins et al., 2006).

1.7 *Stargazer* and *waggler* mice and the AMPA receptor trafficking regulator stargazin (γ2)

The *stargazer* mouse is characterized by distinctive head tossing, ataxia, spike wave seizures and behavioral arrest, all of which are characteristic of absence epilepsy in humans (Arikkath & Campbell, 2003) and arose as a spontaneous mutation in the γ2 gene (Letts et al., 1998). The mutant arises from C57BL/6 inbred strain. Another mutant mouse allelic to *stargazer* is *waggler* which is a recessive neurological mutation on chromosome 15 that arose spontaneously in the MRL/MpJ mouse strain (Chen et al., 1999).

Genetic mapping of the *stargazer* locus originally identified the stargazin gene, which encodes a protein containing four transmembrane domains and a cytoplasmic C-terminal tail, which culminates with a PDZ binding site. Stargazin shares identity with a large family of proteins (Burgess et al., 2001; Klugbauer et al., 2000), and a subset of four (γ-2, γ-3, γ-4, γ-8) can traffic AMPARs (Tomita et al., 2003). These four transmembrane AMPAR regulatory proteins (TARPs) (Tomita et al., 2003) are differently expressed throughout the brain (see Table 4 in discussion). Stargazin shows some identity to the subunit of voltage-dependent calcium channels γ2 (Letts et al., 1998) and is highly expressed in cerebellar granule cells. The mutation arises because of a

retrotransposon insertion in an intron, and results in the complete loss of any detectable protein (Kang et al., 2001). Stargazin (γ2) is the protein mutated in *stargazer* mice and is the first transmembrane protein found to be related to both AMPA receptors subunits and synaptic PDZ proteins, such as PSD-95 (Chen et al., 2000) see Figure 9. The mutation occurred at the C-terminus PDZ binding domain in which stargazin binds to PSD-95. The *waggler* mutation results in a premature termination of the gene γ2 (cacng2) transcript, leading to a substantially lower level of γ2 in the mutant mice (Letts et al., 1998).

The ataxia is associated with complete absence of functional AMPA receptors on cerebellar granule cells; AMPA receptors are made but are not on the surface of the cell (Chen et al 1999; Chen, 2000; Hashimoto et al., 1999), and neurons in forebrain show normal AMPA receptor expression. This suggests either that the cerebellar granule cells are unique in their requirement for stargazin or that other related proteins (TARPs) mediate AMPA receptor trafficking in forebrain.

It has been addressed that not only AMPAR maturation requires stargazin, but also stargazin can control AMPA receptor function by three distinct roles (reviewed Ostern & Stern-Bach 2006; Nicoll et al., 2006). First, stargazin regulates delivery of AMPA receptors to the membrane surface–this function does not require the PDZ-binding domain. Second, stargazin mediates synaptic targeting of AMPARs and this function does require the PDZ-binding C terminus (Chen et al., 2000); third stargazin enhances the function of AMPRs through slowing channel deactivation and desensitization, which are controlled by the ectodomain of stargazin (Tomita et al., 2005; reviewed Osten and Stern-Bach 2006; Nicoll et al., 2006 – see Figure 9 and 10).

By the study of structure and different conformational states of native AMPA receptor complex, the γ2 might be an obligatory protein partner for AMPA receptors. It has been found that members of the stargazin/TARP family of transmembrane proteins co-purified with AMPA-Rs and contributed to the density representing the transmembrane region of the complex (Nakagawa et al., 2005).

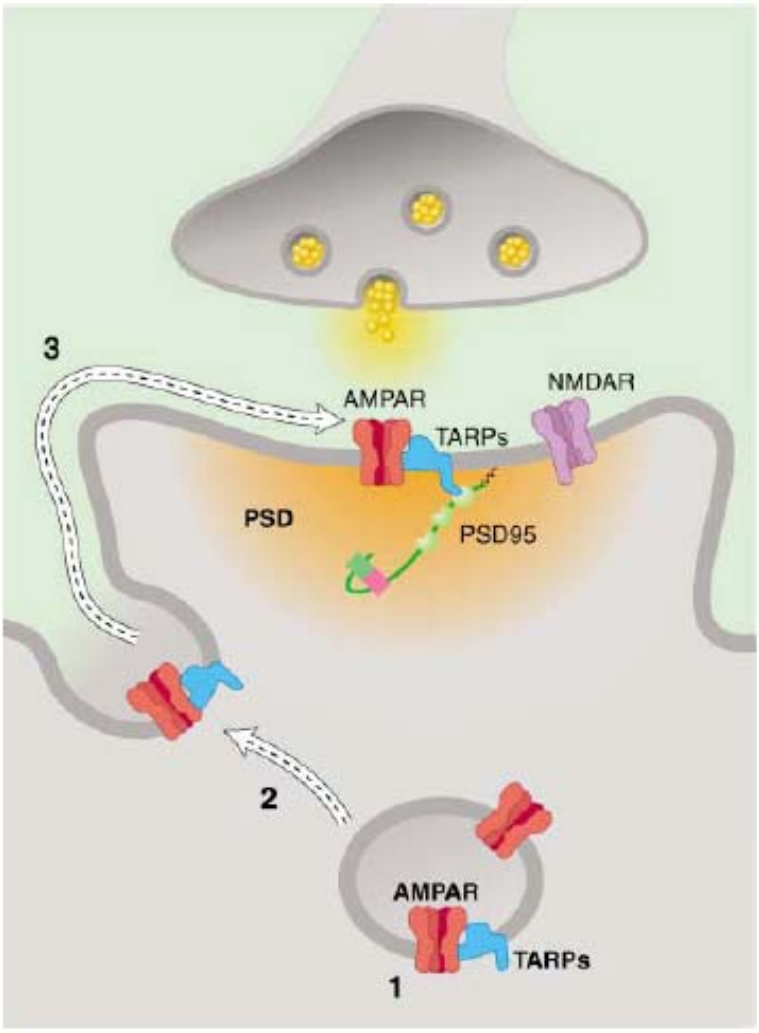


Figure.9 *Roles of stargazin-like TARPs in the trafficking of AMPARs* TARPs (blue) bind to AMPARs (red) early in the synthetic pathway (1) and are required for the trafficking of receptors to the surface (2). Interaction of the C-terminal PDZ binding site of TARPs with PSD-95 at the postsynaptic density (PSD) captures the surface AMPARs at the synapse (3). Reproduced from review of Nicoll et al., 2006

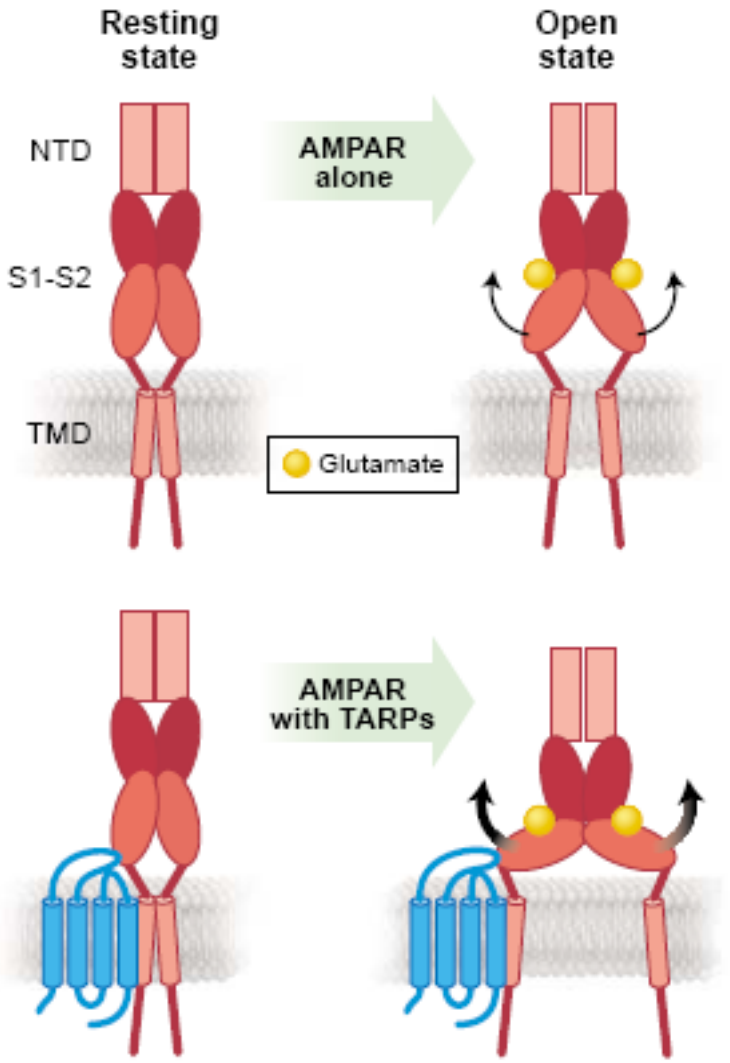


Figure 10. *A model for possible role of TARPs on AMPAR channel opening* AMPAR subunits consist of four domains: a large N-terminal domain (NTD), a ligand-binding pocket (S1-S2), transmembrane domains that form a channel pore (TMD), and a cytoplasmic domain. Upon glutamate binding, S1-S2 closes like a clamshell, which causes channel pore opening. TARPs bind to AMPAR and affect AMPA channel opening either by inducing more closure of S1-S2-to-glutamate binding or more efficient coupling of domain closure to pore opening without any change in S1-S2 closure. Reproduced from the review of Nicoll et al., 2006.

Additional phenotypes of *stargazer* mice

Stargazer and *waggler* mutant mice exhibit a selective reduction of brain-derived neurotrophic factor (BDNF) mRNA expression in cerebellar granule cells (Qiao et al., 1996; Bao et al., 1998), which may effect the maturation or another function of granule cells (see Figure 11 below).

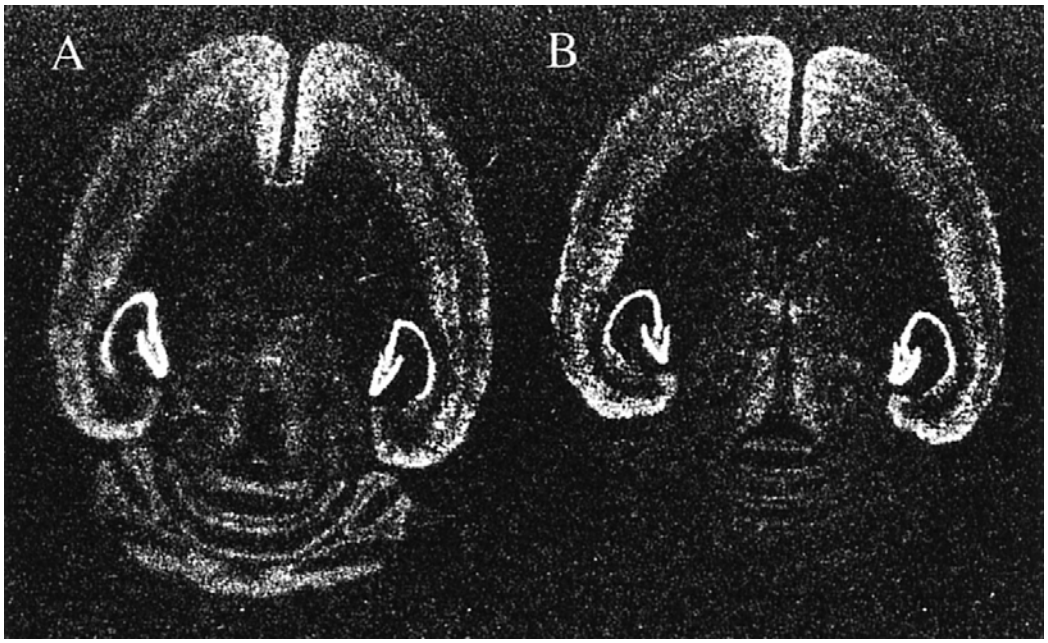


Figure 11. *In situ hybridization of BDNF mRNA expression in WT and waggler mouse brains* No BDNF mRNA expression occurs in the cerebellum of *waggler* mouse. A: wild-type mouse brain (WT); B: *Waggler* mouse brain. reproduced from Bao et al., 1998.

In addition stargazin may play a direct role in the expression, assembly and/or trafficking/targeting of $GABA_A$ receptor subunits expressed in cerebellar granule cells. The expression of α6 and δ subunit mRNA and protein are reduced in granule cells from s*targazer* mice (Thompson et al., 1998; Chen et al., 1999, Ives et al., 2002). By *in situ* hybridization there is a 20% reduction of $GABA_A$ receptor subunit mRNA level in adult *waggler* cerebellum was detected (Chen et al., 1999). It is not clear if the reduced gene expression of BDNF and $GABA_A$ receptor subunits is due to the lack of AMPA receptor-mediated depolarization of stargazer mutant granule cells or is an independent property of the stargazin protein (see Figure 12).

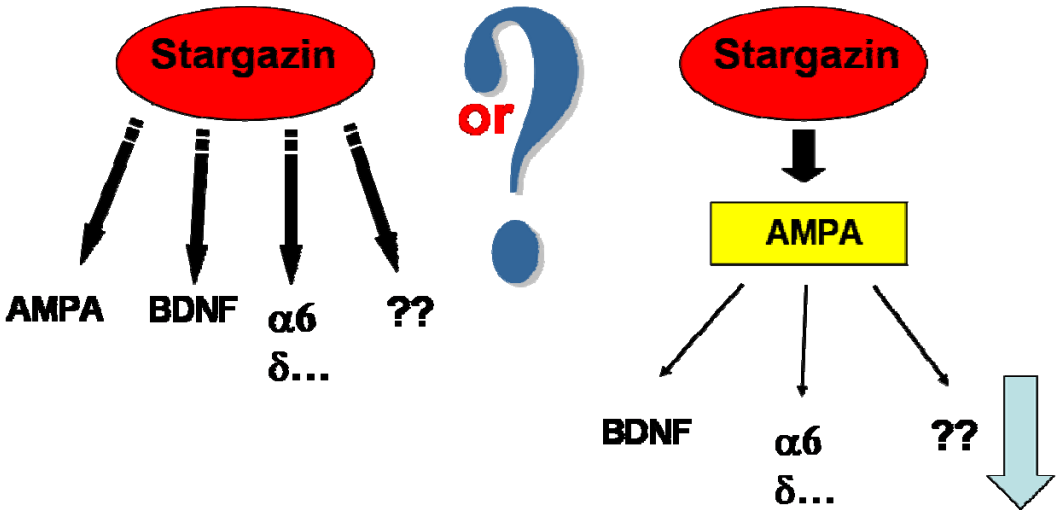


Figure 12. *A schematic of stargazin's possible functions* The Stargazin could have multiple "chaperone" functions and directly influence the levels of AMPA receptors and other proteins such as BDNF and $GABA_A$ receptor subunits; alternatively, the lack of AMPA receptor expression on stargazer granule cells could reduce the extent of membrane depolarization needed to chronically mainatin the expression of numerous genes e.g. BDNF and $GABA_A$ receptor subunits.

1.8 Function of LTP and LTD in cerebellum

LTP and LTD remain prime candidates for mediating learning and memory. Nevertheless their function roles must be determined at those synapses and circuits that mediate a specific type of plasticity. For example, the hippocampus, where LTP and LTD have been most extensively investigated, is necessary for certain forms of spatial learning, and the cerebellum is necessary for certain forms of motor learning. The cerebellar circuitry is essential for acquisition and expression of motor learning (Marr, 1969; Kim and Thompson, 1997; Mauk, 1997). The most well-known form of cerebellar plasticity is certainly Pf-PC LTD (Ito, 2001). Mf-Grs LTP has also been investigated (Maffei et al., 2002, 2003; D'Angelo et al., 1999; Rossi et al., 2002; Armano et al., 2000).

Parallel fiber LTD is generally understood to have three initial requirements for induction. The climbing fiber contributes to LTD induction via Ca^{2+} influx through voltage-gated channels, occurring during the complex spike. The parallel fibers release glutamate to act upon both

mGluR1 metabotropic receptors and AMPA receptors. After initiation, activation of PKC and nitric oxide/cGMP/protein kinase G/phosphatase inhibition pathway has been supposed to be required (Bear and Linden, 2000). Parallel fiber LTD is expressed postsynaptically, as a reduction in the number of functional AMPA receptors produced by clathrin-mediated endocytosis (Wang and Linden, 2000; Xia et al., 2000).

LTP is induced at mossy fiber granule cell synapses (MF-GrC) after a high-frequency stimulation of MFs paired with membrane depolarization. It is similar to that commonly recorded in hippocampal area CA1. This LTP induction needs the activation of NMDA receptors, mGluRs, Ca^{2+} influx and PKC (D'Angelo et al., 1999) and is accompanied by changes of intrinsic excitability of granule cells (Armano et al., 2000). As such, it is quite sensitive to the level of inhibitory input (Armano et al., 2000) which, in this case, is provided by Golgi cells (Maex and De Schutter, 1998). The cerebellum is thought to operate in feed-forward mode anticipating the corrections needed to regulate complex sequenses of movements (Ghez and Thach, 2003,). LTP plays a role in motor coordination and learning. Mf-GrC LTP provides a wide substrate for information storage in the cerebellum. The functional consequences of MF-GrC LTP depend on several factors including the molecular and cellular mechanisms involved, the spatial distribution of plasticity, local network activity (primarily related to endogenous rhythms and synaptic inhibition), and long-range modulation (primarily related to cholinergic, serotoninergic and noradrenergic innervation of the cerebellum). Any abnormal changes of LTP, LTD induction may elicit the cerebellar dysfunction in cellular and behavioral studies (Table 2).

Table 2. Cerebellar dysfunction in mutant mice

Targeted Genes	Cellular abnormality			Behavioral abnormality			
	LTD	CF	MF/GA/PC	VOR/OKR adaptation	Eye-blink conditioning	Motor coordination	Reference
mGluR1	Abs	Multi				Abn	1.
GluRδ2	Abs	Multi				Abn	2.
Gαq	Abs	Multi				Abn	3.
nNOs	Abs			Abs		Abn	4.
PKC-γ	Retained	Multi				Abn	5.
mGluR4	Retained		Abn			Abn	6.
PLC-β2		Multi				Abn	7.
NR2A/2C		Multi	Abn			Abn	8.
GFAP	Abs	Single			Abn	Norm?	9.
PKC inhibitor	Abs	Multi		Abs		Norm?	10.

modified from reference of Ito, 2001. MF/GA/PC, mossy fiber-to-axons of granule cells-to-Purkinje cell transmission; abs, absent; abn, abnormal; multi, multiple innervation of PCs by climbing fibers(CFs); single, normal one-to-one innervation of PCs by CFs; norm?, seemingly normal; nNOS, neuronal nitric oxide synthase; PKC, protein kinase C; GFAP, glial fibrillary acidic protein; References: 1. Aiba et al., 1994; Conquet et al., 1994; Ichise et al., 2000; Kano et al., 1997; Linden et al., 1994; 2. Hirano et al., 1995; Kashiwabuchi et al., 1995; 3. Offermanns et al., 1997; 4. Katoh et al., 2000; 5. Chen et al., 1995; 6.Pekhletski et al., 1996; 7. Kano et al., 1998; 8. Kadotaniet al., 1996; 9. Shibuki et al., 1996; 10. De Zeeuw et al., 1998.

1.9 Cre-loxP system

The Cre/loxP system allows tissue-specific (and in connection with the tet system also time-specific) knockout of such genes which can not be investigated in differentiated tissues because of their early embryonic lethality in mice with conventional knockouts. This system allows researchers to create a variety of genetically modified animals and plants with the gene of their choice being externally regulated (Kuhn et al., 2002; Ghosh et al., 2002). The *cre* gene, short for **c**yclization **re**combination, encodes a site-specific DNA recombinase. Cre is a 38 kDa recombinase protein from bacteriophage P1 which mediates intramolecular (excisive or inversional) and intermolecular (integrative) site-specific recombination between loxP sites (Sauer, 1993). A loxP site (locus of X-ing over) consists of two 13 bp inverted repeats separated by an 8 bp asymmetric spacer region.

One molecule of Cre binds per inverted repeat; or two Cre molecules line up at one loxP site. The recombination occurs in the asymmetric spacer region. Those 8 bases are also responsible for the

directionality of the site. Two loxP sequences in opposite orientation to each other invert the intervening piece of DNA, two sites in direct orientation dictate excision of the intervening DNA between the sites leaving one loxP site behind. This precise removal of DNA can be used to eliminate an endogenous gene or activate a transgene **(**see Figure 13).

1.9.1 Strategy:

Two mouse lines are required for a conditional gene deletion. First, a conventional transgenic mouse line with Cre targeted to a endogenous gene of the specific tissue or cell type, and second a mouse strain that carries a target gene (endogenous gene or transgene) flanked by two loxP sites in the same orientation ("floxed gene"). Recombination (excision and consequently inactivation of the target gene) occurs only in those cells expressing Cre recombinase. Hence, the target gene remains active in all cells and tissues, which do not express Cre (Figure 13). In a first experiment, a group in Canada (Orban et al., 1992), showed that Cre could be used at a high efficiency to excise a transgene *in vivo*. Rajewsky's group in Cologne, Germany (Gu et al., 1994), used Cre expressing mice to inactivate for the first time an endogenous mouse gene.

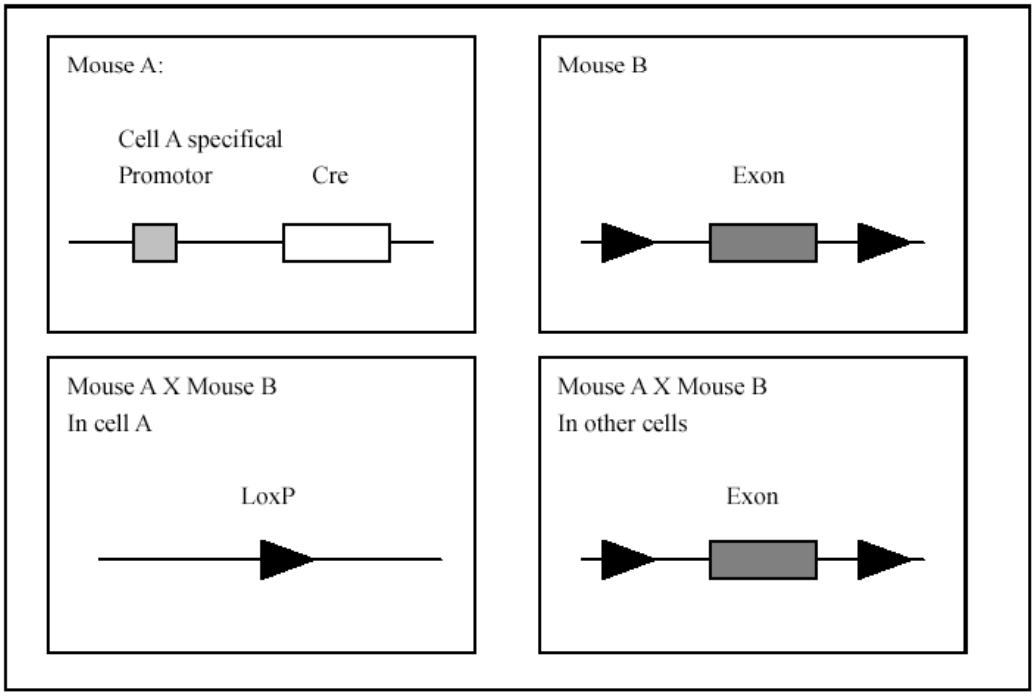
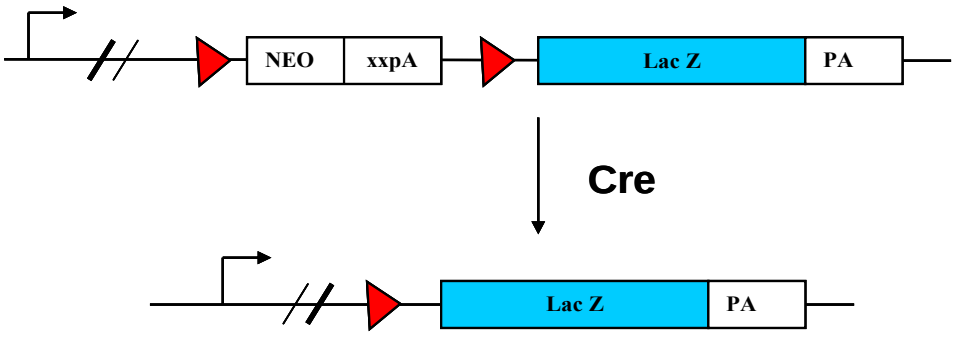


Figure 13. *Targeting gene deleted in the special cells/tissues under the special promoter*

In our study, the Bacα6 Cre mice generated by Dr. Aller et al. have the Cre gene placed into the α6

bacterial artificial chromosome (BAC) transgene. A α6Bac transgene was produced in which the nlsCre was placed into the α6 gene's first coding exon. This Bacα6 Cre transgene contains 14kb α6 gene and probably all the α6 genes' regulatory regions (50kb upstream, 120kb downstream of α6 gene) (Aller et al., 2003). The α6 gene is transcribed in just two cell types: cerebellar granule cells and the lineage-related cochlear nucleus granule cells (Kato, 1990; Lueddens et al., 1990, Laurie et al., 1992). This transgenic line enables the native $GABA_A$ receptor α6 subunit expression to be kept intact in the cerebellar granule cells (Jones et al., 2000). In order to check that the Cre is only present in the cerebellar granule cells in this transgenic mice (Bacα6 Cre), Aller et al. crossed Bacα6 Cre and the ROSA26 Cre reporter strain (R26R); in these reporter mice, a neo gene is flanked by two loxP sites in front of a lacZ reading frame (Figure 14 upper). When Cre is expressed only in the cerebellar granule cells of the offspring, the neo cassette in front of the lacZ was deleted, the lacZ gene expressed and the cerebellar granule cells were intensely lacZ-positive (Figure 14 lower). In $GluRD^{2lox}$ mice (Figure 15) generated by Elke Fuchs and Hannah Monyer (unpublished), two loxP sites flank exon11 of the GluR-D gene. Thus in the offspring of Bacα6 Cre transgene mice and $GluR\text{-}D^{2lox}$ mice GluR-D gene expression will be deleted/knocked out only from the cells in which the Bacα6 Cre is specially expressed (cerebelar granule cells). Ablating exon 11 of GluR-D will remove part of the N-terminus, TM1 and the re-entrant loop TM2, thus effectively destroying the protein. As only GluR-B Flip subunits will be left in granule cells (see Figure 8), and recombinant homomeric GluRB(R) Flip subunits can not leave the Endoplasmic Reticulum (ER). In response to glutamate, removal of GluR-D from granule cells should remove or reduce strongly APMA receptor function and thus mimic partially the *stargazin* mutation (see Figure 12).



