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Stress, caffeine and ethanol trigger transient neurological dysfunction through shared mechanisms in a mouse calcium channelopathy

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Abstract

Several episodic neurological disorders are caused by ion channel gene mutations. In patients, transient neurological dysfunction is often evoked by stress, caffeine and ethanol, but the mechanisms underlying these triggers are unclear because each has diverse and diffuse effects on the CNS. Attacks of motor dysfunction in the Ca_v2.1 calcium channel mouse mutant *tottering* are also triggered by stress, caffeine and ethanol. Therefore, we used the *tottering* mouse attacks to explore the pathomechanisms of the triggers. Despite the diffuse physiological effects of these triggers, ryanodine receptor blockers prevented attacks induced by all of them. In contrast, compounds that potentiate ryanodine receptors triggered attacks suggesting a convergent biochemical pathway. *Tottering* mouse attacks were both induced and blocked within the cerebellum suggesting that the triggers act locally to instigate attacks. In fact, stress, caffeine and alcohol precipitated attacks in Ca_v2.1 mutant mice in which genetic pathology was limited to cerebellar Purkinje cells, suggesting that the triggers initiate dysfunction within a specific brain region. The surprising biochemical and anatomical specificity of the triggers and the discovery that the triggers operate through shared mechanisms suggests that it is possible to develop targeted therapies aimed at blocking the induction of episodic neurological dysfunction, rather than treating the symptoms once provoked.

Keywords

episodic; channelopathy; cerebellum; Purkinje; calcium channel; *tottering*

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Introduction

Recurrent transient neurological dysfunction superimposed on a relatively normal baseline is observed in several disorders such as familial hemiplegic migraine, episodic ataxia, and periodic paralysis. In each case, transient neurological impairments are superimposed on a relatively normal baseline. Although the clinical features of these disorders are diverse, the underlying causes of the condition and the triggers that bring on attacks are strikingly similar. Many of these disorders are associated with ion channel gene mutations, and are therefore known as channelopathies (Bhatia et al., 2000; Cannon, 2006; Ptacek, 1999). Further, regardless of etiology, attacks are induced in many episodic disorders by psychological or physical stress, and the consumption of caffeine or alcohol (Bhatia, 2001; Frucht et al., 2000; Ptacek, 1999).

The mechanisms underlying the induction of neurological dysfunction in patients who are otherwise normal between attacks are unknown primarily because the triggers have a variety of effects on the nervous system. Stress activates the limbic-hypothalamic-pituitary-adrenal axis, causing the release of several neurochemicals, including corticotrophin releasing factor (CRF), adrenocorticotrophic hormone, corticosteroids and catecholamines (Koob, 1999). Caffeine acts as a stimulant through adenosine receptor antagonism, inhibits phosphodiesterase and mobilizes calcium from intracellular stores (Daly, 2007; Muller and Daly, 1993). Ethanol acts through multiple signaling cascades involving GABA, glycine, adenosine and NMDA receptors (Kumar et al., 2009; Newton and Messing, 2006). Each of these biochemical mechanisms may impinge on multiple brain areas. Despite the diverse effects of these triggers, their ability to provoke symptoms across a broad range of episodic disorders suggests the possibility of a common mechanism.

Because the attacks are observed only in intact animals, the trigger mechanisms must be assessed *in vivo*. Mouse mutants that recapitulate both genotypic and phenotypic characteristics of episodic neurological disorders are therefore essential for this type of analysis. The *tottering* mouse mutant exhibits stereotyped attacks of dystonia (Shirley et al., 2008) that are caused by a mutation in the *Cacna1a* gene (Fletcher et al., 1996). Mutations in this gene in man and mouse are associated with aberrant function of the pore-forming α_1 subunit of $\text{Ca}_v2.1$ (P/Q-type) voltage-gated calcium channels (Jen et al., 2007; Wakamori et al., 1998). In humans, *CACNA1A* mutations are associated with various disorders, including episodic ataxia type 2 (EA2) and familial hemiplegic migraine type 1 (Ophoff et al., 1996). Similar to humans, the attacks in *tottering* mice are triggered by stress, caffeine and alcohol (Fureman et al., 2002). Further, drugs that prevent episodic dysfunction in patients, such as 4-aminopyridine, also block attacks in *tottering* mice (Weisz et al., 2005). Therefore, we used the attacks in *tottering* mice as a readout to explore the pathomechanisms of the triggers.

