GENETICS AND CELL BIOLOGY

Ethanol Increases GABAergic Transmission and Excitability in Cerebellar Molecular Layer Interneurons from GAD67-GFP Knock-in Mice

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INTRODUCTION

Acute and chronic ethanol exposure impair the function of the cerebellum, a brain region that controls motor coordination, balance, muscle tone, motor learning and cognition (Botta et al., 2007; Fitzpatrick et al., 2008; Jaatinen and Rintala, 2008; Valenzuela et al., 2010). These functions are mediated by neurons located in the cerebellar cortex. Purkinje cells (PCs), the sole output of the cerebellar cortex, are regulated by rhythmically firing GABAergic interneurons located in the molecular layer (i.e., molecular layer interneurons; MLIs). MLIs can be divided into basket cells (located in the inner third of the molecular layer) and stellate cells (located in the outer two-thirds of the molecular layer) (Fig. 1). Basket cell axons encircle the soma of a PC forming a net-like structure containing axo-axonal paintbrush-like (pinceau) synapses located at the axonal initial segment, a critical site for action potential generation. It has been estimated that a basket cell axon diverges onto ~30 PCs (Barlow, 2002; Ito, 2006). Stellate cells are star-shaped neurons, each making diverging synapses with dendrites of ~10–17 PCs (Barlow, 2002; Briatore et al., 2010). Basket cells are thought to predominantly regulate the spike output of PCs, whereas stellate cells modulate excitatory synaptic inputs to PCs, limiting membrane potential depolarization and voltage-gated Ca2+ channel activation in dendrites (Bao et al., 2010). Collectively, the functions of MLIs include feed-forward and lateral inhibition of PCs (Ito, 2006; Bao et al., 2010), as well as regulation of spontaneous firing of PCs (Haussler and Clark, 1997). Inhibition of PCs by MLIs is required for consolidation of eyeblink conditioning and vestibulocerebellar motor learning (Scelfo et al., 2008; Wulff et al., 2009).

A number of studies have demonstrated that acute ethanol exposure increases GABAergic transmission at MLI–PC synapses. Acute ethanol exposure increased the frequency of spontaneous action potential-dependent inhibitory postsynaptic currents (sIPSCs) in cerebellar slices from mice and rats, an effect that is mediated by an increase in firing of MLIs (Mameli et al., 2008; Hirono et al., 2009). In the presence of tetrodotoxin to block spontaneous firing of MLIs, ethanol also increased the frequency, but not the amplitude, of GABA_A receptor-mediated miniature inhibitory postsynaptic currents (mIPSCs) in PCs (Ming et al., 2006; Kelm et al., 2007; Mameli et al., 2008; Hirono et al., 2009). These findings are consistent with an ethanol-induced increase in quantal GABA release at MLI–PC synapses. Studies from our laboratory showed that ethanol significantly increases the amplitude and decreases the paired-pulse ratio of PC IPSCs evoked by stimulation of stellate cells, but not basket cells (Mameli et al., 2008). These findings suggest that acute ethanol exposure differentially increases evoked GABA release at stellate cell- vs. basket cell-to-PC synapses. Moreover, we showed that the ethanol-induced increase of GABAergic transmission at MLI–PC synapses leads to a decrease in glutamatergic transmission at granule cell-PC synapses (Mameli et al., 2008).