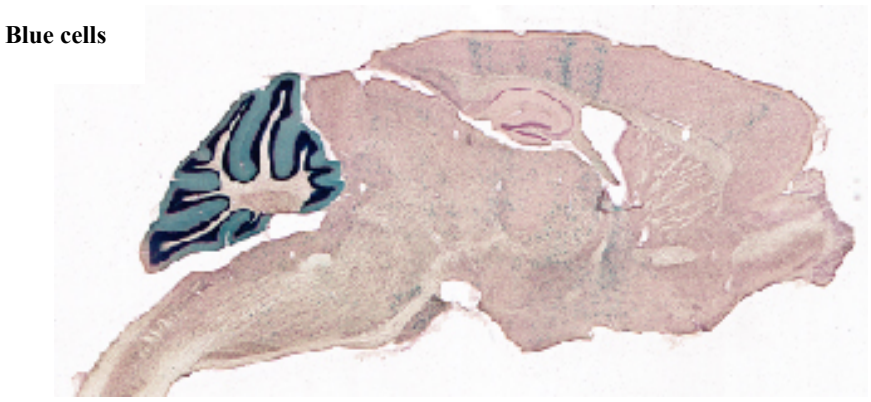


Figure 14. *ROSA 26 gene (upper); lacZ positive cerebellum granule cells (lower) (Aller et al., 2003)*

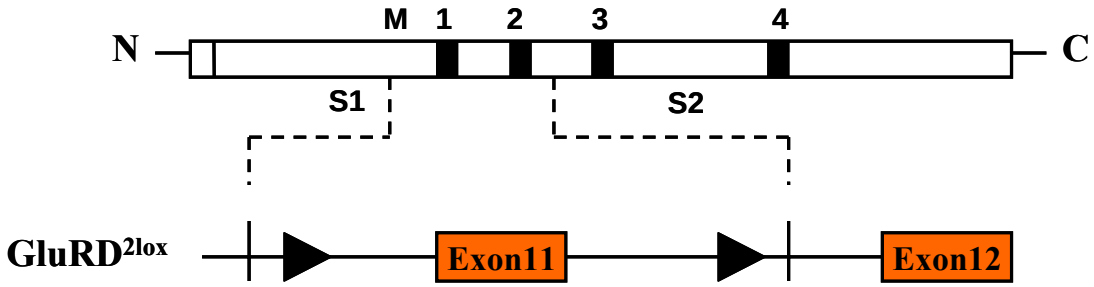


Figure 15. *Structure of the relevant part of the GluRD^{2lox} gene* The arrows are loxP sites. The corresponding protein is shown at the top. Ablating exon 11 will remove part of the extracellular domain, TM1 and the re-entrant loop TM2. S1 and S2: two binding sites (E. Fuchs and H. Monyer, unpublished)

1.10 Project aims of GluR-D conditional knock-out mice (Gr△GluRD)

My project focused on the AMPA receptors in cerebellar granule cells where the excitatory input from mossy fibers is sent, via granule cells, to Purkinje cells and other interneurons. My work aimed to analyse the Gr△GluRD mouse in which we selectively inactivated the AMPA receptor subunit GluR-D gene from the cerebellar granule cells using the loxP-Cre system. The issues I was interested in investigating with the conditional GluRD knock out mice are outlined below:

1. Since the Cre recombinase protein expression was restricted to the nuclei of cerebellar granule cells in the Bacα6 Cre (Aller et al., 2003), I aimed to confirm whether the GluR-D gene is lesioned only from cerebellar granule cells in the Gr△GluRD mouse brains.
2. As we deleted the GluR-D gene in granule cells, the only other AMPA subunit expressed in granule cells is GluRB Flip (see Figure 8). The global GluR-D knock out mice generated by Dr. Fuchs and Prof. Monyer (unpublished) showed more GluR-B protein expressed in the hippocampus. Is there also any compensative change by other AMPA receptor or kainate receptor subunits in granule cells lacking GluR-D?
3. How is the function of AMPA receptors changed in the granule cells lacking the GluR-D subunit? Are AMPA receptor responses abolished? Is there any effect on the evoked NMDA response? We predict that in our mice, the mossy fibre-granule synapses will be silent. The reason is that NMDA receptors can not be activated since there will not be enough AMPA-mediated depolarization to remove the Mg^{2+} block on NMDA receptors. If this prediction is true, we would assume there would be no LTP at the mf-granule cell synapse.
4. Mice with a selective death of granule cells, or mice whose granule cells can not release glutamate are ataxic (Shmerling et al., 1998; Yamamoto et al., 2003) and have impaired motor learning, we were interested in investigating the motor behaviour of these mice lacking AMPA receptors selectively on granule cells.
5. *Stargazer* granule cells, in addition to having no functional AMPA receptors, have no BDNF mRNA (Qiao et al., 1996) and reduced levels of $GABA_A$ receptor subunit α6 mRNA and protein (Thompson et al., 1998; Ives et al., 2002). By making granule cells with no functional AMPA receptors in Gr△GluRD mouse, in effect I produced mice which phenopcopy the *stargazer* mutation, but selectively in granule cells. This enabled us to test whether the atxaia, reduced BDNF and $GABA_A$ receptor expression was due to the stargazer mutation in granule

cells from lack of AMPA receptor-mediated depolarization (as is often assumed) or originates from other effected cell types.

6. AMPA receptors normally mediate depolarization of granule cells. The selective loss of AMPA receptor responses from these cells enabled us to use gene chip technology to determine the target genes whose expression is normally regulated by AMPA receptors.

2 METHODS

2.1 Animals

We used wild type C57/BL6 (WT), Bacα6Cre C57/BL6 (WT-α6Cre), Bacα6Cre X GluR-D^{2lox} C57/BL6 (Gr△GluRD) and GluR-D knock out mice (GDKO) for our study.

The Bacα6Cre C57/BL6 (α6Cre line was generated by Dr. Aller et al., 2003, Department of Clinical Neurobiology, University of Heidelberg). GluR-D^{2lox} and GluR-D knock out mice (GDKO) were generated by Dr. E. Fuchs in Prof. H. Monyer's group (unpublished), 2002 (Department of Clinical Neurobiology, University of Heidelberg). Bacα6CreXGluR-D^{2lox} C57/BL6 (Gr△GluRD) mice were produced by crossing heterozygote α6Cre/ GluR-D^{2lox} and homozygote GluR-D^{2lox} mice; from these litters some mice were Cre positive and homozygous for the GluRDlox allele (granule cell knockouts); all other mice from the litters were used as "wild-type" litter mates. I set up these crosses each time I needed experimental animals.

2.2 Genotyping

1cm cut tails were digested with proteinase K (20mg/ml) in HIRT (define) buffer at 55°C overnight. DNA was extracted and precipitated with protein precipitation solution (define) and isopropanol. The DNA pellets were washed with 70% ethanol, and dissolved in TE buffer. PCR reaction was performed with extracted DNA, Taq polymerase kits, primer GluRD1, GluRD2 / Cre1, Cre2 (sequences shown in the Appendix), dNTPs, with denature temperature at 95ºC for 3 minutes and 94ºC for 30seconds, annealing temperature at 58°C for 45 seconds, the extension is at 72ºC for 1minute, 34 cycles. The genotype was determiend by by 2.5% agarose gel electrophoresis of the PCR products. The genotyping of WT, WT-α6Cre, GrΔGluRD and GluR-D KO mice is shown in Figure 16. With the Cre primers, we got Cre+ and Cre- genotypes, meaning the Cre gene is present or absent in the genome (Figure 16b). The primer GluRD we used is 3' (antisense) of the flanked GluR-D-exon 11, where one loxP site was inserted (Figure 16a). The loxP sequence is about 130 bp long (see diagram of loxP sequence in the Introduction). The size of this flanked sequence with one loxP site is 480 bp, whereas it is 350 bp without the loxP. Our data (Figure 16c) show that the +/+, +/-, -/- genotypes respectively have no loxP on two alleles, loxP on one allele, and loxP on both alleles. From this we confirmed the genotyping of the mice. WT and WT-α6Cre are homozygous without loxP (+/+), GluR-D KO and Gr△GluRD are homozygous with loxP (-/-),

whereas the +/- is heterozygous with one loxP allele called BacGD^{lox}, and one wild-type GluR-D gene.

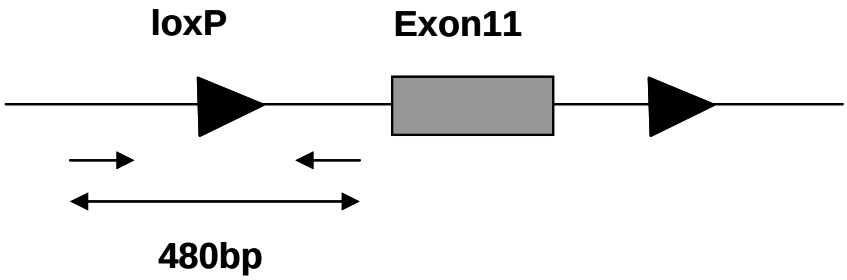


Figure 16a. *Genotyping the transgenic mice*.The schematic procedure of PCR, reaction with primer GluRD, 480bp of PCR products

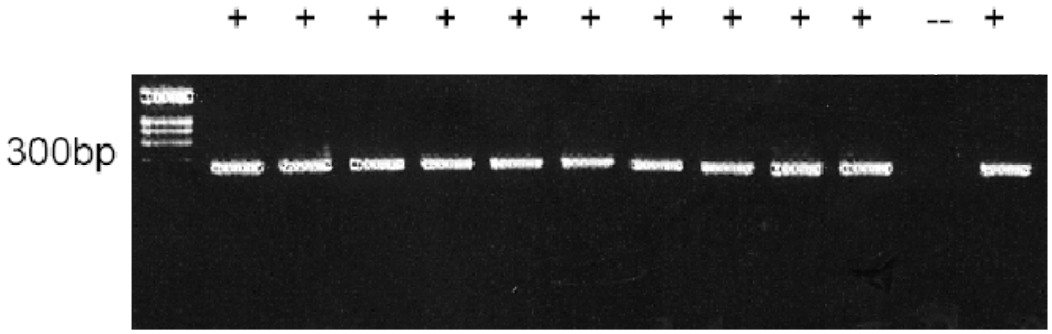


Figure 16b. *PCR with primer Cre*. + Cre positive, – Cre negative

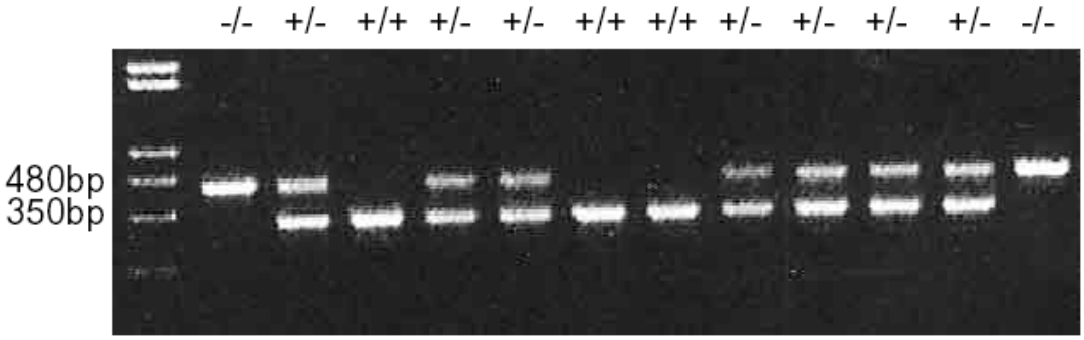


Figure 16c. *Genotyping the mice*: *PCR with primers GluR-D*. —: GluR-D with loxP site; +: GluR-D without loxP site

2.3 *In situ* hybridization

Brains from WT-α6Cre, Gr△GluRD, GDKO and WT-littermates adult were used for *in situ* hybridization analysis (Wisden & Morris, 1994). The mice were anesthetized and killed, the mouse brains were completely excised from the skull and immediately frozen on dry ice, then transferred to –70°C. Tail samples were cut for the confirmation of genotyping. Fresh frozen sections (12μm in thickness) were cut by cryostat (MICROM HM500) at –20°C and mounted on glass slides pre-coated with poly-L-lysine. Sections were allowed to dry at room temperature for half an hour, then transferred into 4% PFA solution, 1xPBS, 70% ethanol and 95% ethanol all diluted with DEPC-treated H_2O, each for 5 minutes. For detection of the mouse AMPA and kainate receptor subunit mRNAs, BDNF, stargazin, GAD65 and GAD67 mRNAs, 45-mer antisense oligonucleotides were synthesized. The antisense sequences of mouse oligonucleotides are discribed in the Materials section (see Appendix). These oligonucleotides were labeled with α^{35}S-dATP using terminal deoxyribonucleotidyl transferase (TdT), reaction buffers, oligonucleotides and ^{35}S dATP at 37°C for 5 minutes (Wisden and Morris, 2001). I stopped the reaction with TNES (define) buffer and spin the labeled oligonucleotides through tSephadex-G25 spin columns by centrifuging at 2300rpm for 2 minutes; I scintillation counted the activity of the labeled oligonucleotides to ensure they were labeled; only oligonucleotides more than 100,000dpm/μl were used. For storage I added DTT and froze the probes at -20°C. I hybridized the labeled probes and fixed sections with hybridization buffer (50% formamide/4xSSC/10% dextran sulphate). In order to distinguish the background and positive hybridiztion I added a 50-fold excess of unlabeled oligonucleotide to the hybridization mixture as a negative control. We covered the sections with parafilm and incubated at 42°C overnight, the slides were washed with the following order the next day: 1xSCC, 1xSCC (at 60°C), 1xSCC, 0.1xSCC, 70% ethanol, 95% ethanol. Sections were exposed to X-ray film for 1 month (protocol, Wisden & Morris, 2001).

2.4 Immunoblot (Western Blotting and protein quantitative analysis)

WT, WT-α6Cre, Gr△GluRD, GDKO mice were decapitated under anesthesia, and brains were removed, and separated into two parts: cerebellum and forebrain. They were homogenized in HEPESs buffer (define buffer) (pH=7.4, with protease inhibitor tablet, 1pill/10ml Hepes buffer). The volume of added HEPES buffer was three times of the weight of the cerebellum/forebrain.

The mixture was centrifuged 2 times to obtain a postnuclear fraction. The protein concentration was determined by the method of Bradfold (Biolab). Equal amounts of the protein (40μg) were fractionated by 10% SDS-PAGE and electrotransfered onto a nitrocellulose membrane. To block the nonspecific binding, Iused 5% milk solution plus 0.1% Tween 20 at 4°C overnight. The blot was immunoreacted with rabbit polyclonal anti-GluRA antibody at 2μg/ml (Chemicon), mouse anti-GluRB antibody at 2.5μg/ml (Chemicon), rabbit polyclonal anti-GluRD antibody at 0.75μg/ml (Chemicon), monoclonal anti-β-actin antibody at 1:1500 (Sigma), respectively, followed by incubation with secondary antibody with anti-rabbit IgG, or anti-mouse IgG (1:8000) and visualized by chemiluminescence (ECL detection system), and exposed to the Hyper film^{TM} ECL. To do the protein quantitative analysis, we extracted protein from the cerebellum of four Gr△GluRD and littermate WT-α6Cre mice respectively, and repeated the immunoblot three times for each mouse. We measured the density of the visible bands of GluR-A, GluR-B, GluR-D, β-actin, respectively and subtracted the background of the film using the Image J 1.31 program. Calculation of the statistical significance of differences was performed using unpaired, two-tailed Student's *t* test.

2.5 Immunocytochemistry staining

2.5.1 DAB staining

Gr△GluRD, GluR-D knock out (GDKO) and WT-α6Cre mice were anesthetized by a subperitoneal injection of a etamin and Xylasin mixture. The chest of the mouse was opened, exposing the pumping heart. We performed intra-leftventricular perfusion with 1xPBS for 5 minutes, 4%PFA (in PBS pH7.4) solution for 15 minutes. Perfused brains were excised and postperfused in 4%PFA for two hours, put into the 1xPBS overnight, then embedded with 4% Agar/PBS. The brains were sliced into 50μm sagittal vibratome sections (LEICA VT 1000s). The free-floating sections were washed with 1xTBS 10 minutes for 3 times, followed by H_2O_2 treatment; I reduced the non-specific background with blocking solution (1xTBS+0.4% triton-X-100 +20%goat serum). The sections were incubated in polyclonal anti-GluRD antibody (final con. 0.67 μg/ml), polyclonal anti-Cre recombinase antibody (1:3000, diluted with 1xTBS+0.4% tritonX100+2% goat serum) at 4°C for 72 hours. After washing with 1xTBS 10 minutes for 3 times, sections were incubated with 2nd antibody of biotinylated goat ani-rabbit IgG (diluted

1:600 in 1xTBS+0,2% tritonX100+ 2% goat serum) at room temperature for 2 hours followed by incubation in avidin-biodinzlated horseradish peroxidase complex (ABC kits, diluted 1:600) for 90 minutes. Peroxidase enzyme reaction was with DAB enhancer plus Nickel as chromogen and H_2O_2 as oxidant. The reaction was stopped with 1xTBS after 5-10 min. For controls, the sections were incubated without 1st antibody, or with 2nd antibody (+ABC, -ABC) and without 2nd antibody (+ABC). The stained sections were mounted on slides. Sections were air-dried, dehydrated in ethanol and xylene, and coverslipped.

2.5.2 Immunoactivity staining with flourescence:

Three brains from each Gr△GluRD and WT-α6Cre mice were perfused with 4% PFA and brains were fixed and sliced using the same procedure as described above. The sections were washed with 1x PBS 10 minutes for 3 times, followed by blocking with solution 1xPBS+0.3% triton-X-100+4%goat serum at room temperature for 1 hour to reduce the non-specific binding. For the first incubation the antibodies used were monoclonal anti-GABA (1:1000, diluted with 1xPBS+0.2% triton-X-100 +2%goat serum), mouse anti-GAD65 (1:300), polyclonal rabbit anti-stagarzin antibody (1: 500) and polyclonal rabbit anti-phosphorylated CREB antibody (1:1000). Sections were incubated with primary antibody at 4°C for 72 hours. The frozen sections, which were sliced to 14 μm by cryostat (MICROM HM500) at –20°C and mounted on glass slides precoated with poly-L-lysine, followed by the fixation with 4%PFA for 5 minutes, were used for the incubation with anti-stagarzin antibody. After washing 10 minutes for 3 times with 1xPBS+0.1%Triton+2%NGS , the sections were incubated with 2nd antibody: anti-mouse Alexa 488 (Invitrogen, 1:500) or anti-rabbit Cy3 (Jackson ImmunoResearch, 1:800) for 2 hours at room temperature, washed again with 1xPBS, H_2O, then mounted on the slides. Sections were air-dried and coverslipped. From the step of incubation with 2nd antibody, the sections were covered with aluminum foil.

2.6 SYBR green-based real-time quantitative PCR (qRT-PCR)

Total RNA from cerebellum and forebrain were isolated from four adult homozygous Gr△GluRD and WT-α6Cre littermates using the ULTRASPEC RNA isolation system (BIOTECX Laboratories, Inc.). The RNA was treated for 15 min at room temperature with DNase I (Qiagen) and cleaned with the RNeasy Mini kit (Qiagen). Extracted RNA was

quantified by Biometer and checked by 1.2% agarose gel electrophoresis. 5 μg of total RNA was reverse transcribed using random hexanucleotide-priming (SuperScript Single-Stranded cDNA Synthesis Kit (Invitrogen)). Real-time PCR was performed with a SYBR® Green PCR Kit (PE Applied Biosystems) using an ABI Prism 7000 sequence detector (PE Biosystems, courtesy of Dr. M. Schwaninger, Dept. of Neurology, Heidelberg). The concentrations of primers were normalized, gene-specific forward and reverse primer pair were mixed. Each primer (forward or reverse) concentration in the mixture was 5pmol/μl.

The cycling conditions were: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescence measurements were recorded during each annealing step. For each PCR, 5 μl of cDNA template was added to 25 μl of the PCR master mixture. All amplification reactions were performed in triplicate. To control for the recovery of intact cellular RNA and for the uniform efficiency of each reverse transcription reaction, a cyclophylin fragment was co-amplified. The efficiency of real-time amplification was determined by running a standard curve with serial dilutions of pooled samples. A linear concentration-amplification curve was established. Quantified results for individual cDNAs were normalized to cyclophylin and the purity of the amplified products was checked by the dissociation curve (Aller et al., 2005).

The primers for real-time PCR were from TIB MOLBIOL Syntheselabor GmbH, Berlin. The GABAα6 oligonucleotide sequences were as used by Heurteaux et al. (2004).

The other oligonucleotide sequences are included in Appendix, section 5.2.3 “Primers for quantitative real-time PCR”.

2.7 Sequence analysis of the GluR-B editing site

Reverse transcription PCR amplication of GluR-B sequences from the cerebella of Gr△GluRD and WT-α6Cre adult mice were performed. Three adult mice from the Gr△GluRD and WT-α6Cre littermates were killed at 2 months age (n=3 mice/group). Intact cerebella were excised and total cellular RNA was extracted and cDNA was synthesized as described in the real-time PCR section (see above) Performing PCR amplification of GluR-B editing site used the primers B52 (10μM, sense primer) and 3’LAMLO (10μM, anti sense primer) which were kindly provided by Dr M.Higuchi at the MPI Heidelberg (primer sequences are described in the

Materials section). Using these primers, and 0.2 µg cDNA, the GluR-B product was amplified with denature temerature at 95ºC, 3min and 1min, annealing temperature 55ºC, 30S, the extension is at 72ºC for 45S, 30 cycles. The 100µl final PCR products were run on 3% agarose gels and extracted with a gel extraction kit (Qiagen). The sequencing reaction took place with denature temperature 94ºC for 3min and 15S, denature at 55ºC for 15S, extention at 60ºC for 4min, 29 cycles, using Big Dye (provided by MPI), an internal sequence primer (provided by M. Higuchi in MPI) and 50ng PCR product extracted from gel as template; after the reaction, the products were precipitated using sodium acetate and absolute ethanol. Dried DNA products were sequenced at the MPI Heidelberg. The strategy of sequencing is shown in Figure17. The primer sequences are given in the Appendix section.

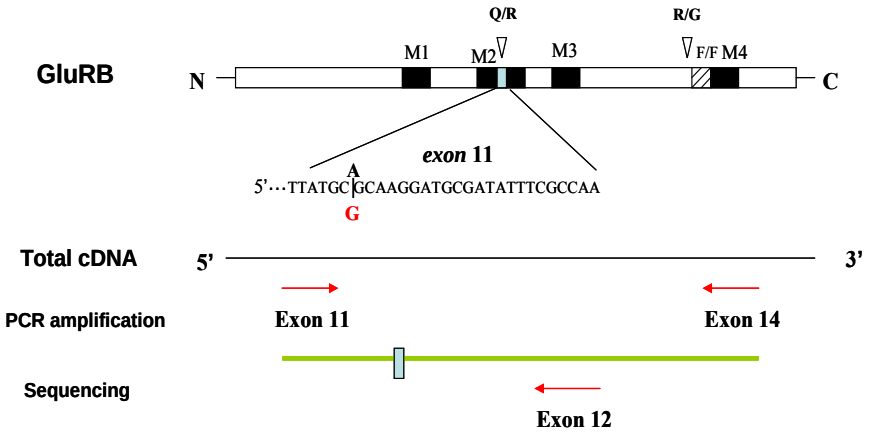


Figure 17. *The strategy of sequencing at GluR-B editing site.* Sense primer located in Exon11; antisense primer at Exon14; primer for sequencing at Exon12

2.8 Gene Expression Profile array (DNA microarray analysis)

Three from each adult Gr△GluRD (BAC) mice and WT-α6Cre (WC) littermates were sacrificed at 2 months age (n=3 mice/group). Intact cerebellums were excised and total cellular RNA was extracted using ULTRASPEC RNA isolation system (BIOTECX Laboratories, Inc.) and RNeasy Mini kit (Qiagen). Extracted RNA was quantified by Biometer and qualified by 1.2% agarose gel. Each amounts of RNA from each group were pooled. Two cRNAs were then independently generated from each pooled RNA preparation.

Amplification, hybridization and scanning were performed at the IFZ BioChip-Labor of the Universitätsklinikum Essen, Germany. Biotinylated cRNA was hybridized on Affymetrix U74a2 murine expression profiling arrays. Data was processed with MAS 5.0 (Microarray Suite) and DMT 3.0 (Data Mining Tool).

Briefly, the integrity of the cRNA amplification reaction was confirmed with LysX spike in controls (Gr△GluRD probes received three times the amount of spike in control compared to WT-α6Cre) and by looking at the 3' to 5' amplification ratios. All reactions fulfilled the set criteria. Subsequently the cRNA was hybridized to Affymetrix U74a2 murine expression profiling arrays and scanned. Integrity of the hybridization was confirmed by evaluation of the average background (<50) of the individual array and by the number of present calls (>50%). After making sure the individual array had no physical damage or hybridization bias (unnatural strong hybridization at some parts) the arrays were normalized to a value of 1000. Significant ($p = 0.05$) up or down of transcripts between the wild-type α6Cre (WC) and Gr△GluRD (BAC) arrays was determined using a Mann-Whitney analysis. For significantly up regulated transcripts (WC→BAC) at least two knock out arrays had to have a detection present call, for down regulated transcripts at least two wt arrays had to have a detection present call for the given transcript.

