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ABERRANT CEREBELLAR GRANULE CELL-SPECIFIC $GABA_A$ RECEPTOR EXPRESSION IN THE EPILEPTIC AND ATAXIC MOUSE MUTANT, *Tottering*

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Abstract—The *Tottering* (*cacna1a^{tg}*) mouse arose as a consequence of a spontaneous mutation in *cacna1a*, the gene encoding the pore-forming subunit of the pre-synaptic P/Q-type voltage-gated calcium channel (VGCC, Ca_V2.1). The mouse phenotype includes ataxia and intermittent myoclonic seizures which have been attributed to impaired excitatory neurotransmission at cerebellar granule cell (CGC) parallel fiber–Purkinje cell (PF–PC) synapses [Zhou YD, Turner TJ, Dunlap K (2003) Enhanced G-protein-dependent modulation of excitatory synaptic transmission in the cerebellum of the Ca^{2+}-channel mutant mouse, tottering. J Physiol 547:497–507]. We hypothesized that the expression of cerebellar $GABA_A$ receptors may be affected by the mutation. Indeed, abnormal $GABA_A$ receptor function and expression in the *cacna1a^{tg}* forebrain has been reported previously [Tehrani MH, Barnes EM Jr (1995) Reduced function of gamma-aminobutyric acid A receptors in tottering mouse brain: role of cAMP-dependent protein kinase. Epilepsy Res 22:13–21; Tehrani MH, Baumgartner BJ, Liu SC, Barnes EM Jr (1997) Aberrant expression of $GABA_A$ receptor subunits in the tottering mouse: an animal model for absence seizures. Epilepsy Res 28:213–223]. Here we show a deficit of 40.2±3.6% in the total number of cerebellar $GABA_A$ receptors expressed ($\gamma 2+\delta$ subtypes) in adult *cacna1a^{tg}* relative to controls. [^{3}H]Muscimol autoradiography identified that this was partly due to a significant loss of CGC-specific $\alpha 6$ subunit-containing $GABA_A$ receptor subtypes. A large proportion of this loss of $\alpha 6$ receptors was attributable to a significantly reduced expression of the CGC-specific benzodiazepine-insensitive Ro15-4513 (BZ-IS) binding subtype, $\alpha 6 \beta \gamma 2$ subunit-containing receptors. BZ-IS binding was reduced by 36.6±2.6% relative to controls in cerebellar membrane homogenates and by 37.2±3.7% in cerebellar sections. Quantitative immunoblotting revealed that the steady-state expression level of $\alpha 6$ and $\gamma 2$ subunits was selectively reduced relative to controls by 30.2±8.2% and 38.8±13.1%, respectively, $\alpha 1$, $\beta 3$ and δ were unaffected. Immunohistochemically probed control and *cacna1a^{tg}* cerebellar sections verified that $\alpha 6$ and $\gamma 2$ subunit expression was reduced and that this deficit was restricted to the CGC layer. Thus, we have shown that abnormal cerebellar P/Q-type VGCC activity results in a deficit of CGC-specific subtype(s) of $GABA_A$ receptors which may contribute to, or may be a consequence of the impaired cerebellar network signaling that occurs in *cacna1a^{tg}* mice. **© 2007 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: cerebellum, $GABA_A$ receptor, Ca_V2.1, *cacna1a^{tg}*, ataxia.

Tottering (*cacna1a^{tg}*) mice exhibit a severe inherited neurological phenotype consisting of ataxia, absence seizures, paroxysmal dyskinesia and intermittent myoclonus that manifests around postnatal day 21 (Green and Sidman, 1962; Noebels and Sidman, 1979). Located on chromosome 8, the *cacna1a^{tg}* locus is characterized by an autosomal recessive single amino-acid change (P601L) in *cacna1a*, the gene encoding the pore-forming subunit of the P/Q-type voltage-gated calcium channel (VGCC), Ca_V2.1 (Fletcher et al., 1996). Ca_V2.1 belongs to the class of high voltage–activated Ca^{2+} channels and couples membrane depolarization to Ca^{2+} entry across the neuronal plasma membrane subsequently triggering a variety of intracellular processes, including neurotransmitter release and gene expression (Sutton et al., 1999). Highly expressed in the cerebellum, Ca_V2.1 channels are responsible for 85–95% and 50% of the total calcium current measured in Purkinje cells (PCs) and cerebellar granule cells (CGCs), respectively (Mintz et al., 1992; Randall and Tsien, 1995).