Materials and Methods

Animals

Mice were bred and housed at Emory University or Johns Hopkins University vivaria. *Tottering* mice (*Cacna1a*^{tg/tg}) congenic with C57BL/6J were originally obtained from The Jackson Laboratory (Bar Harbor, ME). Mice carrying floxed *Cacna1a* alleles (*Cacna1a*^{flox/flox}) were on a C57BL/6J background, as described previously (Todorov et al., 2006). Tg(Pcp2-Cre)2Mpin/J (common name, *L7-Cre*) on C57BL/6J were purchased from The Jackson Laboratory. Mice carrying a null allele (*Cacna1a*^{+/-}) on a mixed C3H-C57BL/6J background were kindly provided by Dr. David Yue (Johns Hopkins University, Baltimore, MD). PCR was used to genotype mice with forward and reverse primers: for the *Cacna1a*⁻ allele, 5'-ATAATAAGTCACCTCTCGTTCTAAAG-3' and 5'-

CTGACTAGGGGAGGAGTAGAAG-3'; for the *Cacna1a*^{fllox} allele, 5'-ACCTACAGTCTGCCAGGAG-3' and 5'-TGAAGCCCAGACATCCTTGG-3'; for the *Cacna1a*^{tg} allele, 5'-GGAAACCAGAAGCTGAACCA-3' and 5'-GAAATGGAGGAATTCAGGG-3'; for *L7-Cre*, 5'-GCGGTCTGGCAGTAAAACTATC-3' and 5'-TCTCTGACCAGAGTCATCCTTAGC-3'. The PCR product for the *Cacna1a*^{tg/tg} allele was sequenced to detect the C1802T mutation.

Male and female mice (8–20 wks of age) were used in all experiments since we have previously shown that *tottering* mouse attacks are comparable across age and between genders (Weisz et al., 2005). Mice had access to food and water *ad libitum* throughout all experiments. All procedures conformed to the NIH Guidelines for the Care and Use of Animals and were approved by the Emory University or Johns Hopkins University Animal Care and Use Committees.

Motor behavior analyses

Mice were transferred from the vivarium to the laboratory at least 4 hours prior to drug challenge or restraint stress drug. For restraint stress, mice were placed in a plastic tube with air holes for 10 min, and then scored for attacks following release. Pretreatment drugs were administered 30 min before challenge with caffeine or ethanol or 10 min before restraint challenge. After drug challenge or restraint stress, mice were observed for 40 min for the presence or absence of an attack of generalized dystonia. Each mouse was scored for 30 sec every 10 min using a disability scoring system previously described (Jinnah et al., 2000), where D0 = normal motor behavior; D1 = slightly slowed or abnormal movements; D2 = fleeting abnormal postures; D3 = moderate impairment, limited ambulation even when disturbed, frequent abnormal postures; D4 = severe impairment, sustained abnormal postures. An attack was defined as a disability score of D ≥ 3. The percent of mice exhibiting an attack during the 40 min following challenge was used as a measure of attack frequency. Observers were blinded to treatment.

To assess the central effects of adenosinergic antagonists, gross locomotor activity was quantified using photocell activity chambers (San Diego Instruments, San Diego, CA). Mice were individually placed into a clean activity chamber and habituated overnight. The following morning mice were treated systemically with drug or vehicle and the total number of beam breaks in the ensuing 40 min period was used for analysis.

To assess the effects of dantrolene on strength and coordination, the cling and pole tests were used. The cling test was performed as previously described (Devanagondi et al., 2007), where mice were habituated for 1 min on a horizontal 20 × 20 cm framed grid of wire mesh suspended above a padded surface. The frame was then rotated 90° to vertical for 1 min, and then rotated another 90° for 1 min so that the mice were inverted. The time to fall was recorded with a 180 sec cut-off. For the pole test, a vertical wooden rod measuring 50 cm in length and 1 cm in diameter affixed to a square base (12 × 12 cm) was used. The base of the pole was placed in the home cage. Mice were placed with the head facing up at the top of the pole. The time for each mouse to turn around, descend the pole and place all four limbs onto the base was recorded. Mice performed 5 trials of training each day for 2 days prior to testing. On the test day, the best time of the 5 trials was used.

Systemic drug administration

All drugs were first tested in normal C57BL/6J mice for deleterious side effects such as sedation or seizures. Each drug was tested on at least 2 separate dates and mice were pseudorandomly assigned to a drug dose or vehicle group. Observers were blinded to treatment. Caffeine, dimethylxanthine (theophylline) and 1,7-dimethylxanthine

(paraxanthine) were obtained from Sigma (St. Louis, MO). ZM241385 and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were obtained from Tocris (Ellisville, MO). Dantrolene was obtained from Proctor & Gamble (Cincinnati, OH). Caffeine, theophylline and paraxanthine were administered subcutaneously (s.c.). All other drugs were administered intraperitoneally (i.p.). Dantrolene was administered 30 min before drug challenge or 10 min before restraint challenge.