2.9 Electrophysiology

The electrophysiology experiments were performed by Dr. Golovko in Dr. Andrei Rossov's laboratory (Clinical Neurobiological department of University Heidelberg, Germany).

2.9.1 Acute slice preparation

The Gr△GluRD mice and WT-α6Cre littermates were decapitated at 42 days (20 mice from each genotype). A sucrose replacement technique adapted from Mann-Metzer and Yarom (1999) was employed for preparation of parasagittal cerebellar slices (250 μm thick). The cerebellum was rapidly removed and placed in ice-cold (~4ºC) artificial cerebrospinal fluid (ACSF: 124mM NaCl, 3mM KCl, 1.25mM NaH_2PO_4, 2.5mM $CaCl_2$, 1.3mM $MgCl_2$, 26mM $NaHCO_3$, 10mM glucose, buffered with 5% CO_2 and 95% O_2, pH=7.4). The cerebellar vermis was sliced sagittally 250um with a vibrotome (Desaka). The slices were incubated in ACSF for at least 30 minutes before being transferred into a submersion recording chamber and constantly perfused (2ml/min)

with standard recording solution that contained (in mM): 125 NaCl, 2.5 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, pH=7.4 when bubbled with 95% O_2 and 5% CO_2. Incubations and all subsequent experiments were done at room temperature.

2.9.2 Patch-clamp recording from cerebellar granule cells

Granule cells were visually identified by infrared differential-contrast video microscopy and their location and firing pattern after current injection. Whole-cell voltage recording were made from granule cells in granule layer of cerebellum. Extracellular stimulation was performed with stimulation pipette. Stimulation pipette was filled with extracellular solution and was positioned in mossy fiber of cerebellum. Recording pipettes of 4-6 M resistance were filled with intracellular solution containing (in mM): 105K-glugonate, 30KCl, 4Mg-ATP, 10 phospho-creatine, 0.3GTP, and 10 HEPES (pH 7.3, 293 mOsm). Extracellular stimulation contains (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 25glucose. For AMPA/NMDA ratio experiments used solution containing 3mM Ca^{2+} and nominally 0 mM Mg^{2+}, in the presence of the $GABA_A$ receptor channel blocker bicuculline (BBC, 5μM) and glycine (10μM). AMPA and NMDA-mediated currents were pharmacologically dissected using AMPA and NMDA receptor channel blockers, CNQX (10μM) and APV (100μM), respectively. AMPA/NMDA ratios were measured ten granule cells from Gr△GluRD and fifteen from WT-Cre cerebella.

Contribution of kainate receptor channels to evoked synaptic currents in Gr△GluRD and WT-α6Cre mice were tested by bath application of GYKI-52466, selective AMPAR blocker. In this experiments extracellular solution contained 2 mM Ca^{2+} and 1mM Mg^{2+}, NMDAR and $GABA_A$ receptors were blocked by APV (100μM) and BCC (5μM) respectively.

Stimulus delivery and data acquisition were performed using PULSE software (HEKA Elektronik, Lambrecht, Germany). Analysis was performed using IGOR PRO software (Wavemetrics Lake Osweg, OR). Calculation of the statistical significance of differences was performed using unpaired, two-tailed Student's *t* test.

2.10 Behavioural studies

The behavioural studies were performed by Dr. E. Fuchs (Department of Clinical Neurobiology, University of Heidelberg, Germany).

GluR-D conditional knockout mice (Gr△GluRD), WT-α6Cre littermates and GluR-D knockout (GDKO) mice (3-4 month old weighting 27-36 g) were maintained at the standard animal facilities in polypropylene macrolon cages with food pellets and tap water available *ad lib*. Lights were on from 6 a.m. to 6 p.m. Temperature and humidity were controlled at 20 ± 1 °C and 50 ± 10%, respectively. All animal tests were approved by the Laboratory Animal Committee of the University of Heidelberg. The person who observed and recorded the behavior was not aware of the genotype of the tested animals. Behavioral and physiological characterization of phenotypes was performed using a modified version (Vekovischeva et al., 2004) of the primary screen described in the SHIRPA protocol (**S**mithKline Beecham Pharmaceuticals; **H**arwell, MRC Mouse Genome Centre and Mammalian Genetics Unit; **I**mperial College School of Medicine at St Mary's; **R**oyal London Hospital, St Bartholomew's and the Royal London School of Medicine; **P**henotype **A**ssessment; Rogers et al., 1997). Eight mice from Gr△GluRD and WT-α6Cre littermates were performed with open field, horizontal bar, rotorod and static rod test to investigate motor coordination and motor learning. For the second trail of motor learning six wild-type, eight WT-α6Cre and seven Gr△GluRD littermate mice were used.

Open field: The open field consisted of a gray PVC enclosed arena (50 x 30 x 18 cm), which was divided into 10 x 10 cm squares. Since our aim was to assess activity and exploration, and not to subject the mice to a strongly anxiogenic situation, normal room illumination was used. Mice were placed individually into one corner of the maze facing the sidewalls and observed for 5 min. The total number of squares crossed, latency to move, total number of rears, and latency to first rear was recorded. Two consecutive tests were run.

Horizontal bar: The bar was a wooden rod 2 mm thick, 38 cm long, held 49 cm above the bench surface by supporting columns at each end. Each mouse, held by the tail, was allowed to grasp the bar with it's forepaws, to a maximum of 30 s or until it reached an end column. Either of these resulted in a maximum score or 5, falls at earlier times received a graded score from 1 to 4.

Static rods: The mouse was placed 2 cm from the open end of a 60 cm long wooden rod supported at the other end by a clamp. Rods were 40 cm above floor level. Three diameters of rods were used: 28 mm, 22 mm and 15 mm, starting with the widest. The time to orientate (turn around 180^{o}, to face away from the open end) on each rod was measured and the transit time to

run the rod and reach the bench end. Any falls were noted, and mice failing to orientate or transit the rod before falling were assigned values of 180 s (the maximum test duration) on that and subsequent tests.

Rotarod: The mice were put on an accelerating rotarod (TSE Systems, Bad Homburg, Germany, model V4.0) which was used with a start speed of 4 rpm. Rotation speed increased from 4 to 40 rpm over a 6 min period.

The mice were trained for 6 days (3-6 trials a day) to stay walking on a rotating rod (TSE Systems, Bad Homburg, Germany, model V4.0) for 180 s while the rotation speed accelerated from 4 to 40 rpm. Repeated testing on the accelerating rotarod is used to assay motor learning in mice (Lalonde et al., 1995; Gerlai et al., 1996; Le Marec and Lalonde, 1997; Paylor et al., 1998). The mice were given daily trails on the rotarod accelerating from 4 to 40 revolutions per minute over a 5-minute period each day. Increasing daily improvement in performance, as measured by increasing latency to fall from the rotarod, indicates motor learning. Rotarod testing was performed on three consecutive days. On each day the mice underwent two sessions, for one session the average time to fall was determined from three trials for each mouse. For the second trail of motor learning test, we compared Gr△GluRD, WT-α6Cre mice with wild-type littermates using the same protocol as described above.

3 RESULTS

3.1 Production of mice lacking AMPA receptor GluR-D in adult cerebellar granule cells.

To generate mice that lack the AMPA receptor subunit GluR-D specifically in cerebellar granule cells (GrΔGluRD), I first crossed BACα6Cre (Aller et al., 2003) and GluR-D2lox (E.Fuchs & H. Monyer unpublished) mice to get α6Cre/GluR-D2lox heterozygotes (see Methods). I then crossed these heterozygotes to get homozygote α6Cre/GluR-Dlox (GrΔGluRD, granule cell knockouts), α6Cre/GluR-D2lox homozygotes ("WT-α6Cre" litter-mate) and GluR-D2lox homozygote mice ("wild-type" litter-mate); all offspring were produced in the expected Mendalian ratio (see Methods section). The α6Cre gene is expressed selectively in cerebellar granule cells (Aller et al., 2003); I used anti-Cre antibody to analysis the Cre expression in the GrΔGluRD cerebellum, in which all granule cell nuclei appeared positive (Figure 20C). The α6Cre gene turns on in the second to third postnatal week after granule cells have completed their migration into the internal granule cell layer (Aller et al., 2003), and so no developmental deficits were anticipated as a result of removing GluR-D from post-migratory granule cells. The morphology of the adult GrΔGluRD brains appeared normal, and there was no obvious reduction in, for example, the density or thickness of the cerebellar granule cell layer (see Figure 18 autoradiographs for an impression of the granule cell layer). Adult GrΔGluRD mice appeared healthy and displayed no overt neurological deficits (see method section, Behavioural studies of GrΔGluRD mice).

I confirmed by *in situ* hybridization that GluR-D exon 11 transcripts were selectively absent from the granule cells of GrΔGluRD mice. In the GrΔGluRD sections hybridized with a GluR-D exon 11-specific probe, there is no GluR-D expression in the cerebellar granule cells of the GrΔGluRD mice, although GluR-D mRNA remains in the Bergmann glial layer of cerebellum and in all other brain areas (Figure 18E and F), such as the thalamus, cortex and hippocampus (HI). In parallel, I used brain from GluR-D exon 11 (-/-) total knock out mice generated by Dr. Elke Fuchs and H. Monyer as a negative control for probe specificity (Figure18G.); when these brains are hybridized with the GluR-D exon 11 probe, no specific signal is obtained (Figure 18G).

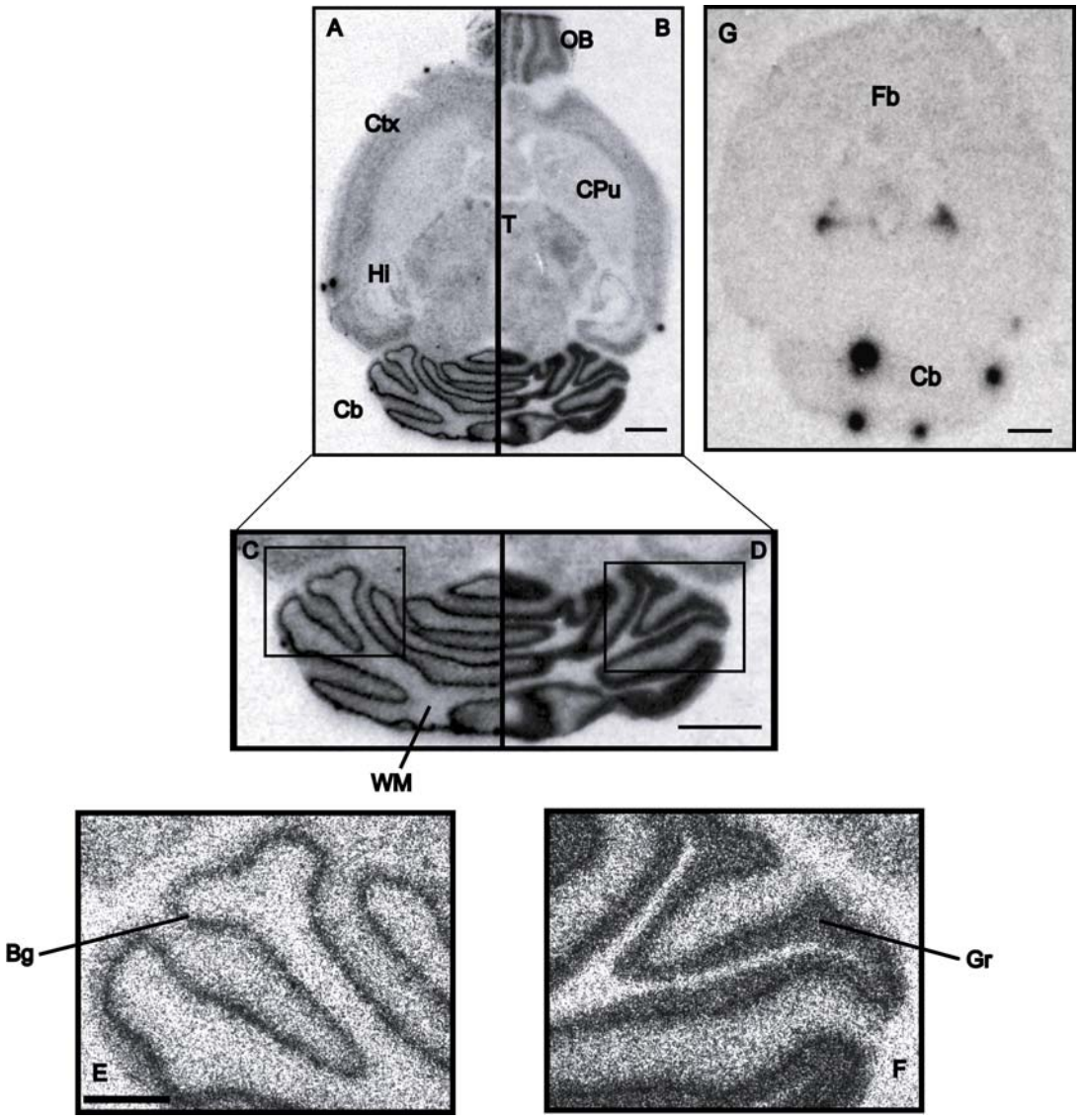


Figure 18. *Removal of GluR-D exon 11 transcripts specifically from cerebellar granule cells as demonstrated by in situ hybridization with an exon 11-specific probe (*X-ray film autoradiograph) GluR-D mRNA in Gr△GluRD mice (A,C,E) and WT-α6Cre mice brain (B,D,F). E and F are the higher magnification from the square region in C and D. G: *Absense of GluR-D mRNA expression in GDKO brain (complete GluR-D KO)* OB: olfactory bulb; Ctx: cortex; T: thalamus; CPu: caudate putamen; HI: hippocampus; Fb: forebrain; Cb: cerebellum; WM: white matter; Bg: Bermannglia cells; Gr: granule cells. Scale bar: 1.5mm; Scale bar in E, F: 0.5mm

In agreement with the pictorial *in situ* hybridization result, by real-time PCR, deleting GluR-D exon 11 specifically from cerebellar granule cells leads to around 50% reduction of mRNA expression in the GrΔGluRD cerebellum (Figure 23). We do not expect a complete absence of GluR-D exon 11 transcripts from GrΔGluRD mice since Bergmann glial cells still strongly express the GluR-D gene (see Figure 18E).

Western blotting confirmed the reduction in GluR-D protein. I first checked the GluR-D protein expression in WT, WT-α6Cre, GrΔGluRD and GluRD-KO mouse cerebella and forebrains (Figure 19). The GluR-D protein is expressed 40% less in GrΔGluRD cerebellum than in WT and WT-α6Cre cerebella (Figure 19, see also Figure 24e and f), and is entirely absent from the global GD-KO mouse cerebellum and forebrain (Figure 19), whereas it is expressed at the same level in the forebrain compared with WT, WT-α6Cre. The specific ablation of the segment encoded by exon11 within the GluR-D gene crippled the formation of GluR-D protein.

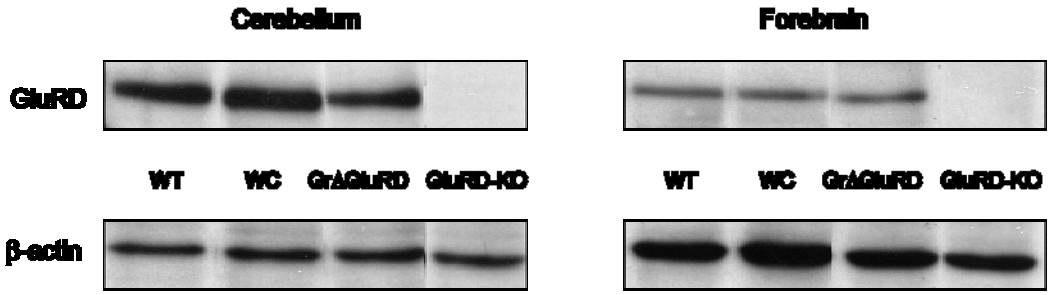


Figure 19. *Immunoblot showing AMPA receptor GluR-D subunit protein expression in WT (wild-type), WC (*WT-α6Cre*), Gr△GluRD, GluRD-KO mice cerebella and forebrains* 20μg extracted protein was electrophoriesed and blotted from cerebella and fore-brains of WT, WC, Gr△GluRD , GluRD-KO mice; β-actin served as the loading control.

However, immunocytochemistry with a GluR-D antibody gave an unclear picture with regard to what happened in the GrΔGluRD granule cell layer. In the wild type mice, using a specific GluR-D antibody (1μg/ml, rabbit, Chemicon), GluR-D immunoreactivity is found in the granule cells and Bergmann glial cells of cerebellum. But immunoreactivity with the light microscope is very dense in the Bergmann glial cell layer compared with the intensity of staining in the granule cell layer (Figure.20); white matter tracts are unstained (Figure 20). In cerebellar sections from the total GluR-D exon 11 knockout (gene ablated in every cell), the intense molecular layer staining vanishes (Figure 20D), leaving a non-specific pattern reminiscent of astrocyte staining (Figure 20D); there is still quite some residual staining of the granule cell layer. In the GrΔGluRD cerebellum, we can see that the GluR-D immunoreactivity remains in the Bergmann glial cell layer, and is reduced in the cerebellar granule cell layer (Figure 20B). The diffuse remaining staining in the GrΔGluRD cerebellar granule cell layer does not correspond to the cell bodies, but it is

background compared with global GluR-D (-/-) knock out staining (Figure 20D). However, the antibody, or our staining conditions, is not perfect; there is a significant background.

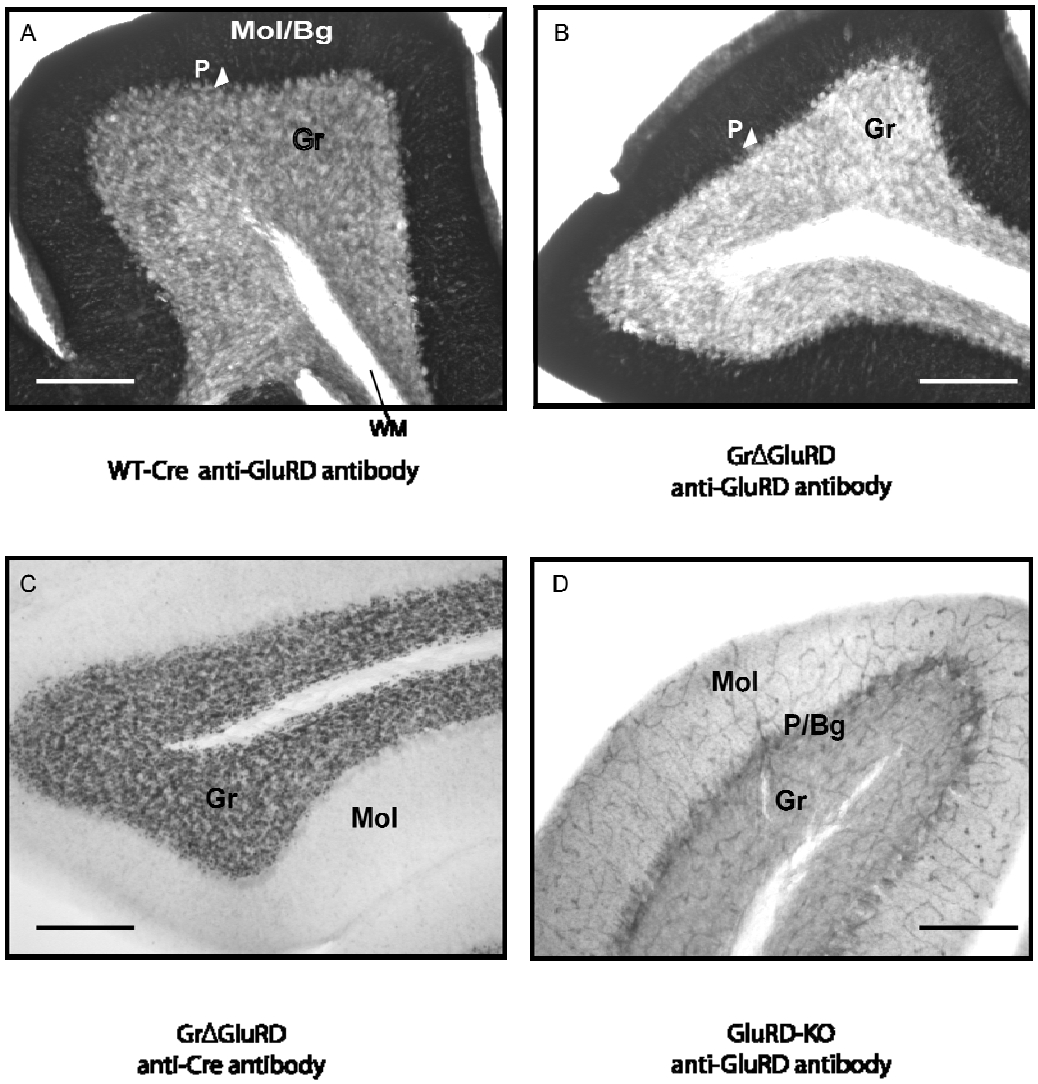


Figure 20. *Immunocytochemistry staining wih anti-GluRD and anti-Cre antibody*

A: WT-α6Cre cerebellum with anti-GluRD antibody; B: Gr△GluRD cerebellum with anti-GluR-D antibody; C: Gr △GluRD cerebellum with anti-Cre antibody; D: GluRD-KO cerebellum with anti-GluRD antibody; WM: White Matter; Gr: Granule cell; P:Purkinje cells; Mol: Molecular layer; P/Bg: Purkinje cell/Bergmann glial cell layer; Scale bar: 250μm

3.2 Electrophysiological analysis of adult cerebellar granule cells lacking GluR-D

Having confirmed the absence of GluR-D from adult granule cells, we asked if the AMPAR component was eliminated. Glutamate activates a mixed population of non-NMDARs (AMPARs and kainate Rs) and NMDARs on cerebellar granule cells (Silver et al., 1992, Cathala et al., 2003). Non-NMDA receptors primarily mediate rapid electrophysiological responses to glutamate, whereas NMDA receptors have a voltage-dependent Mg^{2+} block, and mediate a slower phase of neurotransmission (reviewed Cull-Candy et al., 2001),

Electrophysiology studies were performed on granule cells of WT-α6Cre littermates and GrΔGluRD granule cells in acute cerebellar (adult) slices to examine the significance of the changes in AMPA channel expression. So far we have only collected preliminary data, but already there are some clear differences (Figure 21). By blocking GABAergic synaptic transmission with bicuculline, the EPSCs are present from the whole- cell voltage recording on WT-α6Cre granule cells (Figure 21A). Under the conditions with Ca^{2+} and no Mg^{2+}, AMPA/kainate receptor and NMDAR-mediated currents were pharmacologically dissected using AMPAR and NMDAR channel blockers, CNQX and APV, respectively. AMPA/KainateRs mediate fast EPSCs, NMDAR-mediated component of the EPSC rises slowly and decays slowly on the cerebellar granule cells of WT-α6Cre mice (Figure 21A) and GrΔGluRD mice (Figure 21B). On the granule cells of GrΔGluRD mice, the fast EPSCs mediated by AMPA/KainateRs rise smaller, whereas no changes of NMDAR-EPSCs (Figure 21B). AMPARs and kainateRs may colocalize at the same synapse and respond in parallel to released glutamate. To distinguish AMPARs or kainate Rs, a selective AMPAR blocker, GYKI 53655, was applied. GYKI 53655 abolished the small current, implying that the residual current was due to AMPARs (Figure 21C). The ratio of EPSCs mediated by AMPARs to NMDARs is summarized in Figure 21D. In GrΔGluRD cerebellar granule cells, the non-NMDA receptor response is massively reduced compared with wild-type littermate mice.

