ORIGINAL INVESTIGATION

$GABA_A$ receptors in the dorsal raphé nucleus of mice: escalation of aggression after alcohol consumption

Aki Takahashi · Carolyn Kwa · Joseph F. DeBold ·
Klaus A. Miczek

Abstract

Rationale The dorsal raphé nucleus (DRN), the origin for serotonin (5-HT) in forebrain areas, has been implicated in the neural control of escalated aggression. Gamma aminobutyric acid type-A ($GABA_A$) and type-B ($GABA_B$) receptors are expressed in the DRN and modulate 5-HT neuronal activity, and both play a role in the behavioral effect of alcohol.

Objective The purpose of this study is to examine the interaction between drugs acting on GABA receptors in the DRN and alcohol in their effects on aggressive behaviors.

Method Male CFW mice, housed with a female, were trained to self-administer ethanol (1.0 g/kg) or water via an operant conditioning panel in their home cage. Immediately after they drank either ethanol or water, the animals were microinfused with a GABAergic drug into the DRN, and their aggressive behaviors were assessed 10 min later. Muscimol (0.006 nmol), a $GABA_A$ receptor agonist, escalated alcohol-heightened aggression but had no effect in the absence of ethanol. This effect of muscimol was prominent in the animals that showed alcohol-heightened aggression, but not the animals that reduced or did not change aggressive behavior after ethanol infusion compared to water. On the other hand, the $GABA_B$ agonist baclofen (0.06 nmol) increased aggressive behavior similarly in both water and ethanol conditions. Antagonists of the $GABA_A$ and $GABA_B$ receptors, bicuculline (0.006 nmol) and

phaclofen (0.3 nmol) respectively, did not suppress heightened-aggressive behavior induced by ethanol self-administration.

Conclusion $GABA_A$ receptors in the DRN are one of the neurobiological targets of alcohol-heightened aggression. Activation of the $GABA_B$ receptors in the DRN also produced escalated aggression, but that is independent of the effect of alcohol.

Keywords Alcohol · Escalated aggression · Dorsal raphé nucleus (DRN) · Serotonin (5-HT) · $GABA_A$ receptors · $GABA_B$ receptors

Introduction

More than any other drug, alcohol has been linked to violence and aggression (Miczek et al. 2002, 2004b). Not every individual increases aggressive behavior after alcohol intake, but only a subset of individuals is prone to escalate aggressive behaviors under the influence of alcohol. Preclinical studies using rodents have shown that approximately 30% of individuals escalated aggressive behaviors under the influence of a moderate dose of ethanol (1.0 g/kg) relative to their base level of fighting in the absence of ethanol (Miczek et al. 1992, 1998). This individual difference on alcohol-heightened aggression has been reported in several species including humans, non-human primates, rats, and mice (Fulwiler et al. 2005; Higley et al. 1996; Miczek et al. 1998, 2004b; Virkkunen et al. 1996). The neurobiological basis for the vulnerability for alcohol-heightened aggression has begun to be investigated with a focus on ionotropic receptors.

Most prominently, γ-aminobutyric acid A ($GABA_A$) receptors have been studied extensively as one of the major

A. Takahashi · C. Kwa · J. F. DeBold · K. A. Miczek (✉)
Department of Psychology, Tufts University,
Bacon Hall, 530 Boston Ave,
Medford, MA 02155, USA
e-mail: klaus.miczek@tufts.edu

targets of alcohol (for review, see Kumar et al. 2009). Acute alcohol increases the conductance of Cl⁻ flux through the GABA$_A$ receptors (Aguayo 1990; Allan and Harris 1987; Palmer and Hoffer 1990; Suzdak et al. 1986), and chronic exposure to alcohol changes the composition of receptor subunits in the synaptic membrane (Devaud et al. 1995; Liang et al. 2007; Matthews et al. 1998; Papadeas et al. 2001). Prototypical GABA$_A$ receptor positive modulators (e.g., benzodiazepines, barbiturates, and neurosteroids) can induce behavioral effects that are similar to those of alcohol including anxiolytic, anticonvulsant, sedative, hypnotic, and pro- and anti-aggressive effects (Barnard et al. 1998; Dar and Wooles 1985; Korpi et al. 2002; Liljequist and Engel 1982; Olsen 1982; Rabow et al. 1995; Sieghart 1995). Low to moderate doses of benzodiazepines and some neurosteroids increase aggressive behaviors in a receptor-selective manner in humans and other species; whereas, higher doses have anti-aggressive effects (Arnone and Dantzer 1980; Bond et al. 1995; Christmas and Maxwell 1970; Cole and Wolf 1970; DiMascio 1973; Ferrari et al. 1997; Fish et al. 2001; Miczek 1974; Olivier et al. 1985; Weerts and Miczek 1996; Weisman et al. 1998). This pattern of effects implicates the GABA$_A$ receptor complex as a target for aggression-heightening effects of alcohol. An anatomically discrete analysis is required to identify which brain pathways are regulated by the GABA$_A$ receptors to promote alcohol-heightened aggression.