Intracerebellar microinjection

Intracerebellar microinjections were performed as previously reported (Pizoli et al., 2002; Weisz et al., 2005). Mice were anesthetized with isoflurane and injected with 0.5 μ l of 100 μ M ryanodine (Tocris) or saline vehicle. For cerebellum, coordinates were: -6.5 mm AP from bregma, 0 mm ML from midline, -2 mm V from skull. For lateral ventricle, coordinates were: -0.5 mm AP, 1.25 mm ML, -3 mm V. The wound was sealed with Nexaband topical skin closure (Veterinary Products Laboratories, Phoenix, AZ). Mice were administered caffeine or ethanol approximately 2 min after microinjection. Restraint began approximately 4 min after microinjection. Microinjection sites were histologically verified.

Caffeine microperfusion

Concentric microdialysis probes were used to reverse dialyze caffeine into select brain regions. Mice were anesthetized with tribromoethanol (Sigma), a small incision made and a hole drilled for implantation of the probe, which was constructed as previously described (Fan and Hess, 2007). For cerebellum the coordinates for probe implantation were the same as for microinjections. For striatum the coordinates were: -0.6 mm AP, 1.7 mm ML, -5.4 mm V. After surgery, the probe was connected to a microinjection pump (Harvard Apparatus, Holliston, MA) through a liquid swivel (Instech, Plymouth Meeting, MA) and perfused continuously with artificial cerebrospinal fluid (147 mM NaCl, 3.5 mM KCl, 1.2 mM CaCl_2 , 1.2 mM MgCl_2 , 1 mM NaH_2PO_4 , 25 mM NaHCO_3 , pH 7.4) at a flow rate of 1.5 μ l/min. Mice were allowed 14–16 hr recovery after surgery and then caffeine was added to the perfusate. Percent recovery of amino-acid neurotransmitters *in vitro* was used to determine the relative exchange rate of each probe prior to use *in vivo*. Perfusion sites were histologically verified.

Histology

To verify injection and infusion sites, sections were stained with cresyl violet. For X-gal staining, tissue was incubated in 0.02% Igpal (Sigma), 0.01% sodium deoxycholate, 2 mM MgCl_2 , 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml X-gal in PBS overnight and counterstained with Nuclear Fast Red (Vector Laboratories, Burlingame, CA).

Statistical analysis

Nonparametric logistic regression and chi-square techniques were used to analyze attack frequency data. Student's *t* test was used for cling and pole test data. Analyses were performed using Statview (SAS Institute, Cary, NC).

Results

Common biochemical mechanisms of stress, caffeine and ethanol

Although each trigger has many effects on the CNS, all increase arousal. The stimulatory effects of caffeine are attributed to its action as a nonselective A1/A2a adenosine receptor (ADR) antagonist (Williams and Jarvis, 1988). Stress and ethanol are also implicated in A1 and A2a ADR signaling (da Silva Torres et al., 2003; Minor et al., 2001; Newton and

Messing, 2006), suggesting a common mechanism of action. To determine if adenosinergic signaling mediates neurological attacks, we challenged *tottering* mice with a battery of nonselective and selective ADR antagonists. Centrally active doses were first determined by assessing drug-induced increases in locomotor activity in normal mice. All compounds increased locomotor activity at the doses tested (Table 1). As previously reported (Fureman et al., 2002), caffeine dose dependently induced attacks (Figure 1A). Similar to caffeine, the mixed A1/A2a ADR antagonists, 1,3-dimethylxanthine (theophylline) and 1,7-dimethylxanthine (paraxanthine) dose-dependently induced attacks in *tottering* mice (Figure 1B & C). To determine if these effects were specific to a particular ADR subtype, *tottering* mice were challenged with DPCPX, an A1-selective ADR antagonist, or ZM 241385, an A2a-selective antagonist. Neither DPCPX (Figure 1D) nor ZM 241385 (Figure 1E) induced attacks at any dose tested, even at doses that significantly increased motor activity (Table 1). Although these results suggest that attacks are not instigated through either adenosine receptor subtype alone, it is possible that coincident antagonism of A1 and A2a ADRs, similar to the action of caffeine, is necessary to trigger attacks. However, co-administration of the A1-selective ADR antagonist DPCPX and the A2a-selective antagonist ZM 241385 failed to trigger attacks (Figure 1F), suggesting adenosinergic signaling is not likely involved in the instigation of attacks by caffeine.

