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Contingent and non-contingent effects of low-dose ethanol on GABA neuron activity in the ventral tegmental area

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ABSTRACT

Ventral tegmental area (VTA) GABA neurons appear to be critical regulators of mesocorticolimbic dopamine (DA) neurotransmission, which has been implicated in alcohol reward. The aim of this study was to evaluate the effects of low-dose "non-contingent" intravenous (IV) ethanol (0.01–0.1 g/kg) on VTA GABA neuron firing rate and synaptic responses, as well as VTA GABA neuron firing rate during low-dose "contingent" IV ethanol self-administration. Intravenous administration of 0.01–0.03 g/kg ethanol significantly increased VTA GABA neuron firing rate and afferent-evoked synaptic responses. In the runway self-administration paradigm, presentation of an olfactory cue (S+; almond extract) or no-cue (S−; no odor) in the Start box was paired with IV administration of low-dose ethanol (0.01 g/kg) or saline in the Target box. Runway excursion times decreased significantly in association during S+, and increased significantly during S− conditions. The firing rate of VTA GABA neurons markedly increased when rats received 0.01 g/kg IV ethanol in the Target box. VTA GABA neuron firing increased in the Start box of the runway in association with S+, but not S−. These findings demonstrate that VTA GABA neurons are activated by low-dose IV ethanol and that their firing rate increases in anticipation of ethanol reward.

1. Introduction

The mesocorticolimbic dopamine (DA) system originating in the ventral tegmental area (VTA) and projecting to the nucleus accumbens (NAcc) is considered to be a key area in reward from natural behaviors such as feeding (Phillips et al., 2003), drinking (Agmo et al., 1995), and drug reward (Koob, 1996). Support for a role for this neural circuit in ethanol reward hinges on the evidence demonstrating that local injections of transmitter antagonists into the NAcc and/or afferent circuit systems prevent ethanol self-administration (Hyytia and Koob, 1995; Pettit et al., 1984; Roberts et al., 1996; Vaccarino et al., 1985). Rats will self-administer ethanol directly into the VTA (Gatto et al., 1994) and an ethanol-induced increase of DA release in the NAcc, detected by microdialysis, has been reported extensively (Di Chiara and Imperato, 1988; Weiss et al., 1993; Wozniak et al., 1991; Yoshimoto et al., 1992).

Ethanol increases the firing rate of midbrain DA neurons both *in vivo* and *in vitro* (Brodie et al., 1990a; Gessa et al., 1985). Although VTA DA neurons are excited by ethanol, it has been suggested that their excitation may be attributed to disinhibition produced by a primary inhibitory effect on GABA-containing neurons of the midbrain (Mereu and Gessa, 1985). Accordingly, we have reported that acute ethanol reduces VTA GABA neuron firing rate (Gallegos et al., 1999) and corticotegmental excitatory synaptic responses (Stobbs et al., 2004) *in vivo*, with an IC_{50} of 1.0 g/kg (100 mg% blood alcohol level), a substantial fraction of the EC_{50} for ethanol excitation of DA neurons *in vitro* (Brodie et al., 1999). Moreover, VTA GABA neurons become hyperexcitable during ethanol withdrawal and evince tolerance to ethanol inhibition of firing rate during chronic ethanol (Gallegos et al., 1999), suggesting that GABA neurons in the VTA constitute a critical substrate for the acute and chronic effects of ethanol in the mesocorticolimbic DA system (Diana et al., 2003). Since the discovery that VTA GABA neurons were inhibited by ethanol (0.2–2.0 g/kg), we have observed that the firing rate of many VTA GABA neurons often increases transiently after systemic administration *in vivo*, as well as during ethanol perfusion *in vitro*, before exerting its prolonged inhibitory effects on firing rate and synaptic responses in both preparations. Based on these observations, we hypothesized that VTA GABA neurons might be activated by low doses of ethanol. Thus, the first aim of this study was to characterize the effects of passive injections of intravenous low-dose (0.01–0.1 g/kg) ethanol ("non-contingent" condition) on VTA GABA neuron firing rate and corticotegmental synaptic input. As drug-naïve rats will self-administer ethanol at doses as low or lower than 0.01 g/kg (Gass and Olive, 2007; Kuzmin et al., 1999; Sinden and Le Magnen, 1982), the second aim of this study was to

Abbreviations: Cx, connexin; DA, dopamine; IC, internal capsule; ICPSDs, internal capsule stimulus-induced post-stimulus spike discharges; gap junction, GJ; PSH, peri-stimulus spike histogram.

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characterize the firing rate of VTA GABA neurons during low-dose (0.01 g/kg) ethanol self-administration ("contingent" condition) in the runway paradigm (McFarland and Ettenberg, 1997).

2. Methods

2.1. Animal care and surgery

Male Wistar rats (Charles River Laboratory, Hollister, CA) weighing 300–500 g were housed individually with *ad libitum* access to food and water, and maintained on a reverse 12 h light/dark cycle (OFF 10:00, ON 22:00). Animal care, maintenance and experimental procedures were in accordance with the Scripps Research Institute and Brigham Young University Animal Research Committees (IACUC approved; Animal Welfare Assurance #s A3194-01 and A3783-01). For acute studies, rats were initially anesthetized with Isoflurane (3.0–4.0%) and placed into a stereotaxic apparatus mounted on a pneumatic anti-vibration table (MICRO-g, TMC Corp). Body temperature was monitored and maintained at 37.0±0.1 °C by a feedback-regulated heating pad. The skull was exposed and holes were drilled to accommodate placement of stimulating and recording electrodes. The dura was opened over recording sites to prevent breakage of micropipettes. Isoflurane anesthesia was maintained at 1% following surgery and during electrophysiological recordings.

For chronic studies, a similar methodology was employed; however, aseptic surgical conditions were maintained. Two bundles of eight stainless steel, Teflon-insulated, microwires (50 to 62 μm; diameter of splayed microwire tip was 0.25–0.5 mm, NB Labs, Denison, TX) were lowered bilaterally into the VTA (−5.6 to −6.2 mm anterior–posterior, 0.7–1.0 mm medial–lateral, and 7.0–8.0 mm dorsal–ventral from the cortical surface, relative to bregma; Paxinos and Watson, 1986). Silastic tubing was inserted in the right jugular vein for IV ethanol infusions, as described previously for heroin (Steffensen et al., 1998). The jugular catheter system was flushed daily with heparinized saline to keep the catheter patent. Rats were given at least one week to recover following surgical implantation, and were habituated by daily handling.