In addition to synapsing with PCs, MLIs mutually connect via chemical and electrical synapses, forming a synchronized neuronal network (Sotelo and Llinas, 1972; Kondo and Marty, 1998; Mann-Metzer and Yarom, 2002). MLI-to-MLI synapses are abundant, making up at least a third of all the inhibitory synapses in the molecular layer (Briatore et al., 2010). Feed-forward inhibition, mediated by these synapses, truncates excitatory postsynaptic potentials in MLIs, increasing temporal precision in PCs (Jorntell et al., 2010). Computer modeling and electrophysiological studies suggest that these reciprocal inhibitory connections also contribute to the generation of fast oscillatory activity in the cerebellar cortex (Maex and De Schutter, 2005; Middleton et al., 2008; Jorntell et al., 2010). Although the precise function of these
Subjects were 22–40-day-old male (±) glutamic acid-decarboxylase 67-green fluorescent protein (GAD67-GFP) knock-in mice, which were generously provided by Dr. Y. Yanagawa (Gunma University School of Medicine, Japan) and bred in our animal facility; the generation of these mice has been described elsewhere (Tamamaki et al., 2003). The animals had ad libitum access to water and food (Teklad Rodent Diet, Harlan Laboratories, Indianapolis, IN, USA). These animals express GFP in GABAergic neurons, facilitating the identification of basket and stellate cells. Previous studies showed that GABA brain levels are not significantly different between wild-type and GAD67-GFP knock-in mice at 7 weeks of age, indicating that GAD65 is able to compensate for the decrease in GAD67 activity (Tamamaki et al., 2003).

Immunohistochemical studies

Mice were euthanized by decapitation under deep anesthesia with ketamine (250 mg/kg). Cerebella were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24–48 h at 4°C followed by cryoprotection in 30% sucrose in PBS for an additional 24 h at 4°C. Cerebella were embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA) and flash-frozen by submersion in isopentane cooled in a dry ice/methanol bath. Cerebella were then cryo-sectioned (16 µm) in the sagittal plane and placed in a freezer cooled in a dry ice/methanol bath. Cerebella were embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA) and flash-frozen by submersion in isopentane cooled in a dry ice/methanol bath. Cerebella were embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA) and flash-frozen by submersion in isopentane cooled in a dry ice/methanol bath. Cerebella were embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA) and flash-frozen by submersion in isopentane cooled in a dry ice/methanol bath.

Electrophysiological studies

Electrophysiological recordings were performed using parasagittal cerebellar vermis slices (200 µm) that were prepared as described previously (Botta et al., 2010) with the exception that the artificial cerebrospinal fluid (ACSF) contained 400 µM ascorbic acid to decrease edema (Rice, 1999). Slices were placed in a recording chamber constantly perfused with ACSF equilibrated with 95% O2/5% CO2 and maintained at 32°C using an in-line heater and a heated recording platform (Warner Instruments, Hamden, CT, USA). The flow rate was 2 ml/min. Basket and stellate cells were identified using an Olympus BX51WI microscope equipped with epifluorescence illumination. Basket and stellate cells were defined as GFP-positive cells located in the distal and proximal thirds of the molecular layer, respectively (Fig. 1). Cells located in the middle third of the molecular layer were excluded from the study. All recordings were performed with Axopatch 200B or Multiclamp 700B amplifiers using Clampex 9.2 software (Molecular Devices, Sunnyvale, CA, USA). For whole-cell recordings, pipettes (resistance = 5–7 MΩ) were filled with an internal solution containing (in mM): 110 CsCl, 30 K-glucuronate, 1.1 EGTA, 10 HEPES, 0.1 CaCl2, 4 Mg2-ATP and 0.3 Na-GTP (pH adjusted to 7.3 with CsOH). For recordings of sIPSCs and mIPSCs, the membrane...
potential was held at −70 mV and the ACSF contained 3 mM kynurenic acid (ionotropic glutamate receptor blocker). Miniature IPSCs were recorded under the same conditions with the addition of 1 μM tetrodotoxin. Under these conditions, sIPSCs and mIPSCs were abolished by 10 μM SR95531, confirming that these were mediated by GABA_A receptors (not shown).

The loose-seal cell-attached configuration (seal resistance = 20–100 MΩ) was used to record action currents, as described previously (Botta et al., 2010). This configuration is optimal for long-lasting recordings of spiking activity as it minimally disturbs the intracellular milieu. Unless indicated, these recordings were performed in the presence of a synaptic receptor blocker cocktail containing: 10 μM LY341495 (group II and III mGlur receptor blocker), 100 μM LY367385 (group I mGlur receptor blocker), 10 μM CGP54626 (GABA_A receptor blocker), 10 μM SR95531 (GABA_A receptor blocker), 50 μM DL-AP5 (NMDA receptor blocker) and 1 mM kynurenic acid. For the experiments involving ethanol application, each slice was only exposed once to ethanol to prevent the development of rapid tolerance.