At high concentrations, caffeine acts at ryanodine receptors (RyR) to facilitate the mobilization of calcium from intracellular stores (Kano et al., 1995; Womack et al., 2000). The caffeine analogs theophylline and paraxanthine also promote ryanodine-sensitive increases in the intracellular Ca^{2+} -concentration (Muller and Daly, 1993). Further, *tottering* mice carry a mutation in a calcium channel gene, supporting the idea that calcium-dependent mechanisms may be involved. To test the hypothesis that RyRs mediate attacks, *tottering* mice were treated with systemic injections of the RyR antagonist dantrolene before challenge with caffeine, theophylline or paraxanthine. Pretreatment with dantrolene dose-dependently reduced the frequency of attacks induced by all 3 drugs (Figure 2A–C), suggesting that RyR activation is necessary to induce attacks.

In addition to its action at neuronal RyRs, dantrolene blocks RyRs in skeletal muscle and is used as a muscle relaxant in humans. Because it is possible that dantrolene spuriously inhibited the expression of attacks in *tottering* mice by acting as a muscle relaxant rather than blocking the trigger *per se*, the effect of dantrolene on volitional motor function was examined. Dantrolene did not significantly affect the performance of *tottering* mice on either the cling test or the pole test, which both require strength and motor control (Table 2). These results do not rule out the possibility that dantrolene alters muscle function but do suggest that, at the highest dose used in these experiments, dantrolene did not likely mask *tottering* mouse attacks by weakening muscles.

To determine whether stress triggers attacks through a mechanism similar to caffeine, the ability of dantrolene to block stress-induced attacks was assessed. Pretreatment with dantrolene significantly reduced the frequency of attacks triggered by restraint stress (Figure 2D). Similar experiments using dantrolene to block attacks triggered by ethanol were not feasible due to the sedating effects of this drug combination *in vivo*. Overall, these results suggest that stress and caffeine trigger attacks through similar biochemical mechanisms.

Triggers act in the cerebellum

Localization of (dys)function is critical for understanding pathogenesis; yet stress, caffeine and ethanol act in many brain regions. It is not known whether neurological dysfunction is triggered via diffuse effects, or if attacks are initiated within discrete regions of the brain. We hypothesized that the triggers act locally because the transient motor symptoms arising from mutant $\text{Ca}_v2.1$ channels in humans and mice are associated with cerebellar

dysfunction (Jen et al., 2007; Walter et al., 2006). Therefore, ryanodine, a RyR antagonist, was microinjected into *tottering* mouse brain prior to systemic administration of caffeine. Intracerebellar ryanodine injections significantly reduced the frequency of attacks triggered by caffeine compared to intracerebellar injection of saline vehicle (Fig 3A; $p < 0.05$). However, microinjection of ryanodine into the lateral ventricle had no effect on the frequency of attacks triggered by caffeine whereby four out of five *tottering* mice treated with vehicle exhibited attacks and four out of six *tottering* mice treated with ryanodine exhibited attacks ($p = 0.62$; chi-square test). These results suggest that ryanodine injected into the cerebellum did not diffuse to other brain regions to block attacks.

To determine if the cerebellum is also the site of action for other triggers, we tested the ability of intracerebellar ryanodine injections to block attacks induced by stress or ethanol. Similar to caffeine, intracerebellar injections of ryanodine, but not vehicle, significantly reduced the frequency of attacks induced by stress or ethanol (Figure 3A; $p < 0.01$; chi-square test) suggesting that all three triggers work through the cerebellum.

To determine if attacks are triggered within the cerebellum, we performed localized microperfusions of caffeine in freely moving *tottering* mice (Figure 4A). Microperfusion of caffeine into the cerebellum dose-dependently triggered attacks in *tottering* mice with an average latency of 31 ± 4 min after the start of the infusion, whereas vehicle alone had no effect (Figure 4B). Microperfusion of caffeine into the normal mouse cerebellum did not cause attacks ($n = 4$; not shown). Microperfusion of caffeine into the *tottering* mouse striatum did not induce attacks, even after 80 min (Figure 4B) suggesting that the effects of microperfusion are specific to the cerebellum and that microperfused caffeine was not diffusing throughout brain. Furthermore, pretreatment of *tottering* mice with systemic administration of dantrolene completely blocked attacks triggered by microperfusion of caffeine into the cerebellum (Figure 4C; $p < 0.01$). Thus, attacks were triggered and blocked within the cerebellum.

