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RESEARCH ARTICLE

# Prior social experience affects the behavioral and neural responses to acute alcohol in juvenile crayfish

**Matthew E. Swierzbinski[1,2], Andrew R. Lazarchik[1] and Jens Herberholz[1,2,*]**


## ABSTRACT

The effects of alcohol on society can be devastating, both as an immediate consequence of acute intoxication and as a powerful drug of abuse. However, the neurocellular mechanisms of alcohol intoxication are still elusive, partly because of the complex interactions between alcohol and nervous system function. We found that juvenile crayfish are behaviorally sensitive to acute alcohol exposure and progress through stages that are strikingly similar to those of most other intoxicated organisms. Most surprisingly, we found that the social history of the animals significantly modified the acute effects of alcohol. Crayfish taken from a rich social environment became intoxicated more rapidly than animals that were socially isolated before alcohol exposure. In addition, we found that the modulation of intoxicated behaviors by prior social experience was paralleled on the level of individual neurons. These results significantly improve our understanding of the mechanisms underlying the interplay between social experience, alcohol intoxication and nervous system function.


**KEY WORDS: Invertebrate, Intoxication, Social history, Neural circuit, Neurons**

## INTRODUCTION

Although alcohol is a widely used drug with significant behavioral, health and economic impacts, the cellular mechanisms of alcohol intoxication are still elusive. While other drugs of abuse have specific receptors in the brain, alcohol does not, but instead exerts its effects by targeting multiple neurotransmitter systems, including serotonin and GABA (Mehta and Ticku, 1988; Mihic et al., 1997; Barr et al., 2003; Wolf and Heberlein, 2003; Ferraz and Boerngen-Lacerda, 2008; Lobo and Harris, 2008; Kumar et al., 2009). Behavioral effects of ethanol (EtOH) exposure are conserved across many different species and typically consist of a period of disinhibition or an increase in activity, followed by loss of motor coordination and postural control (Moore et al., 1998; Vonghia et al., 2008; Robinson and Atkinson, 2013). Existing research on the interconnection between alcohol and social experience has largely focused on how alcohol alters various social behaviors that range from dance communication in honeybees to aggression in humans (Bozic et al., 2006; Heinz et al., 2011). In addition, other studies investigated how alcohol consumption is mediated by social factors; for example, subordinate rats and monkeys have been shown to consume significantly more alcohol than dominants (Blanchard et al., 1987; McKenzie-Quirk and Miczek, 2008), and rats and monkeys that were socially deprived (e.g. by housing them individually) also consumed more alcohol compared with animals housed in groups (Wolffgramm and Heyne, 1991; Helms et al., 2012). However, our understanding of the underlying mechanisms is still very limited, and it was recently suggested that in order to improve clinical relevance of addiction science, future studies should focus more specifically on the role of social context including factors such as social exclusion (Heilig et al., 2016). Yet, despite extensive literature in the field of alcohol research, the vast majority of publications focus on the effects of social mediators on drinking behavior, whereas the opposite, i.e. whether (and how) past social experience might shape the neurobehavioral effects of acute alcohol exposure, is significantly understudied.

Prior work using invertebrates as models for addiction research has been very productive (Søvik and Barron, 2013). The behavioral effect of acute (as well as repeated and chronic) EtOH exposure has been well characterized in *Drosophila melanogaster*, which exhibits behavioral alterations that are similar to those of humans (Wolf and Heberlein, 2003). Using powerful genetic approaches in this model revealed some of the genes and neurochemical components correlated with the behavioral responses to alcohol; however, identification of the underlying cellular–molecular mechanisms has been less successful (Kong et al., 2010; Rodan and Rothenfluh, 2010).

Among invertebrates, the suitability of crayfish as a model for studying the effects of drugs of abuse has also been demonstrated. Crayfish experience discrete behavioral changes in response to several widely abused drugs (e.g. amphetamines and cocaine) that are similar to those seen in vertebrates (Panksepp and Huber, 2004). In addition, crayfish that were chronically exposed to alcohol in the surrounding water exhibited tolerance; the inability to quickly right themselves after being turned on their back resumed normal functioning after 2 weeks of continuous exposure (Friedman et al., 1988). Other studies of chronic alcohol exposure in crayfish found that hemolymph (i.e. blood) alcohol concentration reached approximately 10% of the bath concentrations after 2 h of exposure, and alcohol-induced behavioral effects were observed at a blood alcohol content similar to that found for other organisms, including humans (Macmillan et al., 1991; Hausknecht et al., 1992).

In the current study, we expanded on these studies, and – for the first time – investigated the role of social experience on the behavioral and neurophysiological sensitivity to acute EtOH exposure in juvenile crayfish.

## MATERIALS AND METHODS

### Animals

Juvenile crayfish, *Procambarus clarkii* (Girard 1852), measuring between 3.0 and 4.0 cm (from rostrum to telson) were purchased from a commercial supplier (Atchafalaya Biological Supply Co., Raceland, LA, USA) and housed in large communal housing tanks

[1]Department of Psychology, University of Maryland, College Park, MD 20742, USA. [2]Neuroscience and Cognitive Science Program, University of Maryland, College Park, MD 20742, USA.