Data analysis

Electrophysiology recordings were filtered at 2 kHz and digitized at 5–50 kHz. Data were analyzed with Mini Analysis-6.0.3 (Synaptosoft, Decatur, GA, USA). Changes in IPSC frequency were quantified by measuring changes in inter-event interval. The effects of ethanol with respect to baseline in individual recordings were analyzed using the Kolmogorov–Smirnov (K–S) test using a conservative value for significance of P < 0.01. Statistical analyses of pooled data were performed with GraphPad Prizm 4 (GraphPad Software, San Diego, CA, USA). Data were initially analyzed with the K–S, D’Agostino or Pearson omnibus normality tests. If data followed a normal distribution, these were analyzed using parametric tests. If this was not the case, then non-parametric tests were used. For sample time courses of ethanol’s effect on individual cells, data were normalized to the first minute of recording and expressed as percent of this baseline. When indicated, the effect of ethanol was calculated with respect to the average of control and washout responses. Statistical comparison of baseline, ethanol and washout was performed using repeated measures one-way ANOVA followed by Tukey’s posthoc test. The level of significance was P < 0.05 for all analyses of pooled data.

RESULTS

We recorded sIPSCs, mIPSCs and spontaneous action potential firing from basket and stellate cells using the whole-cell patch-clamp and loose-seal cell-attached configurations. The basal characteristics of these events are summarized in Table 1. These basal parameters were not significantly different between basket and stellate cells (P > 0.05 by unpaired t-test).

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<th>Table 1. Summary of basal electrophysiological parameters of molecular layer interneurons</th>
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Values represent mean ± standard error of the mean. Of the number of cells recorded is given in parentheses. Frequency and amplitude of sIPSCs and mIPSCs were sequentially measured in individual cells (i.e. in the absence and in the presence of tetrodotoxin).

Acute exposure to ethanol increases sIPSC and mIPSC frequency in stellate cells

In stellate cells, acute application of 80 mM ethanol also reversibly increased sIPSC frequency (Figs 2A, D and E; 4) but not amplitude (6.57 ± 6.79% change from average of baseline and washout; n = 6; P > 0.05 by one-sample t-test vs. zero; not shown). Figure 2B shows average trace recordings illustrating the lack of effect of ethanol on amplitude. Analysis of cumulative probability plots (Fig. 2C) with the K–S test revealed that 80 mM ethanol significantly (P < 0.01) decreased the inter-event interval in six out of six basket cells with respect to baseline. The amplitude was increased in one, decreased in two and unchanged in three out of six basket cells.

Recordings of sIPSCs include events driven by spontaneous action potential-dependent GABA release, as well as spike-independent events mediated by spontaneous fusion of GABA-containing vesicles with the plasma membrane in axonal terminals. The spike-independent events are known as mIPSCs. We measured the effect of ethanol in the presence of 1 μM tetrodotoxin to isolate mIPSCs. Tetrodotoxin did not significantly decrease either the sIPSC frequency (−15.9 ± 6.6% change from control) or amplitude (−9.0 ± 3.8% change from control) in basket cells (n = 6; Table 1; P > 0.05 by one sample t-test vs. zero; also compare baseline sIPSC and mIPSC frequencies in Fig. 2E). Acute application of 80 mM ethanol reversibly increased mIPSC frequency (Figs 2D–E and 4) but not amplitude (0.19 ± 1.49% change from average of baseline and washout; n = 6; P > 0.05 by one-sample t-test vs. zero; not shown). Analysis of cumulative probability plots (not shown) with the K–S test revealed that 80 mM ethanol significantly (P < 0.01) decreased the inter-event interval in three out of six basket cells with respect to baseline. The amplitude was unchanged in six out of six basket cells.
the frequency (−45.8 ± 4.9% change from control, P < 0.001 by one sample t-test vs. zero) and amplitude (−36.4 ± 10% change from control, P < 0.02 by one sample t-test vs. zero) of sIPSCs in stellate cells (n = 6; Table 1; also compare baseline sIPSC and mIPSC frequencies in Fig. 3E). Acute application of 80 mM ethanol significantly increased mIPSC frequency (Figs 3D–E and 4) but not amplitude (−3.11 ± 1.93% change from average of baseline and washout; n = 6; P > 0.05 by one-sample t-test vs. zero; not shown) in stellate cells. Repeated measures one-way ANOVA indicates that this effect was not fully reversible upon ethanol washout. Analysis of cumulative probability plots (not shown) with the K–S test revealed that 80 mM ethanol significantly (P < 0.01) decreased the inter-event interval in six out of six stellate cells with respect to baseline. The amplitude was increased in two, decreased in one and unchanged in three out of six stellate cells.