Although there is no clear structural interaction with alcohol, GABA$_B$ receptors also appear to play a role in some of the behavioral effects of alcohol. Preclinical and clinical data have shown that the GABA$_B$ receptor agonists and positive modulators reduce alcohol withdrawal symptoms (Addolorato et al. 2006; Colombo et al. 2000; File et al. 1991) and also change self-administration of alcohol (Besheer et al. 2004; Colombo et al. 2003; Daoust et al. 1987; Flannery et al. 2004; Maccioni et al. 2008; Moore et al. 2007; Orru et al. 2005; Walker and Koob 2007). Alcohol can modulate the GABA$_B$ receptor expression in the rat cerebral cortex (Li et al. 2005), and electrophysiological studies have shown that the GABA$_B$ receptor modulates alcohol effects (Ariwodola and Weiner 2004; Wu et al. 2005). GABA$_B$ receptor activation can escalate aggressive behaviors when baclofen was systemically administered or microinjected into the dorsal raphé nucleus (DRN) in mice (Takahashi et al. 2010). Thus, it is possible that GABA$_B$ receptors are also involved in the alcohol-heightened aggression.

The serotonin (5-HT) system has long been implicated in neurobiological mechanisms of escalated aggression or violence (de Boer and Koolhaas 2005; Miczek et al. 2004a; Olivier 2004), and much evidence suggests the 5-HT system as a major target of alcohol-heightened aggression (Cloninger et al. 1989; Virkkunen et al. 1996;

Virkkunen and Linnoila 1993). Gene expression analysis found reduced 5-HT receptor mRNA expressions in several forebrain areas in the male mice that engaged in alcohol-heightened aggression relative to the animals that did not change their aggressive behavior (Chiavegatto et al. 2010). Chronic treatment with an SSRI inhibited the heightened aggression induced by the alcohol without changing the species-typical aggression in mice (Caldwell and Miczek 2008). Therefore, differential activation of the 5-HT system by alcohol may contribute to the individual vulnerability for alcohol-heightened aggression. Forebrain 5-HT is mainly derived from the DRN (Azmitia and Segal 1978; Dahlstrom and Fuxe 1964; Michelsen et al. 2007).In addition to 5-HT cells, a large number of GABA neurons can be found in the DRN, and they modulate the activity of 5-HT neurons (Belin et al. 1983; Gervasoni et al. 2000; Nanopoulos et al. 1982; Wang et al. 1992). Both GABA$_A$ and GABA$_B$ receptors are expressed on the 5-HT neurons in the DRN and inhibit 5-HT cell firing (Bowery et al. 1987). Due to its role in the behavioral action of alcohol, GABA receptors may modulate the DRN 5-HT system after alcohol consumption and thus, change aggressive behaviors. In this study, we modulated the GABA$_A$ or GABA$_B$ receptors in the DRN pharmacologically and examined the resulting changes in alcohol-heightened aggression in male mice.

Methods

Subjects

Male CFW mice (Charles River Laboratories, Wilmington, MA), weighed 21–23 g upon arrival. Resident males were housed in pairs with females in a clear polycarbonate cage (28×17×14 cm) with pine shavings as bedding material. Intruder males were group housed seven to ten per large cage (48×26×14 cm) with corn cob bedding. All animals were maintained in our vivarium with controlled humidity and temperature (35–40%, 21±1°C) on a reversed 12-h-light/dark cycle (lights off at 7:00 AM). Food (Purina, St. Louis, MO) was freely available, whereas availability of water was limited to 3 h per day. All procedures were approved by the Institutional Animal Care and Use Committee of Tufts University. The animals were cared for according to the "Guide for the Care and Use of Laboratory Animals" (National Research Council 1996).

Ethanol self-administration

After 21 h of water restriction, the female and offspring were removed to a holding cage during the experimental session, and a custom designed aluminum panel (16.5× 15.9 cm) was inserted into the resident's home cage

(Miczek and de Almeida 2001). The panel has two nose-poke operanda with drinking troughs (3×5 cm) on the right and left sides of the panel with cue lights positioned above each operandum and one house light at the center top (Med Associates, Georgia, VT). The fluid receptacle was connected to a syringe containing either water or ethanol (6% *w/v*), operated by a syringe pump (Med Associates). The panel and syringe pump were controlled by MED-PC software running on a PC (MED-PC for Windows, v. 4.1; Med Associates). During the session, a house light and a cue light over the active operandum (right or left side, counterbalanced across the animals) were illuminated. A nose-poke response was detected by the photobeam sensor in the operanda, and every fifth response into the active operandum was reinforced by the delivery of 50 μl of fluid into the trough (schedule of reinforcement, fixed ratio five). The animals self-administered water or ethanol 5 days per week between 12:00 and 17:00, with ethanol (1.0 g/kg) being available every third experimental session and water on the intervening days.

Resident-intruder test and alcohol-heightened aggressive behavior

After 3 weeks of being housed with a female, the residents were studied for their aggression toward the same intruder male (Miczek and O'Donnell 1978). The female and the pups were removed, and an intruder was introduced into the home cage of the resident male. Their behaviors were recorded for 5 min after the first attack bite or the intruder was removed after 5 min if no attack occurred. This encounter occurred every other day until the animals showed a stable number of attack bites.