Attacks are mediated by Purkinje cells

Although stress, caffeine and ethanol act as nonselective agents throughout the CNS, the previous results suggest that attacks are triggered locally within the cerebellum. In light of this localized effect, we asked if a single cell type might mediate the response. Purkinje cells provide the sole output of cerebellar cortex and their firing patterns are exquisitely sensitive to changes in $\text{Ca}_v2.1$ currents (Hoebeek et al., 2005; Walter et al., 2006). Therefore, we tested the hypothesis that Purkinje cells mediate the response to triggers by using the conditional floxed *Cacna1a* allele in combination with the *tottering* allele to achieve cell-specific expression (Table 3). Heterozygous *tottering* mice (*Cacna1a*^{tg/+}; $n = 14$) did not exhibit attacks (Campbell and Hess, 1998). Likewise, *Cacna1a*^{tg/flox} mice, which bear one *tottering* allele and one normoactive floxed *Cacna1a* allele (Todorov et al., 2006), did not exhibit attacks ($n = 8$). To isolate the attack-causing genotype to Purkinje cells, the *L7-Cre* transgene, which drives Cre recombinase expression in Purkinje cells (Figure 5), was bred into *Cacna1a*^{tg/flox} mice. These mice carried one normoactive allele and one *tottering* allele everywhere except Purkinje cells, where the *tottering* allele and a null allele were present (*tottering*^{PC-haplo} mice). *Tottering* mice carrying the *L7-Cre* transgene (*tottering*^{PC-Cre} mice) were produced to control for possible effects of Cre recombinase expression in Purkinje cells on the response to the triggers. Mice bearing one *tottering* and one null allele throughout the nervous system (*tottering*^{haplo} mice) were also generated to control for *Cacna1a* gene dose and to determine if other cell types contribute to the response to triggers.

The response to stress in *tottering* mice and *tottering*^{PC-Cre} mice was similar (Figure 6A). Likewise, the response to caffeine in *tottering* mice ($\text{ED}_{50} = 3.19$ mg/kg) and *tottering*^{PC-Cre} mice ($\text{ED}_{50} = 2.96$ mg/kg) was comparable (Figure 6B) and the response to ethanol in

tottering mice ($ED_{50} = 0.69$ g/kg) did not differ from *tottering*^{PC-Cre} mice ($ED_{50} = 0.85$ g/kg; Figure 6). These results demonstrate that the response to triggers is not modified by Purkinje cell expression of Cre recombinase *per se*. All three triggers also induced attacks in *tottering*^{haplo} mice. However, *tottering*^{haplo} mice were more sensitive to the triggers compared to *tottering* mice. Even minor stressors triggered attacks more frequently in *tottering*^{haplo} than in *tottering* mice (Figure 6A). The dose response curves for both caffeine and ethanol ($ED_{50} = 0.10$ g/kg) were shifted to the left for *tottering*^{haplo} mice compared to *tottering* mice (Figure 6B & C), although it was not possible to calculate the ED_{50} for caffeine in *tottering*^{haplo} due to the high vehicle baseline. These results suggest that there is an effect of gene dose whereby mice that are haploinsufficient for the *tottering* allele are more sensitive to the triggers than mice that are homozygous for the *tottering* allele. Stress, caffeine and ethanol also induced attacks in *tottering*^{PC-haplo} mice. These mice were also more sensitive to stressors than *tottering* mice (Figure 6A). The ED_{50} for caffeine was shifted ~5-fold to the left in *tottering*^{PC-haplo} mice ($ED_{50} = 0.62$ mg/kg) compared to *tottering* mice. A similar 5-fold shift in the dose-response for ethanol was observed in *tottering*^{PC-haplo} mice ($ED_{50} = 0.14$ g/kg) compared to *tottering* mice. Overall, mice with the attack-causing genotype in only Purkinje cells had preserved and even exaggerated responses to triggers.

To determine if the triggers of attacks in *tottering*^{PC-haplo} mice also depend on RyR activity within the cerebellum, localized microinjections of ryanodine were conducted. Similar to *tottering* mice, intracerebellar injections of ryanodine significantly reduced the frequency of attacks induced by stress, caffeine or ethanol (Figure 3B; $p < 0.01$, $p < 0.05$ and $p < 0.01$; as determined by chi-square test) indicating that the mechanisms for attacks in *tottering*^{PC-haplo} mice are similar to those of *tottering* mice.

Discussion

Here, we described the biochemical and anatomical targets of stress, caffeine and ethanol in the induction of attacks of neurological dysfunction due to a calcium channelopathy. At the biochemical level, we found that compounds that potentiate RyR activity, including caffeine, theophylline and paraxanthine, triggered attacks. Conversely, dantrolene and ryanodine, which are structurally unrelated RyR antagonists, prevented attacks induced by stress, caffeine or ethanol. Taken together, these results suggest that the triggers act through a common biochemical pathway that includes RyR signaling. At the anatomical level, we found that the attacks are triggered locally. Intracerebellar delivery of ryanodine blocked attacks and intracerebellar infusions of caffeine induced attacks. Further, the results of the conditional genetic manipulations implicate a single cell type – Purkinje cells. Thus, the seemingly disparate triggers share biochemical and anatomical substrates. These results suggest that despite the diverse and relatively diffuse actions of the triggers throughout the CNS, the substrates of attack induction are surprisingly well circumscribed.