Comparison of the effect of ethanol on GABAergic transmission in basket vs. stellate cells

Figure 4 shows a comparison of the effect of 80 mM ethanol on sIPSC and mIPSC frequency in basket vs. stellate cells. In an attempt to correct for current run-down or run-up, these effects were calculated as the ethanol-induced percent change in frequency with respect to the average of baseline and washout responses. Baseline was defined as the averaged normalized frequency at t = 3–5 min, ethanol exposure as the averaged normalized frequency at t = 8–10 min and washout as the averaged normalized frequency at t = 18–20 min (D in Figs 2 and 3). In basket cells, there was no significant difference in the effect of ethanol on sIPSC vs. mIPSC frequency (Fig. 4). Tetrodotoxin did not significantly affect sIPSC frequency or amplitude in these cells (compare baseline sIPSC and mIPSC frequencies in Fig. 2E and see Table 1), suggesting that these events are predominantly action potential independent. In stellate cells, however, the effect of ethanol on sIPSC frequency was significantly greater than on mIPSC frequency (P < 0.01 by two-way ANOVA and Bonferroni’s posthoc test). These results are consistent with the finding that tetrodotoxin has a significant effect on sIPSC frequency in stellate cells (compare baseline sIPSC and mIPSC frequencies in Fig. 3E and see Table 1), suggesting that these cells receive a mixture of action potential-dependent and independent events under our experimental conditions.

Ethanol increases spontaneous action potential frequency in MLIs

The increase in sIPSC frequency produced by ethanol in stellate cells could be a consequence of increased spontaneous firing of neighboring MLIs. To investigate the effect
of ethanol without the influence of synaptic inputs, we performed these studies in the presence of a cocktail of neurotransmitter receptor blockers. Basal spontaneous action potential firing frequency significantly increased in both basket and stellate cells in the presence of this cocktail (Table 1). Acute exposure to 80 mM ethanol had a variable, but significant, effect on the spontaneous action potential firing frequency of these cells; this effect was reversible in some but not all cells (Fig. 5). On average, the ethanol-induced increase in the firing frequency was not significantly different in basket and stellate cells (P > 0.05 by unpaired t-test; Fig. 5F and G). For comparison, we tested the effect of ethanol in the absence of the synaptic blocker cocktail. Ethanol (80 mM) increased the firing frequency of basket cells by 3.66 ± 1.15 Hz (P < 0.05 by one sample t-test vs. zero) and 410.1 ± 384.4% (P = 0.35 by one sample t-test vs. zero; n = 5). Analysis of these data with Grubb’s outlier test identified one outlier (1947.32%). When this point was removed from the dataset, the one-sample t-test gave a P = 0.055. In stellate cells, ethanol increased frequency by 4.71 ± 1.06 Hz (P < 0.01 by one sample t-test vs. zero) and 87.37 ± 17.93% (P < 0.01 by one sample t-test vs. zero; n = 9). These values were not significantly different from those obtained in the presence of the synaptic blocker cocktail (P > 0.05 by unpaired t-test; compare with Fig. 5F and G).

