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Perturbation of chemokine networks by gene deletion alters the reinforcing actions of ethanol

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Abstract

Microarray analysis of human alcoholic brain and cultured cells exposed to ethanol showed significant changes in expression of genes related to immune or inflammatory responses, including chemokines and chemokine receptors. To test the hypothesis that chemokines exhibit previously undiscovered pleiotropic effects important for the behavioral actions of ethanol, we studied mutant mice with deletion of the *Ccr2*, *Ccr5*, *Ccl2* or *Ccl3* genes. Deletion of *Ccr2*, *Ccl2* (females) or *Ccl3* in mice resulted in lower preference for alcohol and consumption of lower amounts of alcohol in a two-bottle choice test as compared with wild-type mice. Ethanol treatment (2.5 g/kg, i.p.) induced stronger conditioned taste aversion in *Ccr2*, *Ccl2* or *Ccl3* null mutant mice than in controls. *Ccr2* and *Ccr5* null mutant mice did not differ from wild-type mice in ethanol-induced loss of righting reflex (LORR), but mice lacking *Ccl2* or *Ccl3* showed longer LORR than wild-type mice. There were no differences between mutant strains and wild-type mice in severity of ethanol-induced withdrawal. Genetic mapping of chromosome 11 for the *Ccl2* and *Ccl3* genes (46.5 and 47.6 cM, respectively) revealed that an alcohol-induced LORR QTL region was contained within the introgressed region derived from 129/SvJ, which may cause some behavioral phenotypes observed in the null mice. On the contrary, known QTLs on Chr 9 are outside of 129/SvJ region in *Ccr2* and *Ccr5* (71.9 and 72.0 cM, respectively) null mutant mice. These data show that disruption of the chemokine network interferes with motivational effects of alcohol.

Keywords

Chemokines; Knockout mice; Ethanol; Intake; Conditioned taste aversion

1. Introduction

Chemokines constitute a superfamily of small proteins (8–14 kDa) that induce chemotaxis, tissue extravasation and modulate the function of leucocytes during inflammation [51]. Approximately, 50 different human chemokines have been described and these chemokines may interact with 18 different chemokine receptors [63,87]. Upon ligand binding, all

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chemokine receptors activate G proteins, leading to the dissociation of the G protein heterotrimer into its α and $\beta\gamma$ subunits. G protein triggering initiates several parallel signaling cascades, which finally trigger Ca^{2+} release from intracellular stores. Transient elevation of intracellular Ca^{2+} is a well-characterized effect of chemokine stimulation and is widely used to study functional interaction of chemokine receptors and chemokines (see [19], for review). Chemokine-induced activation of different types of kinases, such as phosphatidylinositol-3-OH kinase [80], the mitogen-activated protein kinase cascade [83] and activation of the janus kinase-signal transducers and activators of transcription pathway [59] are well-documented.

Chemokines are structurally related, with most containing four invariant cysteine residues. Depending on the arrangement of the first two of these cysteines, chemokines are divided into four subfamilies: CXC (α), CC (β), C (γ) and CX3C (σ) [63]. Recent evidence shows the chemokine family is not unique to mammals, with several members also identified in birds, amphibians and fish, including a primitive vertebrate, the lamprey [46].

As chemoattractant molecules, chemokines regulate cell trafficking through interactions with a set of their receptors [87]. For example, some evidence suggests that the migration of autoreactive immune cells via the blood-brain barrier is an early and critical process during the development of inflammatory CNS lesions of experimental allergic encephalomyelitis and multiple sclerosis, and that this transmigration is regulated by chemokines [38,41]. In addition to chemotaxis, chemokines are also involved in the regulation of T cell differentiation, apoptosis, cell cycle and metastatic processes. Further, chemokines can control the generation of soluble inflammatory products, such as free radicals, nitric oxide, cytokines and matrix metalloproteases [87,53].

Although originally identified on the basis of their ability to regulate the trafficking of immune cells, the biological role of chemokines goes well beyond this simple description of their function as chemoattractants, and they have been shown to be involved in a number of biological processes, including growth regulation, hematopoiesis, embryonic development and angiogenesis [36]. In vitro studies have demonstrated that, for example, SDF-1 α /CXCL12 is a potent chemoattractant for several types of neural cells, including neuronal precursor cells from the external germinal layer [86], cortical neuronal progenitors [47], cerebellar granule neurons [52] and dentate gyrus granular neurons [4]. In vivo studies using genetically manipulated mice deficient in chemokine receptors or their ligand indicate a crucial role for chemokines in the brain development [88]. It is becoming evident that chemokines and their receptors play an important role in glial proliferation in the developing brain [5,60,62]. Chemokines may be also involved in the modulation of synaptic transmission in the brain. For example, the stimulation of mouse and rat CXCR2 receptors by the chemokines Gro- α /CXCL1, Gro- β /CXCL2 and IL-8/CXCL8 modulates fast synaptic transmission and long-term synaptic plasticity of the cerebellum through the activation of the extracellular signal-regulated kinase pathway [31,67]. Such modulation of synaptic activity in the CNS has been broadly described for neurotrophic factors, which are characterized by their important roles at different stages of neuronal development and their capacity to alter synaptic transmission in the brain [13,37]. The similarities between the activities of chemokines on nerve cells to those of classical neurotrophins suggest that neuronal activity may be altered, at least in the cerebellum, as a consequence of neurodegenerative diseases, where chemokine expression is upregulated. Dysregulation of chemokine expression is associated with chronic inflammatory conditions, such as arthritis, atherogenesis, inflammatory bowel syndrome, glomerulonephritis, diabetes, endometriosis, transplant rejection and multiple myeloma [3,29,32,69]. Chemokines and their receptors can be important to the molecular mechanisms of hyperalgesia [17] and diseases, such as Alzheimer's disease [73] and multiple sclerosis [79]. Some studies showed that chemokine

receptors play crucial role in human immunodeficiency virus (HIV) infection [71]. Overall, the chemokines are important components of the endogenous host defense system [54,57].