*Author for correspondence (jherberh@umd.edu)

J.H., 0000-0001-7584-5903

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(76×30×30 cm L:W:H) with 50–100 crayfish until the day of the experiment (Com group) or before they were placed in social isolation (Iso group) for 7–10 days prior to testing. The bottom of communal tanks was filled with ∼2 cm of gravel, which provided small hiding places, and the water was constantly circulated and filtered using an aquarium pump (Tetra Spectrum Brands, Blacksburg, VA, USA). The small isolation tanks (15×8×10 cm L:W:H) were equipped with air stones for water oxygenation (BubbleMac Industries, McAlpin, FL, USA). Both Com and Iso groups were housed on a 12 h:12 h light:dark schedule at a constant temperature of 22±1.5°C. Com animals were fed shrimp pellets (Aqua Pets Americas, Salt Lake City, UT, USA) *ad libitum* twice weekly and Iso animals were fed one shrimp pellet on the day of isolation. Only animals that showed no signs of major bodily injury (i.e. both claws and at least six walking legs intact) and had not molted within 2 days prior to experiments were used. Com crayfish were taken directly from a communal tank with a fish net, weighed on a laboratory scale, and measured with a ruler. Iso crayfish were measured and weighed following isolation before the experiment was performed. After measurements were taken, each individual crayfish was immediately transferred to the experimental (i.e. alcohol-containing) tank. All experiments were performed at about the same time of day and each animal was only used once.

### Freely behaving animals

EtOH baths were created on test day using 100% ethyl alcohol (Ethyl Alcohol 200 Proof, Pharmco-Aaper, Brookfield, CT, USA) and deionized water. Both behavioral and implanted electrode experiments were performed in 500 ml water in tanks (15×8×10 cm). The light in the experimental room was kept constant and disturbance in the room was kept to a minimum.

A digital video camera (Canon XL1-S, Canon, Tokyo, Japan) was used for all video recordings. The camera faced the long side of the tank to allow full view of the animal's behavior. Recording was started before the crayfish was placed gently into the tank using a fish net. The camera was connected to a monitor (20 in; Sony WEGA, Sony, Tokyo, Japan) to observe the animal's behavior from a distance, and data were stored in S-VHS format. Tapes were later reviewed (by different experimenters) on a large TV monitor (27 in; Sony WEGA) using single-frame video analysis.

The effects of different EtOH concentrations on the behavior of Com crayfish were measured by immersing animals, of similar body size and mass (mean±s.d.), in 0.1 mol $l^{-1}$ EtOH ($N$=15, 3.25±0.08 cm, 0.84±0.02 g), 0.5 mol $l^{-1}$ EtOH ($N$=16, 3.19±0.09 cm, 0.85±0.02 g) or 1 mol $l^{-1}$ EtOH ($N$=24, 3.30±0.17 cm, 0.86±0.07 g) and video recording for up to 180 min to capture all intoxicated behaviors. Iso animals ($N$=20, 3.29±0.17 cm, 0.81±0.13 g) were tested in 1 mol $l^{-1}$ EtOH only using the same experimental protocol and results were compared with those for Com crayfish exposed to the same EtOH concentration. For both sets of experiments, the starting time ('onset') of different intoxicated behavior was measured in minutes after the start of the trial for each animal after reviewing the video recordings. The onset of tail-flipping was determined as the first occurrence of a tail-flip (which was always followed by additional tail-flips), and supine posture was measured when the animal first landed on its back and was unable to immediately right itself. At the end of the experimental period (either after 180 min of recording or when the animals remained on their backs for several minutes), the animals were transferred into a new tank with fresh deionized water. Animals displayed normal behaviors after a couple of hours in the new tank.

### Threshold tracking

Lateral giant interneuron (LG) threshold tracking in freely behaving crayfish using implanted wire electrodes was performed in groups of Com and Iso animals of similar size and mass (Com: $N$=11, 3.95±0.18 cm, 1.26±0.21 g; Iso: $N$=11, 3.85±0.23 cm, 1.30±0.15 g). All crayfish were anesthetized on ice for 15 min before surgery. A pair of stimulating electrodes was made from insulated silver wire (A-M Systems, Sequim, WA, USA). The insulation on the end was stripped to expose approximately 5 mm of bare wire (0.127 mm diameter). Under a microscope, small holes were made through the soft membrane on the ventral side of the abdomen close to the last abdominal (A6) ganglion using steel insect pins. Electrodes were carefully inserted into these holes to make contact with the ipsilateral sensory nerves (N2–5) that connect to A6. Once in place, electrode wires were glued (Loctite Super Glue Gel, Loctite, Düsseldorf, Germany) onto the hard exoskeleton near the insertion area. Crayfish (Com or Iso) were then placed in a tank (15×8×10 cm L:W:H) of 500 ml de-ionized water and electrode wires were connected to a pulse stimulator (Isolated Pulse Stimulator 2100, A-M Systems), which allowed for varying amounts of constant voltage to be applied to the sensory afferents.

After a period of recovery from surgery and acclimation, the activation threshold for the LG was found by increasing the stimulating voltage by 0.3 V from a 1 V starting voltage until a stereotyped LG tail-flip was produced. After a 15 min acclimation period, 1 h of LG threshold tracking began; if the animal tail-flipped, the voltage was reduced by 0.3 V and this new stimulus was presented after a 2 min inter-stimulus interval. If the animal did not tail-flip in response to the stimulus, the voltage was increased by 0.3 V. The voltage and corresponding behavior were recorded on paper and used for later analysis. After 1 h of threshold tracking in water, the animal was given a 15 min resting period before 1 h of threshold tracking in 1 mol $l^{-1}$ EtOH began. To create the EtOH bath, 23 g of 100% EtOH was added to the tank and the solution was stirred using a pipette without contacting or disturbing the animal. Stimuli were presented as before (in water) with each animal receiving 30 stimuli over the course of 1 h. After the experiment concluded, animals were removed from the EtOH bath, wires were cut, and animals were returned to individual holding tanks. Most animals recovered and lost the remaining wire tips during the next molt.