RyR signaling was necessary for the induction of attacks by stress, caffeine and ethanol. RyRs mediate the release of intracellular stores of calcium from the endoplasmic reticulum in response to changes in the cytosolic calcium concentration. Acute changes in cytosolic calcium concentrations can have an immediate impact on excitability through the regulation of transmitter release and by mediating the activity of other ion channels and receptors. While it is possible that stress, caffeine and ethanol exert their effects directly at RyR, it is more likely that each trigger disrupts calcium homeostasis through unique mechanisms and the requirement for RyR activity is downstream of the primary effects. Stress induces the release of CRF, which evokes ryanodine-sensitive Ca^{2+} -release from internal Ca^{2+} -stores (Riegel and Williams, 2008; Yamamori et al., 2004). Stress also stimulates noradrenergic transmission to promote calcium release from intracellular stores through α_1 -adrenergic

receptors (Kirischuk et al., 1996; Paladini and Williams, 2004). In fact, α -adrenergic receptor signaling is required for stress-induced attacks in *tottering* mice (Fureman and Hess, 2005). At millimolar concentrations *in vitro*, caffeine induces rapid and sustained increases in cytoplasmic calcium concentrations through RyR activation (Kano et al., 1995; Womack et al., 2000). However, only micromolar concentrations of caffeine are typically achieved *in vivo*. Low caffeine concentrations evoke an increase in the rate of calcium sparks (Kong et al., 2008; Usachev and Thayer, 1997), which are short-lived local increases in intracellular calcium concentrations (Cheng and Lederer, 2008). It is possible that caffeine triggers attacks by promoting calcium sparks. It is also possible that the other diverse actions of caffeine, such as phosphodiesterase inhibition, contribute, although we have ruled out adenosinergic signaling. Ethanol indirectly mediates the disposition of calcium through the regulation of numerous calcium-permeable channels (Crews et al., 1996; Haorah et al., 2007). Thus, RyR blockade may preclude attacks by damping abnormal fluctuations in cell calcium caused by the triggers without direct interaction of the triggers at RyRs. Although additional experimentation is needed to describe the precise role of RyR receptors in the response to triggers, the experiments presented here provide a clear direction for examining shared biochemical features, despite the complex physiological effects of stress, caffeine and alcohol.

Several different lines of evidence converge to suggest that the triggers act specifically within the cerebellum but not other brain regions. Infusion of caffeine directly to the cerebellum, but not the striatum, triggered attacks. Attacks induced by stress, caffeine and ethanol were blocked by intracerebellar injection of ryanodine, but not by microinjection of ryanodine into the lateral ventricle. Finally, attacks persisted even after isolating the attack-causing genotype to Purkinje cells in *tottering*^{PC-haplo} mice suggesting that the abnormal response to the triggers originates specifically within these neurons. However, it is possible that the triggers instigate abnormal presynaptic signals that then provoke abnormal Purkinje cell responses, but not likely because the abnormal activity in *tottering* mouse Purkinje cells is intrinsically generated and independent of synaptic input (Chen et al., 2009; Walter et al., 2006). This finding does not rule out the possibility that other cells contribute. Although restricting the attack-causing genotype to Purkinje cells in *tottering*^{PC-haplo} mice caused an increase in sensitivity to stress, caffeine and ethanol compared to *tottering* mice, *tottering*^{haplo} mice also exhibited a similar increase in sensitivity. Indeed, *tottering*^{haplo} mice appeared even more sensitive to stress than *tottering*^{PC-haplo} and a similar trend for an increase in sensitivity to caffeine and ethanol is also evident for *tottering*^{haplo} mice. The exaggerated response in both *tottering*^{PC-haplo} and *tottering*^{haplo} mice is likely caused by haploinsufficiency of the *Cacna1a* gene in Purkinje cells. The further augmentation of the response in *tottering*^{haplo} mice suggests that other cells may also contribute but abnormal Purkinje cells alone are sufficient for the response to stress, caffeine and ethanol.

The abundant expression of Cav2.1 channels in Purkinje cells may, in part, explain the selective vulnerability of these neurons (Volsen et al., 1995; Westenbroek et al., 1995). The intrinsic pacemaking function of Purkinje cells is mediated by calcium-dependent small-conductance type (SK) potassium channels, which are exclusively activated by calcium entry through Cav2.1 channels (Womack and Khodakhah, 2004). Loss of precision in Purkinje cell firing patterns has been detected in *tottering* mice presumably due to inefficient SK channel activation (Hoebeek et al., 2005; Walter et al., 2006). Furthermore, potassium channel activators correct the irregular Purkinje cell firing patterns in mice (Alvina and Khodakhah, 2010) and ameliorate attacks of cerebellar dysfunction in both EA2 patients and *tottering* mice (Strupp et al., 2004; Weisz et al., 2005). The effects of the persistent subclinical abnormalities in Purkinje cell calcium handling caused by the calcium channel mutation plus the typically inconsequential effects of the triggers combine to precipitate overt behavioral dysfunction in the form of an attack. Thus, rather than indiscriminately

acting throughout the CNS, the triggers likely exploit cell-type specific differences in gene expression and electrophysiological properties.

