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Acute ethanol exposure increases firing and induces oscillations in cerebellar Golgi cells of freely moving rats

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Abstract

Background—Alcohol is a widely abused substance and is responsible for significant morbidity and mortality worldwide. The precise mechanisms underlying ethanol's actions in the CNS remain elusive. *In vitro* studies suggest that GABAergic interneurons are important targets of ethanol action in the CNS. Although ethanol generally acts to inhibit CNS neurons, it appears to cause an increase in GABAergic interneuron excitability. However, it has yet to be demonstrated that ethanol produces this effect in the brain of behaving animals. Here, we demonstrate for the first time that acute ethanol exposure excites a subtype of GABAergic interneuron (cerebellar Golgi cell) in a freely moving animal.

Methods—Electrophysiological recordings were made from microwire arrays implanted in the anterior cerebellum of freely moving rats.

Results—Cerebellar Golgi cells display a slow, irregular, spontaneous action potential firing pattern under control conditions. Ethanol caused dramatic and consistent increases in the rate and regularity of Golgi cell discharges, including a re-distribution of the power in the Golgi cell spike train, such that power became concentrated in the 26.7 ± 7.3 Hz region.

Conclusions—Taken together with our previous findings, these data suggest that a major mechanism of ethanol actions on cerebellar function is an ethanol-induced de-afferentation at the input stage of the cerebellar cortex in the form of granule cell inhibition, and that this inhibition is caused by an increase in Golgi cell firing. It is likely that Golgi cells may play a significant role both in the gating of information transmission to granule cells and in the modulation of the overall excitability of the cerebellum by tonically controlling granule cell activity.

Keywords

alcohol; cerebellum; Golgi; granule; interneuron

Introduction

Ethanol (EtOH) is the most commonly used psychotropic agent in the world's adult as well as adolescent populations. Average per capita annual consumption rates can reach 18 L of

pure EtOH in some countries [Fuhr and Gmel 2011]. The central nervous system (CNS) is vulnerable to the actions of EtOH, which dose-dependently alters neuronal function in the short term, resulting in a wide range of behavioral alterations including euphoria, sedation, ataxia, and impaired consciousness. Susceptible individuals can readily develop physical and psychological dependence as well as serious long-term neurological deficits with repeated use or abuse. The many mechanisms responsible for the actions of EtOH in the CNS are complex and are still poorly understood.

A substantial body of literature has suggested that acute EtOH exposure blocks glutamatergic transmission while it potentiates GABAergic transmission [Weiner and Valenzuela 2006]. In this way, EtOH may tilt the balance between inhibitory and excitatory neurotransmission in favor of inhibition [Siggins et al 2005, Weiner and Valenzuela 2006]. In the cerebellum, the bulk of afferent input comes from glutamatergic mossy fibers targeting granule cells (grc). These mossy fibers convey a wide spectrum of afferent sensory information to grcs, which are major sources of excitation for all other cerebellar neurons. In anesthetized cats, EtOH was found to inhibit grc responses to sensory stimuli [Huang and Huang 2007]. The interpretation of these results was that acute exposure to EtOH can lead to the de-afferentation of the cerebellum by blocking information flow at grcs, the input stage of the cerebellum. There are, however, several points of concern for experimental inquiries into mechanisms of the acute actions of EtOH in the cerebellum. First, a tilt of balance in neurotransmission in favor of inhibition has also been proposed as the basis of action of general anesthesia. Thus, the use of experimental anesthesia in experimental studies of EtOH actions involving animal models could introduce complications in data interpretation. Second, Golgi cells (GoCs) are major cerebellar interneurons in the grc layer that receive afferent inputs from mossy fibers as well as grc and, in turn, provide GABAergic inhibition to grcs. *In vitro* electrophysiological studies with cerebellar slices indicate that the GoC, rather than the grc, may be the primary target of EtOH in the grc layer [Carta et al 2004]. Moreover, although ethanol is known to cause inhibition to many CNS neuronal types, the observed action of EtOH on GoCs (and other GABAergic interneuronal subtypes throughout the brain) is not inhibitory but excitatory [Freund et al 1993, Carta et al 2004, Hirono et al 2009, Yan et al 2009, Botta et al 2010, Wadleigh and Valenzuela, in press]. At present, it is unclear whether EtOH in behaving animals can excite GABAergic interneurons. Here we used GoC recordings to demonstrate, for the first time, *in vivo* evidence consistent with such an excitatory effect of ethanol. We showed that in un-anesthetized and un-restrained rats, acute exposure to EtOH increases GoC firing. Moreover, this effect was accompanied by increased GoC oscillations, a novel mechanism of action of ethanol not previously seen *in vitro*, that can possibly modulate the level of grc excitability both quickly and globally. In this way, cerebellar GoCs may play a significant role both in the gating of information transmission to grcs and in the modulation of the overall excitability of the cerebellum by controlling the tonic level of grc activity.