Once aggressive behavior had stabilized (<20% variation), the residents were assessed for alcohol-heightened aggression. Fifteen minutes before the resident-intruder encounter, the residents self-administered water or 1.0 g/kg of ethanol (in an equal volume, 16.9 ml fluid/kg). Aggressive behavior was examined three times per week separated by 48 h, alternatively after consuming either water or ethanol for a total of three times for each condition. These encounters were videotaped and analyzed with the aid of software described later. Animals were categorized as alcohol-heightened aggressors (AHA) if the rate of attack bites after ethanol consumption exceeded that after water consumption by at least two standard deviations. The remaining mice were designated as alcohol non-heightened aggressors (ANA).

Surgery and cannulation

After the stabilization of attack bites, residents were anesthetized by i.p. injection of a mixture of 100 mg/kg of ketamine HCl and 10 mg/kg of xylazine, and stereotaxically implanted with a 26-gauge guide cannula (Plastics One Inc., Roanoke, VA) aimed at the DRN (AP, −4.2 mm; ML, +1.5 mm; DV, −1.9 mm from bregma; angled 26° from vertical) as calculated from a mouse brain atlas (Paxinos and Franklin 2001). A 33-gauge obturator (Plastics One Inc.) that extended 0.5 mm beneath the tip of the guide cannula was inserted after surgery. The obturator was moved daily to prevent blockage and also for habituating the animals to handling. The animals were housed individually for 5 days to recover and then pair-housed with the same female. To prevent gnawing by the female, the obturator and head mount were covered with a quinine polish (Bite it©). One week after the surgery, the residents were assessed for self-administration of ethanol or water again and for fighting before starting microinjection tests.

Microinjection and aggression test

On the test day, the resident mice self-administered water or 1.0 g/kg of ethanol immediately before the microinjection. The obturator was removed and a 33-gauge microinjector (Plastics One Inc, Roanoke, VA) attached to a PE-50 tubing was inserted into the guide cannula. The microinjector extended 2 mm below the end of the guide to reach the DRN. The other end of the tubing was connected to a Hamilton syringe placed into an infusion pump (CMA Microdialysis, North Chelmsford, MA). The drug was infused in a volume of 0.2 μl over 2 min. The microinjector was left in place for 1 min after the infusion to allow the drug to diffuse completely. Ten minutes after the microinjection, an intruder was introduced, which prompted attacks by the resident mouse. The animal's behaviors were videotaped for detailed behavioral analysis at a later time. An animal received a total of six microinjections, following three drug conditions under both ethanol and water treatments: experiment 1, muscimol (0.006 nmol), bicuculline (0.006 nmol), and saline vehicle; experiment 2, baclofen (0.06 nmol), phaclofen (0.3 nmol), and saline vehicle. All drugs, purchased from Sigma-Aldrich (St. Louis, MO, USA), were dissolved in saline (0.9%). The drug treatments were administered in irregular sequence. The drug doses of muscimol and baclofen were selected based on previous work (Takahashi et al. 2010). The dose of phaclofen was chosen because it inhibited the effect of the 0.06 nmol of baclofen on aggressive behaviors. The dose of bicuculline was chosen on the basis of pilot studies. The selected dose of 0.006 nmol of bicuculline did not have any apparent effects on motor activity; whereas, higher doses of bicuculline (0.01 and 0.06 nmol) produced turning behavior or inhibited motor activity. Previous studies have estimated that the diffusion range of 1 μl muscimol (1 μg/μl) is about

1.7 mm from the injection site (Arikan et al. 2002; Edeline et al. 2002), but another study found that even 0.05 μl of muscimol can diffuse up to 1.4–1.7 mm from the injection site within 15 min (Martin 1991). In the present study, we used low concentrations of drugs with a 0.2 μl-injection volume and slower infusion rate. The animals that received infusions outside of the DRN did not show behavioral effects (Supplementary Table 1). Therefore, the effects of drugs are considered due to its action in the DRN.

Histology

At the end of the experiment, the mice were deeply anesthetized (ketamine and xylazine mixture) and intracardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in phosphate-buffered saline. After post-fixation in the 4% PFA for at least 24 h, the brains were placed into 15% sucrose solution. A microtome was used to slice the brains (60 μm thickness), and the sections were stained with cresyl violet to verify the placement of the cannula. Table 1 summarizes the number of animals used in this study, and Fig. 1 shows the injection site in each animal. Four out of 22 animals in experiment 1 and two out of 16 animals in experiment 2 had infusions, which missed the DRN, and their data were analyzed separately.

Blood ethanol concentration (BEC) measurement

For the analysis of the BECs, we used a new set of animals ($n=7$). Because we found an interaction between muscimol and alcohol-heightened aggression, we examined the effect of muscimol on BEC after 1.0 g/kg of ethanol consumption. Once stable self-administration of water and ethanol was established, the animals were subjected to cannulation surgery under anesthesia. After 1 week of recovery, water and ethanol self-administration was reestablished. On the test day, the animals received muscimol (0.006 nmol) or vehicle microinjection into the DRN immediately after they had consumed ethanol or water. Ten minutes later blood samples were collected from their submandibular vein (250 μl) using animal lancets (MEDIpoint, Inc., NY, USA). Blood was collected in 1.5-ml-centrifuge tubes containing 50 μl of heparin (100 U/ml) on ice. Samples were centrifuged to separate plasma, and supernatants were

stored at −80°C. Plasma ethanol concentrations were measured by using an Ethanol Assay Kit (BioVision, CA, USA) with high sensitivity (ranging from 0.4 to 40 ppm of ethanol).