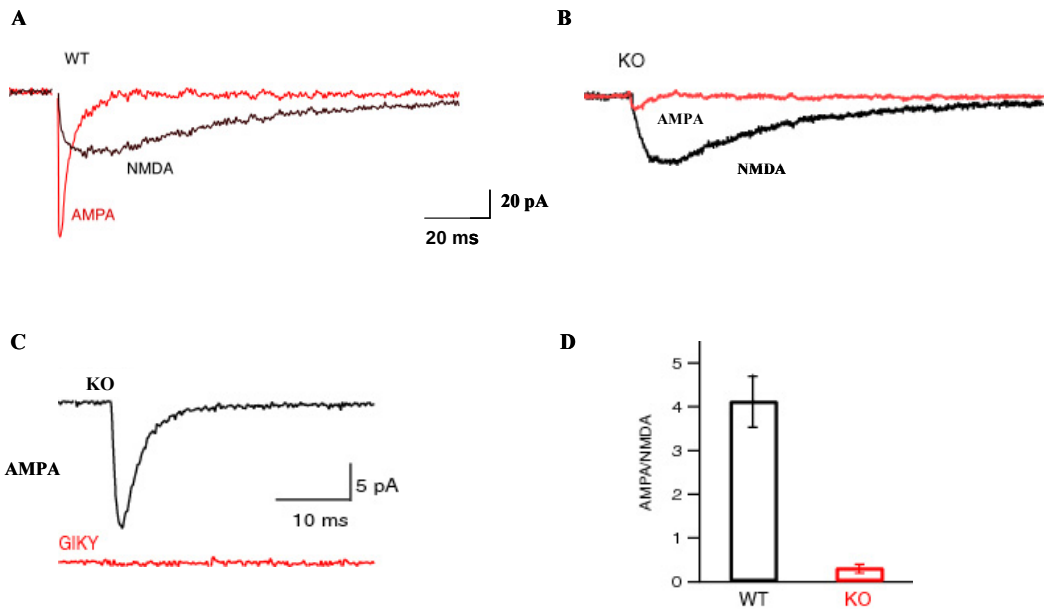


Figure 21. *Patch-clamp recording from cerebellar granule cells in WT-α6Cre mouse and Gr△GluRD cerebella.* AMPA and NMDA mediate EPSCs in WT(WT-α6Cre) mouse (A) and in Gr△GluRD cerebella (B); AMPA response before and after applying GYKI 53655 (C); ration of AMPA/NMDA response in WT(WT-α6Cre) and Gr△GluRD cerebella (D); WT: WT-α6Cre; KO: Gr△GluRD

3.3 AMPA receptor subunit expression in GrΔGluRD mice: possible compensation by increased GluR-B?

What AMPA receptor subunit(s) explains the small GYKI 53655-sensitive current in GrΔGluRD granule cells? To look for possible compensatory changes in the gene expression of other glutamate receptor genes, I compared AMPA receptor subunit gene expression in the cerebellum of WT-α6Cre littermates and the GrΔGluRD mice. As assessed by *in situ* hybridization, the GluR-A, -B and -C AMPA receptor subunit genes are expressed in the adult mouse brain (Figure 22) in similar patterns to that reported for the rat (Keinaenen et al., 1990; reviewed Wisden et al., 2000, see Figure 7 in introduction). In the mouse cerebellum, AMPA receptor subunit GluR-A, -B and-C mRNAs are in the Purkinje cells, GluR-B and-D mRNAs are in the granule cells, GluR-A and GluR-D are in the Bergmann glial cells. Comparing the expression of the other AMPA receptor subunits in the cerebellum of the GrΔGluRD and WT-α6Cre littermate mice, there is no comparable difference of mRNA distribution in the cerebellum for the GluR-A (Figure 22 A, E), -B (Figure 22 B, F) and -C (Figure 22 C, G) subunits. GluR-A mRNA is present in the Purkinje

cells and Bergmann glial layers – there is no induction of GluR-A or GluR-C mRNA in the GrΔGluRD granule cell layer (Figure 22E & G). There is no change in the granule cell expression of GluR-B transcripts, which are abundant in granule cells of both WT-α6Cre and GrΔGluRD (Figure 22 B & F). I further confirmed these results using real-time PCR with GluR-A, -B, and -C primers on cerebellar cDNA. No significant change was noted in AMPAR (-A, -B, -C) transcript levels in response to loss of exon 11 GluR-D transcripts form cerebellar granule cells (Figure 23). Although GluR-B mRNA levels do not obviously change, GluR-B protein levels increase significantly as analysed by Western bloting (Figure 24A and B). The blot shows GluR-A (Figure 24A; a, b), GluR-B (Figure 24A; c,d) and GluR-D (Figure 24A; e,f) protein expression in the cerebellum of WT-α6Cre (Figure 24A; a,c,e) and GrΔGluRD (Figure 24A;b,d,f) mouse, respectively (Figure 24A). GluR-A protein is expressed at the same level in both cerebella (a,b). As I described above that GluR-D expression is less in the conditional knock out mouse (f) than in WT-α6Cre cerebellum (e), whereas GluRB is dramatically upregulated in the cerebellum of GrΔGluRD mouse (d). The quantification of protein expression particularly illuminates the changes of GluR-B and GluR-D occurred in the cerebellum of GrΔGluRD mouse (Figure 24B), in which GluR-D protein gets around 40±2% reduction, is compatible with mRNA distribution (*in situ*), whereas GluR-B protein level increased 38±5%. I was unable to confirm if this GluR-B protein increased specifically in the GrΔGluRD cerebellar granule cell layer. I used an anti GluR-B antibody (1μg/ml), but the positive staining was too weak so that I could not distinguish the exact localization of the GluR-B subunit.

Assuming the GluR-B protein to be increased in the granule cell layer: homomeric GluR-B(R) flop forms channels with only 0.36pS (Swanson et al., 1997) However, GluR-B(R) does not reach the surface of the cell efficiently and is said to mostly retained in the endoplasmic reticulum (Greger et al., 2003, 2006), so maybe increased levels can compensate to drive surface expression. As an adjunct of a possible compensation I looked at editing of GluR-B, as homomeric GluR-B (Q) can reach the cell surface.

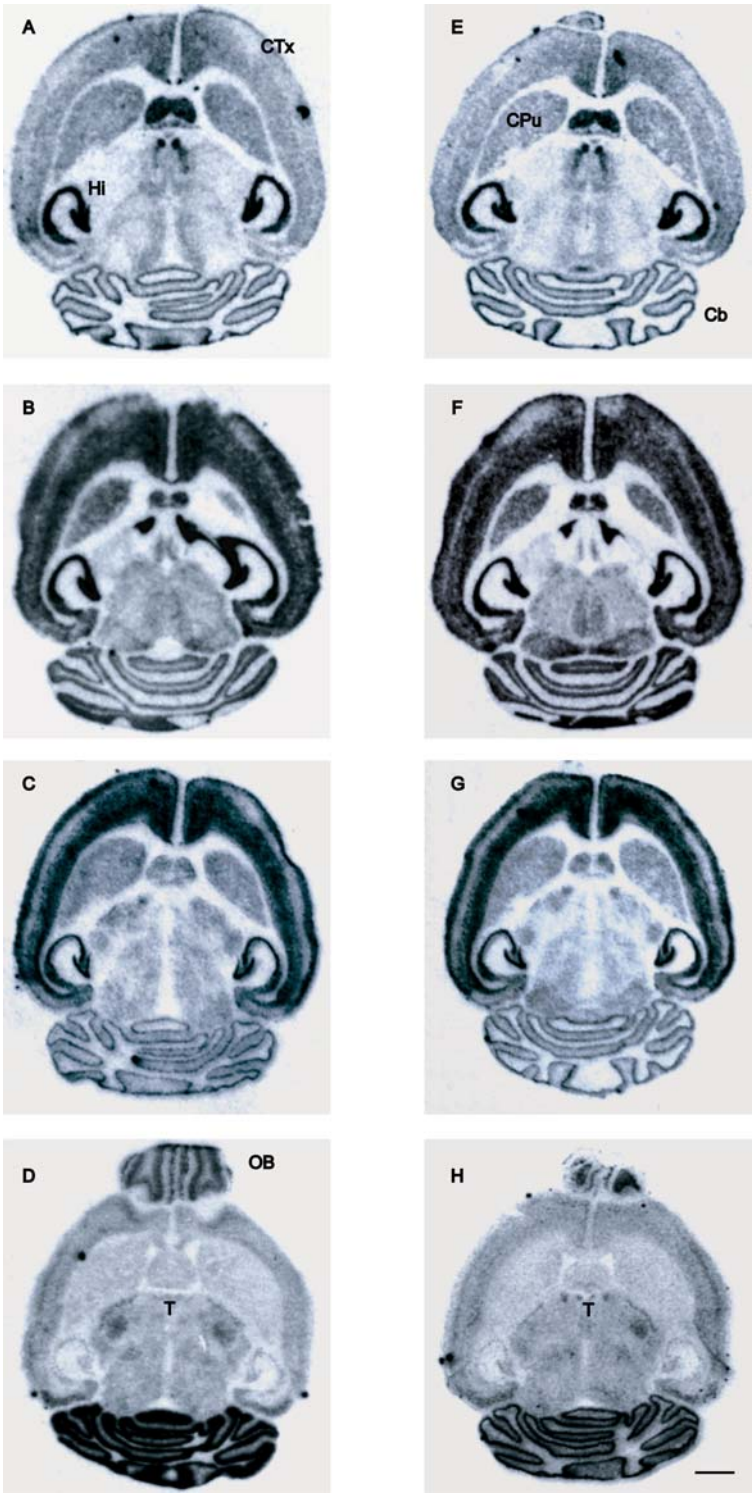


Figure 22. *In situ hybridization of AMPA receptor subunit* The distribution of AMPA receptor subunits mRNA GluR-A (A,E), -B (B,F), –C (C,G), -D (D,H) in WT-α6Cre (A,B,C,D) and Gr△GluRD (E,F,G,H) mice brains; CTx: cotex; CPu: caudate putamen; Hi: hippocampus; Cb: cerebellum; OB: olfactory bulb; T: thalamus; scale bar: 1.5mm

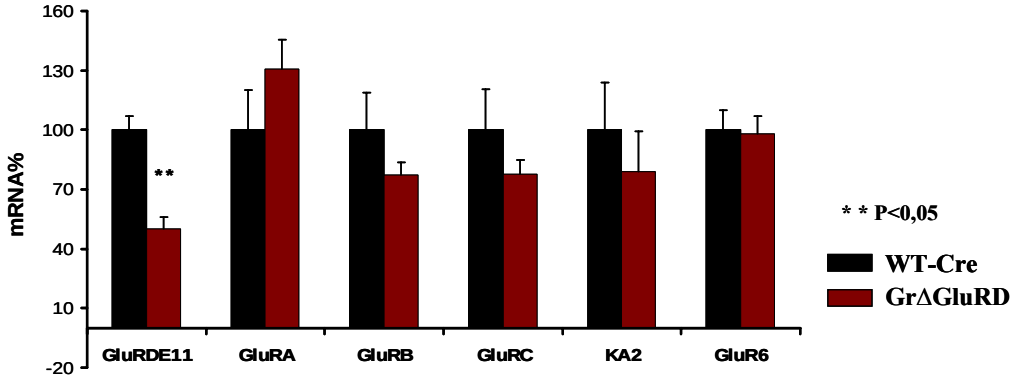


Figure 23. *Real-time PCR of AMPA receptor subunits and Kainate receptor subunits KA-2, GluR-6 mRNA expression in WT-α6Cre(WT-Cre) and Gr△GluRD mice cerebella.* The primer GluRDE11 hybridizes within the exon 11 of the GluR-D gene as an internal control. Data is mean±S.D.

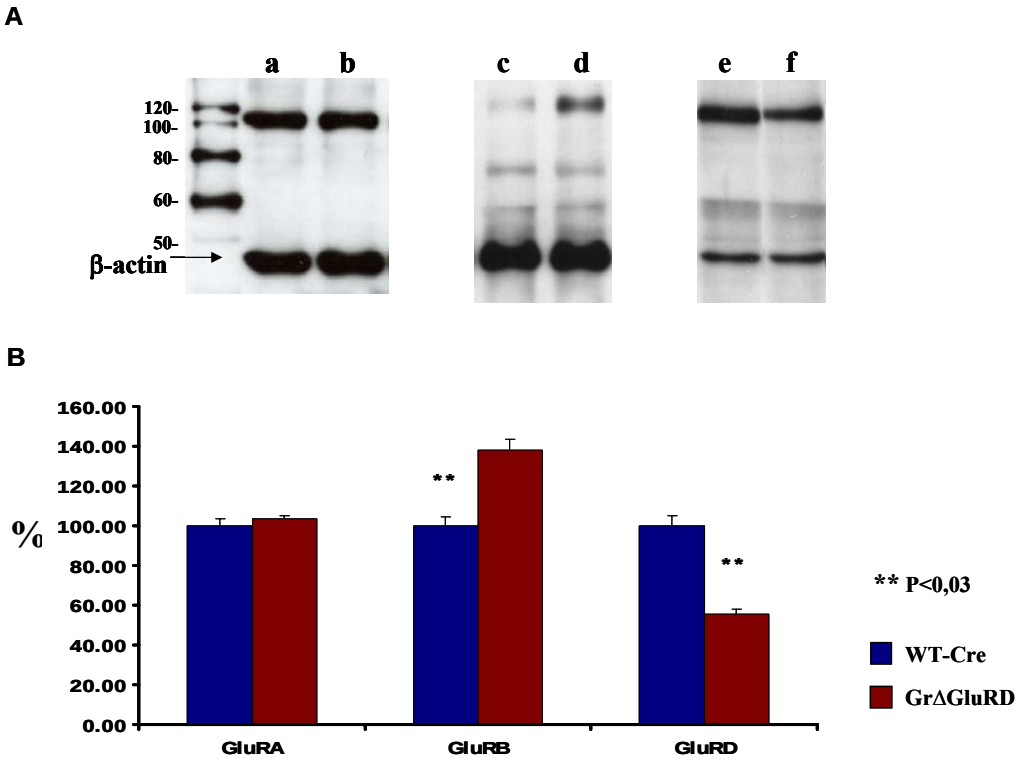


Figure 24. *AMPA receptor subunits protein expression in WT-α6Cre mice and GrΔGluRD mice cerebella*

A*:* Western blot of GluR-A (a, b), -B (c,d), -D (e,f); extracted protein from WT-α6Cre cerebella (a,c,e) and from GrΔGluRD mice cerebella (b,d,f); B: quantitative analysis of GluR-A, -B and -D protein expression in WT-α6Cre mice and GrΔGluRD mice cerebella, blue columns indicate in WT-α6Cre cerebella and red in GrΔGluRD mice cerebella. Data is means±S.D.

3.4 The extent of RNA editing of the AMPA receptor subunit GluR-B is unchanged in GrΔGluRD mouse cerebellum

Post-transcriptional alterations bring functional diversity to AMPAR channels. During RNA editing a single nucleotide is changed in the RNA encoding the R/Q position in the GluR-B subunit: in the M2 loop region, CAG encodes glutamine ("Q") in unedited GluR-B subunit transcripts, whereas in edited GluR-B mRNA, the A nucleotide is edited to G (actually inosine in real RNA, but G in cDNA) and the resulting CGG encodes an arginine ("R") (reviewed by Seeburg, 2002). In HEK 293 cells, homomeric recombinant GluR-B (Q) channels give more robust and considerably larger currents than those from homomeric GluR-B(R) subunits in response to 300 μM glutamate (see Figure 1B & D in Burnashev et al., 1992). Editing at the R/Q site occurs with nearly 100% efficiency in the normal brain (Seeburg, 2002), but during development and during some pathological circumstances the extent of Q/R editing can decrease to produce GluR-B (Q) protein (reviewed by Kittler, 2006). Edited GluR-B(R) subunits are largely unassembled and retained in the endoplasmic reticulum, whereas unedited GluR-R (Q) subunits readily tetramerize and traffic to synapses (Greger et al., 2003, 2006). We thus wondered, given that there was no GluR-A or -C subunits present, if the residual AMPAR current seen in the GrΔGluRD granule cells was due to a decreased GluR-B Q/R editing frequency, combined with the increase in GluR-B protein levels, permitting as a possible compensatory mechanism (GluR-B (Q)/GluR-B(R) channels) to appear on the granule cell surface (and which produce the residual GYKI-sensitive current). To investigate this, I sequenced the GluR-B cDNA population synthesized from WT-α6Cre (Figure 25A) and GrΔGluRD mouse cerebella RNA (Figure 25B) Using the quantitative technique of Melcher et al 1996, R/Q site-selective RNA editing of GluR-B was determined by analyzing the extent of adenosine conversion by direct DNA sequencing of the cerebellar RT-PCR products (see "Methods" section). However, the cDNA sequence of GluR-B mRNA from GrΔGluRD cerebellum showed complete CGG to CAG editing, so not differing from wild-type (Figure 25).

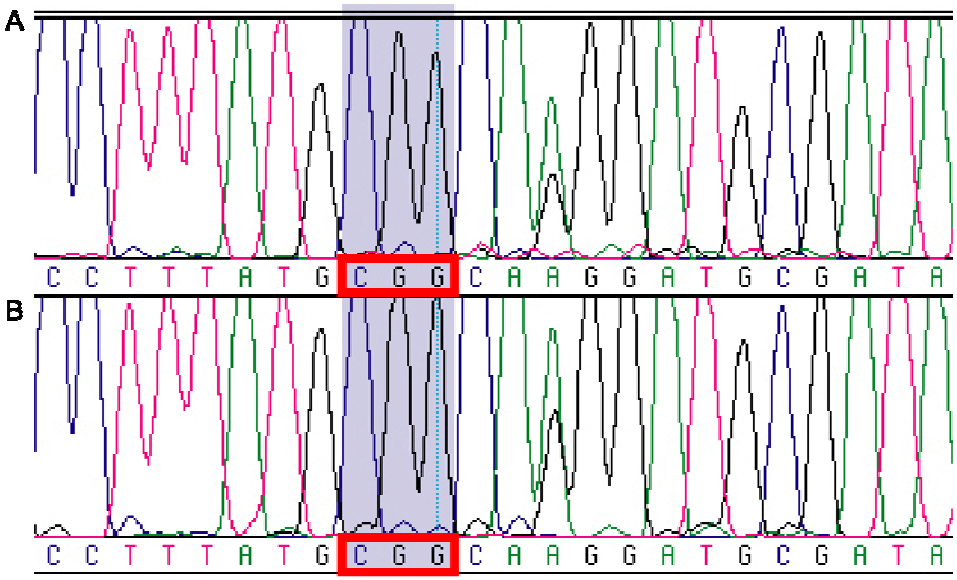


Figure 25. *Sequence of GluR-B mRNA editing site*: A, from WT-α6Cre cerebellum; B, from Gr△GluRD mouse cerebellum. CGG encodes for arginine (R) in edited GluR-B mRNAs**.**

3.5 Kainate receptor expression in GrΔGluRD mice

Kainate receptors are heteromeric and homomeric tetramers of GluR-5, GluR-6, GluR-7, KA-1 and KA-2 subunits. As seen by *in situ* hybridization, these genes are differentially expressed through out the rat brain (Wisden & Seeburg, 1993). The situation is very similar in mouse, and I have shown that the kainate receptor subunit mRNAs are expressed in the same characteristic patterns as rat (Figure 26). Cerebellar granule cells express two kainate receptor genes: GluR-6 and KA-2, and receptors there are likely to be heteromeric GluR-6/KA-2 assemblies. The characterization of native kainate currents in the hippocampus has been made possible by using the antagonist GYKI 53655 that selectively inhibits AMPA, but not kainate receptors (reviewed Osten et al., 2006). However, as GYKI 53655 blocks the residual non-NMDA EPSP in GrΔGluRD granule cells (Figure 21C), the kainate receptor genes did not change their pattern of expression in response to loss of GluR-D (Figure 26), and by real-time PCR the KA-2 and GluR-6 genes are not up-regulated in response to loss of granule cell AMPA receptors (Figure 23), kainate receptors are unlikely to be relevant compensatory factors.

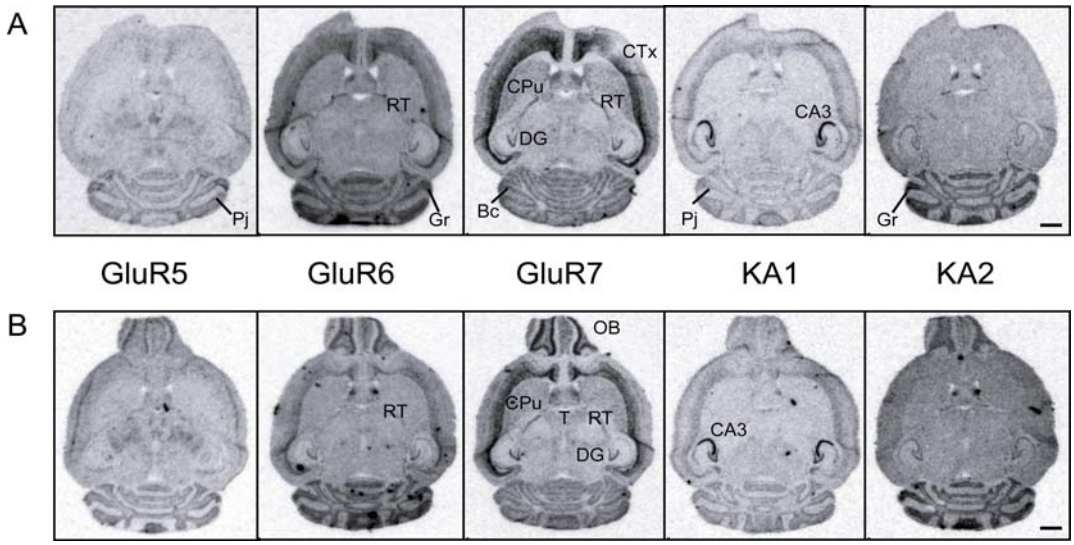


Figure 26. *X-ray film of in situ hybridization: Kainate receptor subunit mRNA distribution in WT-α6Cre mouse (A) and Gr△GluRD mouse brains (B).* Kainate receptor subunits GluR5, GluR6, GluR7, KA1, KA2; Bc, Basket cells; CA3, hippocampal CA3 pyramidal cell layer; CPu, caudate putamen; CTx, cortex; DG, hippocampal dentate gyrus; Gr, cerebellar granule cells; OB, olfactory bulb; Pj, Purkinje cells; RT, reticular thalamic nucleus; T, thalamus. Scale bar: 1.5mm.

3.6 Stargazin protein is selectively reduced in cerebellar granule cells that cannot make AMPA receptor GluR-D subunits

The transmembrane protein stargazin (γ2 or transmembrane AMPA receptor regulating protein, TARP) regulates AMPA receptor trafficking and surface expression in the cerebellum and other neuronal types (reviewed Osten and Stern-Bach, 2006, Nicoll et al., 2006). Since functional AMPA receptors were not present selectively on GrΔGluRD cerebellar granule cells due to the specific ablation of GluR-D subunits in the cerebellar granule cells (see earlier sections, Figure 18), I was able to investigate if stargazin is an obligate protein partner of AMPA receptor subunits in granule cells. Are TARP and AMPA receptor protein levels stoichiometrically linked?

I thus did fluorescent immunostaining of stargazin protein on Gr△GluRD and WT-α6Cre littermate sections (Figure 27). In the hippocampus, and molecular layers of cerebellum, stargazin immunoreactivity was at the same intensity in both wild-type and Gr△GluRD brain sections, whereas stargazin immunoreactivity was considerably less in the cerebellar granule cell layer of Gr△GluRD mice (Figure 27b) compared with WT-α6Cre litter-mate mice (Figure 27B).

This result further elaborates the idea that TARPs associate with AMPA receptors as integral auxiliary subunits. I confirmed this was a phenomenon at the protein level by looking at mRNA levels in adult mouse brain by *in situ* hybridization with a stargazin-specific probe (Figure 28). The same pattern was obtained as that recently reported by Fukaya et al. 2005. The stargazin gene is widely expressed in the brain, with cerebellar granule cells having the highest expression level (Figure 28 – see also Fukaya et al., 2005). As a control, no stargazin mRNA signal could be detected in *stargazer* brains, thus confirming the specificity of the probe (Figure 28). In GrΔGluRD brains, the level of stargazin mRNA in cerebellar granule cells is the same as WT-α6Cre, thus suggesting that loss of stargazin protein is post-transcriptional (Figure 28). Given that stargazin apparently binds all GluR AMPA receptor subunits (Chen et al., 2000), and that in cerebellum about 50% of total GluR-B/C subunits associate with stargazin (Vandenberghe et al., 2005), the substantially reduced stargazin immunoreactivity in GrΔGluRD granule cells might indicate that homomeric GluR-B protein in granule cells is degraded (at odds with the western blot data showing increased GluR-B expression), or that *in vivo* stargazin can not bind GluR-B homomeric receptors effectively; and that stargazin would prefer AMPA receptor heteromers.

Assessing similarity of the cerebellar granule cell GluR-D AMPA receptor ablation with the *stargazer* mutation.

My original plan had been to examine if the multiple effects seen in the cerebellar granule cells of *stargazer* mice (e.g. reduced BDNF, reduced $GABA_A$ receptor subunit expression, reduced GABA expression) was a consequence of the stargazin protein having multiple targets (not just AMPA receptors). I had expected stargazin protein to remain in the absence of AMPA receptor subunits – my hypothesis was that stargazin was also doing other things in addition to AMPA receptor trafficking. However, removing GluR-D subunits from cerebellar granule cells produced a similarly selective depletion of stargazin protein in granule cells to produce a double knockout (see Figure 27). In effect the Gr△GluRD mice were a phenocopy of the *stargazer* mutation confined to granule cells. By looking at the bahaviour of the Gr△GluRD mice, I was thus able to ask if the behavioural deficits in *stargazer* (e.g. pronounced ataxia, head tossing) originated from the cerebellar granule cell defect (as often assumed) or came from other cell type(s) expressing stargazin.