Alcohol is a frequently abused drug that inhibits numerous immune functions of the host (see [64,74], for reviews). Enhanced production of CC chemokines is thought to contribute to the development of alcoholic liver disease. For example, elevated monocyte chemoattractant protein-1 (Ccl2) levels were observed in sera and tissues of patients with alcoholic liver disease [28]. Prolonged consumption of alcohol is associated with elevated serum levels of proinflammatory cytokines and chemokines in humans and in experimental models of alcohol intoxication [8,9,33,56].

It is interesting to note that microarray studies of brain tissue from human alcoholics [48,58] and of cultured human cells exposed to ethanol [76] showed changes in expression of genes related to immune or inflammatory responses, including the chemokines (Ccl2, Ccl3) and the chemokine receptors (Ccr5, Ccr1 motif). However, research to date has focused on changes in chemokines (or cytokines) in response to ethanol administration. We asked a different question: do altered levels of chemokines or chemokine receptors affect the behavioral actions of ethanol? The purpose of the present study was to test the hypothesis that mice deficient in either chemokines Ccl2 and Ccl3 (macrophage inflammatory protein-1- α) or chemokine receptors Ccr5 and Ccr2 will show changes in preference and consumption of ethanol using a two-bottle choice paradigm. Our findings indicate that endogenous chemokine receptors may contribute to oral self-administration of ethanol, perhaps because they reduce aversive reactions to alcohol.

2. Materials and methods

2.1. Animals

Null Ccl3 ($-/-$), Ccl2 ($-/-$), Ccr2 ($-/-$) and Ccr5 ($-/-$) allele mice were created using homologous recombination as previously described [20,44,45,50]. Ccl3 ($-/-$) mice purchased from Jackson Laboratories, Bar Harbor, ME. Founders of Ccl2 ($-/-$) mice were a generous gift from Dr. Barrett J. Rollins (Dana Farber Cancer Institute). Ccr2 ($-/-$) and Ccr5 ($-/-$) null mutant mice were from colonies maintained at the University of Texas by Dr. W. Kuziel. The colony of double knockout Ccl2 ($-/-$) $\times$ Ccr2 ($-/-$) was generated by breeding of mutant parental strains. All mouse colonies were maintained by homozygous breeding. Because all mutant mice were backcrossed on C57Bl/6 background at least for 10 generations, C57Bl/6 mice from a colony maintained at the University of Texas at Austin (original breeders were purchased from Jackson Laboratories, Bar Harbor, ME) were used as the control or wild-type strain.

Mice were at least 10–12 weeks of age at the time of analysis; within each experiment, all mice were of similar age. At the end of some experiments, mice were 18–20 weeks of age. Mice were group housed (three to five per cage) under a 12-h light/12-h dark cycle (lights on at 07:00 h) and provided ad libitum access to food and water unless otherwise specified. Each experiment was performed using naïve mice. Only in Sections 2.2 and 2.3 were mice used several times. All experiments were conducted in an isolated behavioral testing room in the animal facility to avoid external distractions. All experiments were approved by the Institutional Animal Care and Use Committee.

2.2. Alcohol drinking

Mice were allowed to acclimate for 1 week to individual housing. Experiments were carried out in standard 7.5 in. $\times$ 12.5 in. cages in sliding racks. Bottles were placed vertically 3.5 in. from the back wall through two holes in the cage top. The distance between the two bottles was about 1.0 in. A feeder was placed on the front wall. Two drinking tubes were

continuously available to each mouse and tubes were weighed daily. Food was available ad libitum and mice were weighed every 4 days. After 4 days of water consumption (both tubes), mice were offered 3% ethanol (v/v) versus water for 4 days. Tube positions were changed every day to control for position preferences. Quantity of ethanol consumed (g/kg body weight/24 h) was calculated for each mouse. Because the consumption of ethanol in wild-type mice as well as in mutant mice was very stable across days these values were averaged for every concentration of ethanol. Immediately, following 3% ethanol, a choice between 6% ethanol and water was offered for 4 days, then 9% ethanol for 4 days and finally 12% ethanol versus water for 4 days.

2.3. Preference for non-alcohol tastants

Two weeks after the ethanol drinking study, part of the same wild-type and knockout mice were tested for saccharin (sweet) or quinine (bitter) consumption in a two-bottle choice paradigm using an order-balanced experimental design. Mice were serially offered saccharin (0.033 and 0.066%) and quinine hemisulfate (0.03 and 0.06 mM) and intakes were calculated. Each concentration was offered for 4 days, with bottle position changed every day. Within each tastant, the low concentration was always presented first, followed by the higher concentrations. Between tastants mice had two bottles with water for 2 weeks.

2.4. Conditioned taste aversion

Subjects were adapted to a water-restriction schedule (2 h water per day) over a 7-day period. At 48 h intervals over the next 10 days, all mice received 1 h access to a solution of saccharin (0.15%, w/v, sodium saccharin in tap water). Immediately, after 1 h access to saccharin, mice received injections of saline or ethanol (2.5 g/kg). All mice also received 30 min access to tap water 5 h after each saccharin access period to prevent dehydration. Two-hour access to tap water was given during intervening days.

2.5. Acute withdrawal severity

Mice were scored for handling-induced convulsions (HIC) severity 30 min before and immediately before i.p. ethanol administration. The two pre-drug base-line scores were averaged (PRE). A dose of 4.0 g/kg of ethanol in saline was injected i.p. and the HIC score was tested every hour until the HIC level will reach the baseline. Acute withdrawal was quantified as area under the curve but above PRE level [16].

