

## Augmented Stress-Induced Alcohol Drinking and Withdrawal in Mice Lacking Functional Natriuretic Peptide-A Receptors

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**Abstract — Aims:** Preclinical and clinical data suggest an involvement of atrial natriuretic peptides (ANP) in alcohol-associated psychopathology. We now present first data on alcohol drinking behaviour in mice lacking a functional natriuretic peptide-A (NPR-A) receptor. **Methods:** NPR-A<sup>-/-</sup> and wild-type mice were given a free choice between water and increasing concentrations of alcohol (2–16%). A forced swim stress was performed thereafter on three consecutive days to investigate stress-induced alcohol drinking. Additionally, neurobehavioural alcohol withdrawal response was investigated following 14 days of forced-alcohol intake. **Results:** Whereas basal alcohol intake did not differ between NPR-A mutants and wild-type littermates, NPR-A mutants showed an increased stress-induced alcohol intake and aggravated neurobehavioural symptoms of alcohol withdrawal. **Conclusions:** Mice lacking a functional NPR-A receptor represent a useful model to study the role of the ANP system in alcohol-associated pathology. To study the role of the natriuretic NPR-A gene for the modulation of risk of alcohol-related disorders, NPR-A-related polymorphisms should be targeted in clinical studies.

### INTRODUCTION

Voluntary alcohol intake and vulnerability to alcohol addiction have been shown to be modulated by numerous genetic and environmental factors (Goldman *et al.*, 2005; Spanagel, 2009). One major environmental factor that was detected repeatedly to affect voluntary alcohol intake in rodents and humans is stress (Vengeliene *et al.*, 2003; Heilig and Egli, 2006; Uhart and Wand, 2009).

The hypothalamic–pituitary–adrenocortical (HPA)-axis represents the endocrine backbone of behavioural stress response in both humans and rodents. As repeatedly shown, alcohol intake and HPA-axis activity are linked in a bi-directional manner (Kiefer *et al.*, 2002a; Sillaber and Henniger, 2004; Adinoff *et al.*, 2005). Chronic alcohol exposure was shown to be associated with increased synthesis and release of corticotropin-releasing hormone (CRF), and its downstream mediators adrenocorticotrophic hormone (ACTH) and adrenal steroids. However, several central peptide hormones modulating HPA-axis activity have also been shown to be associated with alcohol intake behaviour and severity of alcohol withdrawal symptoms (Kiefer *et al.*, 2001; Jahn *et al.*, 2004; Kiefer and Wiedemann, 2004). One of these peptide hormones, the atrial natriuretic peptide (ANP), was shown to inhibit HPA-axis activity on the hypothalamic, the pituitary and the adrenocortical level (Antoni *et al.*, 1992). In addition to its role in regulating fluid and salt homeostasis, ANP was suggested to have important neuromodulatory functions within the CNS, being involved in anxiety and arousal as well as the sequelae of stress hormone release and the autonomic nervous system activation (Kuhn, 2003). Moreover, an impact of ANP on affective and anxiety

symptoms has been shown repeatedly, and the specific role of ANP in the modulation and termination of stress responses and panic attacks is currently being discussed (Bhattacharya *et al.*, 1996; Wiedemann *et al.*, 2001; Ströhle *et al.*, 2006). Natriuretic peptides act within the CNS via the natriuretic peptide receptors NPR-A, NPR-B and NPR-C (Ruskoaho, 1992) counteracting centrally mediated anxiogenic effects of CRH and Isatin (Kellner *et al.*, 1992; Glover *et al.*, 1995; Medvedev *et al.*, 2005).

Effects of ANP are mainly mediated by its binding to the natriuretic peptide-A (NPR-A) receptor, inducing an activation of guanylyl cyclase-A and a consecutive increase in postsynaptic cGMP (Kuhn, 2003).

In recent years, both pre-clinical and clinical data suggested an involvement of ANP in alcohol-associated pathology. In mice, intracerebroventricular (ICV) injections of ANP have been found to attenuate hyperexcitability during alcohol withdrawal, whereas injections of an antiserum against ANP intensified it (Kovacs, 2003). Correspondingly, human studies reported a dysregulation of ANP plasma concentration during alcohol withdrawal that contributed to symptoms of protracted withdrawal. Detoxified patients with decreased ANP plasma concentrations during alcohol withdrawal suffered from more intense and frequent craving as well as from higher anxiety levels (Kiefer *et al.*, 2002b). In addition, ANP mRNA-expression has been shown to be significantly elevated in alcoholic patients while promoter-related DNA methylation of ANP was significantly decreased. Furthermore, promoter-related DNA methylation of ANP has been found to be significantly correlated to the extent of craving (Hillemacher *et al.*, 2009).