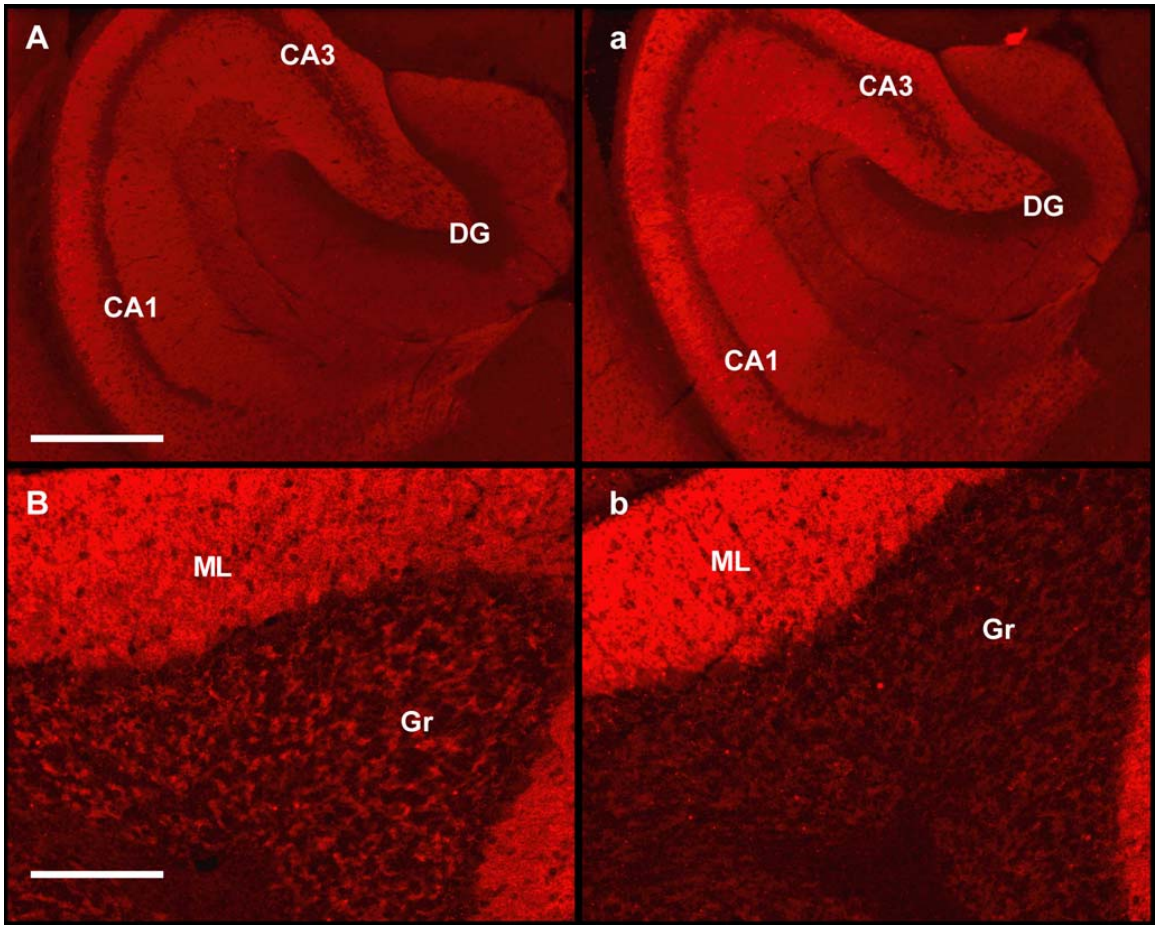


Figure 27. *Stargazin expression in WT-α6Cre (A,B) and Gr△GluD (a,b) mouse brain sections examined by fluorescent immunocytochemistry with a stargazin-specific antibody*

hippocampus (A and a); cerebellar cortex (B and b); CA1 and CA3 region in hippocampus; DG: dentate gyrus; ML: molecular cell layer; Gr: granule cell layer; scale bar: 500μm (in A and a), 100μm (in B and b)

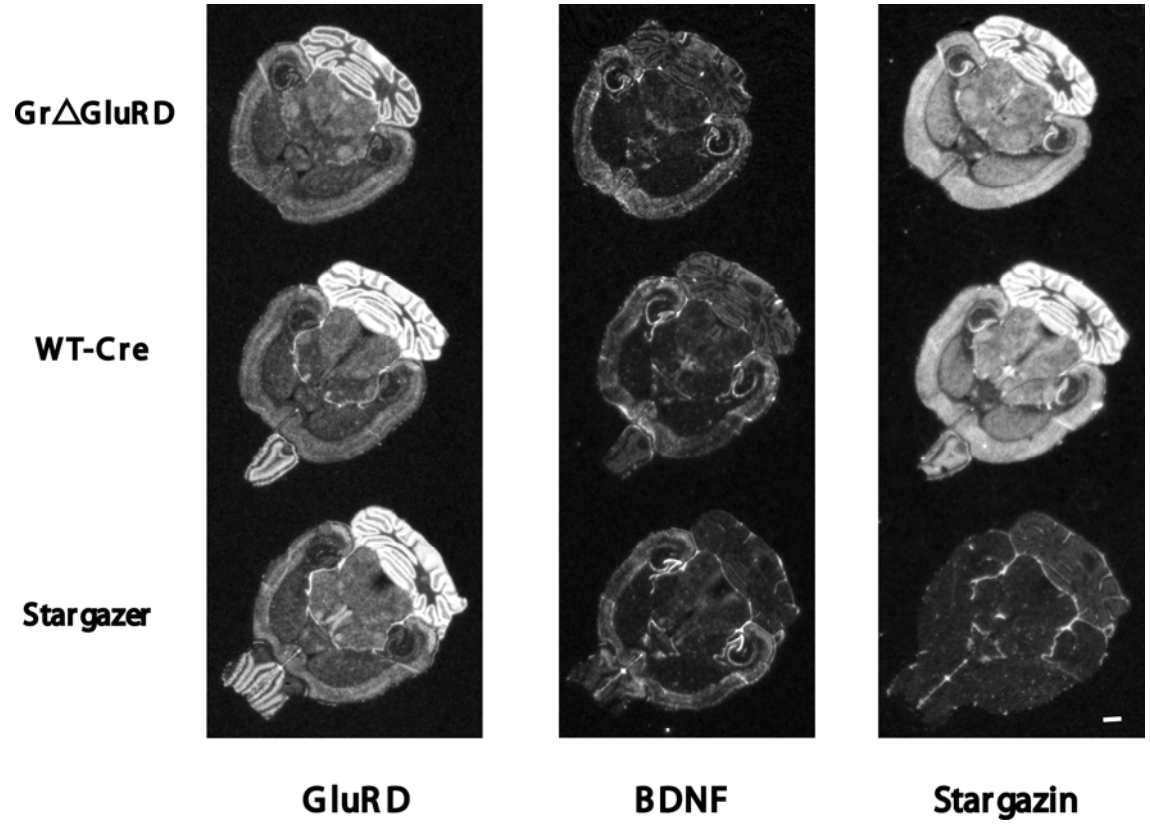


Figure 28. *In situ hybridization to examine and compare expression of GluR-D, BDNF and stargazin mRNAs in GrΔGluRD, WT-α6Cre and Stargazer adult brains* scale bar: 1.5mm

3.7 Behavioural studies on GrΔGluRD mice

Adult homozygote GrΔGluRD mice are apparently healthy; mutants are obtained in the expected Mendelian ratio. SHIRPA analysis (see Methods section) showed that the GrΔGluRD mice have normal weight, normal body posture (no head tossing was observed), respiration rate and spontaneous activity. By contrast, mice with a total GluR-D knockout are ataxic and have impairment in the open field and motor learning test compared to wild-type mice. We were expecting a cerebellar phenotype. To examine this, Dr Elke Fuchs undertook open-field, horizontal bar, rotorod and static rod tests on GrΔGluRD and wild-type littermates (Figure 29).

3.7.1 General motor function and balance: open field and horizontal bar tests

The most standardized general measure of motor function is spontaneous activity in the open filed (reviewed by Crawley, 2000). Equal numbers of male GrΔGluRD mice were compared with the WT-α6Cre littermates (all mice were roughly the same age) in the open-field test. The

number of squares crossed, latency to move, total number of rearings, and latency to first rear were recorded (Figure 29A). The numbers of crossed squares and rears were not significantly different between Gr△GluRD mice and WT-α6Cre mice (Figure 29A, a), whereas the number of rearings in global GluR-D total knock out mice (Figure 29A, b) is significantly decreased compared with the WT-α6Cre littermates.

The horizontal bar test was performed to evaluate the general ability of the mice to balance. The mice were placed on the bar and grasped the bar with their forepaws. The scores were evaluated by the criteria described in section of the methods (2.10). As shown in Figure 29 B, neither Gr△GluRD mice (B.a) nor global GluR-D knockout mice (GluR-KO, B.b) showed any difference compared with WT-α6Cre mice in their ability to balance on the horizontal bar.

Both Gr△GluRD mice and WT-α6Cre littermates were placed on the narrow diameter suspended beams to perform the static rod test. The orientation and transition time on different diameter rods (2,8cm, 2,2cm and 1,8cm) were counted for each mouse. The orientation time of both Gr△GluRD mice and WT-α6Cre on different size rods was the same (Figure 29C), as was the transition time of both genotypes on the rods (Figure 29D). There was no significant difference between genotypes when performing this test.

3.7.2 Gr△GluRD mice have no obvious impairment of motor learning or motor coordination

Cerebellar defects cause performance deficits on the rotarod test (Mason and Sotelo, 1997). The mice have to learn to stay on an accelerating rotating rod (see Methods section). We performed two experiments. First we compared only Gr△GluRD mice with WT-α6Cre littermates (Figure 29E). The Gr△GluRD mice did not show motor deficits during the tests and even performed slightly better (although not statistically different) than WT-α6Cre littermates. During the second trail we also compared WT-α6Cre mice with wild-type littermates (no Cre expression Figure 29F) to assess any changes that might have been induced by Cre alone. Similar results emerged: after 3 sessions the Gr△GluRD mice showed a tendency (although still not significant) to better perform the rotarod test than WT-Cre and wild-type littermates (Figure 29F). The WT-α6Cre and wild-type littermates showed a nearly identical ability in performing the rotarod test, which illustrates that the Cre protein per se does not have any effects on the phenotype. In summary, there is no ataxia or any overt motor deficit observed for Gr△GluRD mice.

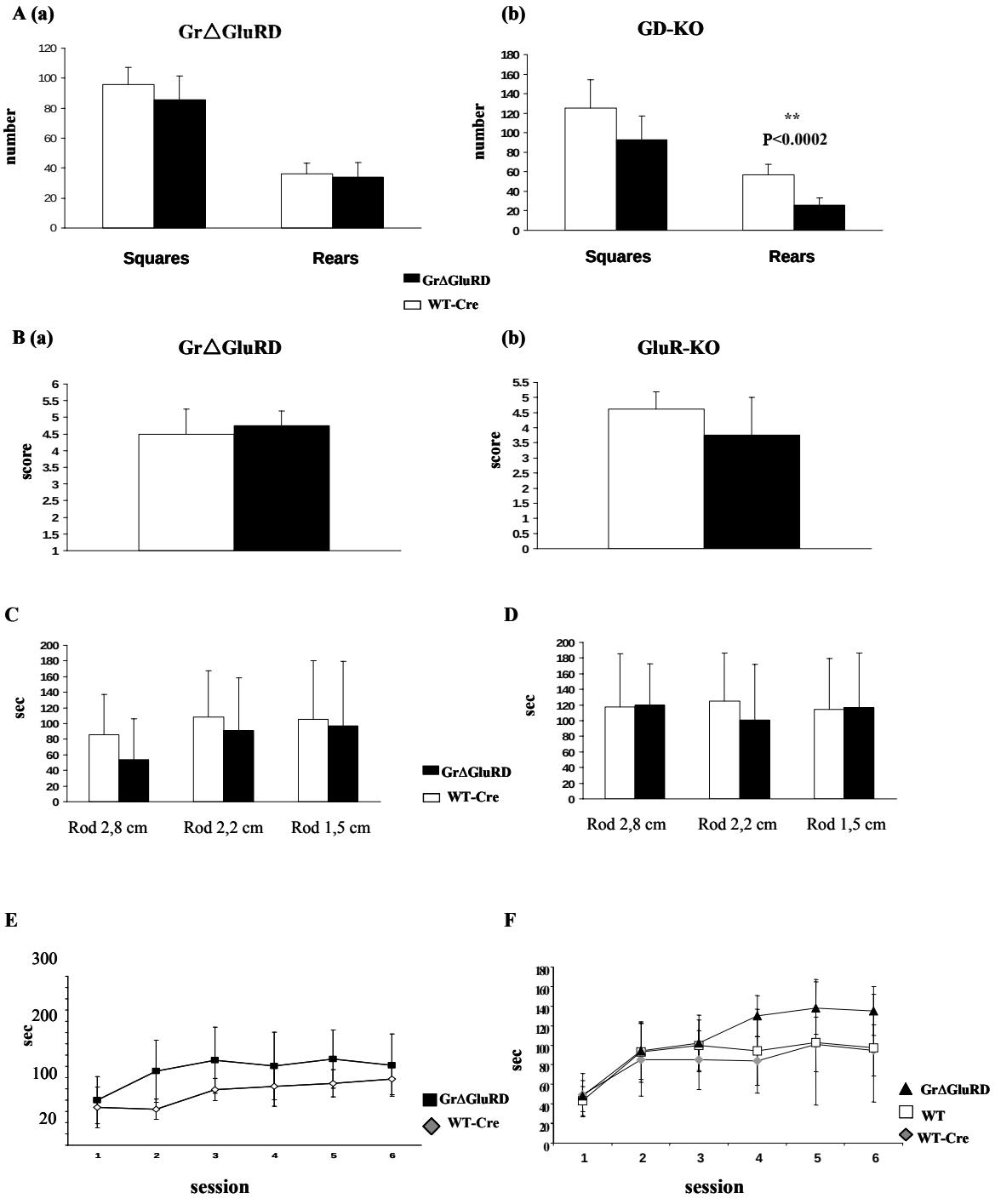


Figure 29. *Behavioral characterization of GrΔGluRD mice* A: open field, (a) Gr△GluRD compared with WT-α6Cre and (b) GluR-D-KO compared with wild-type littermates; B: horizontal bar, (a) Gr△GluRD and (b) GluRD-KO; C: static rod, Gr△GluRD compared with WT-α6Cre, orientation time; D: static rod, transition time; E: first trail of motor learning on the rotatod; F: second trail of motor learning. Error bar: Mean±SEM

3.8 Gene expression changes following ablation of AMPA receptors from granule cells.

The BDNF and $GABA_A$ receptor subunit α6 genes have reduced expression in the *stargazer* and *waggler* mice (Qiao et al., 1996; Chen et al., 1999; Thompson et al., 1998). Indeed the selective absence of BDNF mRNA from *stargzarer* cerebellar granule cells was noted some years before the AMPA receptor deficit, and was an initial defining feature of the mutation (Qiao et al., 1996 – also see Figure 11 in the Introduction). $GABA_A$ α6 subunit mRNA levels are reduced by 20% in *waggler* (Chen et al., 1999). Given that there is reduced/no functional AMPA receptors in the GrΔGluRD cells, and that this mutation basically looks like *stargazer* confined to granule cells, we would predict that the BDNF and $GABA_A$ α6 receptor subunit expression to also be decreased. I tested this directly. *In situ* hybridization (Figure 28) showed that BDNF mRNA is expressed slightly less in the cerebellar granule cells of Gr△GluRD mice compared with WT-α6Cre mice, whereas no BDNF mRNA is detected in the cerebellar granule cells of *stargazer* mice.

Real-time PCR was used to quantify the BDNF and α6 mRNA expression in Gr△GluRD cerebella compared with WT-α6Cre and *stargazer* cerebella (Figure 30). BDNF and α6 expression is significantly reduced in the Gr△GluRD mouse cerebella, but not as much as in *stargazer*; cerebella from the latter have almost no BDNF transcripts and greater than a 50% reduction in α6 gene expression. Thus the Gr△GluRD mutation produces qualitatively the same effect as the *stargazer* mutation – the same genes change their expression in the same direction.

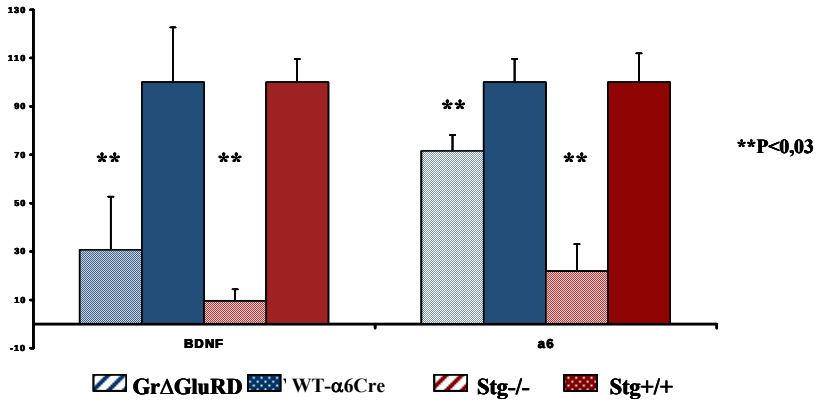


Figure 30. *Quantitative real-time PCR analysis of BDNF and $GABA_A$ receptor α6 mRNA levels in Gr△GluRD mouse cerebella compared with WT-α6Cre and stargazin (Stg -/-), and stargazin wild-type littermate cerebella (Stg +/+),* error bar: Mean±S.D.

3.9 Screening the regulated gene expression due to the deletion of GluR-D gene from granule cells in the cerebellum: Gene chip analysis

A defect in a gene mediating a single aspect of the neuronal cell body, axon, dendrite, neurotransmitter, receptor, transducer, muscle, or skeletal phenotypes may impair motor function(s). The Gr△GluRD mice lack ataxia and any impairment of simple motor learning. Why? A large number of genes, working at many sites, could influence motor behaviours. Compensatory changes in gene expression might explain why Gr△GluRD mice lack behavioural abnormalities. To get more information about possibly regulated genes in Gr△GluRD mice, I undertook gene expression profiling with the help of Stefan Bonn (MPI, Seeburg dept.) (Figure 31), and screened 13,000 genes**.**

At first pass, hundreds of genes have changed expression in RNA from Gr△GluRD cerebella. The transcripts are either at lower levels (shown in the Figure 31 of gene chip analysis A: green indicates less expression; red is higher express) or at higher levels in Gr△GluRD mice (Figure 31: BAC) compared with WT-α6Cre mice (Figure 31: WC). The logarithmic scale (Figure 31B) shows the comparison of gene expression between Gr△GluRD and WT-α6Cre cerebella. Some genes are higher, and some are less expressed in cerebella lacking GluR-D expression in granule cells (Figure 31B: red shows higher expressed genes and green is less expressed). Two internal controls (GluR-D and Cre expression) indicated that some trust could be put in the data: chip analysis shows that GluR-D (termed “alpha4” in the Table) gene expression is reduced by 50% Gr△GluRD cerebellar (showed in table 3 with *), consistent with all other data presented in this chapter.

Table 3 provides information on some genes identified whose transcripts are found at apparently lower levels in Gr△GluRD cerebella. The ratio of means between the Gr△GluRD and WT-α6Cre demonstrates the average intensity of gene expression in three Gr△GluRD mouse cerebella compared with three WT-a6Cre cerebella. The data on up-regulated genes is not shown. Table 3 lists some genes of unknown function and also some presumed artifacts (the enamelin gene which encodes a protein involved in tooth coating, for example, should not be expressed in the brain). BDNF mRNA is not reduced at all according to the chip (Table 3), which must be a wrong result. Indeed, all chip data require independent verification, for example by real-time PCR, and ideally by other methods as well. I now had the challenge presented by all chip studies:

how to follow-up on any of the numerous genes identified, each one a potentially interesting story, or alternatively, red-herrings. In the end, I decided to focus on known genes which might be "logically" linked with a presumed adaptation to no AMPA receptor transmission: glutamate decarboxylase 2 (GAD65) involved in the formation of inhibitory neurotransmitter GABA; some kinases related to calcium signalling; $GABA_A$ receptor subunits and growth factors. To confirm these data, I performed quantitative real-time PCR (see Methods).

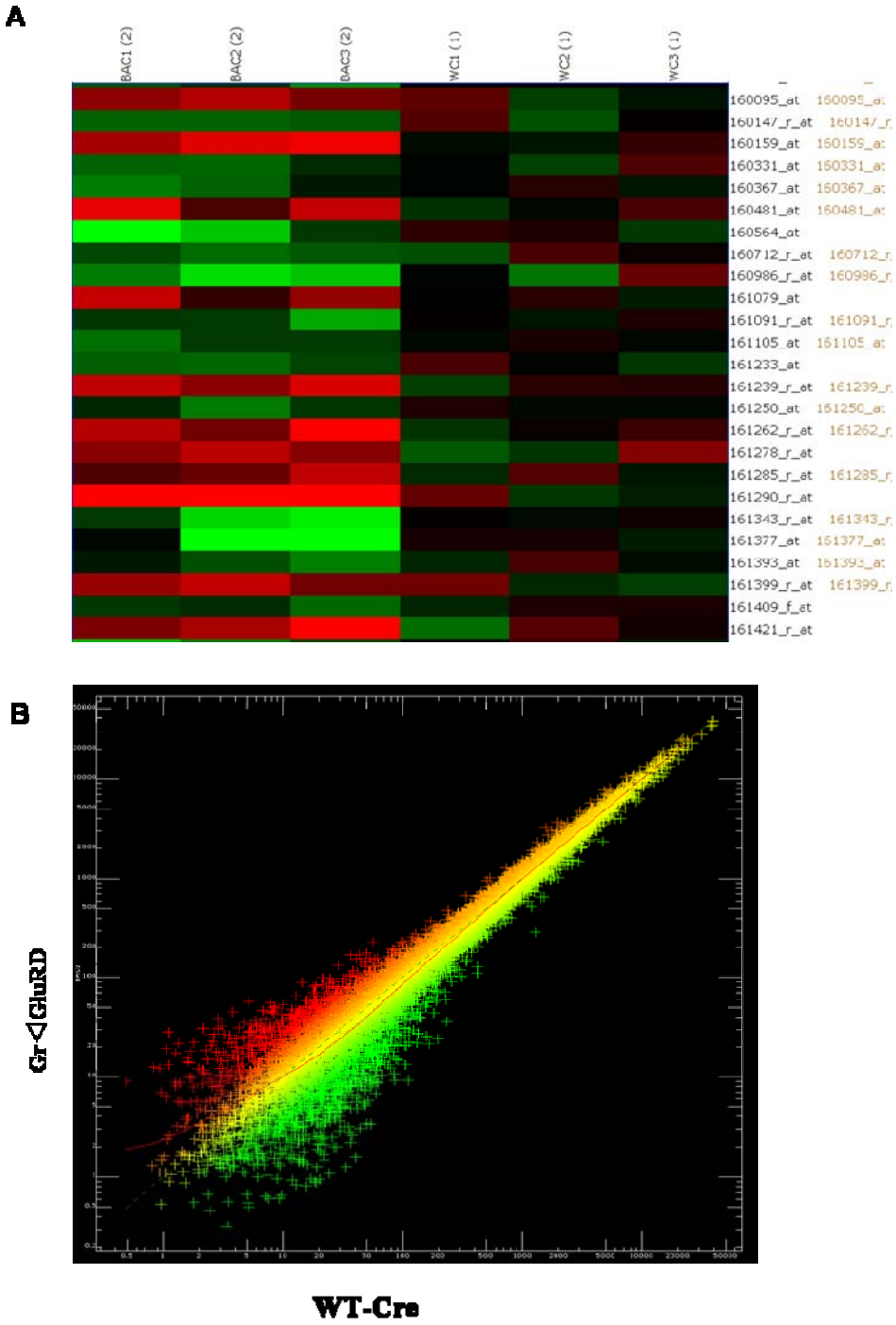


Figure 31. *Gene chip analysis of Gr△GluD cerebellum* A, some significantly regulated genes in Gr△GluRD cerebella (BAC 1, 2 and 3) compared with WT-α6Cre (WC 1, 2 and 3); B, the logarithmic scale of gene expression in Gr△GluRD and WT-α6Cre mouse cerebella. Color indicates the intensity and direction of gene expression; red represents up-regulated genes, green, down-regulated genes; yellow, genes expressed at the same intensity in both WT-α6Cre and Gr△GluRD.

Table 3. Some down-regulated genes identified by chip analysis in cerebella granule cells lacking GluR-D subunit

Ratio of means (Gr△GluRD/WC)	Gene Title
0,33	RIKEN cDNA 1110008H02 gene
0,38	enamelin
0,39	SEC63-like (S. cerevisiae)
0,46	lymphoid nuclear protein related to AF4-like
0,47	zinc finger protein 289
0,49	pleiomorphic adenoma gene-like 1
0,49	importin 4
0,51	glutamate receptor, ionotropic, AMPA4 (alpha 4) *
0,52	glutamic acid decarboxylase 2 *
0,54	nuclear receptor-binding SET-domain protein 1
0,55	myocyte enhancer factor 2C
0,56	synaptotagmin binding, cytoplasmic RNAinteracting protein
0,56	transient receptor potential cation channel, subfamily C, member 1
0,57	ariadne ubiquitin-conjugating enzyme E2 binding protein homolog 1 (Drosophila)
0,58	translocated promoter region
0,58	ryanodine receptor 3
0,59	protein tyrosine phosphatase, receptor type, Npolypeptide 2
0,60	homer homolog 1 (Drosophila)
0,60	RIKEN cDNA 4930429H24 gene
0,60	ARP2 actin-related protein 2 homolog (yeast)
0,61	calcium/calmodulin-dependent protein kinase IV
0,61	dystrophin, muscular dystrophy
.....	.....
0,51	gamma-aminobutyric acid(GABA-A) receptor,subunit gamma1
0,56	gamma-aminobutyric acid(GABA-A) receptor subunit alpha2
0,62	gamma-aminobutyric acid(GABA-A) receptor subunit alpha 6
0,70	protein kinase C, delta
0,77	spermine synthase
0,82	calcium channel, voltage-dependent, beta 2 subunit
0,83	K+ voltage-gated channel, subfamily S, 2
0,88	potassium voltage-gated channel,Shal-related family,member2
0,92	glutamate receptor, metabotropic 8
0,95	brain derived neurotrophic factor (BDNF)
.....	.....

3.10 GAD-65 expression is decreased in Gr△GluRD cerebellum

Here I give one example of a gene identified by chip analysis and how I tried to verify the chip data. One gene I considered straight-away from Table 3 was GAD65. GAD65, an isoform of

glutamate decarboxylase that synthesizes the inhibitory neurotransmitter GABA, is a down-regulated gene identified from gene chip analysis (table 3 with *). GABAergic neurons (Golgi cells, stellate/basket cells and Purkinje cells) from *stargazer* cerebella have reduced GABA immunoreactivity (Richardson and Leitch, 2002). I was also thinking that given that there is predicted to be less excitation onto Gr△GluRD granule cells because the mossy fibres can not excite them so well (or not at all), then granule cells will excite the inhibitory Golgi cells less, and these in turn will down-regulate GABA synthesis – thus normalising the negative feedback loop onto granule cells, possibly an adaptive change (see Figure 39 in discussion). GAD65 is mainly expressed at the presynaptic terminals of GABAergic interneurons, like Golgi cells in cerebellum. Another isoform is GAD67, enriched in the cell soma. The reduction of GAD65 expression in Gr△GluRD mouse cerebella found in the chip analysis data was confirmed by quantitative real-time PCR (Figure 32). GAD65 mRNA expression is reduced around 40% in Gr△GluRD cerebella compared with WT-α6Cre mice, whereas there is no significant difference in the forebrain of these two mouse lines. I looked by *in situ* hybridization with a GAD65-specific probe to see if there was a clear regional/cellular locus that would explain this reduction in GAD65 mRNA levels (Figure 33). Neither the forebrain nor the cerebellum showed obvious differences in GAD65 mRNA expression between Gr△GluRD and WT-α6Cre mice (Figure 33). I was particularly interested to look at the expression of GAD65 in Golgi cells in the cerebellar granule cell layer; these are detectable even on X-ray film autoradiographs as prominent, sparsely distributed, spots in the granule cell layer (Figure 33c, d). The GAD65 spots were clearly present in both genotypes. To quantify any reduction in expression would one have to do silver grain counting using emulsion autoradiography, which I did not do. In any case, there was nothing obvious concerning changed GAD65 gene expression in the "Golgi spots" or elsewhere. *In situ* of GAD67 was also performed. There was no difference of GAD67 mRNA expression in these two mouse brains (data not shown).