Methods

Animals and Preparations

The data were taken from eight adult female Long-Evans rats (260-350 g). Microelectrode arrays were fabricated according to an earlier report [Tsai and Yen 2003]. Briefly, Teflon-insulated microwires (diameter ~50 μm) were assembled into eight- or sixteen-electrode arrays, and implanted into the cerebellar cortex (lobules VI, V) of the experimental animals. Electrode access was made possible via a light-weight connector fixed to the skull. After three days of recovery, the animals were typically found to be alert and in good health indicated by their grooming behavior and their reactions to handling by laboratory staff. The site near and around the electrode head plug was typically free of infection and groomed clean by the animal. When the animals were brought to the recording cage, which was

modified from a cage of the same material and build as their home cage, they soon became accustomed to the recording environment including being tethered by a cable connecting the animal's head plug to the data acquisition electronics (figure 1a). We also monitored the recording cage with a video camera. During a typical recording session, many animals sat quietly with their eyes open. After intraperitoneal injection (2g/kg, ip, 20% EtOH in saline), none of the 8 animals studied lost their righting reflex. Additional experimental details on animals and data analysis can be found in Chang et al [2005].

Ethanol Delivery and Histology

After an initial period of ~15 minutes in the recording cage to allow the animal to settle down, we collected control electrophysiological data over a period of ~30 minutes. EtOH delivery was next carried out via intraperitoneal injection (2g/kg, ip, 20% EtOH in saline). This dose of EtOH is expected to produce blood alcohol levels of ~60 mM within 7-10 min that gradually decay to 50 mM over a period of 1 hr [Nurmi et al 1994, Huang and Huang 2007]. Our post-EtOH recording started at 7.5 minutes after EtOH injection. The entire post-EtOH recording consisted of a period of also ~30 minutes. Anticipating that neuronal activity from un-anesthetized, un-restrained animals will be affected by many more variables than in anesthetized preparations, we tried to adhere to this set of temporal parameters closely. In both control and EtOH conditions, we selected time segments of recordings while the animals were sitting quietly and were not engaged in locomotor, grooming or feeding/drinking activity. In order to avoid potential complications from acute tolerance development, each animal was injected with EtOH only once. We have also carried out recording sessions in which an equivalent volume of saline was injected (ip) and concluded that saline did not change the firing patterns of GoCs (data not shown). At the end of the series of recording sessions, the animal was deeply anesthetized, 5 μ A of current was passed for 30 sec through each of the electrode leads, and the animal was perfused (formalin fixative with 1% potassium ferrocyanide). The brain was removed and kept in 30% sucrose for 5 days before frozen sections (100 μ m) were prepared for cresyl violet histology. The locations of the recording electrodes and tracks were identified via the Gormori reaction and the visualization of iron deposits in serial sections of the cerebellum. The iron deposits readily allowed the localization of recording sites within the molecular layer, granule cell layer, or near the Purkinje cell layer. One example of that is shown in figure 1b. Electrode sites within the grc layer or near the Purkinje cell layer almost always exhibited vigorous neuronal activity while electrode sites within the molecular layer rarely registered neural activity with significant amplitudes.