2.2. Single-unit recordings

For acute studies, extracellular potentials in anesthetized rats were recorded by a single 3.0 M NaCl-filled micropipette (1–3 MΩ; 1–2 μm inside diameter) in Isoflurane (1%) anesthetized rats. Potentials were amplified and filtered with a MultiClamp 700A programmable amplifier (Axon Instruments, Union City, CA). Microelectrode assemblies were oriented into the VTA [from bregma: 5.6–6.5 posterior (P), 0.5–1.0 lateral (L), 7.0–8.5 ventral (V)] with a piezoelectric inchworm microdrive (Burleigh, Fishers, NY). Single-unit activity was filtered at 0.3–10 kHz (−3 dB) and displayed on Tektronix 2200 digital oscilloscopes. Square-wave constant current pulses (50–1000 μA; 0.15 ms duration; average frequency, 0.1 Hz) were generated by an IsoFlex isolation unit controlled by a MASTER-8 Pulse Generator (AMPI, Israel), or by a computer. The internal capsule (IC; from bregma: −1.5 AP, 2.5–3.0 ML, 5.0–6.5 V) was stimulated with insulated, bipolar stainless steel electrodes. We evaluated spikes that had greater than 5:1 signal-to-noise ratio. Extracellularly recorded action potentials were discriminated with WPI-121 (Sarasota, Fl) spike analyzers and converted to computer-level pulses. Single-unit potentials, discriminated spikes, and stimulation events were captured by National Instruments NB-MIO-16 digital I/O and counter/timer data acquisition boards (Austin, TX) in Macintosh-type computers. Potentials were digitized at 20 kHz and 12-bit voltage resolution.

For chronic studies, single-unit activity was recorded from unrestrained rats using a detachable headset containing unity-gain field-effect transistors, one for each of the 16 microwire electrodes. Typically, a few of the 16 electrodes implanted in freely-behaving rats yield usable spike recordings (greater than 3:1 signal-to-noise ratio). The signals were filtered and amplified (1–3 kHz; −3 dB; 1000×) with an Axon Instruments CyberAMP 380 (Foster City, CA), digitized by National Instruments PCI-MIO-16 multi-function data acquisition boards at 20 kHz (16-bit resolution), and processed on-line by customized National Instruments (Austin, Texas) LabVIEW® virtual instrument spike detection software running on a Pentium-type computer (Lee et al., 2001). The duration of each recording session varied according to the performance of the rat. A video recording system consisting of a camcorder (Sony CCD-TR7), videographics cards (Mass Microsystems Colorspace II/FX), MacIntosh Quadra 950 computer, a video monitor, and a videocassette recorder was employed to monitor rat behavior.

2.3. Characterization of VTA GABA neurons

In anesthetized rat studies, all neurons classified as VTA GABA neurons met the criteria established in previous studies for spike waveform characteristics and response to IC stimulation (Allison et al., 2006; Steffensen et al., 1998; Stobbs et al., 2004). Presumed VTA GABA neurons were characterized by short-duration (<200 μs; measured at half-peak amplitude of the spike), initially negative-going, non-bursting spikes, and were identified by the following IC stimulation criteria (Steffensen et al., 1998): short latency (i.e., 2–5 ms) antidromic or orthodromic activation via single stimulation of the IC; and multiple spiking following high-frequency (10 pulses, 200 Hz) stimulation of the IC (ICPSDs; Allison et al., 2006; Lassen et al., 2007; Steffensen et al., 1998; Stobbs et al., 2004). In all studies, stimulation was performed at a level that produced 50% maximum VTA GABA neuron ICPSDs. This was accomplished by determining the current needed to produce the maximum number of ICPSDs at 200 Hz and 10 pulses, and then adjusting the stimulus intensity until 50% ICPSDs were achieved. In freely-behaving rat studies, all neurons classified as VTA GABA neurons met the same criteria for spontaneous activity and spike waveform as cells recorded in anesthetized rats. At the conclusion of the experiment, we passed ±3.0 mA of current for 5.0 s through each of the microwires that contained spikes and evaluated the electrolytic lesion under microscopic examination to determine the location of the electrode. All cells analyzed in freely-behaving rats were located in the VTA.

2.4. Chronic ethanol treatment and assessment of withdrawal

In freely-behaving rats, 14 days prior to performing the ethanol operant runway experiments, and during the days of the runway and self-administration procedures, rats received twice-daily injections of 2.0 g/kg ethanol at 0800 and 1800 h. Rats were recorded in the runway paradigm between 1400 and 1600 h, 6–8 h after the morning injection of 2.0 g/kg ethanol, when VTA GABA neurons have been shown to be hyperexcitable (Gallegos et al., 1999). Observational withdrawal signs were quantified by means of a behavioral rating scale for onset of ventromedial distal limb flexion response, tail stiffness, and abnormal body posture. A subjective 0–2 point scale of severity was utilized for each of three withdrawal signs, with 0 representing undetectable, 1 representing moderately severe, and 2 representing severe withdrawal signs. The ventromedial distal limb flexion response was measured by grasping the rat by the scruff of the neck and checking for retraction of the limbs towards the body. Tail stiffness was characterized by the presence of a rigid, awkwardly bent tail. Abnormal body posture was indicated by broad-based stance or abnormal gait.

2.5. Ethanol operant runway

The operant runway consisted of two distal compartments flanking a 5′ long, 4.5″ wide central alley and arranged in a linear orientation. The interior of each box measured 12″ (width) by 12″ breadth by 16″ (height) and was constructed of opaque 0.25″ acrylic. Each box was fitted with a sliding acrylic door mounted on a modified CD-ROM drive enabling computer control of door openings/closings. A 25 pin commutator was

suspended on a 0.5″ polished stainless steel rod approximately 30″ above the runway and permitted the commutator to slide back and forth above the alley and the distal compartments. A headstage cable assembly was connected to one side of the commutator, and the other side was connected to a patch box that facilitated monitoring of the 16 recording electrodes. The runway consisted of Start, Alley and Target compartments. A Pre-runway box was used to obtain baseline responses before introducing the rats to the Start box in the runway. Acoustic white noise generated by a computer-controlled speaker permeated the space. Movement signals were obtained by piezoelectric sensors mounted under the floor of the Start and Target compartments and were used to determine the runway Alley excursion times. All experiments were recorded on videotape. Rats were placed in the Pre-runway box for 5 min and then moved to the Start box for 5 min when the door to the runway Alley was opened allowing them free access to the Alley and Target box. If the rat did not traverse the Alley to the Target box in 300 s it was walked down the Alley into the Target box and injected with ethanol or saline. The door to the Target box was shut after the rat entered. Ethanol injections in the Target box were paired with an odor cue in the Start box (S+), which consisted of 50 µL of almond oil extract pipetted into a 2″ by 2″ gauze pad in an aerated plastic cassette located in the Target box. Rats received two injections of either 0.01 g/kg IV ethanol (5% solution; ~100 µL in saline, adjusted for body weight) on S+ trials and an equal volume of saline on S− trials with a 5 min interval between injections.