### Intracellular recordings

EtOH was mixed with normal crayfish saline (mmol $l^{-1}$: 202 NaCl, 5.37 KCl, 13.53 $CaCl_2$, 2.6 $MgCl_2$ and 2.4 Hepes). Saline and EtOH saline solutions were superfused over the preparation (see below) at a rate of 5 ml $min^{-1}$ using a Baxter flow control device (Baxter International Inc., Deerfield, IL, USA). Flow rate was verified before each experiment. Excess fluid was removed from the dish (40 ml volume) using a peristaltic pump (Thermo Scientific FH100 Series, Thermo Fisher Scientific, Waltham, MA, USA). All experimental preparations experienced 10 min superfusion of crayfish saline (baseline), 60 min of EtOH exposure at different concentrations, and 60 min of washout with crayfish saline. Intracellular glass electrodes were pulled from glass capillary tubes with filaments (World Precision Instruments, Sarasota, FL, USA; outer diameter: 1 mm, inner diameter: 0.58 mm) using a Sutter micropipette puller (Sutter Instruments, Novato, CA, USA) and backfilled with 2 mol $l^{-1}$ potassium acetate ($CH_3CO_2K$). Electrodes had resistances measuring between 20 and 35 MΩ. LG axons were impaled immediately rostral to the terminal abdominal ganglion (A6). Teflon-coated silver hook electrodes (uncoated

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diameter 0.127 mm) were placed on ipsilateral nerve roots 2–5 of A6. A second extracellular hook was placed more rostral on the ventral nerve cord to record activity of other ascending interneurons and to verify successful impalement of the LG.

Stimuli were presented with an inter-stimulus interval of 90 s and LG threshold was verified to be at a consistent voltage for 10 min during saline superfusion. The stimulus was reduced to 0.2 V below threshold before EtOH superfusion was started. The preparations were exposed to crayfish saline (controls), 20 mmol $l^{-1}$ EtOH or 100 mmol $l^{-1}$ EtOH. During the 60 min of EtOH exposure, the preparation received 40 stimuli and the total number of evoked LG action potentials was recorded. Electrophysiology data were collected using microelectrode amplifiers (Axoclamp 900A, Molecular Devices, Sunnyvale, CA, USA), an A-M systems differential amplifier (Model 1700) and a Grass stimulator (Model S88, Natus Medical Incorporated, Pleasanton, CA, USA) for recording and stimulation using extracellular hook electrodes. A Digidata 1440A (Molecular Devices) was used to digitize analog data. Data were recorded and stored using pClamp 10.4 and Clampfit 10.4 software (Molecular Devices). All electrophysiological recordings were performed in a grounded faraday cage.

For intracellular experiments involving semi-intact preparations, Com or Iso crayfish were pinned down dorsal-side up in a Sylgard-lined dish. The dorsal cuticle and most of the muscles of the abdomen were removed to provide access to the ventral nerve cord and the dorsal LG neurons. The head and thorax were left intact and connected to the abdomen. Com and Iso animals of similar size were used for all experiments: 20 mmol $l^{-1}$ EtOH (Com: $N$=7, 3.46±0.29 cm; Iso: $N$=5, 3.58±0.13 cm) and 100 mmol $l^{-1}$ EtOH (Com: $N$=7, 3.53±0.29 cm; Iso: $N$=6, 3.73±0.23 cm).

For intracellular experiments involving reduced tail preparations, the animals were prepared as described for semi-intact preparations except the abdomen was surgically removed from the head and thorax. Com and Iso animals of similar size were used for all experiments: 20 mmol $l^{-1}$ EtOH (Com: $N$=6, 3.65±0.14 cm; Iso: $N$=6, 3.7±0.2 cm) and 100 mmol $l^{-1}$ EtOH (Com: $N$=6, 3.65±0.21 cm; Iso: $N$=6, 3.68±0.18 cm).

Control animals for semi-intact preparations (Com: $N$=5, 3.76±0.15 cm; Iso: $N$=6, 3.63±0.16 cm) and tail preparations (Com: $N$=4, 3.65±0.1 cm; Iso: $N$=4, 3.68±0.24 cm) were exposed to 1 h of normal crayfish saline instead of EtOH.

### Statistical analysis

IBM SPSS Statistics v23 (IBM, Armonk, NY, USA) was used for all statistical tests. All applied statistical tests and corresponding test results are described in the text.

## RESULTS

To study the acute effects of alcohol on crayfish natural behavior, we observed juvenile crayfish in an aquarium that was filled with EtOH solutions of different concentrations. After the animals were placed into the EtOH bath, their behaviors were recorded and later quantified using single-frame video analysis. Three discrete and unique behaviors following intoxication not typically observed in unexposed crayfish were readily identified as long as the EtOH concentration of the bath was high enough (Fig. S1 and Movie 1). The first behavior was an elevated stance with fully extended walking legs and upheld abdomen that lasted for a few seconds. The next (and more dramatic) change occurred when animals started to produce spontaneous tail-flips, i.e. short swimming sequences mediated by rapid flexions of the abdomen. The final behavioral change was characterized by loss of motor coordination when crayfish fell on their back (into a supine position) and had difficulty returning to an upright posture. These discretely observed behavioral alterations expressed by crayfish in the EtOH bath indicate that internal alcohol levels rise over time and lead to increased behavioral excitability followed by behavioral inhibition, a typical pattern observed in most organisms after alcohol consumption (e.g. Oscar-Berman and Marinkovic, 2007). Interestingly, the EtOH-mediated elevated posture (stance) closely resembled the ‘aggressive posture’ that has previously been described in crayfish after systemic injection of serotonin (Livingstone et al., 1980). This may suggest that some of the early EtOH-induced effects we observed could be mediated through interactions between alcohol and the serotonergic system.