2.6. Loss of righting reflex

Animals were injected with ethanol (3.8 g/kg, i.p.) and the length of ethanol-induced loss of righting response (LORR) was measured. Upon loss of the righting reflex, mice were placed supine in a sleep trough (~90° angle) and the time to regain the righting reflex measured. LORR was defined as inability of a mouse to right itself within 30 s. Return of the righting response (RORR) was defined as the ability of a mouse to right itself twice in 1 min. Sleep time was defined as the time between LORR and RORR.

2.7. Alcohol metabolism

Animals were given a dose of ethanol (4.0 g/kg i.p.) and blood samples were taken from the retro-orbital sinus in 15, 60, 120, 180 and 240 min after injection. Blood alcohol concentration (BEC) values, expressed as mg ethanol/ml blood were determined spectrophotometrically by an enzymatic assay [16].

2.8. Chromosome mapping

Tail DNA was extracted using the Jackson Laboratory method exactly as described (see http://www.jax.org/imr/tail_nonorg.html). Microsatellite alleles of known location for

C57BL/6 mice were tested for polymorphic status in 129X1/SvJ mice using DNA obtained from the Jackson Laboratory and a number of MIT markers (Research Genetics, Invitrogen, Carlsbad, CA). The genomic flanks for each 129X1/SvJ introgressed region containing an induced knockout gene was identified using the following markers: D9Mit355, D9Mit115, D9Mit77, D9Mit281, D11Mit77, D11Mit19, D11Mit21, D11Mit143, D11Mit4, D11Mit38, D11Mit248, D11Mit67, D11Mit99 and D11Mit167 (Research Genetics, Invitrogen, Carlsbad, CA). PCR reactions were completed in an MJR PTC-100 thermocycler using *Tfl* polymerase according to manufacturer's protocol (Epicentre, Madison, WI). Approximately, 20 ng DNA was PCR amplified in a 15 μ l total volume under the following conditions: 94 °C for 2 min, 40 cycles of 94 °C for 30 s, 56 °C for 80 s, 72 °C for 30 s, followed by a 7 min, 72 °C extension and terminal 4 °C hold. DNA fragments were electrophoretically separated in high-resolution agarose (4%) (MetaPhor, Cambrex, Rockland, ME) and visualized using standard ethidium bromide staining and UV detection. Control C57BL6/J and 129X1/SvJ DNA was used to positively identify allele specificity.

2.9. Drugs

All alcohol (Aaper Alcohol and Chemical Co., Shelbyville, KY) solutions were made in saline (20%, v/v) and injected i.p. with a volume of 0.2 ml/10 g of body weight.

2.10. Statistical analysis

Data are reported as the mean $\pm$ S.E.M value. The statistics software program GraphPad Prism (Jandel Scientific, Costa Madre, CA) was used throughout. To evaluate differences between groups, analysis of variance (two-way ANOVA) and Student's *t*-test were carried out.

3. Results

3.1. Ethanol preference

In a two-bottle paradigm in which mice could drink either water or an ascending series of ethanol concentrations (3, 6, 9, 12 and 15%), males lacking *Ccr2* displayed significantly reduced preference for ethanol ($F(1,140) = 36.2$, $P < 0.0001$) and a reduction in the amount of ethanol consumed ($F(1,140) = 12.4$, $P < 0.0001$) (Fig. 1a and c). *Ccr5* ($-/-$) mutant males showed no change in ethanol consumption or preference (Fig. 1a and c). As for males, null *Ccr2* females showed significant reduction in the amount of ethanol consumed ($F(1,115) = 22.4$, $P < 0.0001$) as well as reduction of preference ($F(1,115) = 41.1$, $P < 0.0001$) (Fig. 1b and d). *Ccr5* ($-/-$) knockout females consumed slightly higher amount of ethanol than wild-type females ($F(1,115) = 11.4$, $P < 0.001$, two-way ANOVA) with no difference in preference (Fig. 1b and d). *Ccr2* and *Ccr5* null mutant males consumed more fluid than the control mice ($F(1,140) = 30.2$, $P < 0.0001$ and $F(1,125) = 7.4$, $P < 0.01$, respectively) (Fig. 1e). *Ccr2* ($-/-$) knockout females as well as *Ccr5* null mutant females show greater fluid intake than wild-type ($F(1,115) = 9.2$; $P < 0.01$ and $F(1,115) = 11.6$; $P < 0.001$, respectively) (Fig. 1f).

Although males lacking *Ccl2* showed no significant difference from wild-type either in amount of ethanol consumed or preference for ethanol (Fig. 2a and c), post hoc analysis demonstrated higher consumption of ethanol and higher preference in concentration of ethanol 15% ($P < 0.01$ and < 0.05 , respectively, Bonferroni post *T*-test). *Ccl3* ($-/-$) mutant males displayed a strong reduction in the amount of consumed ethanol ($F(1,140) = 12.1$, $P < 0.001$) and preference ($F(1,140) = 12.5$, $P < 0.001$) (Fig. 2a and c). In contrast to males, *Ccl2* ($-/-$) mutant females showed reductions in the amount of ethanol consumed ($F(1,140) = 19.6$, $P < 0.0001$) and preference for ethanol ($F(1,140) = 17.5$, $P < 0.001$) (Fig. 2b and d). *Ccl3* ($-/-$) knockout females also showed significant decreases in preference ($F(1,140) =$

14.5, $P < 0.001$) and amount of consumed ethanol ($F(1,140) = 27.8$, $P < 0.0001$) (Fig. 2d and e). Ccl3 ($-/-$) mutant females consumed less total fluid ($F(1,140) = 9.5$, $P < 0.01$) and Ccl2 ($-/-$) mutant males more fluid ($F(1,140) = 22.4$, $P < 0.0001$) than control mice (Fig. 2e and f).