2.6. Electrophysiological data analysis and statistics

We acquired, analyzed, and processed data with customized LabVIEW (National Instruments, Austin, TX) software on MacIntosh and Pentium 4 computers. Extracellularly recorded single-unit action potentials were discriminated by a peak detector digital processing LabVIEW algorithm. In anesthetized rats, to determine changes in VTA GABA neuron activity produced by intravenous ethanol in anesthetized rats, firing rate was determined by averaging 5 min epochs of firing rate before and at 5–10 min after ethanol injection by rectangular integration of ratemeter records with IGOR PRO software (WaveMetrics, Lake Oswego, OR). Peri-stimulus and interval-spike histograms were generated off-line using IGOR Pro (WaveMetrics, Lake Oswego, OR) analysis of time-stamped data. The duration (ms) and extent (#events/bin) of post-stimulus permutation of ICPSDs was determined by rectangular integration at specific time points on the peri-stimulus spike histogram using IGOR Pro analysis software. The minimum bin width for peri-stimulus spike histograms was 1 ms and the number of bins was 1000. These parameters allow for detection of all phases of pre- and post-stimulus spike activities before and after ethanol administration. In freely-behaving rats, the firing rate of VTA GABA neurons in the ethanol runway was determined by averaging 3 min of activity via rectangular integration of ratemeter plots with IGOR Pro software (Wavemetrics, Lake Oswego, WA). We averaged 3 min of activity during the middle of the 5 min the rat spent in the Pre-runway box, 3 min of activity during the middle of the 5 min the rat spent in the Start box, during the entire traverse of the runway, and then 3 min immediately after each injection of ethanol in the Target box. Firing rates were expressed as percent of Pre-runway firing rate in the Start, Alley, and Target box during ethanol or saline injections. The results for control and drug treatment groups were derived from calculations performed on ratemeter records and PEHs and expressed as means ± S.E.M. The results were compared by using the Student's paired two-sample *t*-test for matched sample sizes, and a two-way ANOVA (Microsoft Excel) without replication ($\alpha < 0.05$), for unmatched sample sizes. Figures were compiled by using IGOR Pro Software.

3. Results

3.1. Effects of low-dose ethanol on VTA GABA neuron firing rate

Twenty-six VTA GABA neurons were tested *in vivo* for firing rate sensitivity to low-dose (0.01–0.1 g/kg) IV ethanol. Of the neurons tested, 54% of them were activated by 0.01 g/kg ethanol. Fig. 1A shows the effects of saline and IV ethanol (0.01–1 g/kg) on the firing rate of a representative VTA GABA neuron. Compared to saline control (mean saline firing rate = 33.8 ± 3.5 Hz), IV administration of 0.01 g/kg ethanol significantly increased VTA GABA neuron firing rate 86% (Fig. 1B; $P < 0.005$; $t = 4.9$; $n = 14$). Some of the low-dose IV ethanol-sensitive neurons were tested at higher doses. Intravenous administration of 0.03 g/kg ethanol also significantly increased VTA GABA neuron firing rate 187% (Fig. 1B; $P < 0.001$; $t = 9.52$, $n = 6$). Intravenous administration 0.075 or 0.1 g/kg did not significantly alter the firing rate of VTA GABA neurons (Fig. 1B; $P > 0.05$; $n = 5$ each). To assure sensitivity to the inhibitory effects of high dose ethanol, we challenged each neuron that was sensitive to low-dose ethanol to an intraperitoneal injection of 1.0 g/kg ethanol. As previously reported (Gallegos et al., 1999), this dose of ethanol markedly decreased firing rate (Fig. 1B; $P < 0.001$; $n = 6$).

3.2. Effects of low-dose ethanol on VTA GABA neuron evoked discharges: ICPSDs

Concomitantly with firing rate, we evaluated synaptic responses of the VTA GABA neurons that were sensitive to low-dose ethanol by stimulating an excitatory afferent input. Brief, high frequency stimulation of the internal capsule (IC) results in multiple post-stimulus VTA GABA neuron spike discharges (ICPSDs; Steffensen et al., 1998). ICPSDs are blocked by NMDA antagonists (Steffensen et al., 1998; Stobbs et al., 2004), 0.2–2.0 g/kg ethanol (Stobbs et al., 2004), and connexin-36 (Cx36) gap junction (GJ) blockers (Allison et al., 2006). The number of ICPSDs is a monotonic function of frequency, stimulus intensity, and pulse number (Lassen et al.,

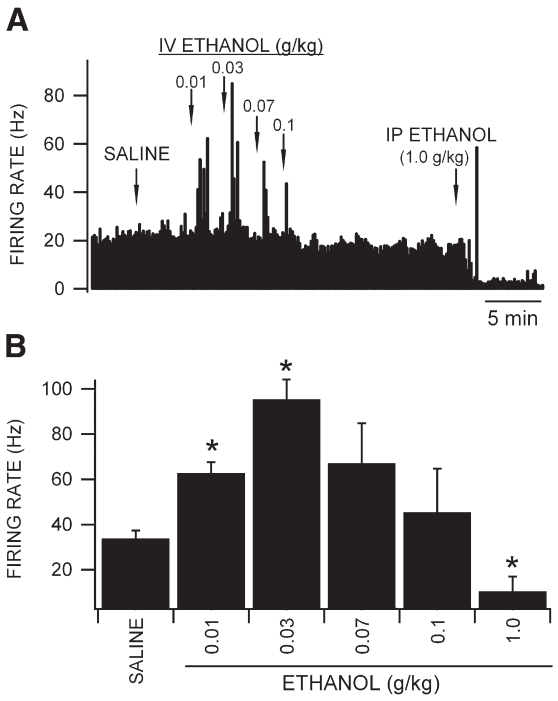

Fig. 1. Effects of low-dose intravenous ethanol on VTA GABA neuron firing rate in anesthetized rats. (A) This ratemeter record shows the effects of intravenous ethanol (0.01–1.0 g/kg) on the firing rate of one VTA GABA neuron recorded in an anesthetized rat. The baseline firing rate of this neuron was approximately 20 Hz. In this neuron, IV saline had no effect, while ethanol (0.01–0.10 g/kg) increased its firing rate, and intraperitoneal ethanol (1.0 g/kg) produced a prolonged inhibition of firing rate (data truncated), as previously reported (Gallegos et al., 1999). However, note the transient increase in firing rate shortly after the ip injection of ethanol, which is often characteristic of ethanol effects on VTA GABA neurons. Arrows indicate time of injection. (B) This graph summarizes the effects of IV and ip ethanol (0.01–1.0 g/kg) on the firing rate of all VTA GABA neurons tested. Compared to saline, IV ethanol at dose levels 0.01–0.03 g/kg significantly increased firing rate while dose levels 0.07–0.1 g/kg did not significantly alter firing rate. Compared to saline, ip ethanol at dose level 1.0 g/kg significantly decreased the firing rate of the same neurons. Asterisks * represent significance level $P < 0.001$.