The development of ataxia and intermittent myoclonus in the *cacna1a^{tg}* mutant mouse has recently been attributed to impaired excitatory neurotransmission at CGC parallel fiber (PF)–PC synapses. A switch from the P/Q-type to N-type VGCCs at the PF presynaptic terminus of *cacna1a^{tg}* has been proposed to underlie this phenomenon, although the signaling mechanism responsible for evoking this switch is unknown (Zhou et al., 2003). A similar switch from P/Q-type to N-type mediated synaptic transmission was observed at hippocampal Schaeffer collateral synapses (Qian and Noebels, 2000) but found not to occur at the peripheral neuromuscular junction (Kaja et al., 2006).

During the convulsive episodes, immediate early gene expression is reported in both PCs and CGCs of the *cacna1a^{tg}* cerebellum implying that these neurons are hyperexcitable (Campbell and Hess, 1998). Invariably, the GABAergic properties of interneurons within hyperexcitable neuronal networks are abnormal and either thought to contribute to the increased network activity or occur as an

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Abbreviations: BZ-IS, benzodiazepine full agonist-insensitive receptor; BZ-S, benzodiazepine full agonist-sensitive receptor; *cacna1a^{tg}*, *Tottering*; CGC, cerebellar granule cell; PBS, phosphate-buffered saline; PC, Purkinje cell; PF, parallel fiber; PKA, protein kinase A; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; VGCC, voltage-gated calcium channel.

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adaptive response to it (e.g. Clark, 1998; Leroy et al., 2004; Payne et al., 2006). Interestingly, aberrant $GABA_A$ receptor expression in the cerebral cortex (Tehrani and Barnes, 1995; Tehrani et al., 1997; Ayata et al., 2000) and the hippocampus (Helekar and Noebels, 1994) of *cacna1a^tg^* has been reported previously, leading us to speculate that $GABA_A$ receptor expression in the cerebellum of *cacna1a^tg^* may also be compromised.

This hypothesis is strengthened by the observations that the *cacna1a^tg^* mutation causes an up-regulation of high voltage-activated Ca_V1 (L-type) channels in the cerebellum that is believed to contribute to the dystonic episodes of *cacna1a^tg^* (Campbell and Hess, 1999). Interestingly, Ca_V1 channels are known regulators of $GABA_A$ receptor expression in rat (Gault and Siegel, 1997) and mouse CGCs (Payne et al., manuscript in preparation) and indeed have been reported to be physically associated with $GABA_A$ receptors in these neurons (Hansen et al., 1992).

Here we present a detailed characterization of $GABA_A$ receptor expression in *cacna1a^tg^* cerebellum in order to test our hypothesis. Some of these data have previously appeared in abstract form (Kaja et al., 2003).

EXPERIMENTAL PROCEDURES

Animals

Tottering mutant mice (*cacna1a^tg^*) and littermate controls (+/+ and +/*cacna1a^tg^*) were raised from breeding stocks supplied by Jackson Laboratories (Bar Harbor, ME, USA) and maintained in the Durham University vivarium on a 12-h light/dark cycle with food and water available *ad libitum*. We observed no overt phenotypic differences between +/+ and +/*cacna1a^tg^* mice, we therefore routinely combined +/+ and +/*cacna1a^tg^* material as our control tissue. Genotyping was performed as previously described (Plomp et al., 2000). Animals used for experiments were 2–6 months of age. Animal husbandry, breeding and experimental procedures performed during these experiments were conducted according to the Scientific Procedures Act 1986.

Materials

$[^3H]$Ro15-4513 (28.3 Ci/mmol) and $[^3H]$muscimol (30 Ci/mmol) were obtained from Du Pont (UK) (Stevenage, Hertfordshire, UK). Horseradish peroxidase–linked anti-rabbit and anti-mouse Ig, ECL Western blotting detection system, Hyperfilm-ECL and Hyperfilm-^{3}H were all purchased from Amersham International (Aylesbury, Buckinghamshire, UK).

Affinity-purified, anti-$GABA_A$ receptor α1(1-14Cys) subunit-specific antibodies for immunohistochemistry were provided by Professor F. A. Stephenson (School of Pharmacy, London, UK); those used for quantitative immunoblotting were generated in-house. α6(1-15Cys) Subunit-specific antibodies were prepared as described previously (Pollard et al., 1995). The β3(345–408), γ2(319–366) and δ(1–44) subunit-specific antibodies were contributed by Professor W. Sieghart (Medical University Vienna, Vienna, Austria) (Pirker et al., 2000).