Our subsequent detailed behavioral analysis focused on only two behavioral changes (tail-flipping and supine position) because they were easily and unambiguously identifiable in all trials. We first tested the effects of three different EtOH concentrations (0.1, 0.5 and 1 mol $l^{-1}$) in the bath and found that EtOH-induced behaviors were expressed in a dose-dependent manner (Fig. S2).

While animals that were exposed to 1 mol $l^{-1}$ EtOH ($N$=24) experienced spontaneous, unprovoked tail-flipping on average after 20.1±0.8 min (mean±s.e.m.), animals in 0.5 mol $l^{-1}$ EtOH ($N$=16) required more than twice the amount of time (45.6±2.6 min), and crayfish exposed to 0.1 mol $l^{-1}$ EtOH ($N$=15) showed an even greater response delay (106.4±18.2 min). The measured behavioral response latencies of all three groups (1, 0.5 and 0.1 mol $l^{-1}$) were significantly different (Kruskal–Wallis test, $P$<0.01). Interestingly, in the group exposed to 0.1 mol $l^{-1}$ EtOH, nine individuals never produced tail-flips during the 3 h recording period, suggesting they never reached the level of intoxication needed to elicit this behavior. A similar picture emerged when latencies for supine position were quantified. Animals in 1 mol $l^{-1}$ EtOH first landed on their backs after 35.9±1.6 min, but this occurred significantly later in 0.5 mol $l^{-1}$ EtOH (71.9±3.8 min). The latencies for the 1 and 0.5 mol $l^{-1}$ groups were significantly different (Mann–Whitney $U$-test, $P$<0.01). Only three of all tested individuals placed into the lowest concentration (0.1 mol $l^{-1}$) EtOH bath displayed a supine position during the 3 h recording period and after much longer latencies (162.2±5.8 min).

As all tested crayfish were taken from a communal tank where they were housed for several weeks with a large number (50–100) of conspecifics, we next investigated whether animals deprived of social contact would produce spontaneous tail-flips and supine position at similar latencies after EtOH exposure (Fig. 1).

Animals were individually isolated for 1 week (Iso group) and tested in a 1 mol $l^{-1}$ EtOH bath ($N$=19); these Iso animals were of equal size and mass to animals that had been communally housed (Com animals). Iso crayfish began showing spontaneous tail-flips on average after 28.4±1.8 min, which is approximately 8 min later and statistically different from Com crayfish (Mann–Whitney $U$-test, $P$<0.01). Iso crayfish also experienced supine position significantly (approximately 7 min) later than Com crayfish (43.1±1.9 min; Mann–Whitney $U$-test, $P$<0.01). This result indicates that crayfish from an environment that provides opportunities for frequent interactions with other conspecifics are more sensitive to acute alcohol exposure than animals that are depleted of any social interactions for 1 week.

Next, we investigated whether the social effects on the interplay between EtOH and behavior were paralleled at the level of the nervous system. As the neural circuits underlying tail-flipping behavior in crayfish have been intensively investigated for years, are well understood and are easily accessible (Edwards et al., 1999;



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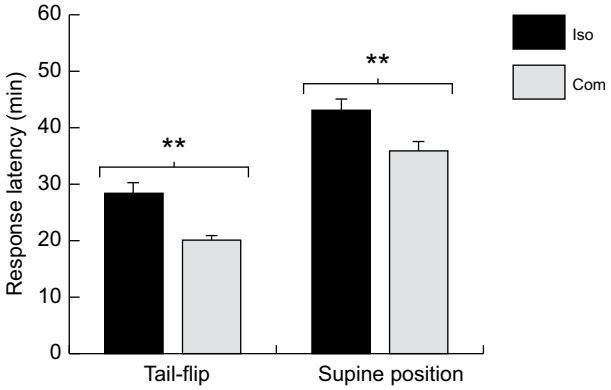


**Fig. 1. Crayfish with recent social experience are behaviorally more sensitive to ethanol exposure than socially isolated animals.** The time to the onset of spontaneous tail-flipping behavior and the first occurrence of the crayfish in a supine position was measured (mean±s.e.m) after exposure to 1 mol $l^{-1}$ ethanol (EtOH) in the water. Comparison of behavioral response latencies between the two social groups showed significantly faster responses in crayfish with recent social experience (Com; $N$=24) than in socially isolated animals (Iso; $N$=20) for both tail-flip (Mann–Whitney $U$-test, **$P$<0.01) and supine position (Mann–Whitney $U$-test, **$P$<0.01).

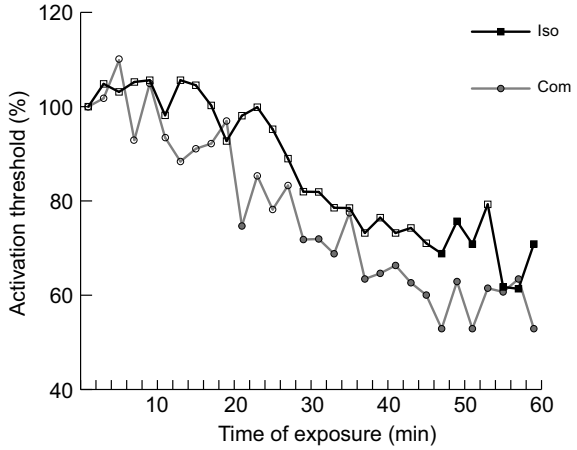


**Fig. 2. EtOH exposure reduces lateral giant interneuron (LG) activation threshold more strongly in Com than in Iso crayfish.** LG activation threshold (mean) was tracked for 60 min in Com ($N$=11) or Iso ($N$=11) animals implanted with electrodes and exposed to 1 mol $l^{-1}$ EtOH. Threshold values were normalized to the starting threshold at the beginning of the EtOH exposure experiment. Filled symbols represent time points that were significantly different from starting threshold (Wilcoxon signed-rank test, $P \leq 0.05$) whereas open symbols represent time points that were not significantly different (Wilcoxon signed-rank test, $P$>0.05).

