

## LETTER TO THE EDITOR

## Genetic rescue of absence seizures

- Crossbreeding GABA<sub>A</sub>  $\delta$  subunit knockout mice with stargazer mice removes the absence seizure phenotype
- Thalamic tonic GABA<sub>A</sub> current is similarly abolished
- Rescue also occurs with acute siRNA knockdown of  $\delta$  subunit
- Ataxic phenotype of stargazer is partially ameliorated

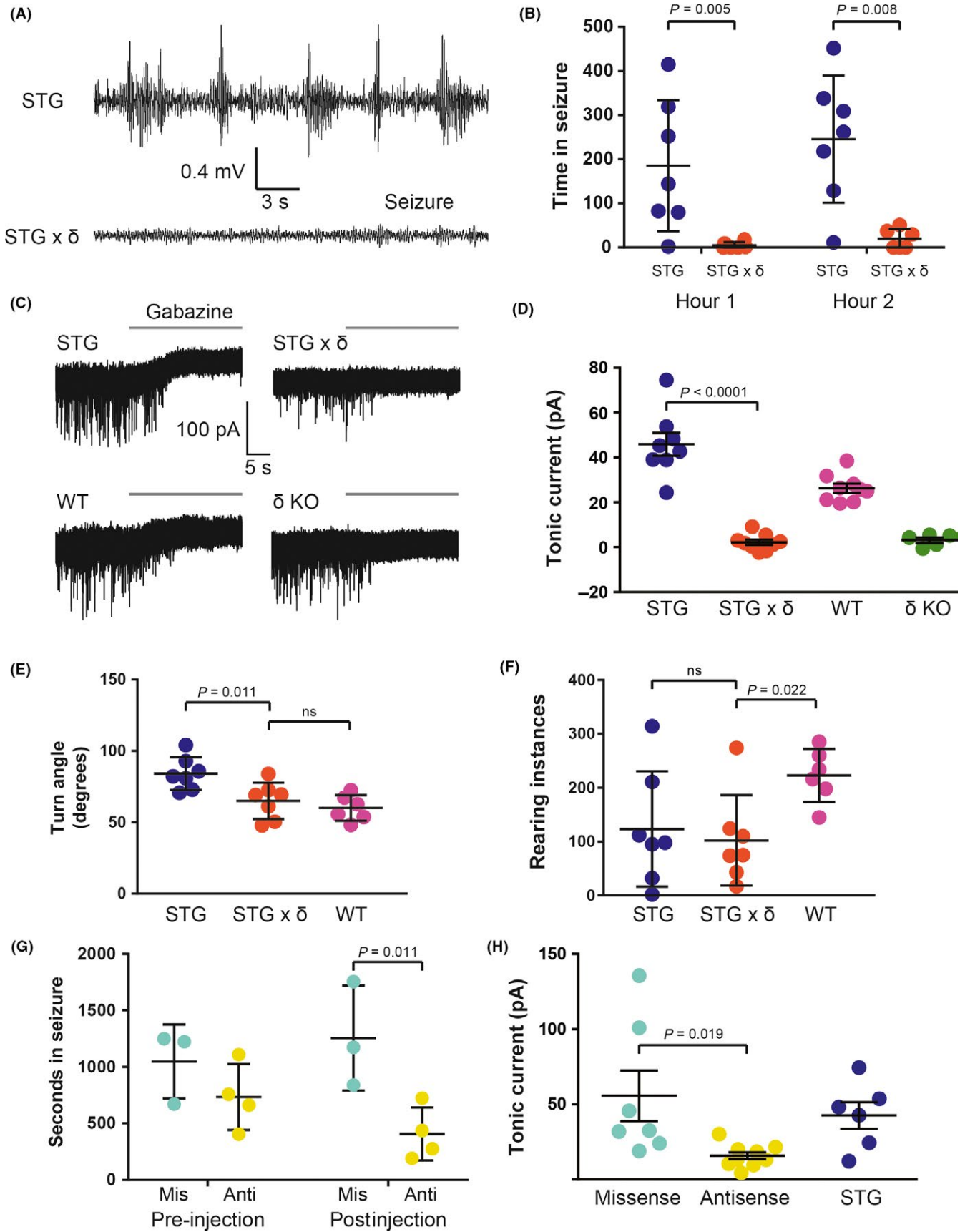
Absence seizures (ASs), the most common form of generalized epilepsy, have significant consequences for patients in the form of impaired attention, mood, and social deficits,<sup>1</sup> and the potential for development into generalized tonic-clonic seizures. Although mechanistic hypotheses of these paroxysmal oscillations are incomplete,<sup>2,3</sup> evidence suggests that an increase in extrasynaptic GABA<sub>A</sub> receptor (eGABA<sub>A</sub>) mediated tonic inhibition in thalamocortical (TC) neurons is sufficient to generate an AS phenotype in multiple rodent models.<sup>4</sup> One of these models is the stargazer (STG) mouse, which has comorbid ataxia and features an early transposon insertion in the voltage-dependent calcium channel (VDCC) subunit gene *Cacng2*, the protein product of which is known as stargazin. Loss of function in this mutated protein results in aberrant thalamic VDCC regulation and impaired cerebellar AMPA-receptor trafficking, which have been implicated in the absence epileptic and ataxic phenotypes, respectively, of the STG mouse.<sup>5</sup> In TC neurons, eGABA<sub>A</sub>Rs invariably contain a  $\delta$  subunit, and tonic GABA<sub>A</sub> current in these neurons has thus been shown to be dependent on the expression of that subunit.<sup>6</sup> Further, knockout (KO) or suppression of the subunit (by RNA interference) reduces both TC tonic GABA<sub>A</sub> current and the occurrence of absence seizures in the GHB ( $\gamma$ -hydroxybutyrate) and GAERS (genetic absence epilepsy rats from Strasbourg) models of ASs, respectively.<sup>4</sup> However, the role of the  $\delta$  subunit and TC tonic GABA<sub>A</sub> in the development of ASs remains to be demonstrated. Consequently, we investigated whether knockout of the  $\delta$  subunit in STG mice, via crossbreeding them with  $\delta$  subunit KO mice, could prevent the development of ASs, thus leading to a “genetic rescue” of the STG epilepsy phenotype.