Behavior analysis and statistics

Detailed behavioral analysis of the videotape record was performed by an observer whose reliability was established using the Observer software (Observer XT 8.0, Noldus, Wageningen, The Netherlands). The frequency of aggressive behaviors (attack bites, sideway threats, tail rattles, and pursuits) and the duration of non-aggressive behaviors (walking, rearing, grooming, and contact) were quantified as operationally defined and illustrated previously (Grant and Mackintosh 1963; Miczek and O'Donnell 1978). Repeated measures one-way ANOVA was performed to examine the effect of ethanol on aggressive and non-aggressive behaviors relative to water using vehicle microinjection data. The effect of drugs on the alcohol-heightened aggression was examined by using repeated measures of two-way ANOVA. In case of significant F values, Holm–Sidak t-tests were conducted as a post hoc analysis to determine which doses of the drug had significant effects compared to the vehicle ($\alpha=0.05$). In this analysis, the effect of the receptor agonist and antagonist were analyzed separately. Three animals in experiment 1 and one animal in experiment 2 could not complete all the injections due to cannula blockage, and those animals were used for analysis of either agonist or antagonist injections which they did complete.

Results

Alcohol-heightened aggression

Table 2 shows the effects of ethanol on aggressive behavior after vehicle microinjection. Self-administration sessions during the microinjection experiments took an average of 3.3 min for water and 3.0 min for 1.0 g/kg of ethanol. Self-administration of ethanol increased attack bites and sideway threats, and significantly reduced tail rattles relative to the measurements after water consumption. Repeated measures

Table 1 Number of animals used in this study

	Target	Drugs	Total animals used for analysis	Misplacement	Excluded[a]
Experiment 1	GABA$_A$ receptor	Muscimol (0.006 nmol), Bicuculline (0.006 nmol)	18	4	9
Experiment 2	GABA$_B$ receptor	Baclofen (0.06 nmol), Phaclofen (0.3 nmol)	16	2	10

[a] Animals were excluded if they died, became sick or stopped fighting after surgery

Fig. 1 Injection sites in the DRN. **a** Representative mouse coronal brain section (20×) that was stained with cresyl violet. **b** Schematic representation of injection sites for experiment 1 (GABA$_A$) and experiment 2 (GABA$_B$) in mouse coronal brain section (Paxinos and Franklin 2001). *Circles* indicate injection sites within the DRN and *triangles* represent injection sites outside the DRN
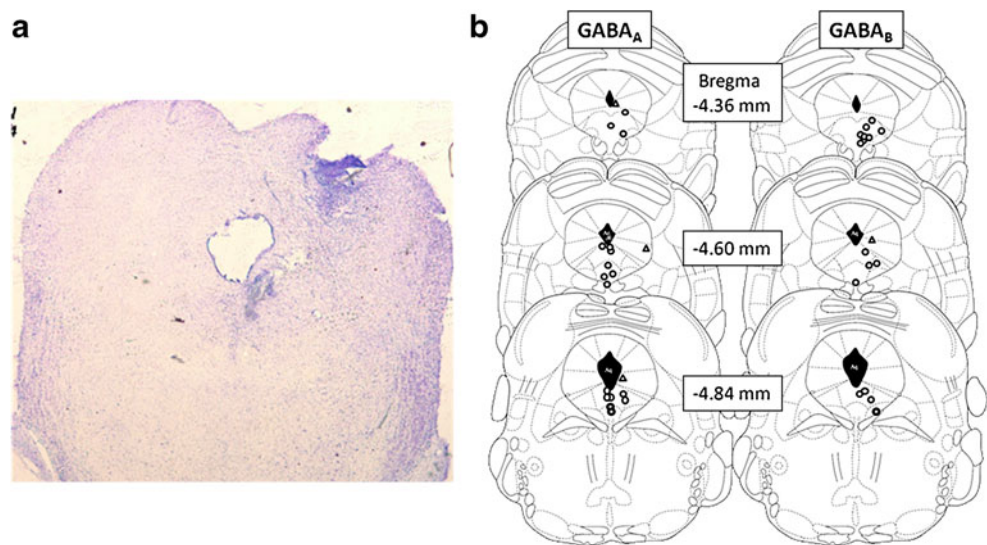
ANOVA showed significant main effects of ethanol on attack bites [$F(1, 34)=4.659$, $p=0.038$], sideway threats [$F(1, 34)=4.457$, $p=0.042$], and tail rattles [$F(1, 34)=8.544$, $p=0.006$]. There was no significant difference between the effects of ethanol and water self-administration on non-aggressive behaviors.

