GABA_A Receptor Expression in the Forebrain of Ataxic Rolling Nagoya Mice

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Abstract
The human CACNA1A gene encodes the pore-forming α1 subunit of Ca_2.1 (P/Q-type) calcium channels and is the locus for several neurological disorders, including episodic ataxia type 2 (EA2), spinocerebellar ataxia type 6 (SCA6), familial hemiplegic migraine type 1 (FHM1) and rare forms of epilepsy [1-4].

Multiple mouse strains exist that carry mutations in the orthologous mouse Cacna1a gene, including Rolling Nagoya (tgrol), carrying the R1262G point mutation in the mouse Cacna1a gene. tgrol mice display a phenotype of severe gait ataxia and motor dysfunction of the hind limbs. At the functional level, the R1262G mutation results in a positive shift of the activation voltage of the Ca_2.1 channel and reduced current density. γ-Aminobutyric acid type A (GABA_A) receptor subunit expression depends critically on neuronal calcium influx, and GABA_A receptor dysfunction has previously been described for the cerebellum of tgrol and other ataxic Cacna1a mutant mice. Given the expression pattern of Ca_2.1, it was hypothesized that calcium dysregulation in tgrol might affect GABA_A receptor expression in the forebrain. Herein, functional GABA_A receptors in the forebrain of tgrol mice were quantified and pharmacologically dissociated using [3H] radioligand binding. No gross changes to functional GABA_A receptors were identified. Future cell type-specific analyses are required to identify possible cortical contributions to the psychomotor phenotype of tgrol mice.

Keywords: Gamma aminobutyric receptor type A; Calcium; Ataxia; Pharmacology; Motor dysfunction; Rolling Nagoya; Cacna1a; Ca_2.1; P/Q-type calcium channel

Introduction
The human CACNA1A gene encodes the α1 subunit of neuronal voltage-gated Ca_2.1 (P/Q-type) calcium channels. Furthermore, CACNA1A is the locus of several genetic neurological diseases, including Episodic Ataxia type 2 (EA2), spinocerebellar ataxia type 6 (SCA6), familial hemiplegic migraine type 1 (FHM1) and rare forms of epilepsy [1-4].

Multiple mouse strains exist that carry mutations in the orthologous mouse Cacna1a gene, including Rolling Nagoya (tgrol), Tottering (tg) and Leaner (tgln); these strains arose spontaneously and exhibit phenotypes of cerebellar ataxia often paired with absence epilepsy and/or other motor phenotypes such as dyskinesia and dystonia [5-7]. Furthermore, transgenic knock-in (KI) mouse models have been generated to harbor the human FHM1 missense mutations R192Q and S218L in the Cacna1a gene [8,9].

The tgrol mouse carries the R1262G mutation that results in a phenotype of pure cerebellar ataxia [6,10]. At the functional level, the mutation results in a loss-of-function phenotype with Ca_2.1 channels exhibiting a positive shift of the activation voltage and reduced current density both in recombinant expression systems and primary culture cerebellar Purkinje cells from tgrol mice [11]. A similar loss-of-function synaptic phenotype was reported for the neuromuscular junction [12].

Numerous studies have investigated anatomy and morphology of the tgrol brain and expression and distribution of neurotransmitter receptors in the tgrol nervous system [10]. However, there is still a controversy regarding the presence and/or extent of cerebellar morphological abnormalities as well as the contribution of striatal dysfunction to the ataxic phenotype of tgrol [13,14].

The rationale for the present study is based on the functional link between neuronal Ca_2+ influx and GABA_A receptor subunit expression [15-19]. In the cerebellum, the loss of GABAergic inhibition may decrease tonic inhibition in cerebellar granule cells (CGCs), leading to ataxia in Angelman syndrome [20]. Similarly, an aberrant GABA_R complement may contribute to the ataxic phenotype of tgrol mice [21-23].

Given the abundant expression of Ca_2.1 channels in the cerebrum, it was hypothesized that functional GABA_A receptor subunit expression may be altered in the forebrain of tgrol mice. Functional GABA_A receptors in the forebrain of tgrol mice were subsequently quantified and pharmacologically dissociated using [3H] radioligand binding.