Herberholz et al., 2002), we first focused on changes in circuit activation expressed in freely behaving animals. We implanted a pair of fine silver wires into the abdomen of both Iso ($N$=11) and Com crayfish ($N$=11) of equal size and mass, and measured changes in the excitability of the LG after exposure to 1 mol $l^{-1}$ EtOH. A single action potential in LG is known to produce a tail-flip response in crayfish, and the LG neurons can be activated with sufficient sensory input from tail afferents in freely behaving animals (Yeh et al., 1996; Krasne et al., 1997). Wires were placed above the sensory nerves of the last abdominal ganglion (A6), and afferents providing sensory input to the LG neuron were stimulated every 2 min with electric shocks. By varying the intensity of the shock just below and just above LG threshold depending on whether the animals tail-flipped or not, we were able to track LG thresholds for 1 h in water and for another hour in EtOH. Over the first hour in water, we observed slight fluctuations in LG circuit excitability, with most Iso crayfish showing a minor increase and most Com crayfish showing a minor decrease in excitability at the end of the first hour when compared with the normalized starting threshold of 100% (Fig. S3). Although the difference was not significant between the two groups, the slight decrease in LG excitability seen in Com animals during the last 10 min of testing in water (104.1±8%) matches results from an earlier study that used a similar method and reported slight increases in LG threshold over time in socially experienced (dominant and subordinate) crayfish (Krasne et al., 1997). However, we also found that LG circuit excitability increased in Iso animals during the last 10 min of the first hour in water (91.1±3.6%), which is a new observation that warrants future investigation and will be discussed again below.

Following exposure to 1 mol $l^{-1}$ EtOH, the LG threshold substantially decreased in both Iso and Com crayfish, indicating that EtOH enhanced the excitability of the LG tail-flip circuit in both animal groups (Fig. 2). In addition, we found that LG threshold decreased more strongly and more quickly in Com than in Iso animals, thus indicating higher neural sensitivity to EtOH in communally housed crayfish and paralleling our previous behavioral results. This shows that the observed differences in behavioral responses of Com and Iso crayfish to EtOH exposure are paralleled by differences in neural circuit excitability.

We then wanted to know whether these socially mediated effects could be observed at the level of a single neuron. We measured intracellular LG threshold changes after EtOH exposure using single-cell electrophysiology in semi-intact preparations. We selected Com and Iso animals as described above and recorded LG activity in the abdomen of mostly intact animals that were pinned down in a Petri dish. The LG neuron was impaled with a fine glass electrode and sensory afferents were stimulated with silver hook electrodes. Using a superfusion system, preparations were exposed to two different concentrations of EtOH (20 and 100 mmol $l^{-1}$) that we estimated to be similar to low and high internal concentrations experienced in our experiments with freely behaving animals. To measure changes in LG excitability, the sensory stimulus was set at a voltage that produced a sizable excitatory postsynaptic potential in LG, but was below threshold. We recorded baseline for 10 min at an inter-stimulus interval of 90 s (with no action potentials observed) and continued the stimulation while EtOH was superfused into the dish and onto the preparation for 1 h (40 stimuli total). This was then followed by a 60 min washout with fresh saline.

Both Iso ($N$=11) and Com animals ($N$=14) of equal size were tested. We found that the effect of previous social experience on LG excitability was preserved in these recordings of semi-intact preparations. LG was brought to threshold in more Com preparations (79%) than Iso preparations (55%) (Fig. 3A), but the largest difference in activated preparations and corresponding LG excitability (i.e. evoked action potentials) was observed with 20 mmol $l^{-1}$ EtOH (Fig. 3B). In fact, fewer Com preparations were activated by 100 mmol $l^{-1}$ EtOH than by 20 mmol $l^{-1}$ EtOH, and they produced fewer LG action potentials (20 mmol $l^{-1}$: 19.7±6.17, 100 mmol $l^{-1}$: 12.43±4.75), indicating that this EtOH concentration may have resulted in a combination of excitatory and inhibitory effects. This was not the case for Iso preparations, however, where higher EtOH concentration generated more activity (20 mmol $l^{-1}$: 8.2±7.24, 100 mmol $l^{-1}$:16.67±6.0). Washout with normal crayfish saline did not change the number of observed action potentials significantly (Wilcoxon signed-rank test, $P$>0.05) for