Before surgery, we characterized the animals for alcohol-heightened aggression and confirmed that some of the animals showed alcohol-heightened aggression, but not others (Miczek et al. 1998). However, not all animals that met our initial criterion for AHA did so after surgery. Therefore, we analyzed the data from all animals together to examine the interaction between ethanol and the GABA receptors in the DRN. Only when we found an interaction between the drug and ethanol, further analysis was performed in AHA and ANA individuals (see muscimol section).

GABA$_A$ receptors in the DRN and alcohol-heightened aggression

Agonist (muscimol, 0.006 nmol)

Muscimol significantly increased attack bites after ethanol consumption, but had no effect in the absence of ethanol (Fig. 2a). Two-way ANOVA showed a significant interaction between muscimol and ethanol [$F(1, 16)=4.537$, $p=0.049$] and the significant main effect of muscimol [$F(1, 16)=6.341$, $p=0.023$] on attack bites. On the other hand, muscimol slightly reduced walking compared to the vehicle [$F(1, 16)=4.756$, $p=0.044$] (Table 3). A significant main effect of ethanol was observed only for the measure of rearing [$F(1, 16)=8.901$, $p=0.009$].

Table 2 Effects of 1.0 g/kg of alcohol on aggressive and non-aggressive behaviors in vehicle microinjection data

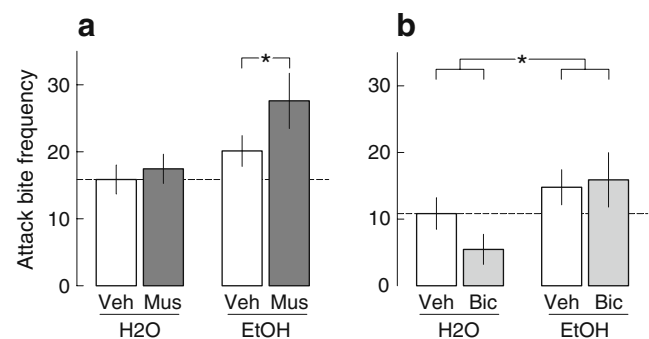|	H$_2$O	EtOH
Aggressive behaviors		
Pursuits	0.7±0.2	1.3±0.3 *
Sideway threats	21.0±2.4	23.6±3.6
Attack bites	17.7±1.6	21.7±1.8 *
Tail rattles	22.4±3.6	12.4±2.0 *
Non-aggressive behaviors		
Grooming	28.7±4.6	32.1±4.3
Rearing	30.1±4.6	25.1±4.1
Walking	55.6±4.1	57.2±3.2
Contacts	14.3±3.4	11.9±2.8

*$p<0.5$

Fig. 2 GABA$_A$ receptor modulation in the DRN and alcohol-heightened aggression. **a** The effect of intra-DRN microinjection of GABA$_A$ receptor agonist muscimol (*Mus*) on the frequency of attack bites after water or 1.0 g/kg of alcohol (*EtOH*) consumption. *Asterisk* indicates significant difference from the vehicle within the same condition ($p<.05$). **b** The effect of intra-DRN microinjection of the GABA$_A$ receptor antagonist, bicuculline (*Bic*), on the frequency of attack bites after water or EtOH consumption. *Asterisk* indicates significant main effect of alcohol

Table 3 Effects of intra-DRN microinjection of GABA$_A$ receptor agonist (muscimol) and antagonist (bicuculline) on aggressive and non-aggressive behaviors after water or 1.0 g/kg of alcohol (EtOH) consumption

Agonist				
	H$_2$O		EtOH	
	Control	Muscimol	Control	Muscimol
Pursuit	0.7+0.2	1.3+0.4	1.2+0.4	1.4+0.6
Sideways threat	15.9+2.4	16.4+2.0	14.7+1.9	16.8+1.9
Attack bite	16.4+2.5	17.8+2.5	20.3+2.8	27.6+4.7 *
Tail-rattle	13.3+3.0	12.2+2.3	10.7+3.4	12.6+2.8
Grooming	29.4+5.7	38.4+12.2	32.6+4.8	40.1+7.1
Rearing[a]	37.3+6.7	42.7+7.6	29.3+5.3	25.6+4.6
Walking[b]	49.3+5.4	44.9+4.6	49.4+4.3	45.5+4.2
Contact	18.1+5.6	14.2+3.2	15.3+5.1	11.9+4.1
Antagonist				
	H$_2$O		EtOH	
	Control	Bicuculline	Control	Bicuculline
Pursuit	0.6+0.2	0.7+0.3	1.3+0.8	0.8+0.3
Sideway threats[a]	15.2+2.4	10.2+2.0	16.5+2.0	18.8+2.5
Attack bite[a]	14.9+2.5	9.8+2.9	20.2+2.8	20.6+2.5
Tail-rattle	13.5+3.0	13.8+2.4	11.1+3.5	14.0+2.1
Grooming	29.5+5.5	24.1+7.0	35.5+5.0	24.1+5.0
Rearing	35.2+5.9	30.3+6.6	28.2+5.7	23.4+7.6
Walking[a]	48.0+5.9	33.3+3.7	49.0+4.5	48.3+4.7
Contact[b]	18.1+6.1	10.8+2.7	14.7+5.3	6.4+2.4

[a] significant main effect of alcohol ($p<.05$)

[b] significant main effect of drug ($p<0.05$)