Anti-β-actin antibody was supplied by Sigma (Poole, Dorset, UK). All other materials were obtained from commercial sources.

Immunohistochemistry

The immunohistochemical localization of $GABA_A$ receptor subunits was carried out by conventional procedures as previously described (Thompson et al., 1992).

Briefly, adult mouse brains (control and *cacna1a^tg^*) were perfusion-fixed with 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were dissected, post-fixed overnight and then cryoprotected by incubation in 10%, 20% and subsequently 30% (w/v) sucrose in 0.01 M phosphate-buffered saline (PBS), pH 7.4, at 4 °C for 48 h. The tissue was then frozen in isopentane (−70 °C), and 30 μm-thick horizontal sections were cut on a Leica CM3050S cryostat (Leica Microsystems, Knowlhill, Buckinghamshire, UK). Free-floating sections were initially treated with methanolic (10%, v/v) hydrogen peroxide (3%, v/v) in 0.01 M PBS, pH 7.4, for 10 min to quench endogenous peroxidase activity. Sections were incubated in 0.01 M PBS, pH 7.4, glycine (0.2%, w/v) and Tween-20 (0.2%, v/v), for 15 min to mop up residual unreacted aldehyde groups from the fixative. Non-specific antibody binding sites on the fixed tissue were subsequently blocked by incubating with goat serum (10%, v/v) in PBS/Tween-20 (0.2%, v/v), pH 7.4, for 30 min. Sections were incubated overnight with anti-$GABA_A$ receptor subunit-specific antibodies at working concentrations of 0.0625–1.0 μg/ml, at 4 °C. Sections were subsequently stained for immunoreactive product by the avidin/biotinylated peroxidase complex method using the Vectastain ABC Elite kit (Vector Laboratories Ltd., Peterborough, UK) and 3′,3-diaminobenzidine tetrahydrochloride as the hydrogen peroxidase substrate. Antibody specificities have been reported previously (Turner et al., 1993; Pirker et al., 2000).

Cerebellar membrane preparation

Individual cerebella were dissected from adult control and *cacna1a^tg^* mice, flash frozen in liquid nitrogen and stored at −80 °C. Eight to 12 cerebella per genotype were pooled and thawed in 50 volumes of ice-cold buffer 1 (50 mM Tris–HCl, pH 7.4, 0.32 M sucrose, 5 mM EDTA, 5 mM EGTA, 0.02% (w/v) sodium azide, 0.5 mM phenylmethylsulphonylfluoride, and 2 μg/ml aprotinin, and 1 μg/ml of both leupeptin and pepstatin A). The tissue was homogenized in a Dounce tissue grinder and centrifuged at 45,000×*g* for 30 min at 4 °C in a Beckmann J2-HS centrifuge (rotor JA-20). The pellet was resuspended in 50 volumes of 50 mM Tris–HCl, pH 7.4, 5 mM EDTA, 5 mM EGTA (buffer 2), re-homogenized and re-centrifuged at 45,000×*g* for 30 min at 4 °C. The resulting pellet was re-homogenized in H_2O, flash frozen in liquid nitrogen and stored overnight at −20 °C in order to release endogenous neurotransmitter.

Subsequently, the membrane preparations were thawed and re-centrifuged at 45,000×*g* for 30 min at 4 °C. They were then re-homogenized in 50 mM Tris–HCl, 150 mM NaCl, pH 7.4, re-centrifuged and re-homogenized in the same buffer. Aliquots were flash frozen in liquid nitrogen.

Protein determinations

Protein concentrations of membrane preparations were determined by the method of Lowry et al. (1951) employing bovine serum albumin as the standard protein for calibration.

Radioligand binding assays

Total, benzodiazepine agonist-sensitive (BZ-S) and benzodiazepine agonist-insensitive (BZ-IS) $[^3H]$Ro15-4513 binding to cerebellar membranes from control and *cacna1a^tg^* mice was performed by a rapid filtration assay essentially as described previously (Thompson et al., 1998).

Briefly, $[^3H]$Ro15-4513, 1–80 nM, was incubated with membranes (50–100 μg protein) in 50 mM Tris–HCl, pH 7.4, 0.15 M

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