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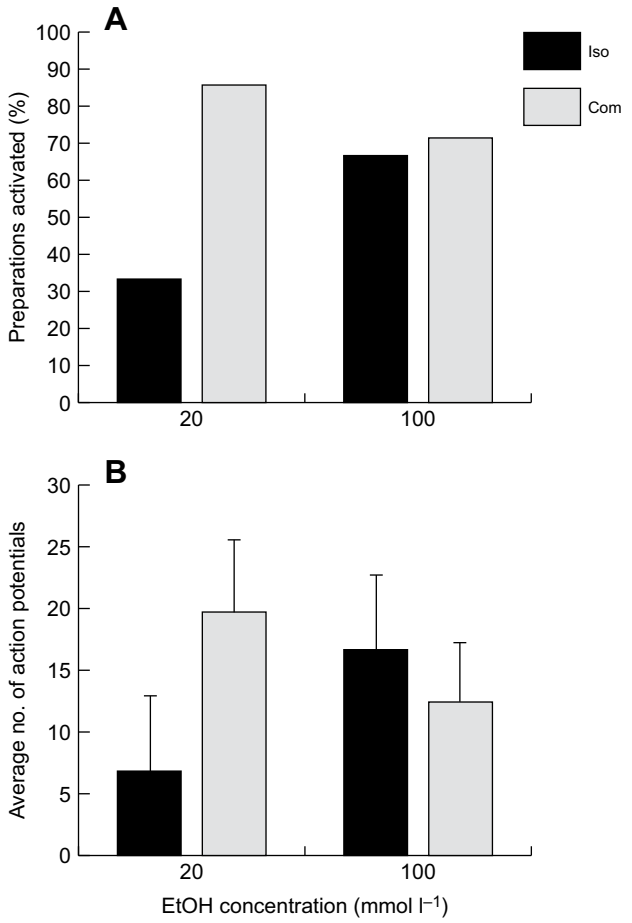


**Fig. 3. Semi-intact Com preparations are sensitive to lower EtOH concentrations than Iso preparations.** Com or Iso preparations were exposed to 20 mmol $l^{-1}$ EtOH (Com: *N*=7, Iso: *N*=5) or 100 mmol $l^{-1}$ EtOH (Com: *N*=7, Iso: *N*=6) dissolved in crayfish saline. (A) Percentage of preparations exposed to EtOH that produced action potentials during exposure. (B) Mean (±s.e.m.) number of action potentials produced in each preparation exposed to EtOH.

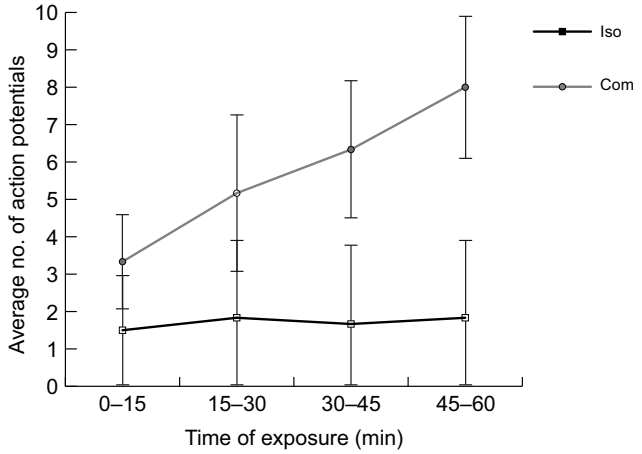


**Fig. 4. EtOH increases LG excitability in Com but not Iso preparations.** Mean (±s.e.m.) number of action potentials produced by Com (*N*=7) and Iso preparations (*N*=5) in four 15 min time intervals during 1 h of exposure to 20 mmol $l^{-1}$ EtOH. Each preparation received 10 stimuli during each time bin. Values in different time bins were compared with the number of action potentials produced during saline baseline before EtOH was applied to the preparations. Filled symbols correspond to values significantly different from baseline (Wilcoxon signed-rank test, $P \leq 0.05$) whereas open symbols represent time points that were not significantly different (Wilcoxon signed-rank test, $P > 0.05$).

Com crayfish (20 mmol $l^{-1}$: 25.57±7.26, 100 mmol $l^{-1}$: 16.33±7.2) or Iso crayfish (20 mmol $l^{-1}$: 8.2±7.95, 100 mmol $l^{-1}$: 10.5±6.17). The resilience of LG to saline washout has also been shown in other studies following exposure to different neuropharmacological agents such as serotonin and picrotoxin (Yeh et al., 1996; Vu et al., 1997).

As the difference between Com and Iso preparations was most apparent in the 20 mmol $l^{-1}$ EtOH condition, we measured whether this difference was expressed at different time points over the course of the 1 h of EtOH exposure. We counted the average number of action potentials of Com (*N*=7) and Iso preparations (*N*=5) in 15 min intervals during EtOH exposure. We found that the number of action potentials increased for each 15 min interval for Com preparations, and it was significantly different from baseline at the first and last two time intervals (Wilcoxon signed-rank test, $P<0.05$ for each time interval). However, no significant increase in the average number of LG action potentials was found for any time point in Iso preparations (Fig. 4). Thus, 20 mmol $l^{-1}$ EtOH elicited a strong excitatory effect in Com preparations, and this effect became more apparent as the concentration of EtOH increased.

This result shows that our observation of EtOH sensitivity in freely behaving animals is paralleled at the single-neuron level. Importantly, the effects of prior social experience on EtOH sensitivity are present in an identified neural circuit. As experiments in the semi-intact preparation are influenced by descending inputs from the brain and thorax onto the local LG circuit, and it has previously been shown that LG excitability is modified by descending GABAergic tonic inhibition (Vu and Krasne, 1993; Vu et al., 1993), we next investigated the effect of EtOH on the LG circuit in an isolated tail preparation. We prepared the animals in a similar way to the semi-intact preparations, but the tail was separated from the thorax, removing any descending neuronal inputs onto the local LG circuit. Stimulation of sensory afferents and electrophysiological recordings were performed identically to the semi-intact preparation experiment.

We repeated the protocol previously used for semi-intact preparations, and measured LG action potentials in Com (*N*=6) and Iso preparations (*N*=6) using the two different EtOH concentrations (20 and 100 mmol $l^{-1}$). Unlike in the semi-intact experiment, where we found significant enhancement of excitability in Com preparations with 20 mmol $l^{-1}$ EtOH, only a small effect of EtOH on LG excitability was observed when the rostral nervous system was disconnected from the abdomen (Fig. 5A).