Data Analysis

Data acquisition and collection were carried out with a Plexon system (Plexon Inc, Dallas, Texas USA, at 40 kHz sampling rate per channel), whose waveform analysis software was capable of separating in real time activities of multiple-units into action potentials attributable to single neurons (typically 2-3). Data on the timing of individual spikes were extracted from the spike train of a given putative single neuron. These timing data were used to obtain mean firing rates, inter-spike-intervals (ISI), ISI histograms, and autocorrelograms. Fast-Fourier Transform (FFT) was carried out on the spike train data to locate major power peaks and their bandwidths. Before- and after-EtOH data were represented as mean $\pm$ SD (error bars in figure 2b) and statistical analyses were performed with the paired-sample t test in figure 2b (one-tailed) and in figure 3c (two-tailed). Cases in which p values <0.05 were considered statistically significant. All calculations and statistical analysis were performed with Matlab and EXCEL.

Results

Identification of Putative

GoCs In the present study, we used a multi-parameter approach to assess the likelihood that we were indeed examining GoCs. First, we required that the action potential waveform attributable to single GoCs must exhibit signal-to-noise ratios of ~10:1 (figure 2a). This was to avoid the possibility of mistakenly including any grcs, which typically display action potentials with signal-to-noise ratios of ~3:1 under similar recording conditions [e.g. with microwires or tungsten microelectrodes, Shambes et al 1978, Hartmann and Bower 2001]. Second, we required that the spike waveform must contain no hint of complex spikes. This was to avoid the possibility of mistakenly including any Purkinje cells. Third, we required that the mean discharge rate in control recordings must be under 20/s [Maex and De Schutter 1998]. This also helped to avoid the possibility of mistakenly including any Purkinje cells, most of which discharge simple spikes tonically at >30/s. Fourth, we required that the blue stain in the Gormori histology must be clearly located within the grc layer. The combined use of these criteria made it unlikely that our pool of putative GoCs would be contaminated by grcs or Purkinje cells. With these criteria, we found ~1 putative GoC per animal.

The Firing Rate of GoCs

EtOH reliably and consistently caused significant increases in the firing rates of neurons in our pool of putative GoC. Before EtOH, the mean GoC firing rate was $8.5 \pm 4.7/s$. The mean firing rate increased to $16.1 \pm 7.7/s$ in the post-EtOH state, a $98 \pm 36\%$ increase ($p=0.0002$, $n=8$, figure 2b). It is of interest to note that treating the before and after EtOH discharge rates of GoC as two paired samples in a one-tailed t test (rather than two independent samples) yielded a highly significant p value despite the seeming large and overlapping error bars in figure 2b. This increase was accompanied by a sharpening of the peak in the ISI histogram (figure 2c). We examined the variations in firing rate (indicated by $[1/ISI_{mean}]$) against the variations in the regularity of GoC spike trains (indicated by the inverse of the standard deviation of the ISI or $[1/ISI_{SD}]$, figure 2d). In the pre-EtOH state, the mean firing rate and firing regularity were tightly correlated (correlation coefficient = 0.985, coefficient of determination = 0.97), indicating that 97% of the changes in these two parameters may be linked – an increase in regularity was reliably accompanied by an increase in firing rate. This should serve to indicate that the mean firing rates of GoCs and their degree of regularity are constrained at least partially so that these two parameters are not free to vary independently. The control GoC firing was quite irregular (figure 2c). This irregularity is unlike the reported Golgi cell firing pattern in slice preparations [Carta et al 2004, Botta et al 2010]. Whereas this difference may be correlated with the differences in the conditions of the mossy fiber afferent system, the exact mechanisms remain to be further explored. EtOH significantly increased the mean firing rate of GoCs but the correlation between firing rate and regularity became less tight than before EtOH (correlation coefficient = 0.82, after EtOH). Perhaps GoC codes information with the timing of its action potentials. As EtOH drives GoC to operate at the higher end of its firing rate (indicated by a higher ISI_{mean}), some of the precision in temporal coding is lost (indicated by a higher ISI_{SD}).