2007). Rats will self-stimulate the IC (Lassen et al., 2007), and the number of ICPSDs is directly proportional to responding for brain stimulation reward (Lassen et al., 2007). Based on these physiological and pharmacological studies, as well as molecular studies demonstrating dye-coupling between VTA GABA neurons (Allison et al., 2006) and Cx36 GJ expression in VTA GABA neurons (Allison et al., 2006; Lassen et al., 2007), it was interpreted that ICPSDs are generated by corticotegmental glutamatergic synaptic input, but sustained by Cx36 GJ-mediated electrical coupling between VTA GABA neurons. We studied the effects of low-dose ethanol on VTA GABA neuron ICPSDs in anesthetized rats (Fig. 2A,B). Compared to saline control (mean saline firing rate = 33.8 ± 3.5 Hz), IV administration of 0.01 g/kg ethanol significantly increased VTA GABA neuron ICPSDs 41% (Fig. 2B; $P<0.02$; $t=2.7$; $n=10$). To assure sensitivity to the inhibitory effects of high dose ethanol, we challenged each neuron with an intraperitoneal injection of 1.0 g/kg ethanol. As previously reported (Stobbs et al., 2004), this dose of ethanol markedly decreased ICPSDs 61.3% (Fig. 2B; $P<0.0001$; $t=8.6$).

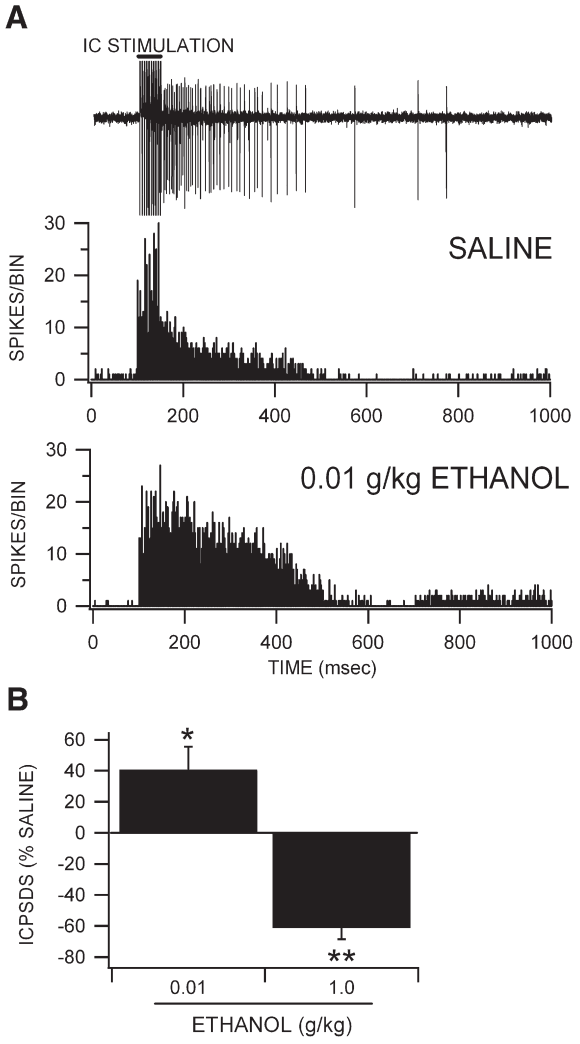

Fig. 2. Effects of low-dose ethanol on VTA GABA neuron ICPSDs. (A) The inset shows a 1.0 s trace of the spike activity of a representative VTA GABA neuron in association with brief (50 ms), high frequency (200 Hz) stimulation of the internal capsule (IC). The horizontal bar indicates the time of the stimulus train. VTA GABA neurons evince multiple spike discharges for hundreds of ms following IC stimulation. These are termed IC-induced post-stimulus spike discharges, or ICPSDs (Steffensen et al., 1998). The peri-stimulus spike histogram (PSH) below the trace shows the average of 12 IC stimulation trials on ICPSDs obtained from this representative VTA GABA neuron following IV saline. The stimulus artifacts in the PSH are omitted to illustrate spikes only. The PSH bottom shows the effects of 0.01 g/kg IV ethanol on ICPSDs in this same neuron. Note that low-dose ethanol increased VTA GABA neuron ICPSDs at this dose level. (B) This graph summarizes the effects of IV low-dose and ip higher dose ethanol on VTA GABA neuron ICPSDs. Low-dose (0.01 g/kg) IV ethanol significantly reduced ICPSDs and higher dose (1.0 g/kg) ip ethanol significantly decreased ICPSDs in the same neurons. Asterisks * and ** represent significance levels $P<0.02$ and $P<0.0001$, respectively.

