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# Changes in the GRIP 1&2 scaffolding proteins in the cerebellum of the ataxic stargazer mouse

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ABSTRACT

Glutamate receptor-interacting proteins (GRIP1&2) and protein-interacting with C kinase-1 (PICK1) are synaptic scaffold proteins associated with the stabilization and recycling of synaptic GluA2-, 3- and 4c-containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs). PICK1-mediated phosphorylation of GluA serine880 uncouples GRIP1&2 leading to AMPAR endocytosis, important in mediating forms of synaptic plasticity underlying learning and memory. Ataxic and epileptic stargazer mice possess a mutation in the CACNG2 gene encoding the transmembrane AMPAR-regulatory protein (TARP)-γ2 (stargazin). TARPs are AMPAR-auxiliary subunits required for efficient AMPAR trafficking to synapses. Stargazin is abundantly expressed in the cerebellum and its loss results in severe deficits in AMPAR trafficking to cerebellar synapses, particularly at granule cell (GC) synapses, leading to the ataxic phenotype of stargazers. However, how the stargazin mutation impacts on the expression of other AMPAR-interacting scaffold proteins is unknown. This study shows a significant increase in GRIP1&2, but not PICK1, levels in whole tissue and synapse-enriched extracts from stargazer cerebella. Post-embedding immunogold-cytochemistry electron microscopy showed GRIP1&2 levels were unchanged at mossy fiber-GC synapses in stargazers, which are silent due to virtual total absence of synaptic and extrasynaptic GluA2/3-AMPARs. These results indicate that loss of synaptic AMPARs at this excitatory synapse does not affect GRIP1&2 expression within the postsynaptic region of mossy fiber-GC synapses. Interestingly, increased GRIP and reduced GluA2-AMPARexpression also occur in cerebella of autistic patients. Further research establishing the role of elevated cerebellar GRIP1&2 in stargazers may help identify common cellular mechanisms in the comorbid disorders ataxia, epilepsy and autism leading to more effective treatment strategies.



## 1. Introduction

The majority of fast excitatory synaptic neurotransmission is facilitated by the activation of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-subtype ionotropic glutamate receptors. Excitatory synaptic strength is dependent on the number of AMPARs present in the synapse. AMPARs are formed from dimer–dimers of GluA subunits (GluA1–4) (Cull-Candy et al., 2006). The presence of GluA2 produces calcium-impermeable (CI)-AMPARs, while the absence of GluA2 results

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in calcium-permeable (CP)-AMPARs (Cull-Candy et al., 2006). The GluA subunit composition of AMPARs also influences their synaptic recycling at the cell surface (Kato et al., 2010; Incontro et al., 2011). Transmembrane AMPAR-regulatory proteins (TARPs) are a family of auxiliary subunits required for the efficient trafficking of AMPARs to the synapse (Tomita et al., 2005). TARPs interact with GluA subunits, anchoring AMPARs in the synapse by binding to the scaffold protein, PSD-95 (Bats et al., 2007).

The postsynaptic density (PSD) region contains numerous PDZ (PSD-95, discs large, zona occludens-1) domain-containing proteins. GluA subunits contain PDZ binding domains, located at the C-terminus that facilitates binding to scaffold proteins such as glutamate receptor-interacting proteins (GRIP1&2) (Dong et al., 1997, 1999) and protein interacting with C kinase-1 (PICK1) (Staudinger et al., 1995, 1997). GRIP1&2 and PICK1 are crucially involved in the localization, stability and recycling of synaptic AMPARs (Gardner et al., 2005; Liu and Cull-Candy, 2005). The rate of GRIP1&2- and PICK1-mediated synaptic AMPAR recycling influences the strength of excitatory synapses contributing to synaptic plasticity underlying the processes of learning and memory. GRIP1&2 and PICK1 interact with the serine880 residue in the C-terminal domain of GluA2, 3 and 4c (Chung et al., 2000; Seidenman et al., 2003). The binding affinity of GRIP1&2 and PICK1 for synaptic AMPARs is dependent on the phosphorylation state of the serine880 residue. PICK1 binds to protein kinase C (PKC), which phosphorylates serine880 thereby reducing the affinity of GRIP1&2 binding and increasing the affinity of PICK1 binding. This results in the internalization of synaptic AMPARs to intracellular sites (Chung et al., 2000; Daw et al., 2000; Osten et al., 2000; Seidenman et al., 2003). The importance in neurotransmission is significant, as deletion of either GRIP1&2 or PICK1 abolishes cerebellar LTD, which can be successfully restored by the exogenous administration of the deleted proteins (Takamiya et al., 2008; Hanley and Henley, 2010; Mao et al., 2010; Volk et al., 2010).

