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Research paper

Increased alcohol consumption in relaxin-3 deficient male mice

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HIGHLIGHTS

- Relaxin-3 KO male mice show enhanced alcohol intake and preference.
- Wild-type and KO mice show similar increased alcohol intake after chronic alcohol inhalation.
- No genotype difference was detected in sucrose or quinine preference.

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ABSTRACT

Relaxin-3 is a neuropeptide expressed in the brainstem, and predominantly localized in the gray matter of the midline dorsal pons termed the nucleus incertus. Relaxin-3-expressing neurons densely project axons rostrally to various forebrain regions including the septum, hippocampus, and lateral hypothalamus. Several relaxin-3 functions have been reported including food intake, stress responses, neuroendocrine function, emotion, and spatial memory. In addition, recently relaxin-3 and its receptor, RXFP3, were shown to regulate alcohol intake using an RXFP3 antagonist and RXFP3 gene knockout mice. In the present study, we investigated alcohol consumption in relaxin-3 knockout mice, and found that male but not female mice significantly drank more alcohol than wild-type mice in the two-bottle choice test. However, after chronic alcohol vapor exposure, wild-type and mutant mice did not show this difference in alcohol intake, although both genotypes exhibited increased alcohol consumption compared with non-alcohol-exposed control mice. There was no genotype difference in sucrose or quinine preference. These results suggest that the relaxin-3 neuronal system modestly affects alcohol preference and consumption.

1. Introduction

The neuropeptide, relaxin-3, with its cognate receptor, the relaxin-3 family peptide receptor 3 (RXFP3), has various functions including food intake, stress responses, hippocampal theta rhythm-dependent behavior, ascending arousal system, anxiety, and depressive-like behavior [12,14,22,23]. Relaxin-3 is predominantly expressed in neurons of the nucleus incertus (NI) of the dorsal pons, with a smaller number of relaxin-3 neurons scattered in other midbrain regions [2,11,23]. We previously demonstrated that relaxin-3 and corticotrophin-releasing factor (CRF) type 1 receptor (CRF1) are coexpressed in the NI, and further, that relaxin-3 mRNA increases after restraint stress [23,28]. Central administration of relaxin-3 induces a response in CRF-expressing neurons in the paraventricular hypothalamic nucleus and increases plasma adrenocorticotropic hormone [28]. The CRF system is not only involved in stress responses but also anxiety and alcohol intake [4,19]. Furthermore, we have also shown that relaxin-3-deficient mice exhibit slight alterations in anxiety-related behavior [29]. Alcohol dependence is related to dysregulation of behavioral responses to stress, as well as the neural reward circuit [5]. Recently, the relaxin-3/RXFP3 system was reported to regulate alcohol intake. In rats, RXFP3 antagonist treatment decreased self-administration of alcohol in a dose-related manner [17]. Moreover, relaxin-3 mRNA levels in the NI correlated with alcohol intake [18]. Male RXFP3 knockout (KO) mice show a stress-induced reduction in alcohol, although they have a similar baseline alcohol preference as wild-type mice [26]. To date, there is no study of alcohol-drinking behavior in relaxin-3-deficient mice. Thus, in the present study, we investigated basal alcohol preference in relaxin-3 KO mice by comparing with wild-type littermates and using the two-bottle choice test. We also examined alcohol intake in a mouse model of

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alcoholism, generated by chronic (20 days) alcohol vapor exposure [32].

2. Materials and methods

2.1. Animals

Generation of relaxin-3 gene KO mice on a C57BL/6 N background has been described previously [29]. Null mutant mice were backcrossed for at least eight generations to the C57BL/6 N background. All wild-type (+/+) and homozygous KO (−/−) mice were obtained from heterozygous crosses. Genotypes were confirmed in offspring by multiple primer polymerase chain reaction performed on tail biopsy samples. Mice were group housed based on sex (four to six mice in each cage) until the experiments. Mice were caged under a 12:12 h light/dark cycle (lights on from 8:00 to 20:00). The room temperature (26 °C) and humidity (40–50%) were controlled and food given *ad libitum* . All experiments were approved by the Animal Care and Use Committee of the Kyoto Prefectural University of Medicine (#M17-73).

2.2. Two-bottle choice for alcohol consumption and preference

The two-bottle choice test was performed as described previously [1]. Wild-type and relaxin-3 KO mice (3–4 months old; both sexes) were individually housed and habituated over 5 days to drinking from two bottles containing water. Mice were then given 3% ethanol (vol/vol) in one bottle and water in the other. The ethanol concentration was increased every 4 days from 3% to 6%, 9%, and finally 12%. The bottle position was changed every 2 days to control for position preference. Mice were weighed every 4 days. Quantity of alcohol consumption (g/kg body weight/24 h) was calculated and the values averaged for each concentration of ethanol. Alcohol preference was calculated for each ethanol concentration by dividing the ethanol solution consumed (mL/day) by the total fluid (ethanol plus water, mL/day) consumed.

