

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 α_1 subunit of Ca_v2.1 (P/Q-type) calcium channels and is the locus for several neurological disorders, including episodic ataxia type 2 (EA2), spinocerebellar ataxia type 6 (SCA6) and Familial Hemiplegic Migraine type 1 (FHM1). Several spontaneous mouse *Cacna1a* mutant strains exist, among them *Rolling Nagoya* (*tg^{rol}*), carrying the R1262G point mutation in the mouse *Cacna1a* gene. *tg^{rol}* 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_v2.1 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 *tg^{rol}* and other ataxic *Cacna1a* mutant mice. Given the expression pattern of Ca_v2.1, it was hypothesized that calcium dysregulation in *tg^{rol}* might affect GABA_A receptor expression in the forebrain. Herein, functional GABA_A receptors in the forebrain of *tg^{rol}* 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 *tg^{rol}* mice.

Keywords: Gamma aminobutyric receptor type A; Calcium; Ataxia; Pharmacology; Motor dysfunction; *Rolling Nagoya*; *Cacna1a*; Ca_v2.1; P/Q-type calcium channel

Introduction

The human CACNA1A gene encodes the α_1 subunit of neuronal voltage-gated Ca_v2.1 (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* (*tg^{rol}*), Tottering (*tg*) and Leaner (*tg^{la}*); 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 *tg^{rol}* 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_v2.1 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 *tg^{rol}* 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 *tg^{rol}* brain and expression and distribution of neurotransmitter receptors in the *tg^{rol}* 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 *tg^{rol}* [13,14].

The rationale for the present study is based on the functional link between neuronal Ca²⁺ 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_A R complement may contribute to the ataxic phenotype of *tg*, *tg^{la}* and *tg^{rol}* mice [21-23].

Given the abundant expression of Ca_v2.1 channels in the cerebrum, it was hypothesized that functional GABA_A receptor subunit expression may be altered in the forebrain of *tg^{rol}* mice. Functional GABA_A receptors in the forebrain of *tg^{rol}* 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 7.4) containing [³H] muscimol binding, 50 mM Tris 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 750×g 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_{max} and K_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_{max} of wt and *tg^{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_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 γ₂ subunit-containing GABA_A receptors, was quantified using [³H] Ro15-4513 and [³H] flumazenil. Total [³H] Ro15-4513 binding was similar between wt and *tg^{rol}* forebrain membranes (n=4, P=0.56) (Figures 2A-C). Binding affinity, expressed as KD, 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_{max} and K_D values did not differ between wt and *tg^{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