Three-month-old F4 to F6 offspring from the breeding of B6C3Fe a/a-Cacng2stg/J stargazer (STG) and B6.129-Gabrdtm1Geh/J ( $\delta$  KO) mice from the Jackson Laboratory were used for genetic rescue experiments. Stargazer mice from the same source were used for siRNA experiments. Data analysis and experimental procedures were similar to those previously described<sup>4,7</sup> and in accordance with the Animals (Scientific Procedures) Act 1986 (UK). Expression of the 2 transgenes (and GABA<sub>A</sub>  $\delta$  subunit) was investigated by PCR

genotyping at postnatal day 21. ASs were detected from the presence of spike-wave discharges (SWDs) on frontoparietal epidural EEG and of behavioral arrest on accompanying video recordings.<sup>4,8</sup> Tonic GABA<sub>A</sub> current was measured in path-clamped TC neurons of the ventrobasal thalamic nucleus in brain slices by adding the GABA<sub>A</sub> blocker gabazine (100  $\mu$ mol/L) in the presence of tetrodotoxin (1  $\mu$ mol/L) and kynurenic acid (3 mmol/L) as previously described.<sup>6</sup>

We found that the STG  $\times$   $\delta$  KO crosses ( $n = 6$ ) averaged 94.3% fewer seizures ( $12.3 \pm 6.1$  compared to  $215 \pm 55.3$  seconds per hour in seizure,  $P < .01$ , Mann-Whitney) than STG ( $n = 7$ ) across 2 hours (Figure 1A,B; Table 1) and that this suppression was accompanied by an absence of tonic GABA<sub>A</sub> current in TC neurons (STG:  $45.8 \pm 5.1$  pA, STG  $\times$   $\delta$  KO:  $2.1 \pm 1.1$  pA,  $P < .0001$ , Student's *t* test; Figure 1C,D). Some parameters of the ataxia phenotype expressed by the STG mice were also affected by the introduction of the  $\delta$  KO. Thus, while the STG  $\times$   $\delta$  cross was similar to wild-type (WT) mice and improved from STG on measures (including turn angle,  $P = .011$ , Mann-Whitney) relating to direction of movement (Figure 1E; Table 1), the effects on other locomotor measurements (including instances of rearing,  $P = .805$ , Mann-Whitney) suggested limited or no recovery (Figure 1F; Table 1). We confirmed the attribution of the ASs suppression to the  $\delta$  subunit KO by microinjection of short interfering RNA, antisense to the subunit, to the ventrobasal thalamic nucleus of 3-month-old STG mice. Mice injected with the antisense siRNA ( $n = 4$ ) had 50.5% fewer seizures than control mice ( $n = 3$ ) injected with missense RNA ( $570 \pm 164$  compared to  $1152 \pm 147$  seconds in seizure over 2 hours,  $P = .011$ , *t* test). This was accompanied by a 71.5% decrease in TC neuron tonic GABA<sub>A</sub> current in antisense ( $n = 10$ ) vs missense-injected ( $n = 7$ ) animals ( $15.8 \pm 2.2$  compared to  $55.7 \pm 16.9$  pA,  $P = .0185$ , *t* test).