Neuronal Oscillations in Golgi Cells

The nominally stochastic discharge patterns of GoCs in the control state were characterized by relatively flat and unremarkable autocorrelograms (figure 3a). EtOH-induced firing regularity was a major change in the post-EtOH autocorrelograms of GoCs, which were characterized by major periods of high degree of correlation between adjacent spikes in the GoC spike trains (figure 3a). The primary period for this enhanced correlation in the spike train was 45.5 ± 14.5 ms ($n=8$). To obtain a more comprehensive knowledge of the bandwidth of GoCs as signal generators, a fast-Fourier-transform (FFT) was carried out on

the spike train data in the time domain to yield a power spectrum in the frequency domain for each putative GoC. A major bandwidth at 26.7 ± 7.3 Hz ($n = 8$) was identified in the post-EtOH power spectra of many GoCs whereas no such peak was found in the power spectra of the same cells in the pre-EtOH state (figure 3b). The frequency of this EtOH-induced bandwidth (26.7 ± 7.3 Hz) was consistent and essentially represented the inverse of the primary period of autocorrelation at 45.5 ± 14.5 ms. If we convert the inverse of the period between major peaks of autocorrelograms to a measure of frequency, the resultant bar graph resembled the bar graph describing the major frequencies of the power spectra (figure 3c). The two frequencies in figure 3c are statistically indistinguishable ($p=0.56$). This suggests that results in figures 3a (autocorrelograms) and 3b (power spectra) reflect the same phenomenon, which may be the principal, if not the only, effect of EtOH on GoC. An interpretation of the EtOH-induced increase in GoC firing rate from $8.5 \pm 4.7/s$ to $16.1 \pm 7.7/s$ was that EtOH caused a re-distribution of the power in the GoC spike train, such that power became concentrated in the 26.7 ± 7.3 Hz region while giving rise to increased regularity.

Discussion

Comparisons with Other Data and Limitations of the Study

As far as we know, this is the first time that the effect of EtOH on the firing patterns of cerebellar GoC was examined at the level of single cells in freely moving animals. We showed that EtOH increases the firing rates and induces oscillations in putative GoCs. This observation is in agreement with *in vitro* results from cerebellar slices that an important action of EtOH on cerebellar circuitry is the excitation of GoCs which cause inhibition of grcs [Carta et al 2004, Botta et al 2010]. The inhibition of grcs can cause acute functional de-afferentation of the cerebellum, dramatically reducing the ability of the grcs to respond to sensory stimuli [Huang and Huang 2007]. Direct comparisons of the findings from various *in vivo* and *in vitro* preparations, however, can be problematic. The temporal nature of the acute actions of EtOH can be complex and different depending on the experimental conditions. For example, while EtOH concentrations could be maintained at a constant level in cerebellar slices, cerebrospinal fluid EtOH variations over time are more complex in whole animal experiments due to metabolic clearance. Acute tolerance may additionally act in the temporal domain differently in the slice preparation compared with the whole-animal preparation over a matter of minutes to tens of minutes. These issues are difficult to resolve but should not affect the major conclusions of the present study concerning the primary actions of acute EtOH in un-anesthetized and un-restrained animals.

Functional Significance of the GoCs

Largely based on cell count data known at the time, Marr [1969] theorized that grcs should exhibit sparse-coding characteristics to achieve optimal information coding at the input stage. This was confirmed by Shambes et al [1978] who found that grc in anesthetized rats responded briskly to tactile stimuli with brief bursts of spikes lasting no more than 10 ms with little or no capacity for tonic activity. Such discharge patterns were also seen in grc responses to auditory as well as visual stimuli in anesthetized cats [Huang and Burkard 1986; Huang and Huang 2007]. In rats anesthetized with Ketamine [Ruigrok et al 2011], grc discharge rates were the lowest among all other cerebellar neuronal types. These are all consistent with the negative feedback synaptic relationship between grc and its GABAergic synaptic partner, the GoC, which appears to be capable of preventing grcs from firing in a tonic manner or at higher rates (but see [Barmack and Yakhnitsa 2008]). D'Angelo [2008] suggested that a major functional role of GoCs is to dynamically gate information transmission of grcs. The tonic or spontaneous firing patterns of GoCs additionally suggest that GoCs may also modulate the overall level of excitability of grcs. Therefore, an emerging picture of GoC electrophysiology is that the phasic component of GoC firing