Ccl2 and Ccr2 null mutant males consume more ethanol than double Ccl2 ($-/-$) $\times$ Ccr2 ($-/-$) knockout males ($F(1,90) = 30.1$; $P < 0.0001$ and $F(1,115) = 13.5$; $P < 0.001$, respectively), but there was no difference in ethanol consumption between Ccl2 ($-/-$) and Ccr2 ($-/-$) knockout males (Fig. 3a). Ccl2 null mutant females consume more ethanol consumption than double Ccl2 ($-/-$) $\times$ Ccr2 ($-/-$) knockout females ($F(1,80) = 4.1$; $P < 0.05$) and Ccr2 ($-/-$) knockout females consume less amount of ethanol than Ccl2 null mutant females ($F(1,90) = 13.3$; $P < 0.001$). There were no differences in ethanol consumption between Ccr2 ($-/-$) knockout and double Ccl2 ($-/-$) $\times$ Ccr2 ($-/-$) knockout females (Fig. 3b). Ccl2 ($-/-$) knockout males show greater preference for ethanol than Ccr2 ($-/-$) null mutant and double Ccl2 ($-/-$) $\times$ Ccr2 ($-/-$) knockout males ($F(1,115) = 20.6$; $P < 0.0001$ and $F(1,90) = 22.0$; $P < 0.0001$, respectively) with no difference in ethanol consumption between Ccr2 ($-/-$) knockout and double Ccl2 ($-/-$) $\times$ Ccr2 ($-/-$) knockout males (Fig. 3c). Ccl2 ($-/-$) knockout females show greater preference and Ccr2 null mutant females show lower preference for ethanol ($F(1,80) = 5.8$; $P < 0.05$ and $F(1,80) = 6.9$; $P < 0.05$, respectively) than double Ccl2 ($-/-$) $\times$ Ccr2 ($-/-$) knockout males. Ccl2 ($-/-$) knockout females show significantly greater preference than Ccr2 null mutant females ($F(1,90) = 34.5$; $P < 0.0001$) (Fig. 3d). Ccl2 ($-/-$) knockout males and Ccr2 null mutant males show greater fluid intake ($F(1,90) = 87.2$; $P < 0.0001$ and $F(1,115) = 172.9$; $P < 0.0001$, respectively) than double Ccl2 ($-/-$) $\times$ Ccr2 ($-/-$) knockout males. Ccl2 ($-/-$) knockout males show greater fluid intake ($F(1,115) = 36.8$; $P < 0.0001$) than Ccr2 null mutant males (Fig. 3e). Ccl2 ($-/-$) knockout females and Ccr2 null mutant females show greater fluid intake than double Ccl2 ($-/-$) $\times$ Ccr2 ($-/-$) knockout females ($F(1,80) = 50.8$; $P < 0.0001$ and $F(1,80) = 128.5$; $P < 0.0001$, respectively). Ccr2 ($-/-$) knockout females show greater fluid intake than Ccl2 null mutant females ($F(1,90) = 29.6$; $P < 0.0001$) (Fig. 3f).

3.2. Preference for non-alcohol tastants

Male and female mice lacking Ccr2, Ccr5, Ccl2 and Ccl3 did not differ from wild-type mice in preference for saccharin solutions (Fig. 4a–d). Male and female mice lacking Ccr5 consumed significantly more fluid (saccharin solution + water) than wild-type mice ($F(1,40) = 5.3$, $P < 0.05$ for males and $F(1,32) = 4.2$, $P < 0.05$ for females) (Fig. 4e and f). There were no significant differences in total intake between Ccl2 ($-/-$) and Ccl3 ($-/-$) knockout mice of both sexes and wild-type mice (Fig. 4g and h).

Male and female mice lacking Ccr2, Ccr5, Ccl2 and Ccl3 did not differ from wild-type mice in preference for bitter solutions of quinine (Fig. 5a–d). Both Ccr5 ($-/-$) and Ccr2 ($-/-$) knockout males showed slightly total intake (quinine solution + water) than wild-type males ($F(1,36) = 5.8$, $P < 0.05$ and $F(1,36) = 9.9$, $P < 0.01$, respectively) (Fig. 5e). Similar increase of total fluid consumption was shown for female mice lacking Ccr5 and Ccr2 ($F(1,32) = 29.9$, $P < 0.001$ and $F(1,36) = 16.5$, $P < 0.001$, respectively) (Fig. 5f). From mice lacking Ccl2 or Ccl3 only male Ccl2 ($-/-$) knockout mice showed significantly higher total intake of fluid compare with wild-type males ($F(1,36) = 19.9$, $P < 0.001$) (Fig. 5g).

3.3. Conditioned taste aversion

For trial 0, all mice consumed different amount of saccharin solution: C57Bl/6 males, 57.0 ± 2.9 g/kg; C57Bl/6 females, 83.0 ± 4.2 g/kg; Ccr2 ($-/-$) males, 61.4 ± 4.1 (g/kg); Ccr2 ($-/-$) females, 93.2 ± 3.2 g/kg; Ccr5 ($-/-$) males, 68.4 ± 4.5 g/kg; Ccr5 ($-/-$) females, 87.6 ± 5.7