I looked next at the protein level using antibodies to GAD65 (Figure 34). The GAD65 protein is mainly found in presynaptic terminals of GABAergic interneurons in the cerebellum: axons of Golgi cells forming part of glomeruli in the cerebellar granule layer; basket and stellate cells in the molecular layer synapsing onto Purkinje cells.

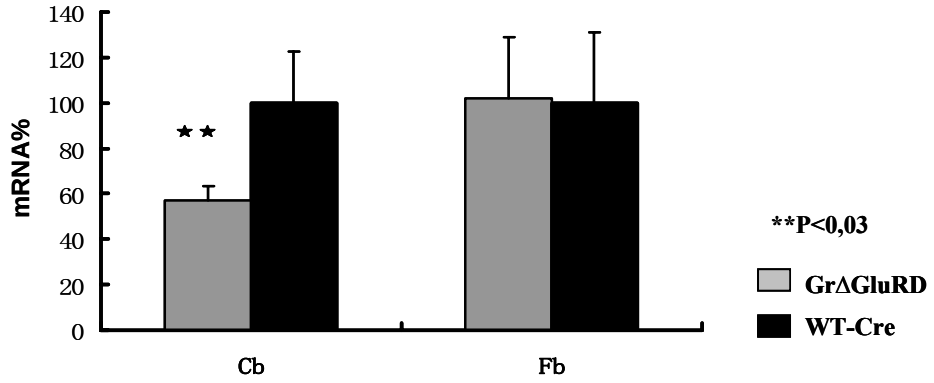


Figure 32. *Quantitative real-time PCR of GAD65 mRNA expression in Gr△GluRD and WT-α6Cre cerebella, forebrains* Cb: cerebellum; Fb: forebrain

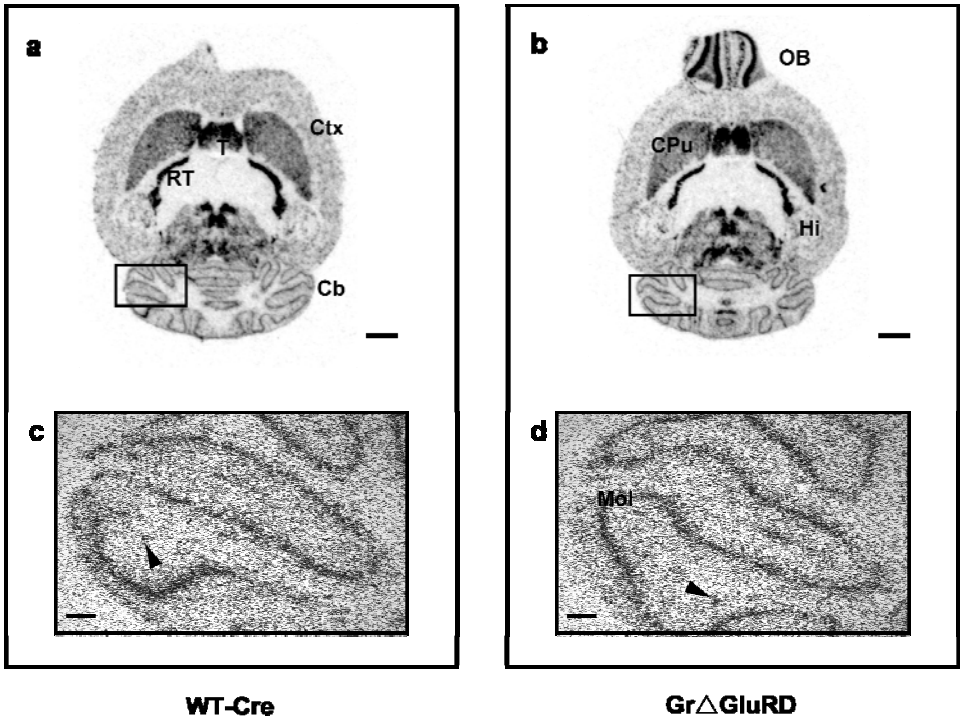


Figure 33. *In Situ hybridization of GAD65 mRNA in WT-α6Cre and Gr△GluRD mouse brains.* WT-α6Cre (a,c); Gr△GluRD (b,d); c and d are the big magnification of the boxed region in a and b respectively; Ctx: cortex; T: thalamus; Hi: hippocampus; Cb: cerebellum; RT: reticular thalamus; arrow shows Golgi cells in the cerebellar granule cell layer; Scale bar, a and b, 1.5mm; c and d, 0.5mm

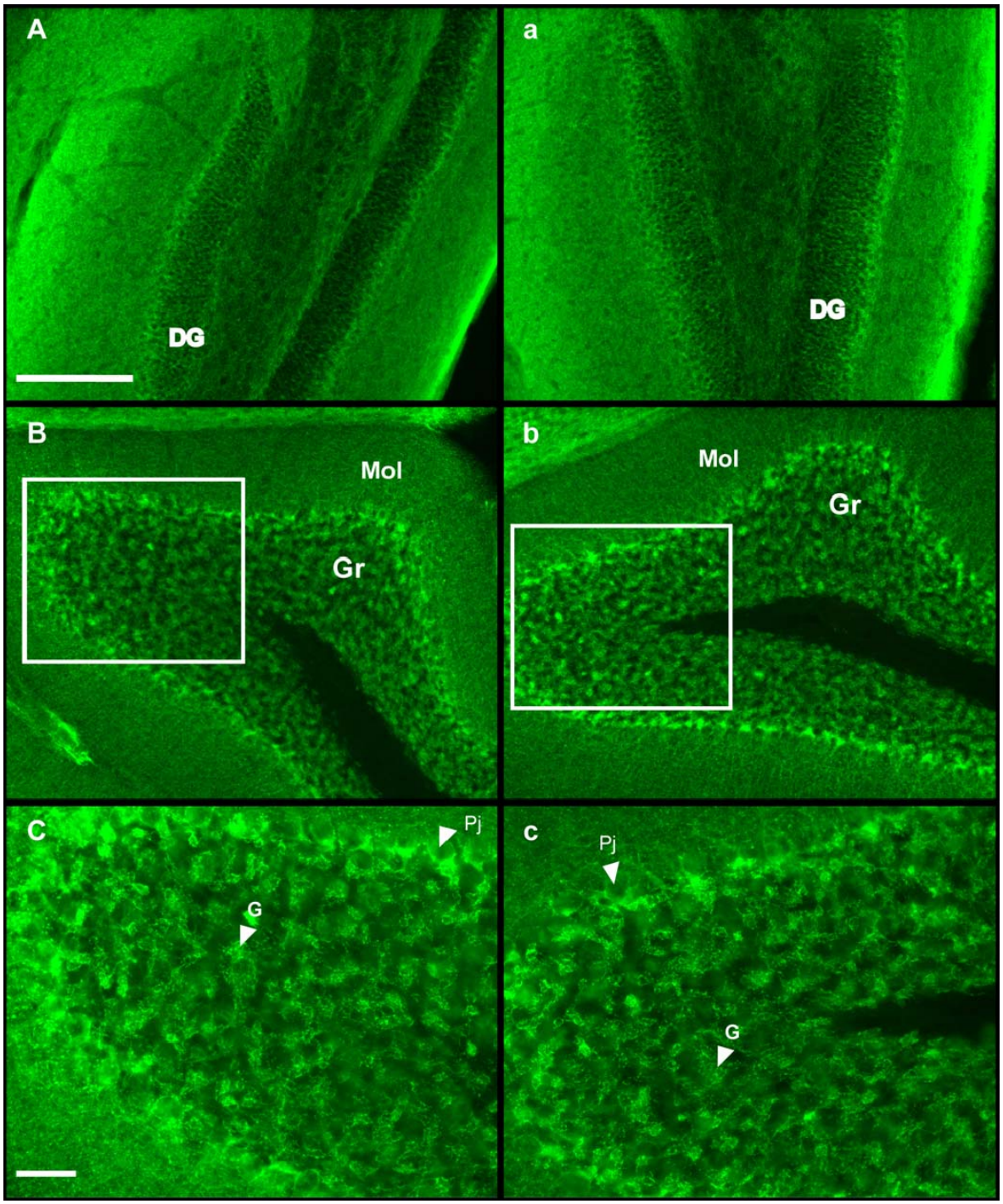


Figure 34. *Immunoflurescent staining of GAD65 expression in WT-α6Cre (A,B,C) and Gr△GluRD brain (a,b,c)* Hippocampus (A and a); cerebellum (B and b); high magnification of cerebellar cortex (C and c) from cerebellar folia (B and b); DG: dentate gyrus; Gr: granule cell layer; Mol: molecular cell layer; Pj, purkinje cell; G, glomeruli; scale bar: 500μm (in A, B and a, b); 50μm (in C and c).

The fluorescent GAD65 immunostaining reveals the GAD65 protein distribution in WT-α6Cre (Figure 34 A,B,C) and Gr△GluRD (Figure 34 a,b,c) hippocampus and a cerebellar folium. The staining of GAD65 in hippocampus serves as a control of intensity between these two mouse lines (Figure 34A and a). In cerebellum GAD65 is mainly found at the glomeruli (Figure 34 C and c) in the granule cell layer, which are formed by terminals of Golgi cells axons, granule cell dendrites and bulbous terminals of mossy fibres – the staining consist of many small spots; GAD65 immunoreactivity is also found decorating the Purkinje cells, marking the axon terminals of basket cells (Figure 34B,C and b,c). However, by immunoflorescence, GAD65 expression does not show any obvious difference reduction in intensity in the Gr△GluRD granule cell and Purkinje cell layers (Figure 34b,c) compared with WT-α6Cre sections (Figure 34 B,C).

However, given that Richardson and Leitsch (2002) reported a significantly decreased GABA content in *stargzarer* cerebellum, I was still curious about GABA levels in the Gr△GluRD cerebellum. Fluorescent immunochemistry staining using a GABA-specific antibody was applied. Figure 35 shows the distribution of GABA in the hippocampus (as a control) and folium of cerebella between WT-α6Cre (Figure 35A,B) and Gr△GluRD mice (Figure 35a,b). In the hippocampus, the signal is diffuse, possibly reflecting the extensive innervation of the pyramidal soma and dendrites by GABAergic interneurons, and the numerous and diverse GABAergic axons of the interneurons. In the cerebellum, GABA immunoreactivity is present in the molecular cell layer, Purkinje cell layer and glomeruli of granule cell layer (see Figure 35, GABA). In the Purkinje cell and molecular cell layers the staining intensity for GABA is the same as WT-α6Cre. At the Purkinje layer, the enrichment of staining likely corresponds to the basket cell terminals surrounding the Purkinje cell soma. In the granule cell layer the granule soma are unlabeled, showing up as many little holes against the staining; in the Gr△GluRD granule cell layer, the intensity of GABA immunoreactivity is less than in the WT-α6Cre granule cell layer (Figure 35B,b).

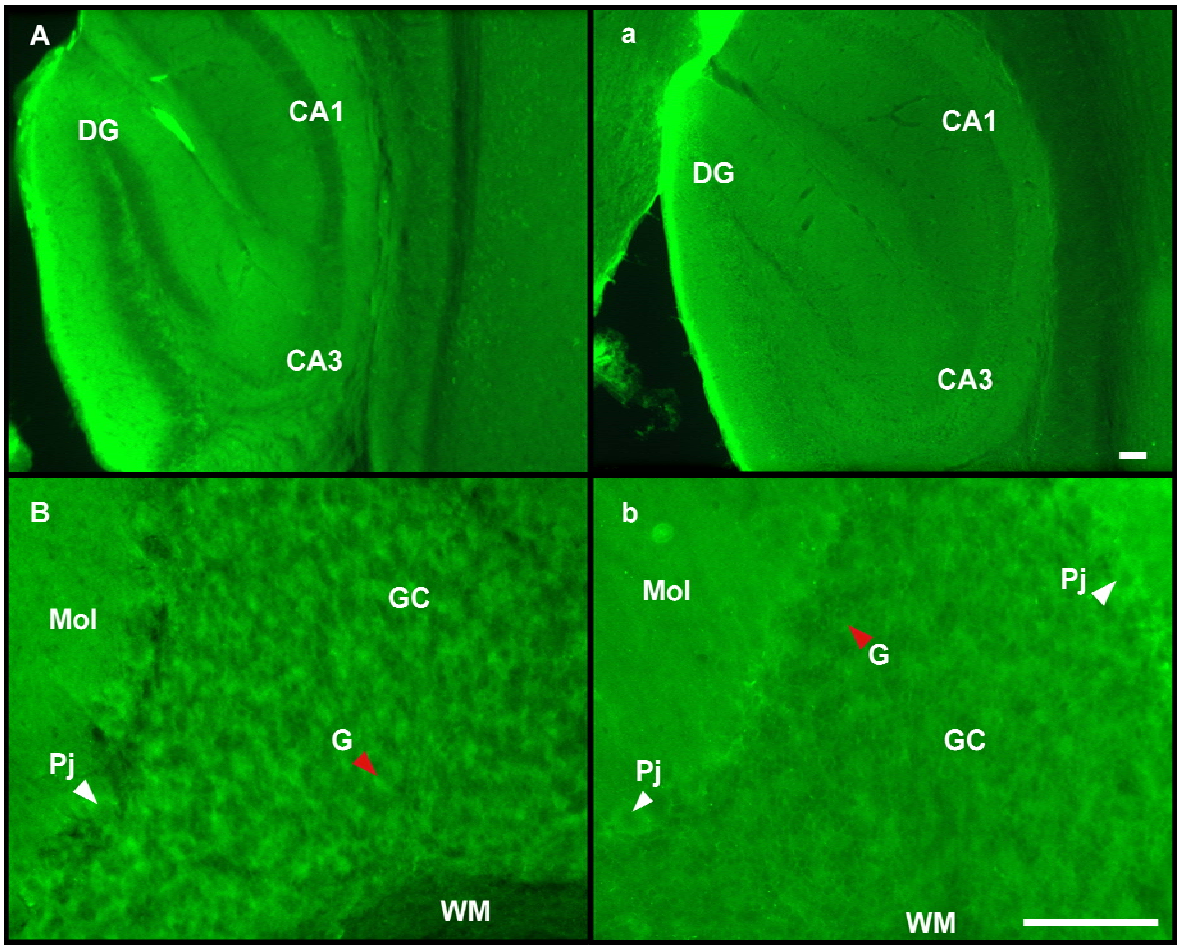


Figure 35. *GABA immunoreactivity in WT-α6Cre (A,B) and Gr△GluRD brains (a,b)* Hippocampus (A and a); cerebellar cortex (B and b); DG: dentate gyrus; CA1 and CA3: hippocampus CA1 and CA3 region; Mol: molecular cell layer; GC: granule cell layer; Pj, Purkinje cell; G, glomeruli; WM, white matter; scale bar: 100μm.

3.11 The amount of phospho-CREB is unchanged in GrΔGluRD granule cells

Another feature I explored in the Gr△GluRD granule cells was whether cAMP/Ca^{2+} signalling to the nucleus might still be intact, given how many genes had changed their expression following AMPA receptor deletion from granule cells. I checked the immunoreactivity of phospho-CREB, which is the proto-activated cAMP-responsive element binding protein, a key regulator of gene expression and primed for activation by phosphorylation on Ser-133. The phosphorylation of CREB is induced by multiple converging pathways involved in calcium signalling, like calmodulin (CaM)/CaM kinase IV pathway, ras/mitogen-activated protein kinase (MAPK) or extracellular signal-regulated protein kinase (ERK)- mediated pathway modulated

by protein kinase A (PKA) and protein kinase C (reviewed Hardingham and Bading, 2003). In the context I am interested in, the CREB pathway would come into play via Ca^{2+} entry through NMDA receptors (and this activity may be strongly reduced in synapses with no AMPA receptors) (Hardingham and Bading, 2003). Additionally, from the chip analysis, CaMKIV, PKC are down-regulated in the Gr△GluRD cerebella (Table 3). This could influence the phosphorylation of CREB. Fluorescent immunochemistry with phosphorylated CREB antibody was performed on perfused sagital brain sections from Gr△GluRD and WT-α6Cre mice (Figure 36).CREB is expressed throughout the CNS of mice, although not evenly in every cell type; he highest levels of CREB were found in the cortex, the hippocampus, striatum and cerebellum. CREB is highly expressed in the dentate gyrus, CA1, CA3 region and cortex of hippocuampus (Figure 36A,a), and in the granule cell layer of cerebellum (Figure 36B,b), but not for example, in cerebellar Purkinje cells. The phospho-CREB is present in the nucleus of cells. No impairment of phospho-CREB expression in Gr△GluRD granule cells is observed, and the nuclei of all cells are strongly immuno-positive (Figure 36B, b).

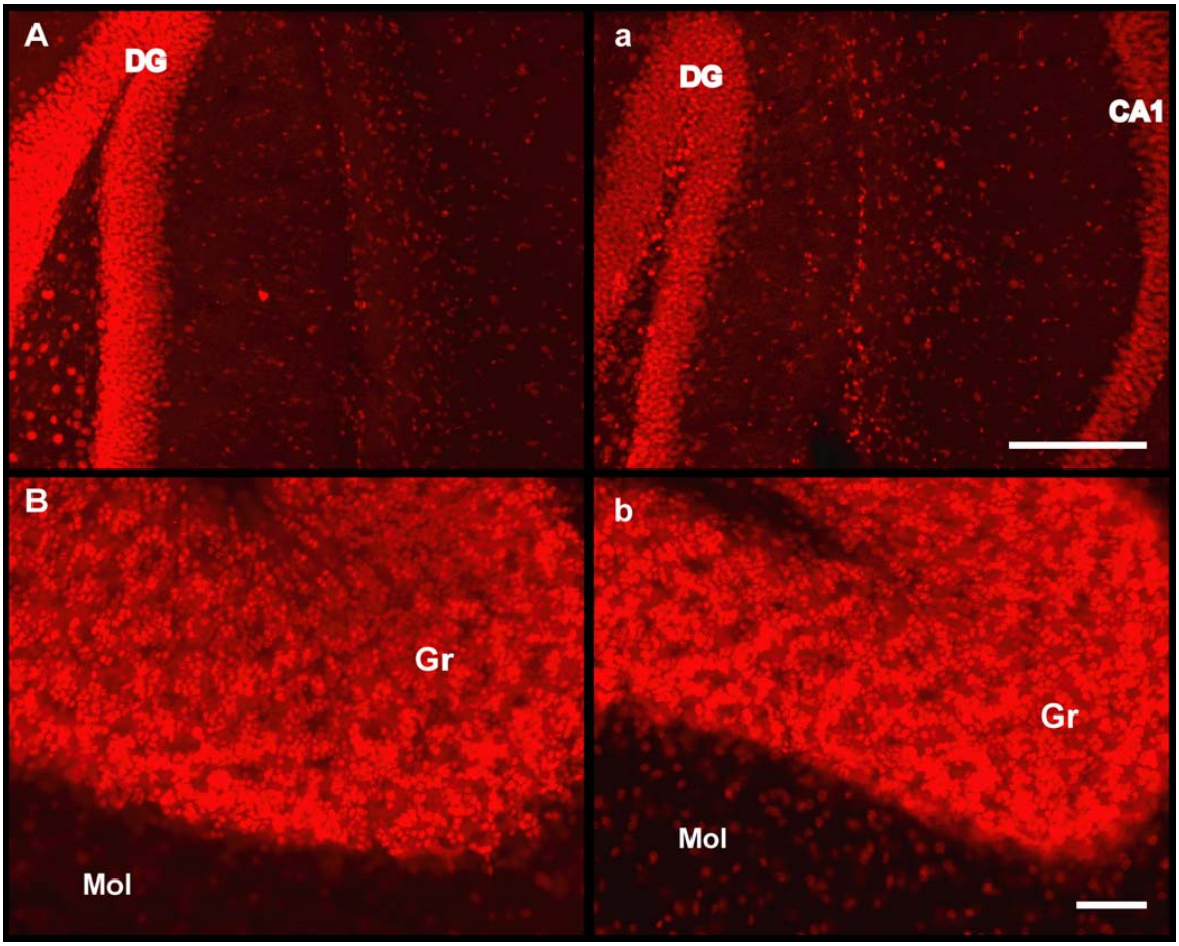


Figure 36. *Immunoflurescent staining of phosphor-CREB expression in WT-α6Cre (A,B) and Gr△GluRD brains (a,b)* Hippocampus (A and a), cerebellar cortex (B and b); DG: dentate gyrus; CA1: hippocampus CA1 region; Gr: granule cell layer; Mol: molecular cell layer; scale bar: 500μm (in A and a); 50μm (in B and b).

4 DISCUSSION

I am interested in AMPA receptor function at the cerebellar mossy fibre-granule cell (mf-gr) synapse. I showed that by genetically deleting GluR-D expression specifically in granule cells (Gr△GluRD mice), the AMPA channels at mf-gr synapses are essentially not functional, and it is possible that "silent" synapses have been established. An intriguing feature is that at the cellular level the Gr△GluRD mice phenocopy mostly the *stargazer* mutation. I found that the stargazin protein, but not its mRNA, is down regulated specifically in Gr△GluRD granule cells. This strongly supports the idea that stargazin is an obligate partner with AMPA receptor subunits, and that this is its most important role. As in stargazer, I found that BDNF and $GABA_A$ receptor $\alpha 6$ subunit mRNA expression decreased in the Gr△GluRD granule cells, and also that GAD65 mRNA and cerebellar GABA content decreased; numerous other genes have been up or down regulated. But the mice have no ataxia or impaired motor behaviour, and so this feature of the *stargazer* phenotype must originate elsewhere in other cell types.

In my project I have looked at the following two issues: (1) by engineering a mutation (virtually no AMPA receptor EPSP) that phenocopies *stargazer* just in one cell type I could gain insights into the effects of the stargazin mutation cell autonomously; and (2) at the circuit/systems level I was interested to investigate if the gr-mf synapse carries essential information for motor learning, as predicted by models (Hansel et al., 2001).

4.1 AMPA receptor expression in cerebellar granule cells

AMPA receptors are hetero-tetramers of GluR-A to D subunits (Nakagawa et al., 2005, see Figure 37). Surveying the whole brain, GluR-D gene expression is highest in the cerebellum, with transcripts found in both granule cells and Bergmann glial cells, with comparatively light expression in the forebrain (Keinaenen et al., 1990; Monyer et al., 1991; reviewed Wisden et al., 2000). GluR-DFlip mRNA is expressed in spinal cord dorsal horn and particularly strongly in motor neurons, together with the GluR-B and GluR-C genes (Tolle et al., 1993). In the forebrain GluR-D expression is often found in GABAergic cells such as the reticular thalamic relay cells and GABAergic interneurons in the neocortex and hippocampus (reviewed Wisden et al., 2000). In forebrain interneurons, GluR-D is often expressed with GluR-A, but with little or no GluR-B, forming Ca^{2+}-permeable receptors with inward rectification (Geiger et al., 1995). This is also the

case in Bergmann glial cells, where the Ca^{2+}-permeable GluR-Aflip/GluR-Dflip AMPA receptors regulate the extent to which glial processes ensheath parallel fibre-Purkinje cell synapses (Iino et al., 2001). By contrast, in cerebellar granule cells, the AMPA receptors are most likely GluR-Bflip/GluR-Dflop tetramers (Brorson et al., 2004). For recombinant GluR-A/GluR-B heteromers, the favoured stoichiometry is a symmetrical arrangement of two GluR-A and two GluR-B subunits; pairs of identical heteromeric dimers preferentially co-assemble, and co-assemblies of the flip and flop isoforms is strongly preferred over homomers - so presumably the same will apply to the GluR-Bflip/GluR-Dflop subunit combination in cerebellar granule cells (Brorson et al., 2004). Furthermore, cerebellar granule cells express two forms of GluR-Dflop ("long" and "short") with different C-termini produced by alternative splicing (Gallo et al., 1992); both GluR-D forms will be missing in Gr△GluRD mice. In response to glutamate, recombinant channels made from the GluR-B(R) flip/GluR-Dflop combination expressed in HEK cells give a surprisingly low single-channel conductance of 1.0 pS (Swanson et al., 1997). This conflict with the conductances found at the actual mf-gr synapse: the conductances of AMPA receptor channels at the mf-gr synapse mostly range between 12 to 20 pS (Traynelis et al., 1993; Silver et al., 1996). High conductance channels carry much of the EPSC's fast component at the mf-gr synapse, with only about 10 AMPA receptor channels activated by a single transmitter packet (Traynelis et al., 1993). This discrepancy between recombinant and neuronal data may be due to the need to co-express in HEK cells the accessory proteins such as stargazin/γ2 with the AMPA receptor subunits to get physiologically relevant receptors (reviewed Nicoll et al., 2006).

4.2 Changes in AMPA receptor subunit levels in response to loss of a partner subunit

Unassembled AMPA receptor subunits presumably find their other subunit partners in the endoplasmic reticulum (ER); in some cell types, such as hippocampal pyramidal cells, which express GluR-A, GluR-B and GluR-C, the assembly must rely on differential affinities of the subunits for each other, with the GluR-B subunit playing a central partner around which assembly proceeds (Greger et al., 2006) (see Figure 37 below).