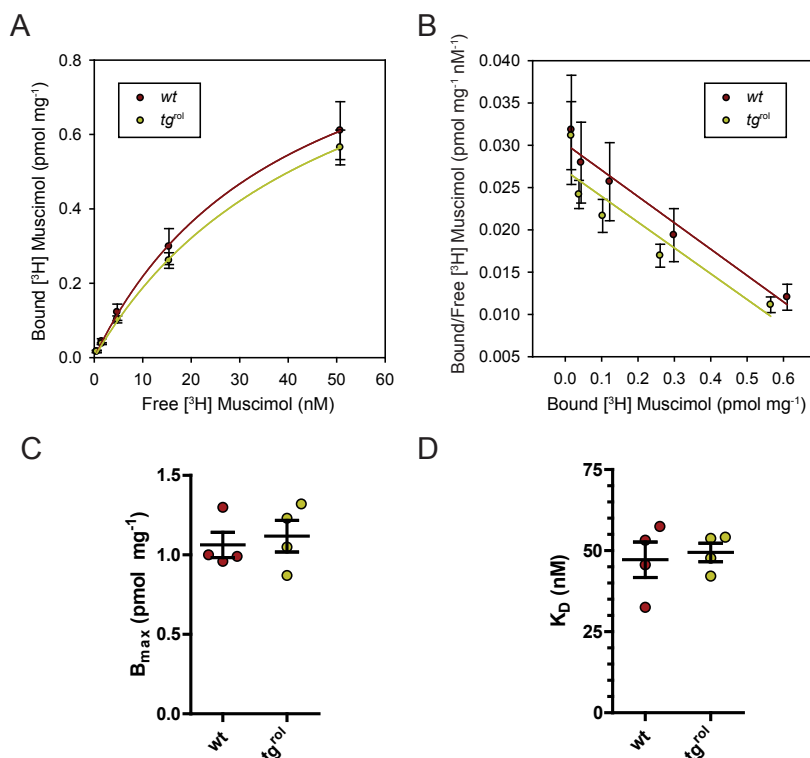


Figure 1: [³H] Muscimol binding.

(A) [³H] Muscimol binding to wt and *tg^{rol}* forebrain membrane homogenates was similar when fitted to a one-site binding curve. (B) Rosenthal transformation was carried out and the Scatchard plot is shown. (C) B_{max} values were not statistically significant different between wt and *tg^{rol}*. Individual values were obtained from fitting a binding curve against the forebrain sample of a single animal. (D) Similarly, no differences in K_D value were obtained.

membranes was quantified. No differences in B_{max} ($n=4$, $P=0.95$) and KD ($n=4$, $p=0.20$) were identified (Figures 3A-D).

Discussion

In this study, GABA_A 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_A receptor ligands to investigate GABA_A receptor pharmacology in forebrain membranes of the *Cacna1a* mutant *tg^{rol}* and *wt* littermate control. In rapid filtration assays, muscimol recognizes all GABA_ARs. In forebrain membranes, the major GABA_A receptor comprises $\alpha_1\beta_2\gamma_2$ 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_ARs and to identify γ_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_{max} and KD 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 ($Ca_v2.1\Delta47$) 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.

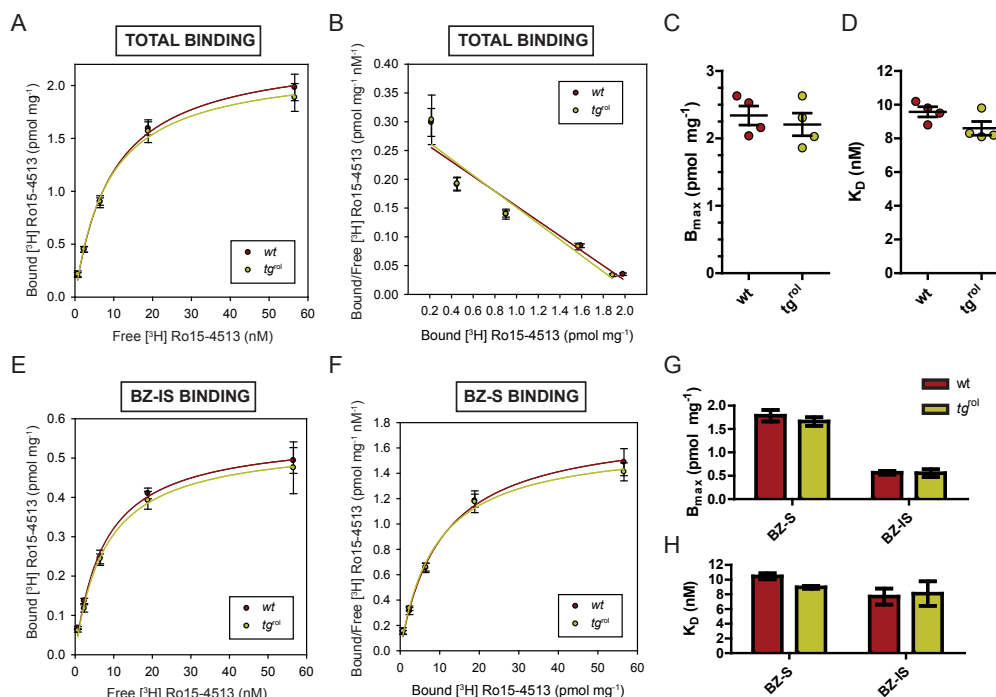


Figure 2: [³H] Ro15-4513 binding.