3.3. VTA GABA neuron firing rate in the runway paradigm

Given the studies demonstrating that rats will self-administer low-dose ethanol (Gass and Olive, 2007; Kuzmin et al., 1999; Sinden and Le Magnen, 1982), we sought to evaluate the firing rate of VTA GABA neurons during ethanol reward in the runway paradigm. However, since only 54% of VTA GABA neurons in anesthetized rats were shown to be activated by low-dose ethanol (0.01 g/kg), and it is much more problematical to characterize VTA GABA neurons in freely-behaving rats without stimulation paradigms (e.g., IC stimulation, etc…), we needed to first determine that the neurons were sensitive to ethanol. After 14 days of twice-daily 2.0 g/kg ip ethanol treatments, rats showed signs of withdrawal including the ventromedial distal limb flexion. Twelve neurons were recorded in four freely-behaving rats during the chronic ethanol injections. Nine of the 12 neurons recorded in four freely-behaving rats were inhibited by acute 2.0 g/kg ip ethanol, became tolerant to ethanol inhibition after 14 days of chronic ethanol, and evinced significant adaptation in baseline firing rate to chronic ethanol ($P<0.05$, $t_{(2,8)}=3.0$; mean baseline rate = 28.1 ± 5 Hz vs mean ethanol withdrawal firing rate = 72.5 ± 13.8 Hz; $n=9$ each), as previously reported (Gallegos et al., 1999). Thus, these nine neurons in the 4 rats (at least 2 neurons/rat) were studied in the runway paradigm with low-dose IV ethanol. During ethanol DAYS 1–6 (S+ condition), an odor cue in the Start box was paired with two 0.01 g/kg IV ethanol injections in the Target box. During saline DAYS 7–9, no odor in the Start box was paired with two equivolumic intravenous saline injections in the Target box (S− condition). Ethanol was reinstated on DAYS 10–13. The Alley run-times over the 13 days of ethanol, saline and ethanol reinstatement are shown for one rat in Fig. 3A. In this rat, the run-time in the Alley for ethanol decreased from 100 s to a plateau around 30 s on DAY 6, increased to 300 s following three days of saline administration, and then decreased following ethanol reinstatement. The mean run-time on DAY 6 of ethanol for the four rats was 25.3 ± 2.9 s. Run-times increased markedly in association with the saline sessions (mean run-time DAY 9 = 285.3 ± 14.7 s), but then decreased again during ethanol reinstatement (mean run-time DAY 13 = 10.3 ± 2.1 s). There was a significant decrease in run-time for ethanol on DAY 6 vs saline on DAY 9 ($P<0.001$, $t_{(2,2)}=17.3$), for DAY 13 ethanol reinstatement vs DAY 9 saline ($P<0.001$, $t=18.6$), and for DAY 13 ethanol reinstatement vs DAY 6 ethanol ($P<0.05$, $t=4.3$).

All neurons studied evinced waveforms and firing properties characteristic of VTA GABA neurons in freely-behaving rats (Fig. 3B). Fig. 3C–F shows the firing rates of three VTA GABA neurons recorded in one of the rats on: DAY 1 (Fig. 3C) when ethanol injections in the Target box were paired with an odor cue in the Start box; DAY 6 (Fig. 3D) following 5 previous days of ethanol injections in the Target box paired with an odor cue in the Start box; DAY 9 (Fig. 3E) following 2 previous days of saline injections in the Target box paired with no odor cue in the Start box; and DAY 13 (Fig. 3F) following 3 days of ethanol reinstatement. On DAYS 1, 6, and 13 the firing rates of the three neurons were markedly enhanced by ethanol injections in the Target box. On DAY 9 the firing rates of the three neurons were enhanced in the Pre-runway box relative to other days and subsided in the Start, Alley and Target box. There was no effect of saline injections on the firing rates of these three neurons. On DAY 13 the firing rates of the three neurons were moderately enhanced in the Start box relative to the Pre-runway box.

Fig. 4 summarizes the effects of paired ethanol and saline injections on the firing rates of the nine VTA GABA neurons recorded in the four rats on DAYS 1, 6, 9, and 13. On DAY 1 the firing rate of the nine VTA GABA neurons was not significantly altered in the Start box ($P>0.05$, $t_{(2,8)}=0.48$) or Alley box ($P>0.05$, $t_{(2,8)}=0.93$) relative to the Pre-