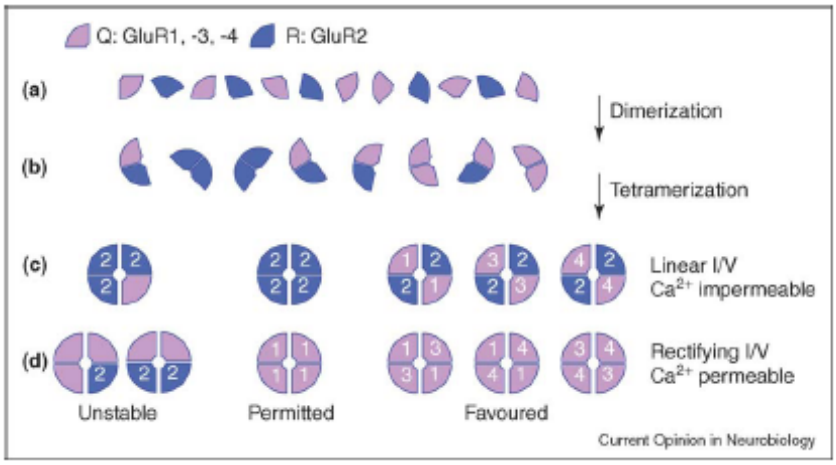


Figure 37. *Schematic representation of the subunit partnerships involved in formation of functional tetrameric AMPARs.* GluR2 (GluR-B) is shown in dark blue (R = arginine at Q/R site, Q = glutamine at Q/R site), other subunits (GluR1, 3 and 4, -A, C and –D respectively) are in purple. Individual subunit types are identified by white numerals. (a) Subunits are synthesised in the ER, then assembled in two stages: dimerization followed by tetramerization. (b) Dimerization; formation of heteromeric dimers is favoured, but homomeric dimers are also allowed. (c) Tetramerization: this results from assembly of a pair of dimers. Studies on GluR1/GluR2 heteromers indicate that pairs of identical dimers preferentially co-assemble, producing a symmetrical stoichiometry. A range of expected subunit combinations is illustrated (c, right hand side), assuming this rule applies to all unedited subunits. (d) A number of Ca2+-permeable combinations is permitted or favoured. Reproduced from a review by Cull-Candy et al., 2006

In adult cerebellar granule cells, the situation is simpler – there are only two subunits, GluR-Bflip and –Dflop; the GluR-D is expressed with two C-terminal variants (long and short) specified by splicing (Gallo et al., 1992). For GluR-B knockout mice in certain cell types (anteroventral cochlear nucleus, deep dorsal cochlear nucleus and stellate/basket cells in the cerebellar molecular layer), GluR-D protein levels significantly increase (Pertralia et al., 2004). In cerebellar granule cells from GluR-B total knockout mice, GluR-D protein levels fall significantly, and presumably GluR-D requires GluR-B for stability (Petralia et al., 2004); by contrast, GluR-B protein levels go up by 38% in Gr△GluRD cerebellum. This phenomenon was also found for the complete GluR-D knockout, with GluR-B immunoreactivity was induced/up-regulated in the hippocampal GABAergic interneurons that had lost GluR-D (Fuchs and Monyer, unpublished). However, GluR-B protein levels in the *stargazer* cerebellum are reduced by about 20% (Nicoll et al., 2006), and so in this respect the Gr△GluRD mice do not

resemble *stargazer*.

4.3 Have we made silent synapses?

Silent synapses have functional NMDA receptors, but no AMPA receptors. They are "silent" because glutamate can not open the Mg^{2+}-blocked NMDA receptor unless the neuron, or the local area of it, has been depolarized by another source. We have shown that elimination of GluR-D protein from cerebellar granule cells strongly reduces AMPA-mediated EPSCs at the mossy fibre to granule cell synapse to virtually nothing (see Figure 21 in the results section). A small amount of GluR-B(R) may exist on the surface of mutant granule cells, as indicated by the residual AMPA-mediated EPSP. However, the edited GluRB(R) has a poor ability to self-assemble (Greger et al., 2006), and GluR-B(R) is largely retained in the endoplasmic reticulum (Greger et al., 2003). I established that the editing efficiency of GluR-B transcripts does not decrease in response to loss of GluR-D from cerebellar granule cells; no GluR-B(Q) subunits are likely to be produced, and it may be unlikely that substantial amounts of GluR-B(R) homomeric receptors would be on the surface of Gr△GluRD granule cells.

But assuming there is some GluR-B(R) on the surface of the Gr△GluRD granule cells, what properties of homomeric channels formed from this subunit could we expect? GluR-B(R) confers unique conductance properties. GluR-B(R)flip homomers would be insensitive to polyamine block and conduct at resting membrane potentials (Washburn et al., 1997), but would have an extremely low single-channel conductance (femtosiemans range, 0.36pS) (Swanson et al., 1997). These low conductances were recorded on recombinant channels without co-expression of stargazin; co-expression with stargazin could promote higher conductance channels (Tomita et al., 2005; Nicoll et al., 2006). Nevertheless, as stargazin levels are strongly reduced in Gr△GluRD granule cells, then it is likely that the recombinant 0.36pS would be close to the *in vivo* value.

An unusual emergent feature of Gr△GluRD mf-gr synapses might arise because GluR-B(R) homomers can passage anions (Burnashev et al., 1996); homomeric GluR-B(R) on granule cell membranes might conduct Cl^-, and might thus confer a small inhibitory signal in response to glutamate, so reversing the normal sign of the synapse. Again, whether the homomeric Glu-R(B) channel in the absence of stargazin would, in practice, generate a physiologically significant

current is unclear.

4.4 GYKI 53655 blockade of the residual AMPA response on Gr△GluRD cells did not unmask a kainate receptor response probably because of the rapid desensitization of these receptors

Rat and mouse cerebellar granule cells express the GluR-6 and KA-2 subunit genes (Wisden and Seeburg, 1993 –see Figure 26 in the results section), but no one has ever detected a kainate receptor response on granule cell soma in slices, and it would be of interest to do so. The characterization of native kainate currents has been made by using the antagonist GYKI 53655 which selectively inhibits AMPA, but not kainate receptors (Donevan and Rogawski, 1993; Wilding and Huettner, 1995; Paternain et al., 1995). With this pharmacological approach, for example, kainate receptor-mediated currents were identified in the hippocampus both at the mossy fiber synapses onto CA3 principal cells, and the Schaffer collateral projections onto CA1 interneurons (reviewed Osten et al., 2006b). Similarly, GluR-5 expression in cerebellar Purkinje cells was identified as producing a small GYKI 53655-sensitive current (Huang et al., 2004). At synapses that express both AMPA and kainate receptors, kainate receptor synaptic currents are typically only 10% as large as those mediated by AMPA receptors and exhibit slower rise and decay kinetics (reviewed Osten et al., 2006b). However, on the face of it the absence of non-NMDA EPSPS after GYKI 53655 application on granule cells lacking GluR-D might suggest that the residual current was AMPA-mediated; GluR6/KA-2 kainate receptors might be located elsewhere on the granule cells, perhaps on their axons or axon varicosities synapsing onto molecular layer cells. At least in *Xenopus* oocytes, recombinant stargazin does not influence GluR-6 kainate receptor function (Chen et al., 2003), so the reduced stargazin immunoreactivity seen in Gr△GluRD granule cells see below) does not explain the absence of the kainate response. However, one important experimental omission is that we did not pre-treat the cells with concanavalin A (ConA); this drug selectively blocks the desensitization of kainate receptors and so allows currents from these elusive receptors to be observed (in the absence of AMPA receptors) (Chen et al., 2003). Indeed, Chen et al confirmed that wild-type or heterozygous *stargazer* cultured granule cells have kainate-evoked responses with pharmacological properties consistent with kainate receptors: in the presence of the AMPA receptor antagonist GYKI 53655, kainate (100 μM) evoked currents (25 pA) in cells that had been pre-incubated for 15 to 20 mins

in ConA. The ConA-stimulated, kainate-evoked, response was blocked by the non-NMDA receptor antagonist CNQX. There was no difference in the size of the kainate receptor-mediated responses in heterozygous *stargazer* granule cells or in granule cells lacking stargazin (Chen et al., 2003).

4.5 Have we blocked the induction of LTP?

The phenomenon of LTP produced by high frequency mf discharge at the rat cerebellar mf-gr synapse has mostly been elucidated by D'Angelo and colleagues (reviewed Hansel et al., 2001; D'Angelo, 2005a; D'Angelo et al., 2005b). Induction of mf-gr LTP requires postsynaptic NMDAR and mGluR activation, an intracellular Ca^{2+} increase, PKC activation, and nitric oxide production. Expression of LTP includes three components: (a) an increase of both AMPA and NMDA synaptic currents, (b) an increase of intrinsic excitability in granule cells, and (c) an increase of intrinsic excitability in mf terminals. Based on quantal analysis, the EPSC increase is mostly explained by enhanced neurotransmitter release. Nitric oxide is a candidate retrograde neurotransmitter which could determine both presynaptic current changes and LTP. According to D'Angelo 2005, "mf-gr LTP provides a wide substrate for information storage in the cerebellum. In the rat cerebellum, there are 10^{11} granule cells and 4 times as many mf-gr synapses. Mathematical models predict that mf-gr LTP improves mutual information transfer, and that the combination of synaptic and non-synaptic changes improves sparse representation of the mf input. In NR2A and NR2C NMDA receptor subunit double knockouts (but not single knockouts) (Kadotani et al., 1996) – AMPA responses are normal at the mf-gr synapse of these mice, but no functional NMDA responses are detected. The NR2A/NR2C double knockouts are not ataxic (they walk normally), and have no tremor; however, the number of rearings was strongly decreased. The double knockouts could do the static beam tasks with no impairment, and the rotorod test at low speeds with no impairment; at higher speeds they were impaired (Kadotani et al., 1996). Thus the double knockouts manage simple coordinated tasks such as walking on the ground or staying on the stationary or slowly running rota-rod. The NR2A/NR2C double KO mice cannot adapt, however, to more challenging tasks such as walking on the narrow bar or staying on the quickly running rota-rod. It is *assumed* that this is because of no NMDA receptors at mf-gr synapses in the cerebellum, and this suggests that integration (LTP?) at mf-gr synapses

is indeed important for general motor function. Can our Gr△GluRD mice provide any further test of this prediction?

NMDA responses can still be evoked in Gr△GluRD granule cells, and the response does not differ in magnitude or kinetics from wild-type. The NMDA receptor genes expressed in adult cerebellar granule cells are NR1, NR2A and NR2C, and the likely receptor subunit combinations will be NR1/NR2A and NR1/NR2C or NR1/NR2A/2C (Monyer et al., 1992). The NR1/NR2C receptors might respond to glutamate in spite of the strongly reduced AMPA EPSCs, because NR2C-containing NMDA receptors have a lower Mg^{2+} sensitivity compared with the more common NR1/NR2A and NR1/2B receptor types (Monyer et al., 1992). Thus NR2C-containing receptors might operate at more negative membrane potentials (reviewed Cull-Candy et al., 2001), allowing them to open even without AMPA receptors. Thus the mf-gr synapse in Gr△ GluRD mice might not be silent. Similar considerations would apply to mf-gr *stargazer* synapses. However, it is not clear what the Mg^{2+} sensitivity of NR1/NR2A/2C receptors would be. This issue needs to be investigated further. Any other modulatory factor(s) eliciting depolarization in Gr△GluRD granule cells would also enable transmission to take place at the silent synapses (e.g. acetycholine).

The very small EPSP found at the mf-gr synapse in Gr△GluRD mice could still undergo plasticity-dependent changes. I argue this because in hippocampal pyramidal cells of the GluR-B and GluR-B/GluR-C double knockout mice, the remaining EPSP can still be potentiated –plasticity is possible (Meng et al., 2003). But stargazin phosphorylation is needed for the expression of LTP (Nicoll et al., 2006), and as stargazin protein is reduced in Gr△GluRD granule cells (see below), LTP should be harder to induce. The key experiment to test this would be to produce theta frequency stimulation of mossy fibres onto Gr△GluRD granule cells to see if any LTP can be produced.

4.6 Why no aberrant motor behaviour in Gr△GluRD mice?

As assessed by the open field, horizontal bar, static rod, and rotarod tests Gr△GluRD mice did not show any impairments compared with WT-α6Cre littermate mice. They do not exhibit alterations or deficits in motor coordination or motor learning, and instead even have tendency to learn better (Figure 29 in the results section). At one level, we might not be so surprised by these

results. Indeed, the whole cerebellar cortex is dispensable, and is certainly not essential for life in a lab cage (Hoshino et al., 2005); a strain of mice, *cerebelless*, can be viably bred although the mice have no cerebellar cortex at all - they have partially defective expression of a transcription factor (***Ptf1a***) involved with specifying cerebellar cortex development (Hoshino et al., 2005). Histologically, this mouse mutant's brain is rather striking – the adult mice have normal sized brains – simply the cerebellar cortex is entirely missing (Hoshino et al., 2005 –see the Figure below).

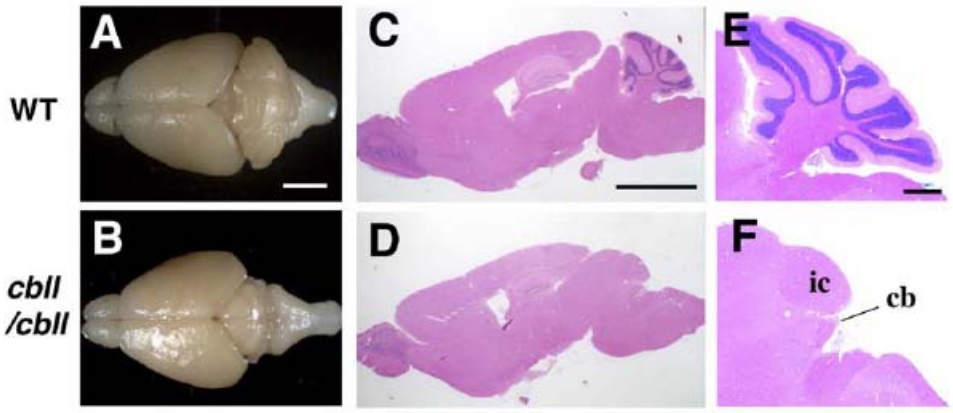


Figure38. *Cerebelless brain.* C,. Cerebellum; ic, inferior colliculus. A-E, wild-type brains cbll/cbl, homozygous Cerebelless brain (reproduced from Hoshino et al., 2005)

Cerebelless mice also have a partially reduced deep cerebellar nuclei (no GABAergic cells, and reduced glutamatergic cells), no inferior olive (which send the climbing fibres to the Purkinje cells and deep cerebellar nuclei) and no pontine nuclei (which provide the mossy fibres to the granule cells and deep cerebellar nuclei). Thus the whole cerebellar system is profoundly mis-functional – and given that there is no inferior olive or pontine nuclei, there cannot be meaningful cerebellar function at all (Hoshino et al., 2005). *Cerebelless* mice have tremor, ataxic gait, and uncoordinated locomotion, but are otherwise viable. How does this translate to the human situation? I can do no better than paraphrase Carpenter, 2003: "In humans, damage to the vestibulocerebellum leads to difficulties of postural coordination, similar to damage to the vestibular apparatus: difficulties in standing upright, dizziness and a staggering gait when

walking. The cerebellum coordinates postural control. If other parts of the cerebellum are damaged, the defects are more generalized and not just postural: a lack of co-ordination of all kinds of movement (asynergia) in association with loss of muscle tone (hypotonia). The patient´s motor system takes too long to respond to sensory information, there is a delay in the feedback loop; dysmetria or overshoot may be seen. When the patient reaches out to touch something, the hand goes too far, presumably because the command to stop the movement is sent to late. A consequence of this is intention tremor; the overshoot is corrected by a movement in the opposite direction, which then itself overshoots, resulting in a new correction, and so on – the result is a oscillation or tremor around the desired position. A related defect is adiadochokinesis – patients are unable to make rapid alternating movements. They cannot issue the command to reverse a movement sufficiently soon after having sent the command to stop it. Altogether, in fact, patients with cerebellar damage have to bring enormously more conscious control into their movements, and it is the time required to think that slows things up. A normal person can walk along, pick things up etc without thinking much beyond willing the final outcome; but a cerebellar patient has to plan and think about the details, not just of what to do, but how to do it" (Carpenter, 2003).

Coming back to the mice I generated, why are no behaviours such as ataxia found in the Gr△ GluRD mice? Rather than no cerebellum, just one synapse type in the cerebellar cortex is impaired. Nevertheless this is a key synapse, providing one of the two main excitatory inputs into the cortex, the other input being the climbing fibre (from the inferior olive) onto the Purkinje cell. If one believes the numerous reviews on cerebellar function (Hansel et al., 2001), it seems hard to believe that motor function is unimpaired after disruption of this synapse. The mossy fibres carry the information transmitted from the pontine nuclei. The mossy fibres and climbing fibres also branch before entering the cortex, and each fibre innervates cells in the deep cerebellar nuclei (DCN) as well as the granule cells and Purkinje cells respectively. Thus it could be that DCN function alone can to some extent bypass the information processing in the mf-granule cell-Purkinje cell loop - this is an old unresolved controversy in the cerebellar research field. However, the theory of cerebellar cortical function all stipulate that the mossy fibres carry information about movements and the climbing fibres carry an error signal. Appropriately timed activation of mossy fibres onto Purkinje cells (via the intermediate granule

cells) and co-activation of the Purkinje cell by a climbing fibre produces long-term depression at the parallel fibre-Purkinje cell synapses. These plastic alterations may somehow be relevant to the encoding of motor memory traces, and should be strongly disrupted in Gr△GluRD mice.

Certainly, based on other mouse mutations, granule cell function seems important for allowing normal motor function on this task. Mice with selective, postnatally-induced, death of cerebellar granule cells develop ataxia (Shmerling et al., 1998; Shimizu et al., 2002). Mice with no GABA input onto their granule cells (because of selective Golgi cell ablation by genetic immunotoxin expression) develop ataxia from which they only partially recover (Watanabe et al., 1998). Golgi cell-lesioned mice initially develop severe ataxia on the rotorod, but after some weeks recover their ability to stay on a rotating rod at slow but not at higher speeds (Watanabe et al., 1998). The granule cells down-regulate their NMDA receptor responses, but AMPA receptor function remains unchanged (Watanabe et al., 1998); the mechanism does not seem to have been further studied since the first publication in 1998 (reviewed Nakanishi, 2005). Further, if synaptic transmission is selectively, reversibly and inducibly blocked from adult granule cells to Purkinje cells by expressing tetanus toxin using the α6 promoter, ataxia is induced for the period at which no transmission is presumed to takes place from granule cells to Purkinje cells (Yamamoto et al., 2003) In calretinin knockout mice, granule cells lacking the calcium binding protein calretinin are hyperexcitable – they fire more action potentials in response to mossy fibre input; Purkinje cells in these mice (which do not express calretinin) fire at higher frequencies, presumably because granule cell activity is higher, and the mice are ataxic (Gall et al., 2003). If calretinin expression is restored selectively to adult calretinin KO cerebellar granule cells using the $GABA_A$ α6 gene promoter (Bahn et al., 1994), the deficit in motor skill is restored, Purkinje cells fire at their normal rates, suggesting that the granule cell defect (hyperexciatability) in the calretinin knockout mice was the cause of the motor defect (Bearzatto et al., 2005). All of these experiments indicate that active granule cell function is needed for normal motor function, as measured by the rotorod task, which makes my mouse mutant more surprising.

Compensation could explain the lack of motor phenotype. Granule cells normally excite the Golgi cells, in addition to Purkinje cells, producing a negative feedback. The Gr△GluRD might fire less, Golgi cells will be less excited, less GABA will be released onto granule cells, allowing them to still fire in response to the reduced mossy fibre input, so normalizing the system. For

example, the down regulation of GAD-65 (possibly in Golgi cells) hat I found, would lead to less GABA being released onto the granule cells.

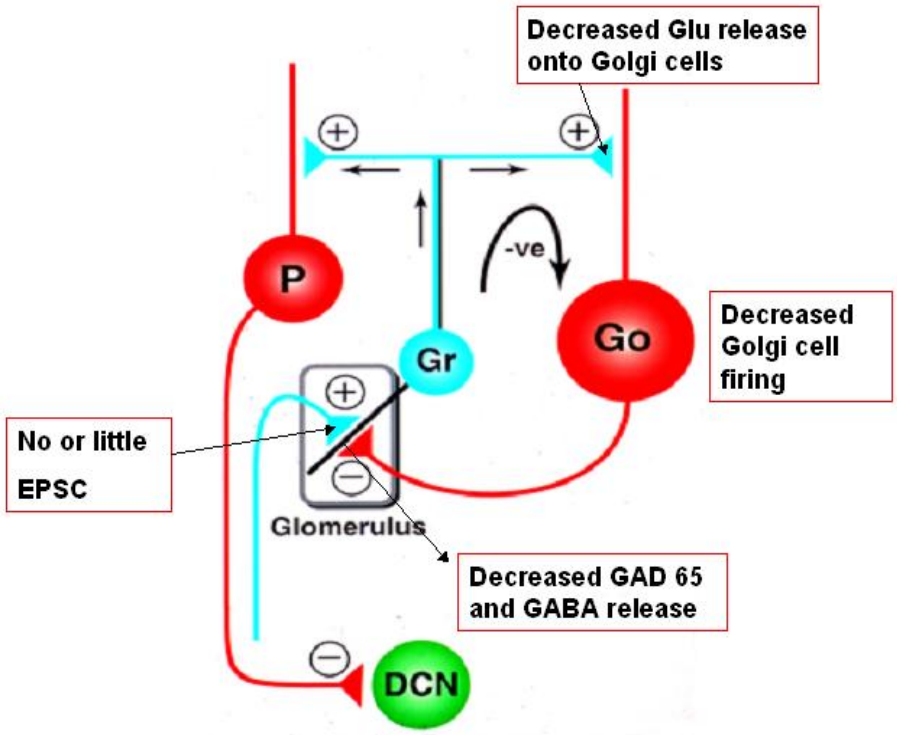


Figure 39. *Possible adaptations to AMPA receptor ablation in cerebellar granule cells.*

By contrast, the total GluR-D knockout mice are ataxic and have impaired motor behaviour (Fuchs and Monyer, unpublished). GluR-Dflip is found in Bergmann glia, where it contributes to Ca-permeable GluR-D/GluR-A receptors that may be vital for normal cerebellar function. The sum of AMPA receptor actions at both mossy-granule and parallel fibre-glia contacts may be important for correct behaviour; or, alternatively, the "locus of ataxia" lies elsewhere in another GluR-D expressing brain region; GluR-D is expressed in many GABAergic interneurons throughout the brain, in the reticular thalamus (responsible for organizing information transfer in the neocortico-thalamaic loop) and in spinal cord motor neurons (Tolle et al., 1993). In any case, as assessed from the phenotypes of the total knockouts, GluR-D is essential at some place(s) the central nervous system for healthy behaviour at the whole animal level: it is not a dispensable

subunit. Interestingly, the *stargazer* mouse is also strongly ataxic, and this feature is usually assumed to arise from the deficient mf-gr transmission, but as I have demonstrated the ataxia must originate elsewhere at any number of other synaptic locations whose transmission is expected to be deficient in stargazer and where the γ2 gene is expressed e.g. Purkinje cells (Fukaya et al., 2005); indeed, the climbing fibre-Purkinje cell synapse, and the parallel fibre-Purkinje cell synapse are both defective (although not silent) in the *stargazer* cerebellum, with reduced AMPA receptor currents (Hashimoto et al., 1999), or perhaps in the vestibular nuclei (Khan et al., 2004) or motor neurons (Tolle et al., 1993).

4.7 Stargazin (γ2)

There is immense interest in clarifying all the roles of stargazin, and it is fair to say this is currently one of the highest profile molecules in the glutamate receptor field (reviewed Nicoll et al., 2006; Osten et al., 2006). Stargazin (γ2) is one of four closely related proteins (the others are γ 3, γ 4 and γ 8) – termed TARPS - believed to be, among other things, integral auxillary subunits of AMPA receptors (reviewed Osten et al., 2006; Nicoll et al., 2006). TARPS are essential for getting AMPA receptors to the surface of the cell, and then to the synapse (because of PSD-95 binding), and they also modify AMPA receptor properties – such as promoting higher single-channel conductance levels (Tomita et al., 2005; reviewed Nicoll et al., 2006). TARPS are part of an integral complex with AMPA receptor subunits. But TARPS have another identity – subunits of voltage-gated Ca^{2+} channels and the entire family, γ1-γ 8, could also function as accessory subunits of these channels; indeed, members such as the muscle-specific γ 1 probably perform entirely this function, and indeed γ 2 as the gene underlying the pathology of the *stargazer* mouse, was initially identified in having this role (Letts et al., 1998, 2003). However, the TARP function was also initially identified by characterizing stargazer granule cells (reviewed by Nicoll et al., 2006), and so I now review these mice in the next section.

4.7.1 *Stargazer* and *waggler* mutations

Stargazer and *waggler* mice have different mutations in the same gene, stargazin (γ2), and together with the disruptions within the introns of this gene, form an allelic series (reviewed Letts et al., 2003). The *stargazer* mutant mouse has an ataxic gait and a distinctive head-lifting (episodic upward head lifting caused by sustained extensor movements of the neck) that gave

rise to its name (Noebels et al., 1990; Letts et al., 2003; Khan et al., 2004). The *waggler* mutant also has an ataxic gait, but lacks the head-tossing motion of *stargazer* (reviewed Letts et al., 2003). The γ 2 gene mutation has pleiotropic effects and it is exceedingly difficult to disentangle cause and effect for the many changes that have occurred in the *stargazer* brains. The γ 2 protein (and some of its close relatives, TARPs) are essential for AMPA receptor trafficking (reviewed in the introduction section); γ2 may also function as an auxillary subunit modulating voltage-gated channels, and may have yet other functions. For example, it is closely related to claudins, and could function in cell-cell adhesion, as confirmed by cell adhesion assays with recombinant proteins (Price et al., 2005). This is not so far-fetched given that in cerebellar granule cells NMDA receptors are enriched at tight junctions (attachment plaques, Petralia et al., 2002).