g/kg; Ccl2 (-/-) males, 70.7±3.8 g/kg; Ccl2 (-/-) females, 96.2±4.1 g/kg; Ccl3 (-/-) males, 51.9±2.9 g/kg; Ccl3 (-/-) females, 65.8±5.1 g/kg. Consumption of saccharin in trial 0 (before conditioning) was greatest for Ccl2 (-/-) ($P < 0.01$, T -test) and Ccr5 (-/-) ($P < 0.05$, T -test) mutant males compare with control C57Bl/6 male mice. Among female mice Ccl2 (-/-) mutant females showed the greatest consumption ($P < 0.05$, T -test), whereas Ccl3 (-/-) knockout females consumed less amount of saccharin solution than wild-type (C57Bl/6) females ($P < 0.05$, T -test). In an attempt to correct for these initial differences in saccharin intake and facilitate presentation of the data, intake was calculated as a percentage of the trial 0 consumption for each subject by dividing the amount of saccharin solution consumed on subsequent conditioning trials on amount of saccharin solution consumed on trial 0 (before conditioning). Ethanol-saccharin pairings produced reductions in saccharin intake across trials, indicating the development of CTA in all genotypes. There were no differences between ethanol-treated groups of Ccr2 (-/-) and Ccr5 (-/-) mutant males from wild-type males. However, in saline-treated groups males lacking Ccr2 consumed more saccharin than wild-type males ($F(1,135) = 27.6$, $P < 0.0001$) (Fig. 6a). Taken together, these results lead to the conclusion that Ccr2 null mutant males develop stronger CTA than wild-type. In contrast, there were no differences between different saline-treated groups of female mice. However, both Ccr2 (-/-) and Ccr5 (-/-) mutant females showed stronger response to ethanol than ethanol-treated wild-type females ($F(1,135) = 28.8$, $P < 0.0001$ and $F(1,165) = 6.1$, $P < 0.05$, respectively) (Fig. 6b).

Ccl3 (-/-) mutant males were not different from wild-type males in their response to ethanol. However, they consumed more saccharin than wild-type males after saline injections ($F(1,100) = 14.6$, $P < 0.001$). There was no difference between saline-treated groups of Ccl2 (-/-) mutant and wild-type males. In contrast, males lacking Ccl2 showed stronger response to ethanol than wild-type males ($F(1,130) = 20.2$, $P < 0.0001$) (Fig. 7a). Both groups of ethanol-treated Ccl3 (-/-) and Ccl2 (-/-) null mutant females displayed stronger response to ethanol than wild-type females ($F(1,110) = 11.7$, $P < 0.001$ and $F(1,125) = 28.0$, $P < 0.0001$, respectively). There was no significant difference in saccharin consumption between females of both mutant strains and wild-type females (Fig. 7b).

3.4. Loss of righting reflex

There were no differences in duration of ethanol-induced LORR between wild-type mice and Ccr2 (-/-) as well as Ccr5 (-/-) knockout mice of the both sexes (Fig. 8a and b). On the contrary, both Ccl2 and Ccl3 null mutant mice showed significantly longer duration of ethanol-induced LORR than wild-type animals (Fig. 8a and b).

3.5. Severity of alcohol-induced withdrawal

No difference between levels of basal HIC for both males and females were revealed. All knockout and wild-type males and females showed similar HIC scores over the entire period of observation (Fig. 9a and b). There were no differences in area under the curves (above the basal level) for HIC during withdrawal either for females [1.64±0.53, C57Bl/6; 1.06±0.42, Ccr2 (-/-); 1.11±0.49, Ccr5 (-/-); 1.39±0.50, Ccl2 (-/-) and 1.55±0.47, Ccl3 (-/-)] or for males [1.55±0.47, C57Bl/6; 1.75±0.48, Ccr2 (-/-); 0.93±0.32, Ccr5 (-/-); 1.95±0.43, Ccl2 (-/-) and 2.19±0.47, Ccl3 (-/-)].

3.6. Metabolism of alcohol

There were no differences in metabolism of ethanol between wild-type and any of the knockout mice (data not shown).

3.7. Chromosome mapping

Using chromosome markers, we identified as polymorphic for the 129/SvJ and C57BL/6J strains, the introgressed regions on chromosomes 9 and 11 were mapped for all four mutant strains. Both *Ccr2* and *Ccr5* genes are located on Chr9 in positions 71.9 and 72.0 cM, respectively. In *Ccr2* and *Ccr5*, null mutant mice, the entire region of Chr 9 from the location of *Ccr5* and *Ccr2* genes up to 67 cM (D9Mit279 marker) was derived from C57BL/6J (Table 2). As a control for backcrossing, another chromosome (Chr 11) in *Ccr5* (–/–) and *Ccr2* (–/–) knockout mice was also positively identified as derived from C57BL/6J (Table 1). Both *Ccl2* and *Ccl3* genes are located on Chr11 in positions 46.5 and 47.59 cM, respectively. In *Ccl2* (–/–) knockout mice 129/SvJ genes contributed a small region between 49 cM (D11 Mit38 marker) and 52 cM (D11Mit284 marker). A larger region from 129/SvJ strain (20–58 cM) was mapped to Chr 11 from *Ccl3* null mutant mice (Table 1). However, again, another backcross control chromosome (Chr 9) in both knockout strains (*Ccl3* and *Ccl2*) was derived from the C57BL/6J strain (Table 2).

4. Discussion

A summary of the behavioral results obtained from all four mutant strains of mice are presented in Table 3. It is clear that deletion of either chemokines (*Ccl2* or *Ccl3*) or chemokine receptors (*Ccr2*) leads to substantial reduction of alcohol consumption and preference. A possible explanation for this reduction could be increased aversion to alcohol. Indeed, all three mutants (*Ccl2*, *Ccl3* and *Ccr2*) demonstrated stronger conditioned taste aversion to ethanol than control mice. Reduced preference for alcohol can be also negatively linked with increased genetic susceptibility to ethanol withdrawal symptoms [61]. However, none of the four mutants tested showed the differences in severity of ethanol-induced withdrawal from wild-type mice. The similarity between alcohol phenotypes of *Ccl2* and *Ccr2* null mutant mice is consistent with the finding that *Ccl2* is one of the major ligands for the *Ccr2* receptor [63]. However deletion of *Ccr2* produced a slightly stronger and clearer alcohol phenotype than deletion of its ligand (*Ccl2*). This enhancement of response to alcohol in *Ccr2* null mutant mice may be a result of reduced expression of mRNA for chemokine receptor *Ccr1* found in mice lacking *Ccr2* [52]. This suggestion is consistent with the finding that mice lacking *Ccl3*, one of the major ligands for *Ccr1* receptor, demonstrated strong avoidance of alcohol.