DISCUSSION

In agreement with previous studies, we found that MLIs receive significant GABA_A receptor-mediated spontaneous inhibitory input (Llano and Gerschenfeld, 1993; Southan and Robertson, 1998). In slices from mice, basket cells were reported to have sIPSC frequencies and amplitudes near 15 Hz and 150 pA, respectively (Southan and Robertson, 1998). In slices from rats, stellate cell sIPSC had frequencies and amplitudes near 0.7 Hz and 132 pA, respectively (Llano and...
The discrepancies between these values and those reported in this study (Table 1) are probably a consequence of differences in experimental conditions, including animal model, composition of the internal solution, slice preparation methodology, recording temperature and age of the animals. Under our recording conditions, we found sIPSC frequency and amplitude not to be significantly different between basket and stellate cells. These events are thought to mainly originate in neighboring MLIs (Kondo and Marty, 1998; Jorntell et al., 2010); however, axon collaterals from Purkinje neurons, and perhaps axons from Lugaro cells, could also contribute to the sIPSCs recorded in basket cells (O’Donoghue et al., 1989; Vincent and Marty, 1993; Jorntell et al., 2010).

Our results extend those studies demonstrating that acute ethanol exposure increases sIPSC frequency at MLI–PC synapses. Work from our laboratory demonstrated that acute exposure to 50 mM ethanol increases sIPSC frequency by 30–40% without affecting amplitude (Mameli et al., 2008). Hirono et al. (2009) reported that 50 and 100 mM ethanol increases sIPSC frequency by ~50 and 75%, respectively. MLIs exhibit ongoing action potential firing activity in the cerebellar slice preparation with a frequency ranging between 0 and 41 Hz (Hausser and Clark, 1997; Hirono et al., 2009). Therefore, it is possible that ethanol increases sIPSC frequency by augmenting spontaneous firing of MLIs. This possibility was assessed by Hirono et al. (2009) who showed that exposure to 50 and 100 mM ethanol increases spontaneous action potential frequency in MLIs by 25 and 75%, respectively. In the presence of synaptic blockers, the effect of these concentrations of ethanol increased to 75 and 125%, suggesting that synaptic inputs limit the effect of ethanol on MLI excitability. In general agreement with these results, we observed that ethanol increases both sIPSC frequency at MLI–MLI synapses and spontaneous action potential frequency in MLIs. These effects are generally similar to those reported by Hirono et al. (2009) in MLI–PC synapses, although the actions of ethanol were more variable and not always reversible under our experimental conditions. Taken together, our studies and those of Hirono et al. (2009) indicate that acute ethanol exposure increases sIPSC frequency at both MLI–PC and MLI–MLI synapses via an increase in spontaneous action potential firing of MLIs. The study of Hirono et al. (2009) suggests that the mechanism of action of ethanol involves potentiation of the hyperpolarization activated cationic current (Ih). Additional studies should be performed to determine whether other conductances are involved in this effect of ethanol, as we found that another cerebellar interneuron, the Golgi cell, is excited by ethanol via inhibition of the Na+/K+ ATPase (Botta et al., 2010). Interestingly, ethanol-induced inhibition of the Na+/K+ ATPase was shown to be enhanced by α1-adrenergic receptor activation in brain synaptosomal preparations (Rangaraj and Kalant, 1980; Rangaraj et al., 1985). Given that these receptors modulate MLI excitability (Hirono and Obata, 2006), it would be interesting to determine whether they play a role in ethanol’s actions on these interneurons.