To further elucidate the involvement of the natriuretic peptide system in neurobehavioural effects of alcohol, we examined free-choice and stress-induced drinking behaviour as well as alcohol withdrawal response in mice lacking a functional

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natriuretic peptide-A (NPR-A) receptor compared to wild-type littermates.

## MATERIALS AND METHODS

### *Animals*

The background of mutant mice carrying a target disruption of the mouse NPR-A gene is on a C57BL/6/129/SvJ mixed genetic background (Lopez *et al.*, 1995). Ten- to twelve-week-old male NPR-A null mutant and wild-type mice were used in our experiments. The animals were singly housed with food and water *ad libitum*. Artificial light was provided daily from 6 a.m. to 6 p.m. (12 h light/dark cycle) with room temperature and humidity kept constant (temperature  $22 \pm 1^\circ\text{C}$ ; humidity  $55 \pm 5\%$ ). The experiments were approved by the Committee on Animal Care Use of the relevant local governmental body and carried out in accordance with the German Law on the Protection of Animals.

### *Free-choice two-bottle alcohol drinking behaviour*

Ten NPR-A null mutants ( $-/-$ ) and ten wild-type ( $+/+$ ) mice were used in these experiments according to standardized procedures (Spanagel and Holter, 1999). After 1 week of habituation, the animals were given continuous free access to increasing concentrations of aqueous alcohol solution. For the first 7 days, the animals were allowed to drink from two bottles containing tap water. Thereafter, one of the bottles was changed to hold a 2% (v/v) alcohol solution for 3 days. For the next 3 days, the alcohol bottle contained a 4% alcohol solution. This was followed by 9 days of an 8% and another 9 days of a 12% alcohol solution. Finally, the concentration was increased to 16% for the following 38 days. The bottles were weighed daily and the position of the bottles was swapped daily as to avoid any preference to any particular side of the cage. The alcohol intake was obtained from the difference in the weight of the bottles from one day to the next, taking into account alcohol density (0.8 g/mL). Finally, the amount of alcohol ingested was expressed as the absolute amount of alcohol consumed with respect to the weight of the animal (grams of alcohol per kilogram).

### *Forced swim stress*

After a habituation period of 4 weeks to 16% alcohol and water, we monitored the voluntary alcohol consumption of all mice during and after repeated swim stress. To investigate stress-induced alcohol drinking, mice were placed in a water-filled glass cylinder (25 cm high, 14 cm wide). The fill height of the glass cylinder was 15 cm. The water temperature was  $20^\circ\text{C}$ . Each daily trial lasted for 5 min and the mice were exposed to the forced swimming on three consecutive days. After each trial, the mice were gently dried and moved back to their home cages with free access to water and alcohol. The bottles were weighed every day to measure daily alcohol intake on the following 3 days after the swim stress.

### *Forced alcohol intake*

Forced alcohol intake (16%) was performed following habituation and stress tests. Water was completely replaced by a 16% alcohol solution, which was offered as the sole drink-

ing fluid for the following 14 days. The alcohol solution was replaced by water on Day 15. After 4 and 8 h without any alcohol consumption (withdrawal), neurobehavioural withdrawal symptoms were evaluated. A similar habituation procedure has been reported elsewhere (Spanagel *et al.*, 1996; Timpl *et al.*, 1998)

### *Ethanol withdrawal severity*

Ethanol withdrawal severity was assessed by scoring the neurobehavioural withdrawal signs on a seven-item scale [protocol has been described by Majchrowicz (1977)]. The metric rating differentiated between no (0), little (1) and severe (2) degree of piloerection, tremor, handling-elicited or spontaneous clonic-tonic seizure, teeth chattering, tail rigidity, vocalizations and wet dog shake (WDS). Mice were placed into clear observation boxes to assess the neurovegetative withdrawal signs for an observation period of 5 min. Each of the seven symptoms was scored as 0, 1 or 2, indicating low, middle or high presence/frequency, respectively. The global withdrawal score was expressed as the sum of the incidence of each sign. During the withdrawal cycle, all mice were scored for withdrawal symptoms after 4 and 8 h of withdrawal.

### *Data analysis*

All data are presented as means  $\pm$  the standard error of the mean (SEM). A significance level of  $P < 0.05$  was used throughout this study. Mean comparisons were performed by using a Student's *t*-test for dependent or independent samples or by a two-way ANOVA for repeated measures when necessary. Normal distribution of data was tested with the Kolmogoroff-Smirnov test. All *post hoc* tests were performed at a reduced level of significance (Bonferroni corrected level), to keep the type I error  $\leq 0.05$ .