*$p<0.05$, in post hoc test after significant alcohol×drug interaction

To examine the interaction between muscimol and ethanol in AHA and ANA individuals, we selected animals that consistently engaged in alcohol-heightened aggression between pre- and post-surgery. Out of 17 animals, four individuals that were categorized as AHA before the surgery also showed increased attack bites (increase>5) after ethanol consumption when given vehicle (Fig. 3a), and four individuals that were designated as ANA before surgery consistently showed no change or reduced attack bites after ethanol consumption (Fig. 3b). Muscimol increased aggressive behaviors after both water and ethanol self-administration in AHA animals (Fig. 3c). Even though the sample size was very small (AHA, $n=4$), repeated measures two-way ANOVA found a significant main effect of the drug [$F(1, 3)=16.963$, $p=0.026$] on the composed aggression scores (attack bites+sideway threats) within this subset of mice. In contrast, muscimol did not produce any effect in the ANA animals (Fig. 3d).

Antagonist (bicuculline, 0.006 nmol)

There was no significant interaction between bicuculline and ethanol in terms of changes in any behaviors (Fig. 2b, Table 3). Only a significant main effect of the drug was detected on the duration of contacts [$F(1, 15)=4.909$, $p=0.043$], and bicuculline reduced the duration of contacts relative to the vehicle. A significant effect of ethanol was observed on attack bites [$F(1, 15)=16.547$, $p=0.001$],

sideway threats [$F(1, 15)=6.265$, $p=0.0242$] and walking [$F(1, 15)=8.373$, $p=0.011$]. There was no difference in the effect of bicuculline between the AHA and ANA individuals (data not shown).

Blood ethanol concentration

Because there was an interactive effect between ethanol and muscimol, we examined the effect of muscimol on BACs using a separate group of animals ($n=7$). After the animals self-administered 1.0 g/kg of ethanol, they received either saline or 0.006 nmol of muscimol into the DRN. Blood samples were collected 10 min after the microinjection, which corresponds to the time when animals were tested for their aggressive behaviors. There was no significant difference between BAC after muscimol microinjection (70.7±4.5 mg/dL) and BAC after saline (73.4±3.9 mg/dL).
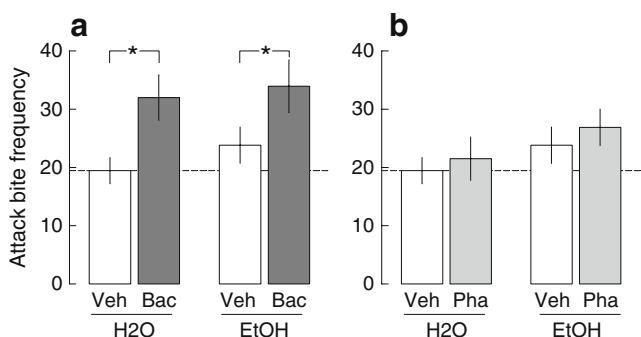
GABA$_B$ receptors in the DRN and alcohol-heightened aggression

Agonist (baclofen, 0.06 nmol)

Baclofen significantly increased attack bites and sideway threats after both water and ethanol self-administration (Fig. 4a, Table 4). However, there was no significant interaction between the effects of baclofen and ethanol was observed on any behaviors. Repeated measure two-way
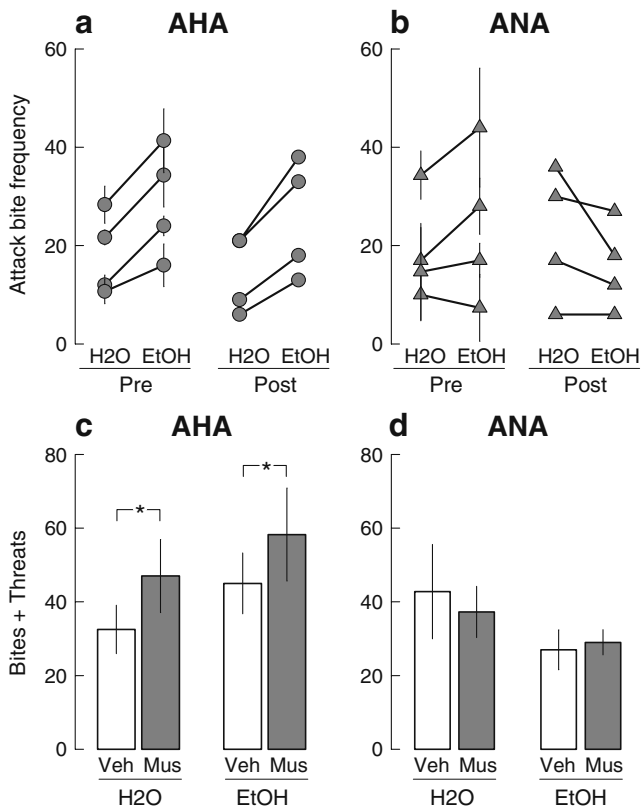
Fig. 3 Interaction between muscimol microinjection into the DRN and alcohol-heightened aggression in AHA and ANA individuals. **a** AHA individuals (*n*=4), *left side* (*Pre*), attack bites before the cannula implantation after water or 1.0-g/kg alcohol self-administration. *Right side* (*Post*), attack bites after water or 1.0-g/kg alcohol self-administration and vehicle microinjection into the DRN. **b** ANA individuals (*n*=4) that were designated as such before surgery and consistently showed no change or reduced attack bites after alcohol self-administration and intra-DRN vehicle treatment. **c** The effect of muscimol in AHA individuals on attack bites after either water or alcohol consumption. **d** The effect of muscimol in ANA individuals on attack bites after water or alcohol consumption. Asterisks indicate significant difference from vehicle within same condition (p<.05)