Materials and Methods

Tissue
Tissue from Rolling Nagoya mice was kindly provided by Drs. Jaap Plomp and Arn van den Maagdenberg (Leiden University Medical Center, Leiden, The Netherlands).

[3H] Radioligand binding assays
[3H] Radioligand binding was essentially performed as described previously [23]. Mice were euthanized by cervical dislocation and forebrain (without olfactory bulb) and cerebellum were dissected into 0.1 M ice-cold phosphate buffered saline (pH 7.  r [3H] muscimol binding, 50 mM Tr 4) and snap frozen in liquid nitrogen. Tissue was

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thawed on ice in 50 volumes assay buffer (50 mM Tris-citrate pH 7.3 for [3H] muscimol binding, 50 mM Tris-HCl pH 7.4 for [3H] Ro15-4513 and [3H] Ro15-1788 binding). Samples were homogenized in a Dounce tissue grinder and centrifuged at 750xg for 10 min at 4°C. Supernatants were subsequently centrifuged at 45,000xg for 30 min, the pellet was washed in 50 volumes assay buffer and re-homogenized. In order to release endogenous neurotransmitter, tissue was incubated for 30 min in 37°C water bath and re-centrifuged. The pellet was then resuspended in 50 volumes assay buffer, flash frozen in liquid nitrogen and stored overnight at -20°C. Immediately prior to experiments, tissue was thawed in a waterbath at ambient temperature, centrifuged and the pellet resuspended 200-fold for [3H] muscimol experiments and 500-fold for [3H] Ro15-4513 and [3H] Ro15-1788 binding. Protein concentrations of membrane preparations were determined by the method of Lowry [24] employing bovine serum albumin as the standard protein for calibration.

**Data analysis and statistics**

Data throughout this manuscript is presented as mean ± s.e.m. Data was analyzed in SigmaPlot v10 (Systat Software, Inc., San Jose, CA) using the one-binding site regression tool with 200 iterations. Overall B\textsubscript{max} and K\textsubscript{D} values were obtained by calculating the mean values obtained from each individual animal. Rosenthal transformations were performed on radioligand binding data and plotted as Scatchard plots for illustration purposes only [23,25,26]. Statistically significant differences were tested for using Student’s t-tests, as appropriate. Statistical significance was defined as P<0.05.

**Results**

**[3H] Muscimol binding**

In order to determine the total number of functional GABA binding sites expressed on forebrain membranes, [3H] muscimol binding was performed. Fitting the binding curve using a single binding-site equation revealed no statistically significant difference between the B\textsubscript{max} of wt and tg\textsuperscript{rol} mice (n=4, P=0.70) (Figures 1A and C). Rosenthal transformations of the data are presented as Scatchard plot for illustration (Figure 1B). The K\textsubscript{D} values for [3H] muscimol binding were similar between genotypes (n=4, P=0.73) (Figure 1D).

**[3H] Ro15-4513 and [3H] flumazenil binding**

Next, benzodiazepine receptor binding, identifying γ\textsubscript{2} subunit-containing GABAA receptors, was quantified using [3H] Ro15-4513 and [3H] flumazenil. Total [3H] Ro15-4513 binding was similar between wt and tg\textsuperscript{rol} forebrain membranes (n=4, P=0.56) (Figures 2A-C). Binding affinity, expressed as K\textsubscript{D}, was not statistically significantly different between genotypes (n=4, P=0.10) (Figure 2D). In order to address the possibility of subunit changes, benzodiazepine-insensitive (BZ-IS) and benzodiazepine-sensitive (BZ-S) binding sites were differentiated pharmacologically (Figures 2E-H). BZ-IS binding was quantified in the presence of 10 µM flunitrazepam (Figure 2E). B\textsubscript{max} and K\textsubscript{D} values did not differ between wt and tg\textsuperscript{rol} (n=4, P=0.92) (Figures 2G and H). Subsequently, BZ-S binding could be calculated mathematically by subtracting BZ-IS binding from total binding (Figure 2F).