To our knowledge, this is the first report of a role for the chemokine network in the motivational effects of alcohol. Several lines of evidence indicate that many cells from the CNS (astrocytes, oligodendrocytes, microglia and neurons) express at least some functional chemokine receptors [see [2,6,14], for reviews]. Thus, the interaction between ethanol and chemokines may occur in the CNS. However, in contrast with chemokine receptors, the expression of chemokines in the CNS is mostly inducible by inflammatory stimuli, meaning that constitutive chemokine expression is hardly detectable in normal brain [21,26]. Therefore, it is unlikely that deletion of chemokines, such as *Ccl2* and *Ccl3* can reduce alcohol consumption by central mechanisms.

In considering possible peripheral actions of chemokines, it is important to note that exposure to pathogens results in complex behavioral and physiological responses, such as fever, disruption of feeding and drinking and reduced social and exploratory behaviors known as ‘sickness behavior’ [23,24,27,39,72]. This ‘sickness behavior’ is usually associated with conditioned or learned taste aversion, i.e. avoidance of a novel flavor (usually, saccharin) associated with administration of the noxious agent. Many of these illness responses are produced by cytokines released by activated immune cells [39,55]. Some data suggest that cytokines may convey peripheral information to the CNS. Interestingly, the aversive effect of peripherally administered cytokines does not involve

their hypophagic effect under normal feeding conditions [7]. The human hangover syndrome has some similarities with described ‘sickness behavior’ [84] and an increased level of cytokines was found in human volunteers during the hangover state [42]. In humans, concentrations of proinflammatory cytokines are also increased after acute consumption of alcohol [82]. Similar increases in concentrations of proinflammatory cytokines were found after chronic ethanol consumption in rats [25,49,81] and mice [70,85] and after acute alcohol exposure [43]. Finally, craving for alcohol in human alcoholics has recently been linked to the production of proinflammatory cytokines [40]. A link between cytokines and chemokines is suggested by reports that decrease in either chemokines or chemokine receptors can change levels of cytokines. Thus, reduction of Ccl2 by antibody neutralization or Ccr2 deficiency resulted in increased cytokine production by lung leukocytes [15,78]. A similar increase of cytokine production was shown also in Ccr5 knockout mice [1]. Significantly, reduced level of exploratory response to novelty in Ccr2, Ccr5, Ccl2 and Ccl3 null mutant mice support the speculation about increased level of cytokines in these mutants (Blednov, unpublished data). Increased levels of cytokines or increased sensitivity to cytokines in mutant mice might lead to increase of conditioned taste aversion to ethanol, and therefore, to decreased ethanol intake.

Most gene-targeted mice, including those used in this study, are developed on a mixed genetic background of inbred mouse strains, usually C57Bl/6J and a 129 substrain. These strains differ in their responses to many drugs, due to the possession of polymorphic alleles [66]. Because all knockout mice used in this study were backcrossed on the inbred C57Bl/6 strain for many generations, the potential confounding effect of the mixed genetic background is reduced. However, in addition to disrupting the gene of interest, homologous recombination places additional 129 “hitchhiking” DNA near the disrupted gene [22,30]. The complete replacement of 129 alleles is limited by the probability of recombination; even after 12 backcrosses a relatively long segment of 129 derived chromosome remains surrounding the mutated gene. If neighboring genes are important for the phenotype of interest, these 129 genes, rather than the null gene, may be responsible for the phenotype.

The 129/SvJ and C57Bl/6 strains show multiple and significant behavioral differences in response to ethanol. Thus, 129/SvJ mice consume less amount of alcohol [10] and demonstrate stronger conditioned taste aversion to ethanol [18] than C57Bl/6J mice. On the other hand, 129/SvJ mice are slightly more sensitive to anesthetic (sedative) effects of alcohol [35] and show more severe ethanol-induced withdrawal than C57Bl/6 [34]. To distinguish between the importance of null genes and “hitchhiking” surrounding genes for the phenotypes of interest, it is necessary to determine the overlap between the known QTLs for these types of behavior and chromosomal regions derived from the parental strains. No alcohol QTL mapping studies have been completed using 129SvJ crosses. Therefore, the replacement of a QTL gene (whether C57Bl/6J, DBA/2J, ILS or ISS) by an uncharacterized 129SvJ allele included in the flanking introgressed region could lead to the incorrect assumption that the null genotype directly influenced the respective phenotype.

On Chr 11, there is one QTLs for ethanol-induced LORR (about 50 cM) [12] and one for conditioned taste aversion for ethanol (40–45 cM) [68]. Our mapping data show that both of these QTLs are inside or bordering the region of Chr 11 derived from 129/SvJ. Therefore, these 129 genes may result in a longer duration of LORR observed in Ccl2 and Ccl3 null mutant mice. An inverse relationship between sensitivity to ethanol-induced sedation and voluntary ethanol consumption has been demonstrated [77]. Thus, the reduced ethanol consumption in Ccl2 null mutant mice could be a result of increased sensitivity to LORR observed in this knockout strain. However, this is not a case for Ccr2 (−/−) knockout mice. Therefore, we might predict an additive reduction in voluntary ethanol intake in double Ccr2 (−/−)/Ccl2 (−/−) knockout mice. However, these mice demonstrated phenotype similar with

only the Ccr2 (−/−) parental strain. This finding is more consistent with relationship between Ccl2 and Ccr2 as agonist-receptor than with an inverse relationship between ethanol-induced LORR and voluntary ethanol consumption.