There is widespread expression of γ 2 in the developing and adult brain (Fukaya et al., 2005). The γ 2 subunit mRNA is detected at high levels in various mouse brain regions (see Figure 28, in the results section). The highest γ 2 expression is found in the cerebellar cortex, where the γ 2 subunit mRNA is expressed strongly in Purkinje cells and granule cells, and moderately in the molecular layer neurons (basket and stellate cells) and deep cerebellar nuclei (Figure 28 in the results section) (Fukaya et al., 2005)

Table 4 : Relative expression levels of stargazin (γ2) family members in the adult mouse brain estimated by visual comparison using both X-ray film autoradiograms and emulsion-dipped sections. (–) not detected; 1, very low; 2, low; 3, moderate; 4, high; 5, very high. Stargazin is γ2; the γ 1 is expressed only in muscle. (Reproduced from Fukaya et al., 2005, Neuroscience Research 53, 376–383)

	γ1	γ2	γ3	γ4	γ5	γ6	γ7	γ8
Cerebellum								
Purkinje cells	–	5	–	–	–	–	5	–
Granule cells	–	5	1	1	–	–	4	–
Stellate/Basket	–	3	4	3	–	–	3	–
Bergmann glia	–	–	–	4	4	–	–	–
Deep cerebellar nuclei	–	3	1	1	–	–	4	–

4.7.2 Development

Stargazin mRNA is detected abundantly through out the mouse brain at the earliest developmental time point, E13, examined (Fukaya et al., 2005). The gene is expressed strongly at all other later time points, including in the developing postnatal cerebellum (although the

expression in e.g. the developing external granule cell layer was not described in detail). In the mouse cerebellum there is expression already from P1 and there appears to be a peak in stargazin cerebellar expression at P14 (Fukaya et al., 2005).

In stargazer cerebellum, there is a delayed disappearance of external granule cells (delayed migration of granule cells) during development at P15 and but with eventually normal granule cell density in adult mutants, but some granule cells in the adult layer have a nuclear chromatin staining pattern characteristic of immature cells (Qiao *et al*., 1998).

4.7.3 Effects of *stargazer* mutation on the cerebellum

All types of neuron are affected in the *stargazer* cerebellum (Richardson and Leitch, 2002, 2005). In *stargazer*, the cerebellar inhibitory neurons have significantly reduced levels of GABA immunoreactivity (40-50% of wild-type), and assuming the immunoreactivity to be specific (i.e. a good antibody), a decreased GABA content compared with wild-type controls (Richardson and Leitch, 2002). Furthermore, the density of inhibitory synapses between Golgi interneurons and granule cells and also between basket and Purkinje cells in *stargazer* mutants is reduced to approximately half that in wild-type controls (Richardson and Leitch, 2002). The *stargazer* mutation has an even more pronounced effect on the phenotype of granule cell neurons in the cerebellum (Richardson & Leitch, 2005). There is a profound decrease in the levels of glutamate-immunoreactivity (up to 77%) in *stargazer* compared with WT controls. The distribution profile of presynaptic vesicles is also markedly different: *stargazer* has proportionally fewer docked vesicles and fewer vesicles located adjacent to the active zone ready to dock than WTs. Furthermore, the thickness of the postsynaptic density (PSD) at mossy fiber-granule cell (mf-gc) and parallel fiber-Purkinje cell (PF-PC) synapses is severely reduced (up to 33% less than WT controls). The number of excitatory synapses, however, appears unchanged (Richardson & Leitch, 2005).

There are some striking molecular and electrophysiological changes in the *stargazer* cerebellum: no AMPA receptors, but normal NMDA receptors on the surface of granule cells suggestive of silent synapses, reduced excitatory transmission at the parallel fibre-Purkinje cell and climbing fibre-Purkinje cells synapses (Hashimoto et al., 1999), reduced GABA-A receptor subunits expression (α6, β3) corresponding to a type of GABA receptor important for tonic (extrasynaptic

inhibition) (Thompson et al., 1998); changed kinetics of IPSCs onto granule cells (Chen et al., 1999), and absence of BDNF mRNA (and therefore BDNF production) from cerebellar granule cells (Qiao et al., 1996). *Stargazer* cultured cerebellar granule cells have an upregulated ER unfolded-protein response (Vandenberghe et al., 2005), suggestive of accumulated mis-folded proteins (e.g AMPA GluR-B). Collectively, all these results would suggest that the entire cerebellum is dysfunctional in *stargazer*.

4.7.4 Whole animal effects of *stargazer* mutation

In addition to the pronounced ataxia, both *stargazer* and *waggler* perform poorly in eye blink conditioning tasks, possibly but not necessarily because of cerebellar defects (Bao et al., 1998). *Stargazers* have a defect in their vestibular nuclei in addition to the cerebellar problems (Khan et al., 2004). For *stargazer*, the ataxia appears at P14. S*targazers* have long-lasting and frequently recurring absence seizure episodes, characterized by repeated spontaneous spike-wave discharge activity. This is first detected from around P18 days and continues throughout life; mice live a normal age span (reviewed Letts et al., 2003). The cerebellum is the only brain region in *stargazer* that shows no seizure activity (Qiao et al., 1998).

4.7.5 Stargazin, AMPA receptors, GABA-A receptors and BDNF: who does what and when?

Are all of these complex changes the result of stargazin's unique role in trafficking and maintaining AMPA receptor function (see Figure 9 and 10 in the introduction). Without AMPA receptor transmission at the mf-gr cell synapse, the cerebellar circuitry might undergo numerous adaptive changes (Richardson & Leitch, 2002, 2005). In particular, does AMPA receptor depolarization normally maintain BDNF expression levels? It is tacitly assumed that it does, because of one study showing that AMPA receptors are linked to Lyn tyrosine kinase to drive BDNF gene expression (Hayashi et al., 1999). Certainly this assumption seems reasonable, but it has not been tested. This signaling pathway would be missing in both the Gr△GluRD and stargazer granule cells. An additional consideration in interpreting the phenotype is that loss of stargazin is throughout development and the γ2 gene is expressed strongly throughout development (Fukada et al., 2005). For example, AMPA receptors are expressed on migrating wild-type granule cells (Smith et al., 2000). In *stargazer*, the AMPA receptor function on these migrating cells might be disrupted. However, this is only one possible example of a

developmental effect that could confound interpretation of the adult phenotype. We abolished AMPA function only in the adult granule cells.

Alternatively, are these many changes in the cerebellum indicative of multiple roles for stargazin? Could the protein be working mainly or solely on AMPA receptors in the cerebellum, or does it have other targets? We hoped in this study, that by mimicking and inducing one specific feature of the stargazin mutation at a specific point in development, namely ablation of AMPA receptor responses in granule cells after they have finished their postnatal migration, to possibly disentangle these effects. What we found is that stargazin dissapears from Gr△GluRD granule cells, suggesting that with loss of AMPA receptors stargazin's main role is to associate with AMPA receptors, further strengthening the idea that main job, at least in granule cells is an AMPA receptor auxillary subunit. We also found that BDNF and GABA-A α6 decrease in these cells. We thus assume that these other changes are secondary, either as adaptations or pathological losses (e.g. no BDNF), which are normally maintained by AMPA receptor drive. In spite of this the mice have no impaired motor behaviour or head tossing, and so these features of the stargazer phenotype originate elsewhere.

4.7.6 We have functionally phenocopied the *stargazer* mutation, but confined the effect to cerebellar granule cells: stragzin´s primary role is to traffic AMPA receptors

This is a complicated game of "wheels within wheels" and cause and effect. Who caused what? In retrospect I have phenocopied the stargazer mutation cell autonomously, induced specifically in post-migratory granule cells. Based on my immunohistochemistry findings I conclude that the primary role of stargazer (γ2) is binding AMPA receptor subunits. If there is no GluR-D protein, there is no stargazer. Nicoll et al. 2006 pointed out that a critical question is whether TARP-dependent trafficking shows any GluR subunit specificity, writing that "a central challenge will be to determine what role TARPs might have, if any, in the subunit specific control of AMPAR trafficking". It seems that stargazin cannot bind to the remaining GluR-B subunit alone, or this GluR-B is degraded. From the AMPA receptor knockout on granule cells, we presume that the decrease in BDNF and a6 gene expression in Gr△GluRD granule cells is thus due to lack of AMPA receptor-mediated signalling, and that tyrosine kinase signalling from AMPA receptors most likely upregulates BDNF (Hayashi et al., 1999). Thus the reduced GAD65 and GABA content in *stargazer* (Richardson and Leitch, 2002) is thus likely to be an adaptation

elsewhere in the circuitry (perhaps in Golgi cells) to the loss of AMPA receptor signalling on granule cells, or from the loss of BDNF release from granule cells, as we see the same trends in Gr△GluRD cerebella. Note: BDNF works not only to promote granule cell migration and maturation during development, but it increases the GABA content and glutamic acid decarboxylase (GAD) activity in cultured striatal neurons (Mizuno et al., 1994; Ventimiglia et al., 1995).

4.8 Future plans and open questions

1. We should confirm if the mf-gr cell synapses onto Gr△GluRD granule cells really are "silent"; or do the NR1/NR2C (or NR1/NR2A/NR2C) NMDA receptors with their lowered Mg^{2+} sensitivity permit activation at more hyperpolarized potentials and allow some transmission? Is LTP detectable? Concanavalin-A pre-treatment of Gr△GluRD granule cells should be done to look at any possibly changed magnitude of response of kainate GluR-6/KA-2 receptors.
2. Given that I have produced a deficit selectively in just one synapse type, which may remove exciatatory input (and conditional reflex) to the granule cell layer of the cerebellar cortex, is there a detectable deficit in cerebellar learning assayed by a highly sensitive motor learning test, e.g. eye-blink conditioning?
3. Given that GAD56 is down-regulated and there is less GABA in the Gr△GluRD cerebellum, do granule cells excite Golgi cells less, and is GABA release reduced onto the granule cells as part of resetting of the negative feed-back loop? Is the level of tonic (extrasynaptic) and synaptic inhibition via $GABA_A$ receptors still the same onto the granule cells? I would predict that it has been strongly reduced.
4. Indeed, are Golgi cells still firing onto granule cells? (ascending mossy fibres also stimulate Golgi cells, so possibly)
5. As an important internal control, we need to confirm that other cell types, like Purkinje cells, stellate/basket cells in the molecular layer are not affected and have normal AMPA receptors. This confirms that the disruption is unique to granule cells.
6. Is GluR-B protein internal in granule cells? Is it degraded? This would need to be checked by EM immunocytochemistry.

7. EM to look at structural parameters: e.g. numbers of inhibitory synapses, density of PSD.
8. We asked using gene chip analysis if there had been changes in the expression of other genes in Gr△GluRD mice, although I was not able to finish this part of the study as each gene whose expression had potentially changed would have to have been verified by real-time PCR and we had no resources for this. Assuming that AMPA receptor input is strongly reduced onto the Gr△GluRD granule cells, our system provides a good opportunity to examine how AMPA and NMDA receptors regulate gene expression in a defined class of cells. Although CREB phosphorylation might indicate changed AMPA receptor signalling to the nucleus (Hardingham and Bading, 2003), immunostaining to phospho-CREB was unchanged in nuclei of Gr△GluRD granule cells compared with wild-types; however, there are many parallel routes that could induce CREB phosphorylation, of which AMPA/NMDA signalling is just but one (Hardingham & Bading, 2003), so this lack of change does not mean too much. Although gene chip data *per se* are non hypothesis-driven – merely a collection of data, they provide a non-biased platform for further hypothesis generation. I would wish to further evaluate the gene chip data; identify interesting genes, confirm the changes by real-time PCR and try to see if any of these changes lead to hypothesises about cerebellar circuit function or homeostasis.

5 APPENDIX

5.1 Materials

Common chemicals were purchased from the following companies:

Amersham Biosciences, Freiburg	MBI Fermentas, St.Leon-Rot
AppliChem, Darmstadt	Merck, Darmstadt
Becton & Dickinson,Heidelberg	New England Biolabs (NEB)
Bio-Rad, München	PEQLAB, Erlangen
Biozym, Hameln	Roche, Mannheim
Fluka, Neu-Ulm	Roth GmbH, Darmstadt
Invitrogen, Karlsruhe	Sigma-Aldrich, Münche

5.1.1 Special Chemicals

30% Acrylamid/Bis solution	Bio-Rad
Agar	Invitrogen
Agarose	Invitrogen
APS (Ammonium Persulfate)	Bio-Rad
BSA:(Bovine Serum Albumin)	Biolabs
Bradford (dye reagent concentrate)	Biolabs
Dextran Sulfate	Merck
DAB (3,3-Diaminobenzidine Tertrahydrochloride)	Sigma
DEPC (Diethylpyrocarbonat)	ROTH
DTT(Dithiothreitol)	Sigma
EDTA	Merck
Ethanol	Sigma-Aldrich
Ethidiumbromide	Serva
Formamide	Merck
Glycine	Applichem
Hepes	ROTH
Hydrogen peroxide 30%	ROTH

Methanol	Merck
Milk powder	ROTH
Nickel cloride	Merck
PFA (Paraformmaldehyde)	Sigma-Aldrich
Poly-L-lysine hydrobromide	Sigma
Potassium chloride	Applichem
Potassium dihydrogen phosphate	Applichem
Protein pricipitation	Gentra
Sodium acetate	Merck
Sodium chloride	Applichem
SDS (Sodium Dodecyl Sulfate)	Merck
Sodium citrate	Applichem
Sodium hydrogen phosphate	Applichem
TEMED (N,N,N'N'-Tetra-methylethylenediamine)	Bio-Rad
Tris base	ROTH
Tris-acetate	Applichem
Tritonx100	Merck
Tween20 (electrophoresis reagent)	Sigma

5.1.2 Enzymes

PK (Protein Kinase)	Sigma
Taq polymerase kits (Cat.No. 10342-053)	Invitrogen
Terminal Transferase kits (Cat.No.3333574)	Roche
DNase I	Qiagen

5.1.3 Antibodies

Polyclonalantibody-rabbit anti-glutamate receptor A	Chemicon
Polyclonal antibody-rabbit anti-glutamate receptor B	Chemicon
Polyclonal antibody-rabbit anti-glutamate receptor D	Chemicon
Monoclonal anti-β-actin antibody	Sigma
Anti-Rabbit Ig**,** antibody linked whole peroxidase horseradish	Amersham
Anti-Mouse Ig, antibody linked whole peroxidase horseradish	Amersham

Biotinylated anti-rabbit IgG(H+L)	Vector laboratories
Monoclonal anti-GAD65 antibody	Chemicon
Polyclonal antibody anti-Stargazin	Upstate
Monoclonal anti-GABA antibody	Swant
Polyclonal antibody anti-phosphorated CREB	Upstate
Cy^{TM}3- conjugated AffiniPure Goat Anti-Rabbit IgG	Jackson ImmunoResearch
Alexa Fluor 488 goat anti-mouse IgG	Invitrogen Molecular Probes

5.1.4 Markers

DNA size marker: pRK7 plasmid DNA cut with Hinf I	MBI
Protein marker: BenchMarkTM Prestained Protein ladder	Invitrogen

5.1.5 Radioactive Compounds

^{35}S dATP Specific activity: 46.3TBq (1250Ci)/mmol	Amersham
Radioactive concentration: 12.5mCi/ml	

5.2 Nucleotides and primers

Deoxyribonucleotides (dNTP´s)	MBI

5.2.1 Oligonucleotides for *in situ* hybridization

MWG-Biotech AG con: 0.3pmol/ml

GluRA: 5’-GTC ACT GGT TGT CTG ATC TCG TCC TTC TTC AAA CTC TTC ACT GTG-3’

GluRB: 5’-TTC ACT ACT TTG TGT TTC TCT TCC ATC TTC AAA TTC CTC AGT GTG-3’

GluRC: 5’-AGG GCT TTG TGG GTC ACG AGG TTC TTC ATT GTT GTC TTC CAA GTG-3’

GluRD: 5’-TTG GTC ACT GGG TCC TTC TTT TCC ATC CTC AGG CTC TTC TGT GTC-3’

KA1: 5’-CTT GTA GTT GAA CCG TAG GAT CTC AGC CAA CTC CTT GAG CAT GTC-3’

KA2: 5’-TTC CAC TCT GGC CTT GGC TGG GAC CTC GAT GAT CCC ATT GAT CTG-3’

GluR5: 5’-CCG GGG TTG GTT CCA TTG GGC TTC CGG TAA AGG ATG CTA ATG CCC-3’

GluR6: 5’-TTG CAG GGA TGA GCA TCA TAC CAC TCG TAA GGG CTG AAT CTG GCG-3’

GluR7: 5’-ACT GGG GTT GGT GCC ATT GGG CTT GCG GTA TAA GAT GCT CAC TCC-3’

BDNF mouse1:5’-AAG GAA AAG GAT GGT CAT CAC TCT TCT CAC CTG GTG GAA CTC AGG-3’

BDNF mouse2: 5’-GTG CCT TTT GTC TAT GCC CCT GCA GCC TTC CTT GGT GTA ACC CAT-3’

STG1: 5’-CAT TTG AAC ACC TCG ATC AAA CAG CCC CAT AAT TCT TCA TTA TAT-3’

STG2: 5’-GGC TGT GTT GGC GTG GAG AGA GTC CTT GCT GTC CTT CTG CAT ACA-3’

GAD65: 5’-AAC CCT CCACCC CAA GCA GCA TCC ACG TGC ATC CAG ATC TTA TAC-3’

5.2.2 Primers for genotyping

Invitrogen Custom Primers, con. 10μM

GluRD1 (intron 10, sense):	5’-CTA GTG CGA AGT AGT GAT CAG G-3’
GluRD2 (intron 11, antisense):	5’-CAC TAT GTC TCA GTT CTC TCA AG-3’
Cre1 (sense):	5’-GAC CAG GTT CGT TCA CTC ATG G-3’
Cre2 (antisense):	5’-AGG CTA AGT GCC TTC TCT ACA C-3’

5.2.3 Primers for quantitative Real-time PCR

TIB MOLBIOL Syntheselabor GmbH, Berlin

GluRA-F:	5’-CTT TGC CTT TTT CTG CAC CG-3’
GluRA-R:	5’-GCC GCA TGT TCC TGT GAT T-3’
GluRB-F:	5’-CAG TGC ATT TCG GGT AGG GA-3’
GluRB-R:	5’-GGG AGC AGA GAA AGC ATT GGT G-3’
GluRC-F:	5’-CCA TCA GCA TAG GTG GAC TT-3’
GluRC-R:	5’-GGT AGT TCA AAT GGA AGG GC-3’
GluRDE11-F:	5’-AGT GAC CAA CCT CCC AAT GA-3’
GluRDE11-R:	5’-ACC ATA CGC CTC CAA CAA TC-3’
KA2-F:	5’-AGA ACT CGC GGT ACC AGA CG-3’
KA2-R:	5'-GAA GGC ATA GCG GGA GTT GA-3'
GluR6-F:	5'-CAT GAC AGT TTT GAG GCC AC-3'
GluR6-R:	5'-GCA AAT GGA CTG GAC AGC AT-3'
α6-F:	5’-CGC CCC CTG TGG CAA-3’

α6-R:	5’-TAC TTG GAG TCA GAA TGC ACA ACA-3’
BDNF-F:	5'-GCT GGA TGA GGA CCA GAA GGT
BDNF-R:	5'-GAG GCT CCA AAG GCA CTT GA
GAD65-F:	5'-GTA TAA GAT CTG GAT GCA CG-3'
GAD65-R:	5'-GAG GAT TCC ATG TCA CAG AG-3'
Cyclophylin-F:	5’-AGG TCC TGG CAT CTT GTC CAT-3’
Cyclophylin-R:	5’-GAA CCG TTT GTG TTT GGT CCA-3’

5.2.4 Primers for sequence of GluRB editing site

Invitrogen Custom Primers con: 10μM

GluRB-F (exon11):	5’-GCG AAT TCA CAC AAA GTA GTG AAT CAA CT
GluRB-R (exon14):	5’-GCG GTA CCT CGT ACC ACC ATT TGT CTT TTC A
GluRB-R (exon12):	5’-GCT TAG ACG GAT CCT CAG CAC

5.3 Special Articles

Nitrocellulose transfer membrane	Schleicher & Schuell Bioscience
Film: Hyper film^{TM}ECL	Amersham Bioscience
Film: Kodak	Biomark
ABC kit A	Vector laboratories
ULTRASPEC RNA isolation system	BIOTECX Laboratories, Inc.
RNeasy Mini kit	Qiagen
Single-Stranded cDNA Synthesis Kit	Invitrogen
SYBR® Green PCR Kit	PE Applied Biosystems
Gel extraction kit	Qiagen
Software:	
MAS 5.0 (Microarray Suite)	Affymetrix
DMT 3.0 (Data Mining Tool)	

5.4 General buffers and other materials:

ECL solution (ECL plus Western Blottiong detection)	Amersham Bioscience

Ponceau S staining solution	Sigma
Goat serum	Sigma
Loading buffer: Laemmli sample buffer	Bio-Rad

4% Agar: diluted with 1xPBS

DEPC.H2O: 1ml DEPC (Diethylpyrocarbonat) per 1L Millipore water then autoclaved

4%PFA: Paraformmaldehyde disolved with DEPC.H_2O

Tissue wet solution for *in situ* hybridisation:	50% formamide
	4xSCC
HIRT lysis buffer:	10mM Tris
	100µM EDTA
	0,5% SDS
20x TAE Puffer	800 mM Tris/HCl
	400 mM NaOAc
	40 mM EDTA
	adjust to pH 8.3 with acetic acid
20x SSC	3M NaCl
	0,3M Natrium Citrate
TNES buffer:	0.14M NaCl
	20M Tris
	5mM EDTA
TE buffer: pH: 7,6	1M Tris
	10µM EDTA
Hybridization buffer for *in situ*:	50% formamide
	10% dextran sulfate
	4xSSC
Hepes buffer:	25mM Hepes
	150mM NaCl
	adjust to pH 7.4
1xPBS:	137mM NaCl
	2.7mM KCl

	10mM KH_2PO_4
	2mM Na_2HPO_4
TBS buffer:	50mM Tris
	0.9% Nacl
Nickel Acetate:	0.175M Sodium Acetate
	8% Nickel cloride
1xRunning buffer for Western blot :	25mM Tris-Cl
	250mM Glycine
	0.1% SDS
1xTransfer buffer for Western blot:	25mM Tris base
	250mM Glycine
	10% Methanol

5.5 Gels

2,5% Agarose gel for genotyping:	Agarose
	1x TAE buffer
	Ethidium Bromide: 0,008%
10% SDS-PAGE gels for protein assay:	30% Acrylamid/Bis solution
	10% SDS
	TEMED
	10% APS
	1.5M Tris

6 ABBREVIATIONS

α	Alpha
^{35}S-dATP	Adenosine triphosphate labeled with ^{35}S in gamma position
A	Ampere
ABP	AMPA-binding protein
ACSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl–4-isoxazole propionate
ATP	Adenosine triphosphate
ATPO	(R,S)-2-2amino-3-[5tertbutyl-3-(phosphonomethoxyl)-4-isoxazolyl
β	Beta
BAC	Bacterial artificial chromosome
BDNF	Brain-derived neurotrophic factor
bp	Base pair
BSA	Bovine serum albumin
C	Celsius
CaMKII	Calcium/calmodulin-dependent protein kinase II
cDNA	Complementary DNA
CGC	Cerebellar granule cells
CNS	Central Nervous System
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
Cre	Cre recombinase
CREB	cAMP-responsive element binding protein
CTZ	Cyclothiazide
DAG	Diacylglycerol
DCN	Deep cerebellar nuclei
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DNQX	6,7-Dinitroquinoxaline-2,3-dione
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
ERKS	Extracellular signal-regulated kinases
et al.	et alii
EDTA	Ethylenediamine tetraacetic acid
EPSCs	Excitatory postsynaptic currents
γ	gamma
G proteins	signal transducing GTP-binding proteins
GABA	γ-aminobutiric acid
GAD65, 67	glutamate decarboxylase 65 (GAD2) and 67 (GAD1)
GRIP	glutamate receptor-interacting protein
GYKI	1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine
HEPES	N-(2-Hydroxyethyl)piperazine-N'-ethanesulfonic acid
I	Current

IgG	Immunoglobulin G
ionotropic receptors	iGluRs
IPSCs	Inhibitory postsynaptic currents
IP3	Inositol-1-4-5-triphosphate
Kb	Kilobase
KO	knock-out
lacZ	β-galactosidase
LoxP	Locus of crossing over (for) phage P
LTP	long-term potentiation
LTD	long-term depression
μ	Micro
m	Milli
M	Methionine
M	Molar
Min	minute
MAPK	mitogen-activated protein kinase
MF-Grs	mossy fiber-granule cell synapses
mGluRs	G-protein coupled metabotropic receptors
mRNA	Messenger RNA
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NSF	N-ethylmaleimide-sensitive factor
P	Pore domain
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDZ domain	Post synaptic density/disc large/zona occludens-1 homology domain (**P**SD-95/SAP-90, **D**iscs-large, **Z**O-1 homologous domain)
PEG	Polyethilenglycol
PFA	Paraformaldehyde
PEPA	4-［2-(phenylsulfonylamino) ehtylthio］-2,6 –difluorophenoxyacetamide
Pl3	phosphatidylinositol 3-kinase
PIP2	Phosphatidyl-1-4-5-inositol-biphosphate
PICK-1	PKC-interacting protein
PLC	Phospholipase C
PKA	Cyclic AMP-dependent protein kinase A
PKC	Protein kinase C
PSD	Post synaptic density
PSP	Postsynaptic Density Proteome
PVP	Polyvinylpyrollidone
Q	glutamine
R	Arginine
RNA	Ribonucleic acid

RT	Room temperature
S	Serine
S	second
SAP97	synapse-associated protein-97
SDS	Sodium-dodecyl-sulfat
SNARE	soluble N-ethylmaleimide-sensitive factor attached protein receptor
Stg	Stargazin
T	Threonine
T	Time
TARPs	AMPAR regulatory proteins
TBS	Tris-buffered saline
TE	Tris/EDTA buffer
TENS	Tris-EDTA-Sodium chloride-SDS buffer
TM	Transmembrane domain
Tris	Tris(hydroxymethyl)aminomethane
Tris-HCl	Tris(hydroxymethyl)aminomethane-hydrochloride
U	Unit
V	Volume
V	Voltage
V	Volt
VDCCs	voltage-dependent Ca^{2+} channels
W	Watt
Wt	Wild-type
WT-α6Cre	Wild-type α6Cre
Y	Tyrosine

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