## RESULTS

### *Basal alcohol drinking behaviour*

The mice were exposed to increasing concentrations of alcohol. At all concentrations (2, 4, 8, 12 and 16%), no significant increase was detected in the amount of alcohol consumed by the NPR-A $^{-/-}$  mice relative to the wild-type group [factor genotype:  $F(1, 18) = 0.9$ ;  $P = 0.3$ , or percent alcohol  $\times$  genotype interaction  $F(4, 72) = 0.3$ ;  $P = 0.8$ ]. There were no significant effects in alcohol preference of both genotypes (data not shown); however, both genotypes showed an increased alcohol intake at the 16% concentration compared to lower percent solutions [percent ethanol effect  $F(4, 72) = 28$ ;  $P < 0.01$ ; Fig. 1]. The mean body weights of the animals did not differ throughout the study (data not shown).

### *Stressed-induced alcohol drinking*

Swim stress application for 3 days did not change the alcohol intake in control mice. A two-way ANOVA did not indicate any significant effect [stress effect  $F(2, 36) = 1.4$ ;  $P = 0.63$ ; stress  $\times$  genotype interaction  $F(2, 36) = 1$ ;  $P = 0.4$ ; genotype effect  $F(1, 17) = 2.6$ ;  $P = 0.1$ ]. However, *post hoc* tests (Tukey) indicated that in the NPR-A $^{-/-}$  mice, the intake increased significantly during the stress days (baseline versus stress  $P < 0.05$ ) (Fig. 2). In the 3 days subsequent to the swim stress, the

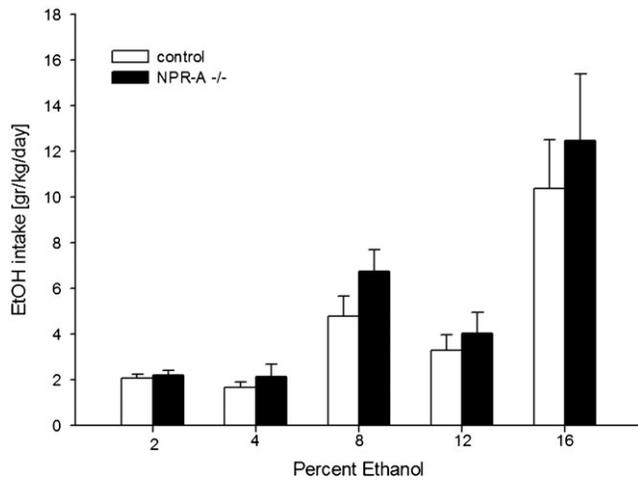


Fig. 1. Voluntary alcohol self-administration in NPR-A<sup>-/-</sup> and wild-type mice. Differences were not significantly different in alcohol intake in NPR-A<sup>-/-</sup> ( $n = 10$ ) and wild-type ( $n = 10$ ) mice during the acquisition phase in a two-bottle free-choice paradigm.

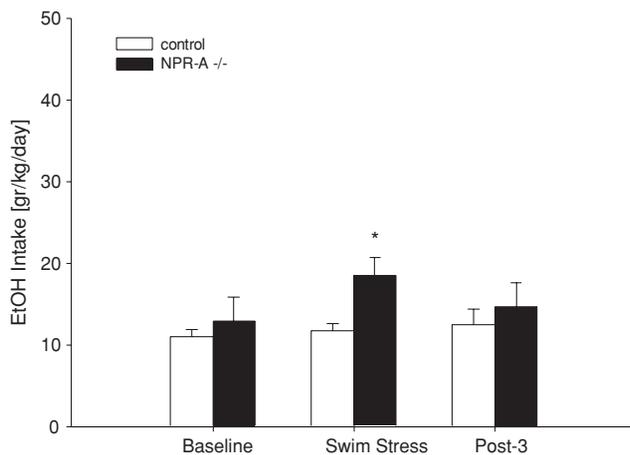


Fig. 2. Effects of swim stress on voluntary alcohol (16%) intake in NPR-A<sup>-/-</sup> ( $n = 10$ ) and wild-type ( $n = 10$ ) mice, shown as an average over 3 days. Intake was measured daily 3 days before (shown as mean  $\pm$  SEM, baseline) and post-stress. Following stress NPR-A<sup>-/-</sup> mice drank alcohol more voluntarily compared with wild-type animals ( $P < 0.05$ ).

alcohol consumption decreased in both genotypes to the basal levels.

#### Alcohol withdrawal assessment

Forced alcohol intake was performed to induce alcohol withdrawal. The mean alcohol consumption did not differ significantly between NPR-A<sup>-/-</sup> mice and wild-types during forced alcohol-drinking. During forced drinking (16% alcohol; 14 days), NPR-A<sup>-/-</sup> mice drank  $14.75 \pm 1.27$  g/kg/24 h compared with  $15.94 \pm 2.07$  g/kg/24 h for wild types.