Moreover, the percentage of Com preparations (17%) and Iso preparations (17%) that were activated by 20 mmol $l^{-1}$ EtOH no longer differed. There was also no significant difference in the average number of LG action potentials evoked with this EtOH concentration (Com: 0.17±0.17; Iso: 1.5±1.5). Thus, when we compared the number of action potentials in tail preparations with the previously recorded number for semi-intact preparations at this concentration, we found that removal of descending inputs from the brain and thorax caused a significant decrease in LG excitability, but only in Com preparations (Mann–Whitney *U*-test, $P<0.05$).

This suggests that preserving 'normal' descending inputs onto the LG facilitates its sensitivity to EtOH, but more in Com than Iso preparations. After superfusion of 100 mmol $l^{-1}$ EtOH into the dish, we observed more activated preparations, with slightly more for

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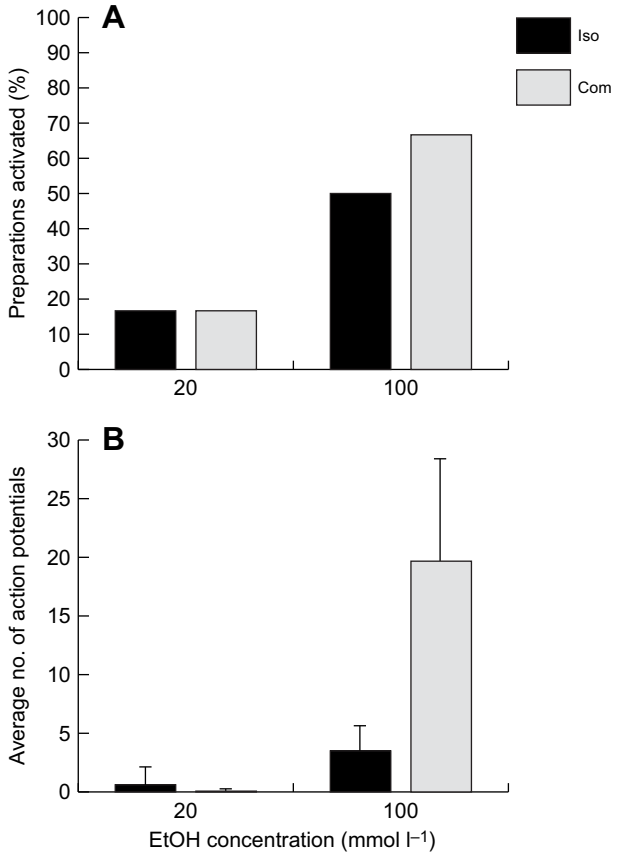


**Fig. 5. Sensitivity of LG to EtOH is reduced in tail preparations, but socially mediated differences remain.** Abdomens of juvenile crayfish were separated from the head and thorax to remove any descending inputs from these areas of the nervous system. (A) Percentage of Com and Iso preparations that produced action potentials during exposure to 20 mmol $l^{-1}$ EtOH (Com: *N*=6, Iso: *N*=6) and 100 mmol $l^{-1}$ EtOH (Com: *N*=6, Iso: *N*=6). (B) Mean (±s.e.m.) number of action potentials produced by all preparations.

Com (67%) than for Iso (50%). In addition, although the difference between Com and Iso preparations was not significant (Mann–Whitney *U*-test, $P>0.05$), an effect of prior social experience on EtOH sensitivity was observed at this higher EtOH concentration; Com preparations (19.67±8.51) fired many more action potentials than Iso preparations (3.5±2.08) in 100 mmol $l^{-1}$ EtOH (Fig. 5B). Washout with fresh saline reduced the number of action potentials in Com (7.83±6.53) but not Iso preparations (7.17±6.58); however, the difference was not significant (Wilcoxon signed-rank test, $P>0.05$). This is similar to results from a previous study of LG excitability in crayfish. Yeh et al. (1996) reported that washout of serotonin was successful in preparations from animals following agonistic pairings, but not in socially isolated animals.

Together, these results suggest that EtOH exerts both global and local effects on LG excitability. The sensitivity to EtOH increases when descending inputs from the brain are present, and the increase is more pronounced in Com than in Iso crayfish. This result allows us to speculate that EtOH might interact with the well-known brain-derived GABAergic tonic inhibition that regulates LG excitability in freely behaving animals (Krasne and Teshiba, 1995; Krasne and Edwards, 2002), and differences in the GABAergic system brought about by different social experiences mediate this effect. It does not exclude other mechanisms, of course, and specifically interactions of EtOH with other neurotransmitter systems, but this needs to be investigated in future experiments.

Motivated by our finding regarding the suggested interplay between social experience and brain-derived descending inputs onto LG, we performed a final set of experiments to explore whether descending neural inputs to the LG circuit would be present even in the absence of alcohol exposure, and whether this effect might be mediated by prior social experience. We superfused Com and Iso preparations, both semi-intact (*N*=6 for both groups) and tail only (*N*=4 for both groups), with crayfish saline for 1 h (i.e. replacing EtOH with saline for the entire experiment) while recording changes in excitability of the LG neuron. Interestingly, we found that several semi-intact preparations (Com: 33%; Iso: 50%) with intact descending inputs produced LG action potentials during the 1 h saline treatment (Com: 5.83±4.74; Iso: 9.83±5.58). However, in tail preparations without any descending inputs to LG, only one Iso preparation produced two action potentials during the entire hour of saline treatment, and LG never reached threshold in any of the Com preparations (Fig. S4). This shows that LG excitability increases over time when the circuit is repeatedly stimulated and only saline is superfused over the preparation; however, the increase is more pronounced when descending inputs onto the local circuit are intact and, most surprisingly, it is stronger in socially isolated animals than in communally housed animals. It is notable that the increase in LG excitability observed in Iso preparations in these experiments mirrors our finding in freely behaving animals that LG became more excitable in Iso animals after the first hour of stimulation in water alone (i.e. before EtOH). This result is opposite to what we observed after EtOH application: Com animals were more sensitive than Iso animals to EtOH, suggesting that social effects on alcohol intoxication are observed despite a naturally occurring tendency of LG excitability to increase in socially isolated animals. Because these differences were most pronounced in intact animals and semi-intact preparations, they are most likely related to differences in brain-derived descending inputs to the LG circuit. Although we currently cannot fully explain this result, it is intriguing and warrants further investigation.