TARP-$\gamma$2 (stargazin) is the prototypical TARP identified in the ataxic and epileptic stargazer mutant mouse, which has a spontaneous recessive mutation in the CACNG2 gene encoding stargazin (Noebels et al., 1990). TARPs have a complimentary distribution in the brain allowing for regional and cellular AMPAR diversity, with stargazin being the predominant TARP present in the cerebellum (Sharp et al., 2001; Tomita et al., 2003; Fukaya et al., 2005). TARPs can be grouped as type I ($\gamma$2, $\gamma$3, $\gamma$4 and $\gamma$8) or type II ($\gamma$5 and $\gamma$7) depending on the length of the C-terminus of the TARP, which influences the efficiency of AMPAR trafficking from the endoplasmic reticulum to the cell surface and their insertion into the synapse (Tomita et al., 2003; Kato et al., 2008). In the absence of the dominant type I TARP, other type I TARPs are capable of mediating AMPAR trafficking and insertion, highlighting a compensatory mechanism (Menuz et al., 2008; Coombs and Cull-Candy, 2009). In the cerebellum, granule cells (GCs) express type I $\gamma$2 and type II $\gamma$7 (Kato et al., 2008; Coombs and Cull-Candy, 2009). Therefore, in stargazer GCs the absence of $\gamma$2 results in a complete loss of GluA2-AMPAR trafficking to the excitatory mossy fiber (MF)–GC synapse, resulting in functionally silent synapses, which contributes to the ataxic phenotype (Hashimoto et al., 1999; Chen et al., 2000). This raises the question of how the absence of stargazin and the subsequent failure of GluA2-AMPAR trafficking to the synapse impacts on synaptic scaffold protein levels. We hypothesized that GRIP1&2 and PICK1 levels may be changed in the cerebellum of stargazers, which is one of the brain regions most severely affected by the stargazin mutation with the greatest loss of AMPAR expression at excitatory synapses (Chen et al., 2000; Shevtsova and Leitch, 2012).

## 2. Results

### 2.1. *GRIP1&2 but not PICK1 levels are increased in the cerebellum of stargazers*

To establish whether scaffold protein levels are indirectly altered as a result of the stargazin mutation, Western blot analysis of GRIP1&2 or PICK1 in the cerebellum of stargazer mice was conducted on whole tissue cerebellar cortex samples. GRIP1&2 and GRIP1 immunopositive bands at 130 kDa (Dong et al., 1999; Srivastava and Ziff, 1999) and a PICK1 positive doublet at 45–55 kDa were detected by antibodies against GRIP and PICK1 (Staudinger et al., 1995) (Fig. 1a–c). The double bands for PICK1 in Western blot have been reported before and attributed to the sensitivity of PICK1 to proteolysis *in vivo* (Staudinger et al., 1995) (Fig. 1c). Preadsorption of GRIP and PICK1 antibodies with an excess of the respective antigens eliminated these bands demonstrating the specificity of GRIP1&2 and PICK1 immunolabeling (Fig. 1d).

Quantitative analysis of whole cerebellar tissue showed that GRIP1&2 levels were significantly increased by 71% in the cerebella of stargazers (Fig. 1e: $1.71 \pm 0.2$, mean $\pm$ SEM, $p < 0.01$, $n = 9$ pairs) compared to matched control littermates. All protein bands were normalized to $\beta$-actin loading controls and analyses were conducted on raw unadjusted original images. PICK1 protein bands were analyzed in the same cerebellar tissue samples and showed no significant difference in expression levels between stargazers and controls (Fig. 1e: $1.10 \pm 0.1$, $p > 0.05$, $n = 9$ pairs). Subsequently, GRIP1 antibody was used to identify specifically which GRIP protein (GRIP1 or GRIP2 or both) was increased in the stargazer cerebella. Western blot band densities indicated that the increase might in part be due to an increase in GRIP1 expression (Fig. 1b). However, GRIP1 levels present in the controls were too low to quantify therefore, the GRIP1&2 antibody was used in all subsequent experiments. Hippocampal tissue was used as a positive control, as this brain region is known to express high levels of GRIP and PICK1 (Dong et al., 1999; Srivastava and Ziff, 1999) (Fig. 1a–c; right panels). Quantitative analysis of whole hippocampal tissue showed no significant difference in GRIP1&2 expression ($1.00 \pm 0.1$, $p > 0.05$, $n = 9$ pairs) or PICK1 expression ($0.94 \pm 0.1$, $p > 0.05$, $n = 9$ pairs) (Fig. 1e).

### 2.2. *Increased cerebellar GRIP1&2 levels are associated with synapses in stargazers*

To establish if the elevated expression of cerebellar GRIP1&2 occurred within the synapto-dendritic compartment,