provides the dynamic gating of information transmission to grcs immediately following the occurrence of a GoC action potential while the tonic level of GoC firing determines the overall level of activity of grcs – the faster GoC fires, the less frequent grcs can provide continuous streaming or up-dating of information.

Further Implications on the Mean Firing Rate of GoCs

In un-restrained rats, grc clusters fire almost continuously with short bursts at higher rates [Hartmann and Bower 2001]. The continuing firing of grcs is at odds with the previously mentioned experimental observations of Shambes et al [1978] and Huang and Burkard [1986] from anesthetized animals. In the context of current theories on the function of grc, ongoing grc firing in freely moving animals should represent grc responses from a continuous stream of sensory stimuli. The principal insight of Marr [1969] on sparse coding of grcs is likely to be still correct but data from awake animals further suggest that grcs report frequently, presumably to provide the cerebellum with up-dated information from one moment to the next. Continuous streaming of afferent information is important to active animals, which require their mossy fiber afferents to constantly transmit impulses to the cerebellum. In the presence of EtOH, increases in ongoing GoC firing rates should result in decreases in ongoing grc firing rates [Carta et al 2004, Botta et al 2010]. The grcs may still retain reduced abilities to respond to stimuli in a phasic manner under EtOH or anesthesia [Shambes et al 1978, Huang and Burkard 1986, Huang and Huang 2007] but may be severely limited in their capacities to fire tonically in order to provide ongoing updates to the cerebellum. This condition, at least under EtOH, appears to be largely a direct result of increases in the tonic firing rates of GoCs.

Neuronal Oscillations in Golgi Cells

In the present study, EtOH-induced increases in GoC firing rates were always accompanied by enhanced neuronal oscillations in GoC (figure 3a, b). The observed major power of the GoC oscillation after EtOH falls in the beta range (5-30 Hz), a range that was suggested to be important in movement modulation, particularly in movement anticipation (but not during movement execution) [Dugue et al 2009, Hartmann and Bower 1998]. GoC oscillations were also suggested to be linked to experimental cerebellar ataxia [Cheron et al 2004]. In both of these observations, the consequences of GoC oscillations, most likely also accompanied by increased GoC firing and dramatically reduced grc firing, are consistent with a lack of normal movement, either in movement anticipation in normal animals [Hartmann and Bower 1998] or in ataxia [Cheron et al 2004]. GoC oscillations are, therefore, unlikely to reflect normal cerebellar function. It is possible that GoC oscillation represents a novel mechanism of restrictive gating of information transmission to grcs in the cerebellar cortex. A network of GoC linked via gap junctions and distributed over a large region of the cerebellum may facilitate the spread of GoC oscillations to areas spanning over several lobules [Dugue et al 2009, Vervaeke et al 2010, De Souza and De Schutter 2011], possibly in a manner not unlike the cardiac muscles (also a system with oscillatory capabilities based on the functions of gap junctions). In view of the recognized role of GoCs in restrictive gating of the mossy-fiber-granule-cell system, a potential tendency for GoC oscillations to spread to a vast area of the cerebellar cortex, and the fact that GoC oscillations were only observed after EtOH in the present study, we hypothesize that the enhanced and EtOH-induced restrictive gating by GoCs may be related to a novel endogenous mechanism to quickly and effectively shut down the cerebellum such as during alternating sleep and wakefulness cycles. It is logical that the cerebellum should have evolved the capacity to modulate its excitatory tone quickly and in a global manner with the consequence of such modulation ultimately manifested at the level of grcs and Purkinje cells. Additional details concerning the effect of extra-cerebellar centers regulating sleep and wakefulness and the GoC are largely unclear at present.