(A) Total [³H] Ro15-4513 binding was not affected by the *tg^{rol}* mutation in *Cacna1a*. (B) Rosenthal transformation was carried out and the Scatchard plot is shown. (C) B_{max} values did not differ between *wt* and *tg^{rol}*. (D) Similarly, K_D values were similar between *wt* and *tg^{rol}*. (E) [³H] Ro15-4513 binding in the presence of 10 μ M flunitrazepam defined BZ-IS binding sites did not differ between genotypes. (F) BZ-S [³H] Ro15-4513 binding was determined by subtraction of the estimated number of BZ-IS binding sites from total [³H] Ro15-4513 specific binding sites were similar between *wt* and *tg^{rol}* mice. (G-H) B_{max} and K_D values did not differ between *wt* and *tg^{rol}* mice.

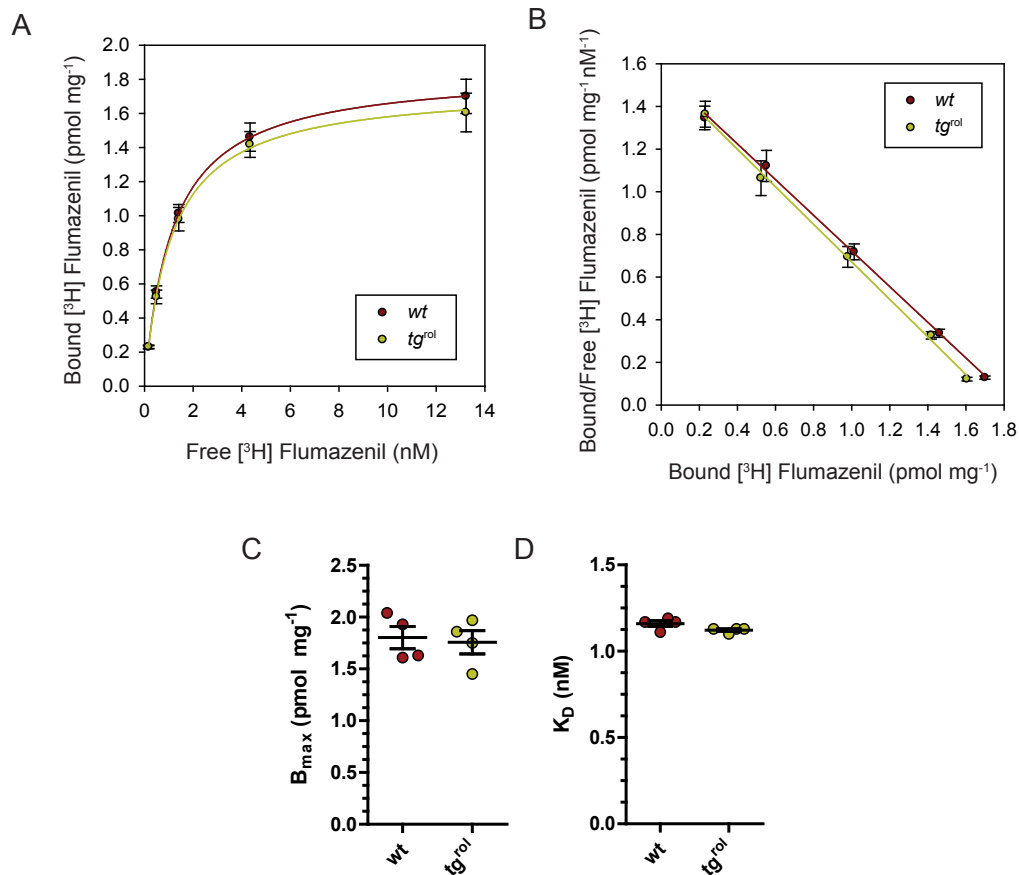


Figure 3: [³H] Flumazenil (Ro15-1788) binding.

(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) B_{max} and K_D values did not differ between wt and *tg^{rol}* mice.

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