In addition to increasing sIPSC frequency, ethanol increased mIPSC frequency, but not amplitude, at MLIs, suggesting that it increases quantal GABA release from axonal terminals. The effect of ethanol on mIPSCs was significantly lower than its effect on sIPSCs in stellate, but not basket cells. These findings suggest that ethanol exerts a mixed effect on inhibitory synapses at stellate cells, involving changes in both action potential-dependent and -independent GABA release. In contrast, basket Fig. 5. Acute ethanol increases basket and stellate cell firing frequency. (A) Sample trace and (B) scatter plot indicating the firing rate (Hz) before (b), during ethanol (80 mM) application (e) and after washout (w/o) in basket cells. (C and D) Same as above but for stellate cells. (E) Sample time courses illustrating the ethanol-induced increase in the firing frequency (%). (F and G) Summary graphs illustrating the effect of ethanol on spontaneous action potential firing in basket and stellate cells, calculated with respect to the average of baseline and washout responses (n = 10; *P < 0.05, **P < 0.01 by one sample t-test). BC, basket cell; SC, stellate cell.
cells predominantly receive spontaneous action potential-independent GABAergic input under our recording conditions; therefore, ethanol increases GABA release in these neurons at the level of quantal release. Given that ethanol increased spontaneous firing of both basket and stellate cells, this finding indicates that spontaneous GABAergic input to basket cells in the parasagittal slice preparation originates in a different cell type, whose excitability is not affected by ethanol. We hypothesize that the source of this input could be the Lugaro cell or PC collaterals (Jorntell et al., 2010). The effect of ethanol on mIPSC frequency at MLI–MLI synapses (~20% with 80 mM ethanol) is similar to its effect on MLI–PC synapses (10–50% with 50–100 mM) (Ming et al., 2006; Kelm et al., 2007; Mameli et al., 2008; Hirono et al., 2009). The mechanism of action of ethanol at MLI axonal terminals has been previously determined to be complex, involving effects on the phospholipase C/inositol-1,4,5-trisphosphate/Ca2+/protein kinase C and cAMP/protein kinase A pathways (reviewed in Kelm et al., 2011).

It is interesting that ethanol increases GABAergic transmission both at MLI–PC and MLI–MLI synapses. The increase in GABAergic transmission at MLI–MLI synapses would be expected to decrease excitability of MLIs, opposing changes in action potential-dependent GABA release at MLI–PC synapses. However, ethanol could simultaneously increase GABA release at PCs and MLIs by predominantly potentiating spontaneous quantal transmitter release, which occurs independently of changes in the excitability of MLIs. The acute effect of ethanol on the probability of GABA release could be significant enough to decrease parallel fiber excitatory input to PCs (Mameli et al., 2008). Future studies should assess the impact of the ethanol-induced increase of GABAergic transmission at MLI–MLI synapses on parallel fiber excitatory inputs to interneurons and on feed-forward inhibition in the cerebellar cortex.

The ethanol-induced increase in GABAergic transmission at MLI–MLI synapses is likely to have significant consequences for the physiology of the cerebellar cortex. Recent data suggest that gamma oscillations in the cerebellar cortex are driven by rhythmic GABA\textsubscript{A} receptor-dependent inhibition mediated by MLIs (Middleton et al., 2008). Induction of gamma oscillations via cholinergic stimulation of MLIs caused membrane potential hyperpolarization and a decrease in spike rate in PCs (Middleton et al., 2008). It would be interesting to determine whether the ethanol-induced excitation of MLIs also increases gamma oscillations, leading to similar changes in PC excitability.

The ethanol-induced increase of GABAergic transmission could also impair synaptic plasticity in MLIs. Co-stimulation of parallel fiber and climbing fiber inputs to stellate and basket cells produces long-term potentiation of parallel fiber inputs that results in an enlargement of cutaneous receptive fields in these interneurons (Jorntell and Ekerot, 2003). MLI long-term potentiation requires an elevation of intracellular Ca\textsuperscript{2+} levels that probably depends on activation of Ca\textsuperscript{2+}-permeable AMPA receptors, NMDA receptors and voltage-gated Ca\textsuperscript{2+} channels (Rancillac and Crepel, 2004; Jorntell et al., 2010). The reciprocal form of synaptic plasticity, long-term depression, has also been detected in MLIs and it too depends on Ca\textsuperscript{2+} influx (Soler-Llavora and Sabatini, 2006). Increased GABAergic transmission at MLI–MLI synapses is likely to reduce activation of ligand- and voltage-gated Ca\textsuperscript{2+} channels, which could alter the induction of these forms of synaptic plasticity. Future studies should examine whether acute ethanol exposure affects plasticity at basket and stellate cells.

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