Fig. 4 GABA$_B$ receptor modulation in the DRN and alcohol-heightened aggression. **a** The effect of intra-DRN microinjection of the GABA$_B$ receptor agonist, baclofen (*Bac*), on the frequency of attack bites after water or 1.0 g/kg of alcohol (*EtOH*) consumption. **b** The effect of intra-DRN microinjection of the GABA$_B$ receptor antagonist, phaclofen (*Pha*), on the frequency of attack bites after water or EtOH consumption. Asterisks 2indicate significant difference from the vehicle within the same condition (p<.05)

ANOVA showed a significant main effect of the drug on attack bites [$F_{(1, 14)}$=11.591, p=0.004] and sideway threats [$F_{(1, 14)}$=5.094, p=0.041]. Both ethanol and baclofen reduced the frequency of tail rattles. Significant main effects of the drug [$F_{(1, 14)}$=9.674, p=0.008] and ethanol [$F_{(1, 14)}$=5.191, p=0.039] were observed on tail rattles. There was no significant effect of baclofen on non-aggressive behaviors (Table 4).

Antagonist (phaclofen, 0.3 nmol)

There was no significant interaction or main effect of phaclofen on any behaviors (Fig. 4b, Table 4). Only the main effects of ethanol were significant on the frequency of tail rattles [$F_{(1, 15)}$=7.642, p=0.014] and grooming [$F_{(1, 15)}$=5.544, p=0.033].

Discussion

A moderate dose of alcohol can promote heightened aggressive behaviors in a certain proportion of the population, and this phenomenon is observed in several species including humans, monkeys, rats, and mice (Fulwiler et al. 2005; Higley et al. 1996; Miczek et al. 1998, 2004b; Virkkunen et al. 1996; Winslow and Miczek 1985). The present results support a role of GABA$_A$ receptors in the DRN in the aggression-heightening effect of alcohol in mice. Previously, Fish et al. (2001) showed that systemic administration of the neurosteroid, allopreg-nanolone, which acts as a positive allosteric modulator at GABA$_A$ receptors, enhanced the pro-aggressive effect of alcohol in mice. A low dose of ethanol (0.6 g/kg) enhanced the pro-aggressive effect of allopregnanolone in the animals who engaged at higher levels of aggressive behavior after ethanol consumption compared to their basal fighting in the absence of ethanol (alcohol-heightened aggressors, AHA), whereas this interactive effect was not observed in alcohol-non-heightened aggressors (ANA). The current data provide evidence that the GABA$_A$ receptors in the DRN may be critical for the individual vulnerability to alcohol-heightened aggression. The GABA$_A$ agonist, muscimol, when microinjected into the DRN, escalated the frequency of attack bites only after ethanol consumption, but not in the absence of ethanol. Therefore, the strong activation of GABA$_A$ receptors in the DRN by ethanol plus muscimol can induce higher vulnerability to alcohol-heightened aggression. Interestingly, when AHA and ANA individuals were separately analyzed, intra-DRN muscimol escalated attack bites only in AHA animals, but not in ANA mice. One implication of these results appears to be that AHA animals may be characterized by higher GABA$_A$ receptor activation due to ethanol relative to ANA animals. It is

Table 4 Effects of intra-DRN microinjection of GABA$_B$ receptor agonist (baclofen) and antagonist (phaclofen) on aggressive and non-aggressive behaviors after water or 1.0 g/kg alcohol (EtOH) consumption

Agonist				
	H$_2$O		EtOH	
	Control	Baclofen	Control	Baclofen
Pursuit	0.7+0.2	0.9+0.3	0.9+0.3	1.7+0.6
Sideway threats[a]	27.7+4.4	41.7+7.1	32.0+7.6	32.9+4.3
Attack bite[a]	20.1+2.2	32.5+3.8	23.9+3.3	33.7+4.8
Tail rattle[b,a]	31.1+7.3	10.0+3.4	14.4+2.6	5.8+1.6
Grooming	27.3+8.4	18.7+4.8	32.4+8.0	17.9+3.0
Rearing	17.4+4.6	16.6+4.8	18.2+4.1	10.3+3.0
Walking	60.0+5.7	71.3+6.6	62.8+4.9	71.5+7.4
Contact	17.9+7.0	15.4+5.1	12.9+4.1	21.5+11.1
Antagonist				
	H$_2$O		EtOH	
	Control	Phaclofen	Control	Phaclofen
Pursuit	0.6+0.2	0.9+0.3	0.8+0.3	1.6+0.6
Sideway threats	27.0+4.6	28.1+4.4	31.7+7.2	29.6+5.1
Attack bite	19.4+2.4	21.8+3.6	23.5+3.1	26.6+3.1
Tail rattle[b]	31.3+7.6	26.0+6.3	13.6+2.5	15.4+4.0
Grooming[b]	27.4+8.7	22.4+5.0	34.9+7.9	43.7+6.7
Rearing	18.3+4.9	20.3+5.6	17.6+3.9	17.5+4.9
Walking	61.1+6.0	65.3+6.9	62.5+4.6	66.9+6.4
Contact	16.9+7.4	11.9+4.4	14.6+4.2	9.7+3.7