During alcohol withdrawal (4 and 8 h), significant effects of genotype were found regarding severity of neurobehavioural withdrawal symptoms (Fig. 3). The withdrawal score containing the degree of piloerection, tremor, handling elicited or spontaneous clonic-tonic seizure, teeth chat, tail rigidity, vocalizations and wet dog shake (WDS) was significantly increased in

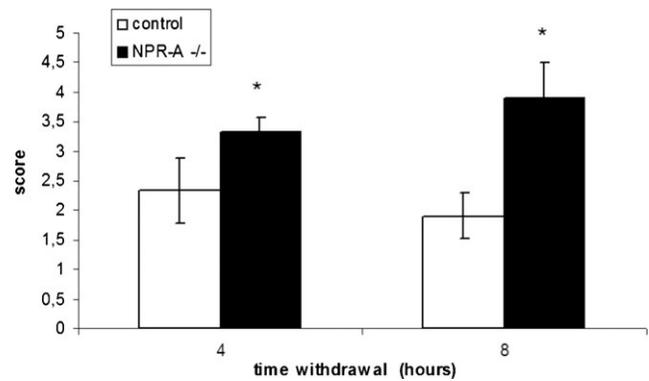


Fig. 3. Neurobehavioural withdrawal symptoms. Neurobiological withdrawal symptoms are significantly increased in NPR-A mutants compared to their wild-type littermates (4 h: wild-type versus NPR-A<sup>-/-</sup>,  $P = 0.008$ ; 8 h: wild-type versus NPR-A<sup>-/-</sup>,  $P = 0.001$ ). Between the two different time points of withdrawal, no significant differences were found in between each genotype. Columns represent means  $\pm$  SEM.  $n = 10$  mice per group.

NPR-A<sup>-/-</sup> mice compared to their wild-type littermates suggesting an increased alcohol withdrawal sensitivity in NPR-A<sup>-/-</sup> mice relative to wild-types (control versus NPR-A<sup>-/-</sup>: 4 h:  $P = 0.008$ ; 8 h:  $P = 0.001$ ).

## DISCUSSION

The main result of our study was that NPR-A<sup>-/-</sup> mutant mice that did not differ in basal alcohol intake from wild-type littermates but significantly increased alcohol consumption compared to wild-types following repeated swim stress. Moreover, NPR-A<sup>-/-</sup> mutant mice displayed a significantly increased intensity of neurobehavioural withdrawal signs compared to the wild-type mice. Our observations are consistent with the findings of Kovacs who reported that ICV injection of ANP attenuated, whereas antiserum against ANP intensified hyperexcitability during alcohol withdrawal (Kovacs, 2000, 2003), and with human studies suggesting a dysregulation of the ANP plasma concentration during alcohol withdrawal that contributes to symptoms of protracted withdrawal (Kiefer *et al.*, 2002b; Hillemecher *et al.*, 2009). ANP hence was suggested to be involved in the severity of alcohol withdrawal (Kovacs 2003; Hillemecher *et al.*, 2009).

Since alcohol intake is known to counteract effects of stress (Conger, 1956; Le *et al.*, 1998), stress attenuation effects of alcohol have been studied within neurobiological pathways such as the HPA-axis and corticotropin-releasing hormone (CRH) system, mesolimbic dopaminergic pathway and glutamatergic neurotransmission (Kiefer and Wiedemann, 2004; Heilig and Egli, 2006; Vengeliene *et al.*, 2008). ANP is known to inhibit the HPA-axis activity (Antoni *et al.*, 1992). Since the biological effects of ANP are mainly mediated by binding to the natriuretic peptide-A (NPR-A) receptors in NPR-A<sup>-/-</sup> mice, the increased alcohol intake following stress might represent a compensation of the impaired ANP-HPA-axis inhibiting effect in NPR-A mutants. This hypothesis is based on the observation that ANP has an important function in counter-regulating states of stress-related HPA-axis hyperstimulation, like cholecystokinin tetrapeptide (CCK-4)-induced panic anxiety (Wiedemann *et al.*, 2001). Pre-treatment with ANP was

shown to reduce panic anxiety, to attenuate ACTH and cortisol secretion, and to inhibit sympathetic stimulation (Wiedemann *et al.*, 2000, 2001).

Alcohol consumption behaviour under basal conditions (free-choice drinking) was not affected in our study. However, we present data suggestive for an involvement of the NPR-A receptor gene in stress-related alcohol consumption and in withdrawal responsiveness as an example for a gene  $\times$  environment interactions. After repeated swim stress, NPR-A<sup>-/-</sup> mice significantly step up their alcohol consumption compared to wild-type mice.

Mice lacking a functional NPR-A receptor may represent a useful animal model to address the question of whether a dysfunctional natriuretic peptide receptor system influences alcohol self-administration and individual vulnerability to alcohol drinking. Mutations in the natriuretic NPR-A gene may constitute a genetic risk factor for increased stress-induced alcohol drinking and alcohol withdrawal response.

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