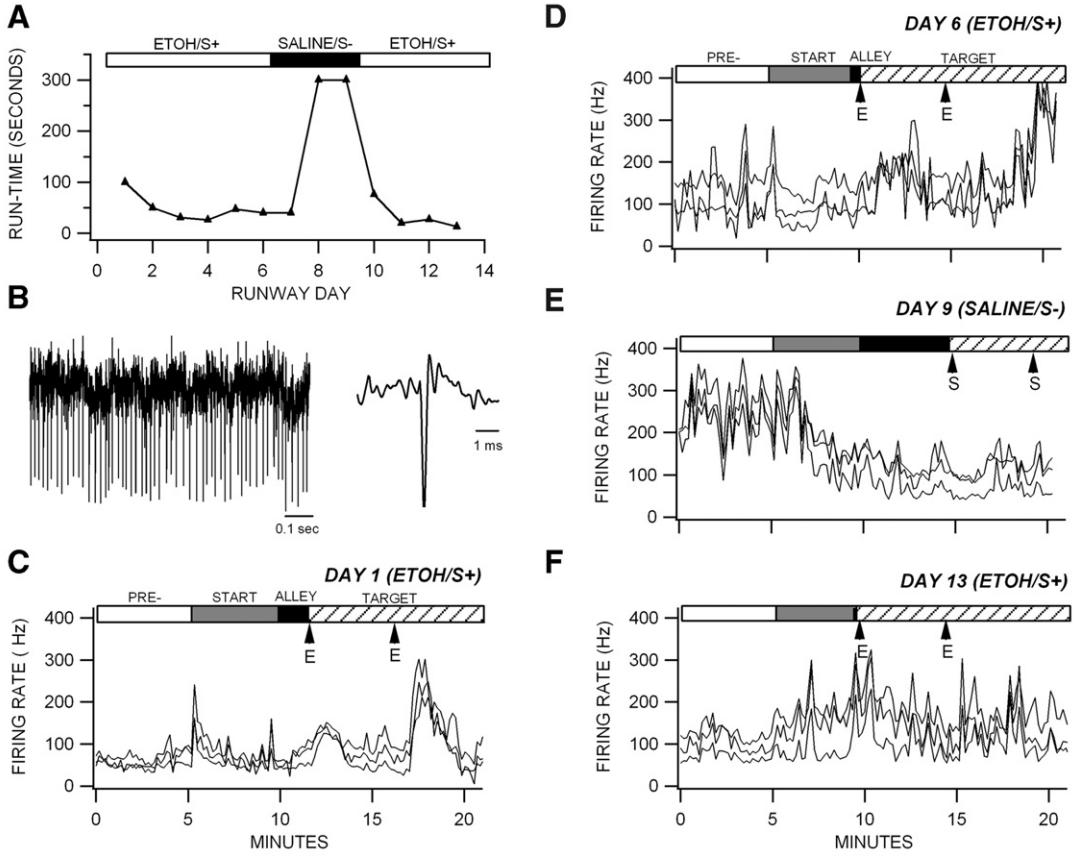

Fig. 3. Ethanol runway and VTA GABA neuron activity. (A) This graph shows the runway Alley run-times from the Start box to the Target box for one rat for each of 13 sessions (one session/DAY). The horizontal bar above the plot shows the days of ethanol or saline paired with an odor cue (S+; almond extract) or no odor cue (S−), respectively. Run-times decreased from DAYS 1–6 when the odor cue predicted the ethanol injections, increased from DAYS 7–9 when no odor predicted the saline injections, and decreased again when the odor was presented and ethanol (ETOH) injections were reinstated in the Target box on DAY 10. (B) The inset left is a 1.0 s unfiltered recording in the Pre-runway box of one of the three VTA GABA neuron spikes in this rat. Note the fast firing rate of this neuron (60 Hz). The inset right is an expanded view of one of the spikes shown in the trace at left demonstrating the short-duration, negative-going spike waveforms typical of VTA GABA neurons. (C) This graph shows a ratemeter record of the firing rates of three neurons recorded in this rat on DAY 1 of the runway paradigm. Ethanol in the Target box was paired with an odor cue in the Start box (S+ condition). The horizontal bar above shows the location of the where the rat was during the session. The rat was placed in the Pre-runway box for 5 min, transferred to the Start box with an odor cue (almond extract), and then the shutter door was opened after another 5 min allowing access to the Alley and the Target box. The firing rates of the three GABA neurons were increased during handling and transfer from the Pre-runway box to the Start box. After 5 min in the Start box, the shutter door was opened and the rat ran down the Alley to the Target box in 100 s, where the shutter door was closed, and the rat was injected immediately with 0.01 g/kg IV ethanol (denoted by an E; 5% solution; ~100 μL in saline, adjusted for body weight) via a jugular catheter. A second dose of 0.01 g/kg intravenous ethanol was administered 5 min later. Note the enhancement of firing rate associated with each ethanol injection (arrowheads denoted by an E for ethanol). (D) This graph shows a ratemeter record of the three neurons in the same rat on DAY 6 following 5 days of ethanol injections in the Target box following an odor cue in the Start box (S+). Note that the firing rates of the three neurons increased slightly in the Start box and Alley and markedly in association with the 0.01 g/kg low-dose ethanol injections in the Target box. The rat took 26 s in the Alley to reach the Target box from the Start box. (E) This graph shows a ratemeter record of the three VTA GABA neurons in the same rat on DAY 9 following 3 days of saline injection in the Target box following a no-odor cue in the Start box (S− condition). Note that the firing rate of the neurons was elevated in the Pre-runway box relative to DAY 1 or DAY 6 and decreased to a plateau level in the Start box, Alley, and Target box, where two injections of saline were administered (arrowheads denoted by an S for saline). The rat took 306 s in the Alley to reach the Target box from the Start box. (F) This graph shows a ratemeter record of the three VTA GABA neurons in the same rat on DAY 13 following 3 days of ethanol reinstatement (S+). Note that the firing rate returned to the level before the saline injections, that the firing rate in the Start box and Alley was greater than in the Pre-runway box, and that the firing rate increased modestly in association with the two ethanol injections in the Target box. The rat took 13 s in the Alley to reach the Target box from the Start box.

runway box. However, the firing rate of VTA GABA neurons was significantly enhanced in association with the intravenous injections of 0.01 g/kg ethanol ($P<0.001$, $t_{(2,8)}=4.59$) in the Target box. On DAY 6 (S+) the firing rate of VTA GABA neurons was significantly increased in the Start box ($P<0.05$, $t_{(2,8)}=2.17$), the Alley box ($P<0.05$, $t_{(2,8)}=2.52$), and in association with the intravenous injections of 0.01 g/kg ethanol in the Target box ($P<0.001$, $t_{(2,8)}=4.62$), relative to the Pre-runway box. On DAY 9 (S−) the firing rate of the VTA GABA neurons was not significantly affected in the Start box ($P>0.05$, $t_{(2,5)}=0.8$), the Alley box ($P>0.05$, $t_{(2,5)}=0.9$), or in association with the intravenous injections of isovolumic saline in the Target box ($P>0.05$, $t_{(2,5)}=1.7$), relative to the Pre-runway box. The mean baseline firing rate of the VTA GABA neurons in the Pre-runway box was significantly increased on saline DAY 9 compared to DAY 1 ($P<0.05$, $t_{(2,8)}=2.2$; mean DAY 9 Pre-runway ethanol firing rate = 149.2 ± 32.4 Hz), DAY 6 ($P<0.05$, $t_{(2,8)}=2.3$) and DAY 13 ($P<0.05$, $t_{(2,8)}=2.1$). On DAY 13 (S+ reinstatement) the firing rate of VTA GABA neurons was significantly increased in the Start box ($P<0.05$, $t_{(2,8)}=2.52$), the Alley box ($P<0.05$, $t_{(2,8)}=2.71$), and in association with the intravenous injections of 0.01 g/kg ethanol in the Target box ($P<0.05$, $t_{(2,8)}=3.2$), relative to the Pre-runway box.

4. Discussion

The notion that local circuit VTA GABA neurons inhibit DA neurons might explain our findings that inhibition of GABA neurons by moderate- to high-dose ethanol (0.2–2.0 g/kg) would lead to enhancement of DA activity and DA release in limbic structures (Gallegos et al., 1999). However, we show here that very low doses of intravenous ethanol (0.01–0.03 g/kg) enhance the firing rate and evoked synaptic activity (i.e., ICPSDs) of approximately 50% of the VTA GABA neurons tested. As mentioned previously, we often observe transient activations of VTA GABA neurons even with high-dose ethanol injections, perhaps due to an initial low-dose ethanol excitatory effect before equilibration. Why then does low-dose ethanol excite some VTA GABA neurons? As