Our findings are in general agreement with the hypothesis that thalamic tonic GABA<sub>A</sub> current has a crucial role in the mechanism of ASs in rodents. They also demonstrate the possibility of a “genetic rescue,” or prevention of seizure onset, by the abolition of tonic GABA<sub>A</sub> inhibition in TC neurons throughout development. Consequently, the development of the AS phenotype from the stargazin mutation is entirely dependent upon the availability of the  $\delta$  subunit, despite the direct role of the stargazin protein in VDCC regulation and AMPAR trafficking, 2 systems that may hitherto have been plausibly hypothesized to influence AS expression.<sup>9</sup> By contrast, it is apparent that the ataxia also present in STG mice is not fully attributable to the  $\delta$  subunit and may instead be related to the impairment of cerebellar



**FIGURE 1** Genetic rescue of stargazer absence seizures by thalamic tonic GABA<sub>A</sub> current abolition. A, Sample frontoparietal EEG traces from stargazer (STG) mice and STG mice crossed with  $\delta$  subunit KO (STG  $\times$   $\delta$ ) mice, demonstrating spike-wave discharges (SWDs, pale red areas) in the former and their absence in the latter. B, Total time spent in seizure in each of 2 recording hours for STG (blue,  $n = 7$ ) and STG  $\times$   $\delta$  (red,  $n = 6$ ) mice demonstrating near-complete abolition of ASs in the latter sample group (Mann-Whitney,  $P < .01$ ). In this and similar plots, horizontal lines indicate mean and SEM. C, Sample intracellular current traces from STG, STG  $\times$   $\delta$ , wild-type (WT, violet), and delta subunit knockout ( $\delta$  KO, green), mouse thalamocortical (TC) neurons showing the effect of tonic GABA<sub>A</sub> current block by administration of 100  $\mu$ mol/L gabazine. WT and STG mouse neurons display gabazine-induced outward current, indicating the existence of the tonic GABA<sub>A</sub> current, whereas  $\delta$  KO and STG  $\times$   $\delta$  mouse neurons do not. D, Tonic GABA<sub>A</sub> current amplitude (pA) in STG ( $n = 8$ ), STG  $\times$   $\delta$  ( $n = 9$ ), WT ( $n = 9$ ), and  $\delta$  KO ( $n = 5$ ) TC neurons illustrate elevated current in STG relative to WT and decreased current in both  $\delta$  KO and STG  $\times$   $\delta$  cells (t test,  $P < .0001$ ). E, Mean turn angle through 1-h open field session for STG ( $n = 7$ ), STG  $\times$   $\delta$  ( $n = 7$ ), and WT ( $n = 6$ ) mice, demonstrating larger turns in STG compared to both STG  $\times$   $\delta$  and WT mice (Mann-Whitney,  $P = .0156$ ). F, Total instances of rearing throughout the same 1-h open field session, showing similar counts for STG and STG  $\times$   $\delta$  (Mann-Whitney,  $P = .805$ ), and a decrease in the latter group relative to WT. G, Time spent in seizure during predrug control and postinjection for STG mice with GABA<sub>A</sub>  $\delta$  subunit missense (cyan,  $n = 3$ ) and antisense (yellow,  $n = 4$ ) siRNA oligonucleotides injected into the ventrobasal thalamus, showing decreased time spent in seizure after antisense injection. H, Tonic GABA<sub>A</sub> current in TC neurons from pairs of mice from the same oligonucleotide-injected groups ( $n = 6$  and  $n = 9$  cells), and STG mice ( $n = 6$  cells), demonstrating decreased tonic GABA<sub>A</sub> current in the antisense-injected mice compared to missense-injected or naïve STG

**TABLE 1** Properties of stargazer and stargazer  $\times$   $\Delta$ KO seizures and locomotion

	STG		STG $\times$ $\delta$		WT	
	Mean	SEM	Mean	SEM	Mean	SEM
Seizure						
Time in seizure (s) <sup>a</sup>	215.4	55.3	12.3	6.1		
Number of seizures <sup>a</sup>	82.2	22.5	6.2	3.7		
Seizure duration (s) <sup>a</sup>	8.5	2.3	2.5	1.4		
Movement						
Distance moved (cm)	5496	1084	8058	887	11789	1357.7
Velocity (cm/s)	4.6	0.9	6.7	0.7	9.8	1.1
Time moving (s) <sup>a</sup>	803.6	25.7	1027	19.5	942.8	57.1
Turn angle (°) <sup>a</sup>	84.2	3.9	64.9	4	60	3.7
Angular velocity (°/s) <sup>a</sup>	-106.5	12.3	-56.7	6.2	-47.1	6.1
Meander (°/cm) <sup>a</sup>	-52	4.6	-25.5	2.2	-26.3	4.7
Heading (°)	150.3	33	152.3	36.6	272.7	23.1
Rearing frequency	123.4	36.4	102.4	34.4	223	20.1

Mean and SEM values of seizure (per h) and locomotion properties for STG ( $n = 7$ ) and STG  $\times$   $\delta$  ( $n = 6$ ) mice.