Although there are QTLs on Chr 9 for alcohol preference drinking: 29 cM [11], 10–35 cM [75] and approximately, 9–56 cM [65], all of these are outside of the 129/SvJ region found in Chr 9 of Ccr2 and Ccr5 null mutant mice. A QTL for conditioned taste aversion for high dose of ethanol (4 g/kg) is located on Chr 9 (49 cM) [68] and is also outside of the 129/SvJ region of Chr 9 of Ccr2 and Ccr5 null mutant mice. The results of this analysis clearly show that decrease of ethanol consumption and stronger conditioned taste aversion to ethanol observed in Ccr2 knockout mice is caused by Ccr2 deficiency and not by 129 “hitchhiking” region on Chr 9. It is important to note that additional mapping of Chr 11 in Ccr2 and Ccr5 knockout mice as well as Chr 9 in Ccl2 and Ccl3 knockout mice showed that these chromosomes belonged to the C57Bl/6J strain and replacement of portion of 129/SvJ genetic background by C57Bl/6J genetic background during backcrossing was complete in all strains, including Ccl3.

Of course, an unanswered question is whether compensatory changes in expression of other genes occur as a result of deletion of Ccr2, Ccr5, Ccl2 and Ccl3 genes. This issue has yet to be explored with these mice.

In summary, these studies provide the first evidence that the chemokine network is important for the motivational effects of ethanol.

5. Summary

Deletion of chemokine receptors (Ccr2) or chemokines (Ccl2 and Ccl3) in mice resulted in lower preference for alcohol and consumption of lower amounts of alcohol in a two-bottle choice test as compared with wild-type mice. Ethanol treatment (2.5 g/kg, i.p.) induced stronger conditioned taste aversion in Ccr2, Ccl2 or Ccl3 null mutant mice than in controls. Ccr2 and Ccr5 (chemokine receptor) null mutant mice did not differ from wild-type mice in ethanol-induced loss of righting reflex. In contrast, mice lacking Ccl2 or Ccl3 showed longer LORR than wild-type mice. There were no differences between mutant strains and wild-type mice in severity of ethanol-induced withdrawal. Genetic mapping of chromosome 11 showed that QTL for alcohol-induced LORR is inside the region of derived from 129/SvJ; thus, these 129 genes may cause some of the ethanol behavioral phenotypes observed in Ccl2 and Ccl3 null mutant mice. On the contrary, similar QTLs on Chr 9 are outside of 129/SvJ region in Ccr2 and Ccr5 null mutant mice. These data show that disruption of the chemokine network interferes with ethanol-induced behaviors, including reduction of ethanol consumption and an increase of the aversive properties of alcohol.

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Fig. 1.

Voluntary ethanol consumption in Ccr2 (-/-) and Ccr5 (-/-) knockout mice. (a) Males: amount of consumed ethanol (g/kg). (b) Males: preference for ethanol. (c) Males: total fluid intake (g/kg). (d) Females: amount of consumed ethanol (g/kg). (e) Females: preference for ethanol. (f) Females: total fluid intake (g/kg); $n = 12-15$ for each male genotypes and $n = 10-18$ for each female genotypes.

Fig. 2.

Voluntary ethanol consumption in Ccl2 (-/-) and Ccl3 (-/-) knockout mice. (a) Males: amount of consumed ethanol (g/kg). (b) Males: preference for ethanol. (c) Males: total fluid intake (g/kg). (d) Females: amount of consumed ethanol (g/kg). (e) Females: preference for ethanol. (f) Females: total fluid intake (g/kg); $n = 10-20$ for each genotype and sex.

Fig. 3.

Voluntary ethanol consumption in *Ccr2* ($-/-$), *Ccl2* ($-/-$) and double *Ccl2* ($-/-$)/*Ccr2* ($-/-$) knockout mice. (a) Males: amount of consumed ethanol (g/kg). (b) Females: amount of consumed ethanol (g/kg). (c) Males: preference for ethanol. (d) Females: preference for ethanol. (e) Males: total fluid intake (g/kg). (f) Females: total fluid intake (g/kg); $n = 10-15$ for each male genotypes and $n = 8-10$ for each female genotypes.

Fig. 4.

Voluntary saccharin consumption in *Ccr2* ($-/-$), *Ccr5* ($-/-$), *Ccl2* ($-/-$) and *Ccl3* ($-/-$) knockout mice. (a) Males: preference for saccharin, *Ccr2* ($-/-$) and *Ccr5* ($-/-$) knockout mice. (b) Females: preference for saccharin, *Ccr2* ($-/-$) and *Ccr5* ($-/-$) knockout mice. (c) Males: preference for saccharin, *Ccl2* ($-/-$) and *Ccl3* ($-/-$) knockout mice. (d) Females: preference for saccharin, *Ccl2* ($-/-$) and *Ccl3* ($-/-$) knockout mice. (e) Males: total fluid intake (g/kg), *Ccr2* ($-/-$) and *Ccr5* ($-/-$) knockout mice. (f) Females: total fluid intake (g/kg), *Ccr2* ($-/-$) and *Ccr5* ($-/-$) knockout mice. (g) Males: total fluid intake (g/kg), *Ccl2* ($-/-$) and *Ccl3* ($-/-$) knockout mice. (h) Females: total fluid intake (g/kg), *Ccl2* ($-/-$) and *Ccl3* ($-/-$) knockout mice; $n = 10-12$ for each male genotypes and $n = 8-10$ for each female genotypes.