2.3. Two-bottle choice for non-alcohol consumption and preferences

Wild-type or KO mice were also examined for sucrose and quinine consumption [24]. One bottle always contained water, and the other the tastant solution. Mice were offered 1.7 and 4.3% sucrose every 3 days. They were then offered quinine hydrochloride (0.03 and 0.1 mM) every 3 days, and the average intake in each mouse calculated. Body weight of the mice was also measured every 3 days.

2.4. Chronic alcohol exposure model and alcohol-drinking behavior test

Chronically alcohol-exposed mice were produced as previously described [32]. Briefly, mice were exposed to 22–27 mg/L of ethanol vapor for 20 days using an intermittent 3–4 h/day schedule that mimics the cyclical pattern of alcohol consumption. After alcohol exposure, mice were withdrawn from alcohol for 5 h in the normal experimental condition. For the alcohol-drinking behavior test, mice were provided with 10% (vol/vol) ethanol solution for 4 h and consumption measured. Total amount of alcohol intake was represented as alcohol (g)/body weight (kg).

2.5. Light–dark transition test

The light–dark transition test was performed as previously described [29]. The apparatus used for the light/dark transition test comprised a cage (30 × 15 × 15 cm) divided into two equally sized sections by a partition with a door (Melquest, Toyama, Japan). One chamber was brightly illuminated (400 lux) and the other chamber dark (2 lux). Mice were placed into the dark side and allowed to move freely between the two chambers with the door open for 10 min. The total number of transitions, time spent in each compartment, and first latency of movement to the light side were recorded automatically by the apparatus (SCANET-40, Melquest).

2.6. Elevated plus maze test

The elevated plus-maze consisted of two open arms (30 cm × 5 cm) and two enclosed arms of the same size, with 13-cm high transparent walls. The arms and central square were constructed from white plastic sheets, elevated to a height of 50 cm above the floor. To minimize the likelihood of animals falling from the apparatus, 5-mm-high Plexiglas sides were used for the open arms. Arms of the same type were arranged at opposite sides to each other. Each mouse was placed in the enclosed arms, and its behavior recorded during a 10-min test period. The number of entries into, and time spent on the open and enclosed arms were recorded. For data analysis, the following three measures were used: number of total entries, percentage of entries into open arms, and time spent on open arms (seconds). Data acquisition and analysis were performed automatically using SMARTV3.0 (Panlab SL, Barcelona, Spain).

2.7. Statistical analysis

Data are presented as mean values ± S.E.M. Consumption of alcohol and non-alcohol tastants was analyzed by two-way analysis of variance with Bonferroni post hoc analysis using the statistics software, Stat View® (SAS Institute, Cary, NC, USA). In the light–dark transition test and elevated plus maze test, data were analyzed by Student's *t* -tests. Differences were considered significant if $P < 0.05$.

3. Results

3.1. Alcohol consumption and preference

To study the role of relaxin-3 in alcohol consumption, voluntary alcohol and water intake in male and female wild-type and relaxin-3 KO mice was examined. Mice were offered a choice of either water and an ascending ethanol concentration series (3, 6, 9, and 12%) in a two-bottle choice protocol. Male mice showed a genotype difference in alcohol consumption ($F(1, 136) = 7.551$, $P = 0.0068$, main effect of genotype; and $F(3, 136) = 11.071$, $P < 0.0001$, main effect of concentration) (Fig. 1A). However, wild-type mice did not show an increase in alcohol intake from 6% ethanol concentration. In contrast, relaxin-3 KO mice showed a gradual increase in alcohol intake in a dose-related manner. Specifically, there were significant genotype differences in alcohol intake at 9% ($P = 0.0365$) and 12% ($P = 0.00068$) alcohol concentration. Female mice showed an increase in alcohol intake according to increased ethanol concentration; however, there was no difference between wild-type and relaxin-3 KO mice ($F(1, 172) = 2.158$, $P = 0.1436$, main effect of genotype; and $F(3, 172) = 19.119$, $P < 0.0001$, main effect of concentration) (Fig. 1B). Regarding alcohol preference, wild-type male mice exhibited decreased preference at 9 and 12% ethanol, while relaxin-3 KO mice maintained the level observed at 6%, even at higher concentrations. Accordingly, there were significant genotype differences in alcohol preference ($F(1, 136) = 8.492$, $P = 0.0042$, main effect of genotype) (Fig. 1C). Female KO mice had a slightly higher alcohol preference ($F(1, 172) = 4.341$, $P = 0.0387$, main effect of genotype), although post hoc tests found no genotype differences at any ethanol concentration (Fig. 1D). There was no difference in