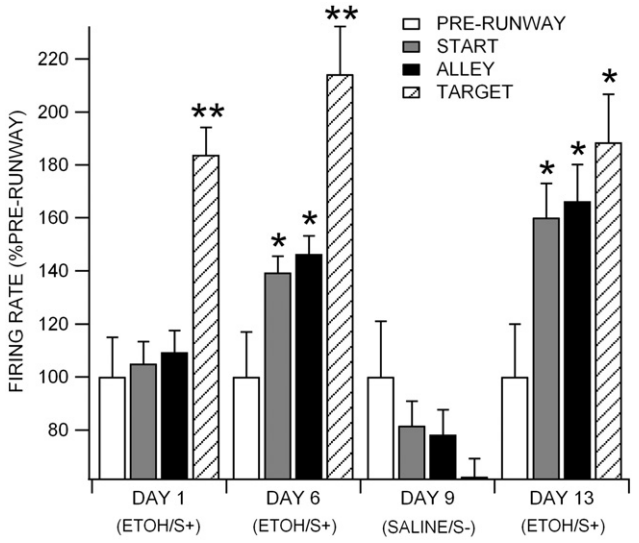

Fig. 4. Summary of VTA GABA neuron firing rate in the ethanol runway. This graph summarizes the firing rate data for the nine VTA GABA neurons recorded in four rats on DAYS 1, 6, 9 and 13 of the operant runway. Firing rates are expressed as percent Pre-runway firing rate for each DAY. On DAY 1, the firing rate of the nine VTA GABA neurons studied was not affected in the Start box or the Alley box relative to the Pre-runway box firing rate; however, the firing rate was enhanced 84% by the 0.01 g/kg intravenous ethanol (ETOH) injections in the Target box. On DAY 6, VTA GABA neuron firing rate increased 39% in the Start box, 46% in the Alley, and 114% by the intravenous ethanol injections in the Target box. On DAY 9, VTA GABA neuron firing rate decreased in the Start, Alley, and Target boxes where intravenous saline was administered, but was not significantly altered. On DAY 13, VTA GABA neuron firing rate increased 60% in the Start box, 66% in the Alley, and 88% by the intravenous ethanol injections in the Target box. Error bars represent S.E.M. values around the mean and asterisks * and ** equal significance levels $P<0.05$ and $P<0.001$, respectively.

approximately 50% of VTA GABA neurons are excited by DA (Stobbs et al., 2004), and DA neurons are excited by ethanol (Brodie et al., 1999), one possible explanation is that DA regulation of a subpopulation of GABA neurons may be operational at some ethanol dose levels, but not at others.

VTA GABA neurons appear to be more sensitive to acute and chronic ethanol than DA neurons. For example, we have shown previously that acute ethanol inhibition of VTA GABA neuron firing rate (Gallegos et al., 1999) and evoked synaptic activity (Stobbs et al., 2004) are nearly one order of magnitude greater than ethanol excitation of DA neuron firing rate (Brodie et al., 1999; Brodie et al., 1990b). Moreover, as demonstrated here, some VTA GABA neurons are activated at dose levels as low as 0.01 g/kg, three orders of magnitude more sensitive than DA neurons. We are working under the model that there are subpopulations of GABA neurons in the VTA, one that inhibits DA neurons, and one that is activated by DA neurons, and that the differential effects of low- and high-dose ethanol on these neurons result from reciprocal connectivity between GABA and DA neurons in this area of the midbrain. Of particular interest, DA not only enhances the firing rate of VTA GABA neurons, but enhances electrical coupling between them (Stobbs et al., 2004). The spike discharges evoked by IC stimulation (i.e., ICPSDs) are a physiological reflection of this electrical coupling mediated by Cx36 GJs (Allison et al., 2006; Lassen et al., 2007), which are also differentially sensitive to acute low- and high-dose ethanol, with enhancement by low-dose ethanol, and inhibition by high-dose ethanol (Stobbs et al., 2004). Thus, VTA GABA neuron excitability is regulated not only by DA, but by corticotegmental inputs to VTA GABA neurons that enhance coupling between them. We have recently reported that the degree of electrical coupling between VTA GABA neurons is directly proportional to responding for brain stimulation reward (Lassen et al., 2007). We can only speculate that electrical coupling between VTA GABA neurons may play a role in ethanol reward.

It had been our previous experience that rats will not self-administer high-dose ethanol, regardless of ethanol history. Thus, given the reports demonstrating that rats self-administer low doses of IV ethanol in operant paradigms (Gass and Olive, 2007; Kuzmin et al., 1999; Sinden and Le Magnen, 1982), along with our findings that low doses of ethanol excite VTA GABA neurons, we were compelled to evaluate whether low-dose ethanol might be rewarding in the ethanol runway paradigm, and how VTA GABA neuron activity might correlate with the behavior. Rats learned to discriminate an olfactory stimulus predicting ethanol availability (S+) from one predicting the absence of ethanol availability (S−). Presentation of the S+ resulted in significant shorter run-times to obtain the IV low-dose ethanol reinforcer, suggesting that a discriminative cue produced ethanol-seeking behavior in ethanol-dependent rats using a low dose of IV ethanol as the reinforcer. Presentation of the S+, but not S−, increased VTA GABA neuron activity preceding the ethanol reinforcer, similar to what we have demonstrated previously with brain stimulation reward and heroin self-administration (Steffensen et al., 2001; Steffensen et al., 2006), suggesting that VTA GABA neurons play a critical role in the orientation to rewarding stimuli, including ethanol reward. We found that S+ trials were associated with an increase in VTA GABA neuron activity in the Target box after administration of low-dose ethanol, similar to what we found in anesthetized rats at 0.01 g/kg. These effects could be mediated through a direct pharmacological effect on the withdrawal-related hypersensitivity of these neurons, as higher doses of non-contingent ethanol administered to ethanol withdrawn rats often resulted in significant enhancement of VTA GABA neuron activity (Gallegos et al., 1999). There was often a delay in the onset of the ethanol-induced increase in VTA GABA neuron activity in the Target box, which complicates interpretations regarding the pharmacological effects vs the behavioral effects of ethanol on VTA GABA neurons in freely-behaving rats. While a direct pharmacological effect on VTA GABA neurons remains plausible, the delay suggests that the enhancement in firing rate may be due to indirect behavioral effects, as we have previously reported that the discharge activity of these neurons is closely linked to cortical arousal (Lee et al., 2001). Low-dose ethanol may be producing a general state of cortical arousal that is reflected in the enhancement of VTA GABA neuron activity. Our findings support earlier studies in operant paradigms (Gass and Olive, 2007; Kuzmin et al., 1999; Sinden and Le Magnen, 1982) demonstrating that very low doses of IV ethanol exhibit reinforcing properties. Although blood samples were taken from the IV catheter and submitted to analysis by the Sigma Enzymatic Method they fell below the detectable limit of this method (i.e., 1 mg%). Indeed, with the Sigma Enzymatic Method, as well as other methods, the detection limit for ethanol is constrained to 1 mg% due to "volatile reducing substances" and other endogenous alcohols. Thus, the determination of BALs at the dose level/injection (0.01 g/kg), concentration (5%), volume (~100 μL) and dosing schedule (twice) used in this study was problematical, as has been discussed in the recent study of Gass and Olive (2007) wherein IV ethanol self-administration was demonstrated at an even lower dose/injection of IV ethanol (0.001 g/kg). Accordingly, further studies are needed to identify the blood alcohol levels (BALs) generated by low-dose IV ethanol self-administration. Nonetheless, this dose of ethanol was clearly rewarding, as runway times decreased markedly with ethanol and increased with isovolumic saline injections, showing extinction and then reinstatement of the running behavior with ethanol.