Future mechanistic probes

Additional studies must be designed to determine the mechanism of action of EtOH excitation of cerebellar GoC. For example, Botta et al [2010, 2011] have suggested that ethanol enhances Go excitability by modulating the sodium/potassium pump based on results from cerebellar slice preparation and computational simulation. *In vivo* characterization of these studies may be feasible but not without difficulties. The sodium/potassium pump is ubiquitously present in all systems, and the functions of many of these systems, including the cardiovascular system *in vivo*, are sensitive to doses of ouabain as low as 10-100 nM [Rossoni et al 2001, Zhang et al 2009], the same concentration range used in Botta et al [2010]. The role of gap junctions between GoCs and EtOH action is another potential area of further investigation. A mouse model lacking the gene for connexin36 (Cx36 KO) is available. Gap junctions, however, are also ubiquitous within the CNS, including the inferior olivary complex and Purkinje cells [Rash et al 2000]. In addition, compensatory modifications of the GABAergic system as well as other cytological and electroresponsive properties of in Cx36 KO are significant [De Zeeuw et al 2003, Voss et al 2010], and may contribute to how these Cx36 KO mice react to EtOH. A study on the effect of EtOH on motor behavior in Cx36 KO mice has yielded complex findings as it was difficult to isolate the EtOH effect on motor coordination versus motor learning [Steffensen et al 2011]. Ideally, the identification of the role of gap junctions in the mechanism of action of EtOH on GoCs would require the conditional mutation of Cx36 in GoCs of adult animals to avoid potential developmental complications of the loss of this important protein.

The Cerebellum and Acute Ethanol Intoxication

We have shown that acute EtOH can cause dramatic increases in the firing rate of cerebellar GoCs, accompanied by the presence of neuronal oscillations in un-anesthetized and unrestrained rats. We suggest that this may be a primary mechanism through which EtOH can cause an effective de-afferentation of the cerebellar cortex, leading to cerebellar functional deficits. Law enforcement with respect to drunk driving already relies routinely on many cerebellar functional disturbances including abnormality in balance, equilibrium, and coordination. The full impact of acute EtOH intoxication is expected to be far more extensive as the cerebellum is now known to serve a broad range of motor as well as non-motor, cognitive functions [Schmahmann 1997, Strick et al 2009]. The results of the present study help to identify that the effect of EtOH on cerebellar GoCs may be a key step leading to cerebellar function alterations during acute intoxication.

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Figure 1.

(a) Example of a rat in the recording cage (connecting cable indicated by arrow), (b) a series of lesions (arrows) indicating the locations of the tip of the microwires. These lesions helped to identify the recording site within different layers of the cerebellar cortex. ML: Molecular layer, gcL: granule cell layer.

Figure 2.

Electrophysiological properties of Golgi cells (GoC). (a) Examples of GoC spike trains before and after EtOH. The firing rate was faster after EtOH. (b) Bar histograms showing mean rates of GoC discharge before and after EtOH. Data were represented as mean $\pm$ SD. The firing rates were significantly faster after EtOH (* $P < 0.05$). (c) Interspike-interval histograms of a representative GoC before and after EtOH. The narrowing the peak in the ISI histogram indicated that EtOH increased the regularity of discharges. (d) The mean firing rate [$1/ISI_{mean}$] and the firing regularity [$1/ISI_{SD}$] were strongly correlated.

Figure 3.

a. Autocorrelograms of GoC spike trains displayed major peaks only after EtOH. The autocorrelograms were obtained with *NeuroExplorer*, by setting bin=1ms, range from -1024 - 1024ms. (b) Power spectrum of GoC spike trains displayed a major peak only after EtOH. FFT was carried out using Matlab with 2048 points and a resolution frequency range from 5 -150Hz. (c) Major frequencies in Hz derived from GoC autocorrelograms and the power spectra of GoCs, both after EtOH. There was no statistically significant difference between the two frequencies ($p=0.56$).