[a] significant main effect of drug ($p<0.05$)

[b] significant main effect of alcohol ($p<.05$)

possible that AHA animals have a higher GABA$_A$ receptor expression in the DRN compared to ANA. This hypothesis can be addressed in future studies by comparing GABA$_A$ receptor expression in the DRN between AHA and ANA animals. In this study, we used previously determined optimally effective doses of ethanol (1.0 g/kg) and muscimol (0.006 nmol). Future studies will address the dose-effect functions for alcohol and muscimol in order to investigate whether the alcohol-muscimol interaction is specific to the AHA individuals or whether ANA individuals can also show heightened aggression with different dose combinations.

In contrast, the GABA$_A$ antagonist bicuculline, at a subconvulsant dose, did not inhibit alcohol-heightened aggression. It is possible that bicuculline did not block the site of action for ethanol on GABA$_A$ receptors and that 1.0 g/kg of ethanol was sufficiently effective to promote heightened aggression even in the presence of a low dose of bicuculline (0.006 nmol). Interestingly, bicuculline inhibited several behavioral acts in the absence of ethanol (e.g., attack bites and walking), but those effects of bicuculline were rescued by ethanol consumption.

Low doses of alcohol act preferentially via selected GABA$_A$ subtype compositions including extrasynaptic GABA$_A$ receptors that contain $\alpha_4\beta_{2/3}\delta$ and $\alpha_6\beta_{2/3}\delta$ subunits (Hanchar et al. 2005; Liang et al. 2007; Sundstrom-Poromaa et al. 2002; Wallner et al. 2006; Wei et al. 2004); however, considerable debate continues about

the selectivity of the subunit requirement for specific alcohol actions (Korpi et al. 2007). Also, chronic alcohol treatments reduced the expression of α_1 subunits of GABA$_A$ receptors and increased or decreased the expression of α_4 subunits in the amygdala and cerebral cortex, respectively (Devaud et al. 1995; Matthews et al. 1998; Papadeas et al. 2001). It is possible that GABA$_A$ receptor subunit compositions in the DRN differ between AHA and ANA mice, and this difference contributes to the sensitivity to the pro-aggressive property of alcohol. Systemic administration of GABA$_A$-α_1 subunit preferring antagonist β-CCt inhibited alcohol-heightened aggression, suggesting the α_1 subunit as a functional target (de Almeida et al. 2004). However, β-CCt also reduced species-typical aggression, and the α_1 subunit-preferring agonist zolpidem failed to potentiate pro-aggressive effects of ethanol. Further exploration with more selectively acting compounds will be required to identify the subtypes of GABA$_A$ receptor that specifically modulate alcohol-heightened aggression.

GABA$_B$ receptors have also attracted considerable interest, primarily because agonists and positive modulators of this receptor suppress the intake of alcohol and other drugs (Besheer et al. 2004; Brebner et al. 2002; Colombo et al. 2004; Flannery et al. 2004; Maccioni et al. 2005, 2008; Orru et al. 2005; Walker and Koob 2007). Therefore, we examined the interaction between ethanol and the GABA$_B$ receptors in the DRN. We found that the local administration of the GABA$_B$ receptor agonist into the DRN did not

enhance the aggression-heightening effects of ethanol, but escalated aggressive behavior independent of whether the animal consumed ethanol or water. This finding is consistent with our previous study without any self-administration of ethanol or water (Takahashi et al. 2010). It appears that different neurobiological mechanisms underlie alcohol-heightened aggression (GABA$_A$ dependent) and baclofen-escalated aggression (GABA$_B$ dependent). Further investigation will be required to address how GABA$_A$ and GABA$_B$ receptors in the DRN promote different types of aggressive behaviors. GABA$_B$ receptors are localized on presynaptic terminals of afferent neurons (e.g., GABAergic and glutamatergic) in addition to post-synaptic 5-HT neurons (Bowery et al. 2002; Cryan and Kaupmann 2005), and we have shown that GABA$_B$ receptors on presynaptic terminals may be the critical target for the pro-aggressive effect of baclofen (Takahashi et al. 2010). In contrast, GABA$_A$ receptors seem to modulate mainly postsynaptic serotonergic activity in the DRN; microinfusion of the GABA$_A$ receptor agonists into the DRN consistently inhibited 5-HT neuronal activity (Colmers and Williams 1988; Gallager and Aghajanian 1976; Innis and Aghajanian 1987; Judge et al. 2004). Thus, GABA$_A$ and GABA$_B$ receptors may modulate either pre- or post-synaptic neurons or different subsets of 5-HT neurons in the DRN.

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