Although recording VTA GABA neurons in freely-running rats in the ethanol operant runway was technically challenging, we were able to successfully record the same VTA GABA neurons for more than 30 days, and under extraordinary recording conditions (i.e., running behavior). Although not quantified, rats consistently exhibited an increase in their activity after low-dose ethanol injections, as they would routinely try to jump up and climb out of the Target box following the second dose of 0.01 g/kg IV ethanol. The activity of VTA GABA neurons clearly reflected this hyper-excitable state. However, as mentioned above, it was difficult to determine if the enhancement of VTA GABA neuron firing

rate in the Target box was associated directly with the injections of ethanol or with the behavioral state induced by the injections of ethanol. It was also difficult to determine whether the escape behavior was related to an anxiogenic response similar to the properties of IV cocaine described by Ettenberg in the operant runway (see Ettenberg and Geist, 1991). Regardless, the responses of VTA GABA neurons correlated with the behavioral effects, if not temporally, at least spatially within each box. It is important to point out that while the dose (0.01 g/kg) and volume injected (100 µl) in these studies were low, the ethanol concentration (5%) might produce local effects on the vascular endothelium surrounding the catheter and influence the animal's behavior. However, the fact that the escape behavior was only observed after the second dose of 0.01 g/kg IV ethanol (at least 5 min after the initial dose) suggests that these effects of ethanol were likely mediated by its pharmacological properties on the CNS and not to direct and immediate effects of alcohol on local vascularity.

As mentioned previously, drug naïve, ethanol non-preferring rats will self-administer doses of ethanol as low, or lower, than the dose used in this study (0.01 g/kg; Gass and Olive, 2007; Kuzmin et al., 1999; Sinden and Le Magnen, 1982). Our findings support these studies with additional evidence showing that low-dose IV ethanol is rewarding, perhaps via its activating properties on VTA GABA neurons. However, interpretations regarding the causal role of VTA GABA neurons in ethanol self-administration are problematical due to the correlative nature of spike and behavioral data. Regardless, the well-known behavioral activating properties of low-dose ethanol may contribute to its rewarding properties, as there is a positive relationship between ethanol preference and ethanol-induced motor stimulation (Waller et al., 1986). What relevance might low-dose intravenous ethanol self-administration have to human alcohol abuse? Since humans typically consume ethanol orally, the intravenous mode of ethanol delivery does not model the human condition well. However, there are well known difficulties with animal models of human ethanol consumption. For example, at least in out-bred animals, the aversive properties of ethanol require the use of sweetened solutions to obtain responding, with subsequent fading-out of the sweetener to obtain significant ethanol responding (Samson et al., 1988). Thus, IV delivery methods have been used to avoid the aversive properties of oral ethanol, as they allow for a more rapid onset of the pharmacological effects of ethanol, a more direct assessment of its reinforcing effects, and eliminate the need for lengthy sweetener substitution procedures (Gass and Olive, 2007). Notwithstanding the fact that the typical route of administration of ethanol in humans is oral, there are reports that some poly-drug users will also self-administer ethanol intravenously (Mahdi and McBride, 1999). Although IV ethanol does not accurately model human ethanol consumption, it is considered as a more direct way to study the reinforcing effects of ethanol given its direct delivery to the bloodstream, rapid onset of effect, and the distinct advantage of more accurate and reliable correlations between behavioral responding and the timing of neuronal activity.

The mesocorticolimbic DA system has been the main target of studies characterizing the neural substrates mediating the role of environmental control of drug- or food-motivated behaviors [e.g., see Gratton and Wise, 1994; Phillips et al., 1993; Schultz, 1986]. McFarland and Ettenberg have used the operant runway paradigm to characterize the ability of rats to use environmental (olfactory) stimuli as discriminative cues to predict the availability or unavailability of a reinforcer in the goal or Target box of an operant runway (McFarland and Ettenberg, 1998; McFarland and Ettenberg, 1997). Their data suggest that non-DA mechanisms play an important role mediating drug-predictive stimuli that promote drug self-administration (McFarland and Ettenberg, 1997). A lack of DA involvement in drug reinforcement has also been demonstrated for oral ethanol self-administration (Kiianmaa et al., 1979; Myers and Quarfordt, 1991; Rassnick et al., 1993) and ethanol conditioned-place preference (Cunningham and Noble, 1992; Risinger et al., 1992). Furthermore, ethanol self-administration is blocked by intra-amygdalar injections of $GABA_A$ agonists in dependent rats (Roberts et al., 1996) and $GABA_A$ antagonists in non-dependent rats (Hyytia and Koob, 1995). These studies provide compelling evidence that DA-independent mechanisms also play an important role in ethanol reinforcement.

In summary, we found that VTA GABA neurons are activated by low-dose IV ethanol in ethanol-naïve rats, and that ethanol-dependent rats will learn to run for low-dose IV ethanol, especially during ethanol reinstatement, when the salience of the predictive cue would be greater. In addition, olfactory cues in the Start box, that predict the availability of ethanol in the Target box, enhance VTA GABA neuron activity in the Start box. It remains for future studies to determine if VTA GABA neurons play a role in the behaviorally-activating properties of low-dose IV ethanol. They would seem to be ideal candidates for causing or reflecting cortical activation considering their correlation with cortical activity (Lee et al., 2001), their widespread projection to the cortex and cortical inputs (Steffensen et al., 1998), as well as their potential synchrony with other ventral brain GABA neurons via GJ-mediated electrical coupling (Allison et al., 2006; Lassen et al., 2007; Stobbs et al., 2004). Interestingly, low concentrations of ethanol enhance, while higher concentrations of ethanol inhibit, low-threshold spiking and T-type calcium currents in GABAergic thalamic relay neurons (Mu et al., 2003). Thalamic neurons have been shown to be electrically coupled, express Cx36 GJs, and evince ICPSDs, similar to VTA GABA neurons (Lassen et al., 2007). Thus, similar to thalamic GABAergic relay neurons, VTA GABA neurons may be involved in cortical activation as well as being differentially affected by low- and high-dose ethanol. This report provides a springboard to future studies that will evaluate the role of T-type calcium channels in mediating the low-dose effects of ethanol on VTA GABA neurons.

Acknowledgements

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