Understanding the mechanisms underlying the induction of transient neurological dysfunction is essential for the development of therapies that eliminate or reduce the incidence of attacks. Though dantrolene is most commonly used clinically to treat spasticity, the drug's ability to stabilize abnormal neuronal calcium handling (Chen et al., 2008; Liu et al., 2009; Nagatomo et al., 2001) and to prevent attacks in *tottering* mice and seizures in other mice (Nagatomo et al., 2001; Swiader et al., 2002) suggests that it could be used similarly to block episodic neuronal dysfunction in patients. Indeed, compounds that regulate calcium signaling, particularly verapamil which is an L-type calcium channel blocker, have been used as treatments for hemiplegic migraine, severe myoclonic epilepsy of infancy and paroxysmal dyskinesia (Hsu et al., 2008; Iannetti et al., 2009; Links et al., 1998; Szczaluba et al., 2009). Despite the global CNS effects of the triggers, the surprising biochemical and anatomical specificity of the triggers suggests that it is possible to develop targeted therapies aimed at blocking the induction of episodic neurological dysfunction, perhaps by damping or stabilizing calcium signaling in target neurons, rather than treating the symptoms after they arise.

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Highlights

- RyR signaling is necessary for the induction of attacks by stress, caffeine and ethanol in *tottering* mice
- stress, caffeine and ethanol act specifically within the cerebellum but not other brain regions, to trigger attacks in *tottering* mice
- Abnormal Purkinje cells mediate the response to stress, caffeine and ethanol to instigate neurological dysfunction in *tottering* mice

Figure 1. Response to adenosine receptor antagonists

A–C. Systemic injections of the nonselective adenosine receptor antagonists caffeine, theophylline or paraxanthine dose-dependently induced attacks in *tottering* mice ($n = 10$ – 24 /dose). **D–F.** Systemic injections of the A1 adenosine receptor-selective antagonist DPCPX or the A2a adenosine receptor antagonist ZM 241385 did not induce attacks when administered alone or in combination ($n = 10$ – 16 /dose). ** $p < 0.01$, *** $p < 0.001$ as determined by logistic regression analysis.

Figure 2. Effect of dantrolene on caffeine- and stress-induced attacks

A–C. Systemic administration of the RyR antagonist dantrolene dose-dependently reduced the frequency of attacks in *tottering* mice triggered by 5 mg/kg caffeine, theophylline or paraxanthine (n = 10–17/dose). **D.** Dantrolene dose-dependently blocked attacks triggered by restraint stress (n = 11–12/dose). *p < 0.05, **p < 0.01, ***p < 0.001 as determined by logistic regression analysis.

Figure 3. Effect of intracerebellar ryanodine microinjections on *tottering* mouse attacks
Mice were pretreated with intracerebellar microinjections of ryanodine (100 μM) or vehicle prior to restraint stress or systemic injections of caffeine (5 mg/kg) or ethanol (1 g/kg). **A.** Ryanodine significantly reduced the frequency of attacks triggered by stress, caffeine or ethanol in *tottering* mice (n = 5–12/dose). **B.** Ryanodine significantly reduced the frequency of attacks triggered by stress, caffeine or ethanol in *tottering^{PC-Haplo}* mice (n = 5–7/dose), which carrying an attack-causing genotype in only Purkinje cells. *p < 0.05, **p < 0.01 as determined by chi-square test.

Figure 4. Effect of intracerebellar caffeine infusion on *tottering* mouse attacks

Vehicle or caffeine was reverse dialyzed into cerebellum or striatum of awake-behaving mice. **A.** Typical location of a cerebellar microperfusion probe. Scale bar, 500 μ m. **B.** Intracerebellar infusions of caffeine dose-dependently induced attacks in *tottering* mice ($n = 7-9/\text{dose}$). $*p < 0.05$, $***p < 0.001$ as determined by logistic regression analysis. **C.** Microperfusions of 2.5 mM caffeine into the *tottering* mouse striatum ($n = 6$) did not elicit any motor abnormalities. **C.** Systemic administration of 20 mg/kg dantrolene (+Dan), but not vehicle (+Veh), blocked attacks induced by intracerebellar microperfusions of caffeine ($n = 4-5/\text{dose}$). $*p < 0.05$, $**p < 0.01$ as determined by chi-square test.