Fig. 5. Voluntary quinine consumption in *Ccr2* (-/-), *Ccr5* (-/-), *Ccl2* (-/-) and *Ccl3* (-/-) knockout mice. (a) Males: preference for quinine, *Ccr2* (-/-) and *Ccr5* (-/-) knockout mice. (b) Females: preference for quinine, *Ccr2* (-/-) and *Ccr5* (-/-) knockout mice. (c) Males: preference for quinine, *Ccl2* (-/-) and *Ccl3* (-/-) knockout mice. (d) Females: preference for quinine, *Ccl2* (-/-) and *Ccl3* (-/-) knockout mice. (e) Males: total fluid intake (g/kg), *Ccr2* (-/-) and *Ccr5* (-/-) knockout mice. (f) Females: total fluid intake (g/kg), *Ccr2* (-/-) and *Ccr5* (-/-) knockout mice. (g) Males: total fluid intake (g/kg), *Ccl2* (-/-) and *Ccl3* (-/-) knockout mice. (h) Females: total fluid intake (g/kg), *Ccl2* (-/-) and

Ccl3 ($-/-$) knockout mice; $n = 10$ – 12 for each male genotypes and $n = 8$ – 10 for each female genotypes.

Fig. 6. Ethanol-induced conditioned taste aversion in *Ccr2* ($-/-$) and *Ccr5* ($-/-$) null mutant mice. (a) Males: $n = 14-16$ for saline injection for all genotypes; $n = 13-23$ for groups with ethanol injection. (b) Females: $n = 10-19$ for saline injection for all genotypes; $n = 9-20$ for groups with ethanol injection.

Fig. 7.

Ethanol-induced conditioned taste aversion in *Ccl2* ($-/-$) and *Ccl3* ($-/-$) null mutant mice.

(a) Males: $n = 7-13$ for saline injection for all genotypes; $n = 12-14$ for groups with ethanol injection. (b) Females: $n = 7-14$ for saline injection for all genotypes; $n = 12-15$ for groups with ethanol injection.

Fig. 8. Duration of ethanol-induced LORR in Ccl2 (-/-), Ccl3 (-/-), Ccr2 (-/-) and Ccr5 null mutant mice. (a) Males: * $P < 0.05$; ** $P < 0.01$, statistically significant differences from wild-type mice (Student's t -test); $n = 24$ for wild-type mice and $n = 11$ – 13 for all knockout strains. (b) Females: ** $P < 0.01$; *** $P < 0.001$, statistically significant differences from wild-type mice (Student's t -test); $n = 10$ – 11 for all strains.

Fig. 9. Severity of ethanol-induced withdrawal in Ccl2 (-/-), Ccl3 (-/-), Ccr2 (-/-) and Ccr5 (-/-) null mutant mice. (a and b) HIC scores for male mice; $n = 7-8$ for all strains. (c and d) HIC scores for female mice; $n = 7-8$ for all strains.

Table 1

Mapping recombination boundaries for insertion of 129/SvJ regions in the C57Bl/6J background on Chr11

Genotype	Sex	Markers (location in cM)									
		Mit21	Mit4	Mit38	Mit284	Mit99	Mit167				
		20	37	49	52	58	71				
Cer5 (-/-)	M	B6	B6	B6	B6	B6	B6				
	F	B6	B6	B6	B6	B6	B6				
Cer2 (-/-)	M	B6	B6	B6	B6	B6	B6				
	F	B6	B6	B6	B6	B6	B6				
Cer3 (-/-)	M	129	129	129	129	129	B6				
	F	129	129	129	129	129	B6				
Cer2 (-/-)	M	B6	B6	129	129	B6	B6				
	F	B6	B6	129	129	B6	B6				

n = 5 for each sex and each genotype; B6-C57Bl/6 strain; 129-129/SvJ strain.

Mapping recombination boundaries for insertion of 129/SvJ regions in the C57Bl/6J background on Chr9

Table 2

Genotype	Sex	Markers (location in cM)									
		Mit218	Mit2	Mit32	Mit355	Mit201	Mit281	Mit279			
		4	17	35	49	63.4	65.5	67			
Cer5 (-/-)	M	B6	B6	B6	B6	B6	B6	B6	B6		
	F	B6	B6	B6	B6	B6	B6	B6	B6		
Cer2 (-/-)	M	B6	B6	B6	B6	B6	B6	B6	B6		
	F	B6	B6	B6	B6	B6	B6	B6	B6		
Ccl3 (-/-)	M	B6	B6	B6	B6	B6	B6	B6	B6		
	F	B6	B6	B6	B6	B6	B6	B6	B6		
Ccl2 (-/-)	M	B6	B6	B6	B6	B6	B6	B6	B6		
	F	B6	B6	B6	B6	B6	B6	B6	B6		

n = 5 for each sex and each genotype; B6 – C57Bl/6 strain; 129 – 129/SvJ strain.

Table 3
Summary of effects of deletion of Ccr2, Ccr5, Ccl2 and Ccl3 genes on ethanol-related behaviors

Behavior	Ccr2		Ccr5		Ccl2		Ccl3	
	M	F	M	F	M	F	M	F
Ethanol intake (preference)	↓	↓	0	0	0	↓	↓	↓
Ethanol intake (g/kg)	↓	↓	0	↑	0	↓	↓	↓
Conditioned taste aversion	↑	↑	0	↑	↑	↑	↑	↑
Loss of righting reflex	0	0	0	0	↑	↑	↑	↑
Acute withdrawal	0	0	0	0	0	0	0	0

(↓) Decreased behavior in comparison with wild-type; (↑) increased behavior in comparison with wild-type; 0: no change in comparison with wild-type; M and F denote male and female.