Lastly, [3H] flumazenil (Ro15-1788) binding to forebrain...
membranes was quantified. No differences in $B_{\text{max}}$ (n=4, $P=0.95$) and $K_D$ (n=4, $P=0.20$) were identified (Figures 3A-D).

**Discussion**

In this study, GABA$_\alpha$ receptor binding sites in the cerebrum of wt and $tg^{rol}$ mice were quantified by $[^3H]$ ligand binding. We utilized highly selective, well-established GABA$_\alpha$ receptor ligands to investigate GABA$_\alpha$ receptor pharmacology in forebrain membranes of the Cacna1a mutant $tg^{rol}$ mice. The $B_{\text{max}}$ and $K_D$ values obtained for GABA A ligands (data not shown), as described previously [23]. Rapid filtration assays, muscimol recognizes all GABA A Rs. In forebrain membranes, the major GABA A receptor comprises $\alpha_3\beta_2\gamma_2$ subunit, accounting for approximately 50% of all GABA A receptors [27,28]. $[^3H]$ Ro15-4513 and $[^3H]$ flumazenil (Ro15-1788) were used as ligands for the benzodiazepine binding site of GABA Rs and to identify $\gamma_2$ subunit-containing receptors [29,30]. Specificity of ligands was confirmed by using 10-fold excess concentrations of unlabeled ligands to displace $[^3H]$ ligands (data not shown), as described previously [23]. Rapid filtration assays did not reveal any differences between the number and pharmacology of functional GABA A receptors in forebrain membranes of wt and $tg^{rol}$ mice. The $B_{\text{max}}$ and $K_D$ values obtained for GABA A receptor binding were similar to those reported previously [31,32]. At the molecular level, the $tg^{rol}$ mutation (R12642) is located in the domain III voltage-sensor region of the Ca V2.1 protein and results in a positive shift of the activation voltage of the channel and overall reduced current density of the P/Q-type current [11], thought to result in impaired neurotransmission and transmitter secretion [10,12]. Ca V2.1 channels are distributed widely throughout the mammalian central nervous system [33]. The rationale for this study was derived from the regulation of GABA A receptor subunits by Ca$^{2+}$ influx [15-19] and that striatal dysfunction contributing to ataxia may result from GABAergic changes in the forebrain.

There could be several reasons for the absence of GABA A receptor abnormalities in the forebrain in the presence of the $tg^{rol}$ mutation: 1) Compensatory Ca$^{2+}$ channel expression may restore intracellular Ca$^{2+}$ signaling leading to normal Ca$^{2+}$ influx. Unfortunately, there is no data available to date to support or reject this hypothesis. At the neuromuscular junction, where Ca V2.1 channels are the exclusive mediators of acetylcholine release, no compensatory Ca$^{2+}$ channel expression was found [23]. 2) Region and/or cell type-specific changes may be occluded when quantifying binding to membrane preparations. Future studies employing autoradiography are needed to confirm our results presented herein. 3) Effects of Cacna1a mutations are dependent critically on the specific splice isoform of the Ca V2.1 channel. For instance, FHM1 mutations in Cacna1a exhibit greater hyperpolarizing shifts in voltage-dependence when expressed in the short (Ca V2.1Δ47) versus the long C-terminal variant (Ca V2.1+47) [34]. Cerebellar splice variants may be more susceptible to the effects of the $tg^{rol}$ mutation and results in disruption of Ca$^{2+}$ signaling and thus cause the ensuing cerebellar GABA A receptor dysfunction in $tg^{rol}$ mice [21].

In conclusion, we did not identify any gross changes in GABA A receptor pharmacology and expression in the forebrain of $tg^{rol}$ mice. Future cell type-specific analyses are required to confirm cortical contributions to the psychomotor phenotype of $tg^{rol}$ mice.
Figure 3: [3H] Flumazenil (Ro15-1788) binding. (A) [3H] Flumazenil binding did not reveal any quantitative differences between binding to wt and tg rol forebrain membrane homogenates. (B) Scatchard plot is shown for illustration. (C-D) Bmax and KD values did not differ between wt and tg rol mice.

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References


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