Figure 5. X-gal staining as a reporter for Cre recombinase expression in mice carrying the L7-Cre transgene

The L7-Cre transgene was bred onto the Cre-reporter mouse line, Rosa26, which is permissive for β -galactosidase expression in the presence of Cre recombinase. **A–D.** X-gal staining was localized to Purkinje cells. Scale bars, 100 μ m

Figure 6. Attacks are mediated by Purkinje cells

The L7-Cre transgene, which expresses Cre recombinase only in Purkinje cells was bred into mice bearing one *tottering* allele and one floxed allele (*Cacna1a^{tg/flox}*) to generate *tottering^{PC-haplo}* mice, which carry an attack-causing genotype only in Purkinje cells. Mice bearing one *tottering* allele and one null allele (*tottering^{haplo}*) and *tottering* mice carrying the L7-Cre transgene (*tottering^{PC-Cre}*) were also tested as controls. **A.** Environmental challenges. Restraint induced attacks in all genotypes. *Tottering^{PC-haplo}* mice (n = 11–12/challenge) and *tottering^{haplo}* mice (n = 14/challenge) were more sensitive to the other environmental challenges than *tottering* mice (n = 11/challenge) and *tottering^{PC-Cre}* mice (n = 7/challenge). **B.** Caffeine dose-dependently induced attacks in all genotypes. *Tottering^{PC-haplo}* mice (n = 11–12 per dose) and *tottering^{haplo}* mice (n = 13–14/dose) were more sensitive to the effects of caffeine than *tottering* mice (n = 10–11/dose) and *tottering^{PC-Cre}* mice (n = 7/dose). **C.** Ethanol dose-dependently induced attacks in all genotypes. *Tottering^{PC-haplo}* mice (n = 10–16/dose) and *tottering^{haplo}* mice (n = 12–14/dose) were more sensitive to the effects of ethanol than *tottering* mice (n = 9–11/dose) and *tottering^{PC-Cre}* mice (n = 7/dose). *p < 0.05, **p < 0.01, ***p < 0.001 as determined by logistic regression analysis.

Table 1

Effect of adenosinergic antagonists (5 mg/kg) on the locomotor activity of normal mice

Drug (ADR subtype)	Beam breaks (% vehicle)
Caffeine (mixed)	281 ± 33 ***
Theophylline (mixed)	223 ± 42 *
Paraxanthine (mixed)	346 ± 72 **
DPCPX (A1)	234 ± 54 *
ZM 241385 (A2a)	281 ± 36 ***
DPCPX + ZM 241385 (A1+A2a)	369 ± 63 **

Data are expressed as mean ± SEM and were analyzed with Student's *t* test (n = 6–11/drug).

*
p < 0.05,

**
p < 0.005,

p < 0.001 compared to vehicle treatment.

Table 2

Effect of dantrolene (20 mg/kg) on the motor performance of *tottering* mice

	Time after injection	
	30 min	60 min
Pole Test (sec)		
Vehicle	17 ± 2	13 ± 1
Dantrolene	14 ± 2	15 ± 1
Cling Test (sec)		
Vehicle	150 ± 6	142 ± 2
Dantrolene	156 ± 7	144 ± 6

Data are expressed as mean ± SEM and were analyzed with Student's *t* test (n = 9–10/group). There were no significant differences between vehicle and treated mice..

Table 3

Nomenclature of experimental and control mice

Common Name	Genotype	Description	Phenotype
Normal	<i>Cacna1a</i> ^{+/+}	Normal C57BL/6J mice	Normal
<i>Tottering</i>	<i>Cacna1a</i> ^{tg/tg}	Two <i>tottering</i> alleles	Attacks
Heterozygous <i>tottering</i>	<i>Cacna1a</i> ^{+/tg}	One <i>tottering</i> and one normal allele	Normal
Heterozygous <i>tottering</i> -floxed	<i>Cacna1a</i> ^{tg/flox}	One <i>tottering</i> and one normal floxed allele	Normal
<i>Tottering</i> ^{PC-Cre}	<i>Cacna1a</i> ^{tg/tg} ; <i>L7-Cre</i> ^{+/-}	<i>Tottering</i> mice expressing Cre recombinase in Purkinje cells	Attacks
<i>Tottering</i> ^{haplo}	<i>Cacna1a</i> ^{tg/-}	One <i>tottering</i> and one null allele throughout	Attacks
<i>Tottering</i> ^{PC-haplo}	<i>Cacna1a</i> ^{tg/flox} ; <i>L7-Cre</i> ^{+/-}	One <i>tottering</i> and one null allele in Purkinje cells but otherwise heterozygous throughout	Attacks