## DISCUSSION

We have shown here for the first time (to the best of our knowledge) that the acute effects of alcohol on single neurons and corresponding behavior are modulated by prior social experience. Our results suggest that these differences are based on interactions of EtOH with known neurotransmitter systems, possibly 5-HT and GABA. Previous work in crayfish has shown that the effects of 5-HT on LG excitability depend on the animal's social status (Yeh et al., 1996), and thus we hypothesize that exposure to different social environments (communal housing versus social isolation) causes lasting changes to neurotransmitter systems, possibly by altering distributions of 5-HT and GABA receptors, and thereby creating new or different targets for EtOH. Thus, acute alcohol exposure affects individuals differently because their nervous system changes according to their social environment, which alters the interactions between alcohol, brain receptors, neural activity and corresponding behavior.

Although crayfish are known to interact frequently with conspecifics when housed in communal tanks, and they form dominance relationships and social hierarchies through pairwise agonistic encounters (e.g. Issa et al., 1999; Herberholz et al., 2007, 2016; Graham and Herberholz, 2009), we cannot exclude the possibility that other factors, such as the difference in personal space between communally housed and isolated animals, may have contributed to our observed effects. For example, more crowded conditions could have led to higher gill ventilation rates and thus

higher alcohol intake in Com animals. This seems unlikely, however, because Com and Iso animals were removed from their respective tanks and behaviorally tested for EtOH effects in a new tank under identical conditions, and ventilation rates in crayfish return to baseline quickly after disturbance (Schapker et al., 2002). Moreover, we obtained similar results in reduced preparations of Com and Iso crayfish, including isolated nerve cords, which clearly point toward neuronal changes underlying the difference between the two groups. Nonetheless and despite the established relationship between social experience, neurotransmitter action and LG excitability in crayfish, further experimentation is needed to investigate other possible contributions to the observed behavioral differences.

We do not yet know the exact neurocellular mechanisms of interactions of EtOH with the LG circuit; however, the accessibility of the circuit as well as its suitability for neurochemical investigations makes the crayfish a great model for future alcohol research. Our observation that EtOH exerts a local effect on the LG is promising because the circuit is anatomically and functionally fully characterized (e.g. Antonsen et al., 2005; Liu and Herberholz, 2010), and both the excitatory and inhibitory actions of alcohol can be measured in one tractable circuit. Neurotransmitter systems such as GABA and 5-HT are likely involved and their individual and combined roles can be elucidated with neuropharmacological manipulations. In addition, as the sensitivity to alcohol was reduced after removal of descending inputs from the brain onto the LG circuit, we can begin to form and test discrete hypotheses. For example, it is possible that after removal of tonic descending inhibition from the brain, GABA receptors on the LG become available targets for alcohol, which leads to stronger inhibitory effects. Thus, higher EtOH concentrations are needed to bring the LG to threshold because alcohol-induced inhibition dominates its excitatory effects under these conditions. As the socially mediated differences were preserved in these experiments, (currently unknown) alterations of GABA receptors in socially isolated and socially experienced animals may at least in part be responsible for this effect.

Although somewhat speculative at this point, it is tempting to suggest that the reduced sensitivity to alcohol we observed in socially isolated crayfish underlies the increase in drinking behavior that has been widely reported in socially isolated mammalian species (Wolffgramm and Heyne, 1991; Helms et al., 2012). If social isolation causes a suppression of the alcohol-induced acute neurobehavioral response, it would be reasonable to expect that humans and non-human animals increase drinking after social isolation (or 'exclusion') as a result of the lower sensitivity to the cellular effects of alcohol. It is currently unclear whether our results in crayfish generalize to neural circuits and corresponding behavior in mammals, but they can certainly inform those studies, and in the long term will hopefully contribute to the development of better options for the treatment and prevention of the negative consequences of alcohol abuse.

Although our current study was primarily concerned with the effects of EtOH on tail-flip latencies and corresponding neural circuitry, it has opened the door for investigation of other important questions related to alcohol intoxication, e.g. its evolutionarily conserved effects on locomotor behavior, learning and memory, aggression, and decision making (Paulus and Geyer, 1997; Liden et al., 2010; Alcaro et al., 2011; Luchiari et al., 2015). Lastly, crayfish have been shown to exhibit behavioral manifestations such as drug seeking, withdrawal, and tolerance related to other substances of abuse (Huber et al., 2011), and thus additional neurobehavioral experiments that extend beyond the acute effects of alcohol exposure seem feasible. This is an exciting avenue for future studies because of prior findings illustrating that differences in sensitivity to acute alcohol consumption in humans strongly predict later alcohol dependence (Mayfield et al., 2008).

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#### Competing interests
The authors declare no competing or financial interests.

#### Author contributions
J.H. and M.E.S. designed the study. M.E.S. and A.R.L. conducted experiments. All authors performed data analysis and statistical testing. J.H. and M.E.S. wrote the manuscript.

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