<sup>a</sup>Amelioration of seizure or ataxic characteristic in STG  $\times$   $\delta$  mice ( $P < .05$  in Mann-Whitney test).

AMPA trafficking by the mutation of stargazin. However, our results also do not rule out a role for tonic GABA<sub>A</sub> inhibition in the remaining locomotor phenotype due to the presence, in nonthalamic regions, of eGABA<sub>A</sub>Rs without the  $\delta$  subunit.<sup>10</sup>

It is also noteworthy that, despite the elevated tonic GABA<sub>A</sub> current in STG mice compared to WT, the current is completely abolished with the introduction of the  $\delta$  KO. In other words, the mechanisms, downstream of the stargazin mutation, that increase tonic GABA<sub>A</sub> inhibition in STG mice are completely dependent on the  $\delta$  subunit and do not involve, for example, the insertion of other

subunit configurations of eGABA<sub>A</sub>Rs. This is in stark contrast to the GABA<sub>A</sub> receptor  $\gamma$ 2R43Q mouse model, in which ASs are associated with a complete lack of thalamic tonic GABA<sub>A</sub> current.<sup>11</sup> The discrepancy may suggest that there exist multiple routes, including opposite disruptions of inhibitory balance, that result in ASs. This is supported by the existence of the same mutation in some human subjects with ASs.<sup>12</sup> Moreover, the siRNA experiments demonstrate that the suppression of ASs by interference with the TC tonic current is effective whether delivered throughout development or acutely. Finally, the recent observation of elevated thalamic GABA in the thalamus of a

child with absence epilepsy lends support to the potential translational application of this mechanism of rescue.<sup>13</sup>

In conclusion, this study is the first example of a genetic rescue of an absence seizure phenotype, confirming the centrality of TC neuron tonic GABA<sub>A</sub> current in the mechanisms of rodent ASs, and therefore suggesting that therapeutic interventions for these seizures based on the disruption of this current, specifically via eGABA<sub>A</sub> receptor containing the  $\delta$  subunit, hold particular promise.

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#### REFERENCES

1. Tenney JR, Glauser TA. The current state of absence epilepsy: can we have your attention? *Epilepsy Curr.* 2013;13:135-140.
2. Paz JT, Huguenard JR. Microcircuits and their interactions in epilepsy: is the focus out of focus? *Nat Neurosci.* 2015;18:351-359.
3. McCafferty CP, David F, Venzi M, et al. Cortical drive and thalamic feed-forward inhibition control thalamic output synchrony during absence seizures. *Nat Neurosci.* 2018, in press.
4. Cope DW, Di Giovanni G, Fyson SJ, et al. Enhanced tonic GABA<sub>A</sub> inhibition in typical absence epilepsy. *Nat Med.* 2009;15:1392-1398.
5. Letts VA. Stargazer—a mouse to seize!. *Epilepsy Curr.* 2005;5:161-165.
6. Cope DW, Hughes SW, Crunelli V. GABA<sub>A</sub> receptor-mediated tonic inhibition in thalamic neurons. *J Neurosci.* 2005;25:11553-11563.
7. Noldus LP, Spink AJ, Tegelenbosch RA. EthoVision: a versatile video tracking system for automation of behavioral experiments. *Behav Res Methods Instrum Comput.* 2001;33:398-414.
8. Marescaux C, Vergnes M, Depaulis A. Genetic absence epilepsy in rats from Strasbourg—a review. *J Neural Transm Supplementum.* 1992;35:37-69.
9. Lacey CJ, Bryant A, Brill J, Huguenard JR. Enhanced NMDA receptor-dependent thalamic excitation and network oscillations in stargazer mice. *J Neurosci.* 2012;32:11067-11081.
10. Brickley SG, Mody I. Extrasynaptic GABA(A) receptors: their function in the CNS and implications for disease. *Neuron.* 2012;73:23-34.
11. Mangan KP, Potter WB, Nelson AB, et al. Tonic inhibition is abolished in GABA<sub>A</sub> receptor  $\gamma$ 2R43Q knock-in mice with absence epilepsy and febrile seizures. *BioRxiv* [Epub ahead of print]. 2017.
12. Wallace RH, Marini C, Petrou S, et al. Mutant GABA(A) receptor gamma2-subunit in childhood absence epilepsy and febrile seizures. *Nat Genet.* 2001;28:49-52.
13. Leal A, Vieira JP, Lopes R, et al. Dynamics of epileptic activity in a peculiar case of childhood absence epilepsy and correlation with thalamic levels of GABA. *Epilepsy Behav Case Rep.* 2016;5:57-65.