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Age-related differences in anxiety-like behavior and amygdalar CCL2 responsiveness to stress following alcohol withdrawal in male Wistar rats

Kathryn M Harper[1], **Darin J Knapp**[1,2], **Meredith A. Park**[1], and **George R Breese**[1,2,3,4,5]

[1] Bowles Center for Alcohol Studies, The University of North Carolina at Chapel Hill, School of Medicine, CB#7178, 3006 Thurston-Bowles Building, Chapel Hill, NC 27599-7178, USA

[2] Department of Psychiatry, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

[3] Department of Pharmacology, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

[4] Curriculum in Neurobiology, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

[5] The UNC Neuroscience Center, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Abstract

Rationale—Behavioral and neuroimmune vulnerability to withdrawal from chronic alcohol varies with age. The relation of anxiety-like behavior to amygdalar CCL2 responses following stress after withdrawal from chronic intermittent alcohol (CIA) was investigated in adolescent and adult rats.

Methods—Adolescent and adult Wistar rats were exposed to CIA (three 5-day blocks of dietary alcohol separated by 2 days of withdrawal) at concentrations that created similar blood alcohol levels across age. Twenty-four hours into the final withdrawal, half of the rats were exposed to 1 h of restraint stress. Four hours post-stress, rats were used for behavior or tissue assays.

Results—Anxiety-like behavior was increased versus controls by CIA in adolescents and by CIA + stress in adults. CCL2 mRNA was increased versus controls by CIA in adolescents and by CIA and CIA + stress in adults. CCL2 co-localization with neuronal marker NeuN was decreased versus controls by CIA in adolescents and by CIA + stress in adults. CCL2 co-localization with astrocytic marker GFAP was decreased versus controls by CIA and CIA + stress in adolescents, but experimental groups did not differ from controls in adults. CCL2 co-localization with microglial marker Iba1 was decreased versus controls by stress alone in adolescents and by CIA + stress in adults.

Conclusions—Changes in CCL2 protein might control behavior at either age but are particularly associated with CIA alone in adolescents and with CIA + stress in adults. That the

Darin J Knapp darin_knapp@med.unc.edu.

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number of CeA neurons expressing CCL2 was altered after CIA and stress is consistent with CCL2 involvement in neural function.

Keywords

Ethanol; Neurons; Astrocytes; Microglia; Adolescent; Adult

Introduction

Adolescents exposed to chronic intermittent alcohol (CIA) demonstrate greater persistence of anxiety-like behavior than adults during withdrawal from CIA exposure (Wills et al. 2008, 2009). The CIA protocol consists of three blocks of 5 days of ethanol diet separated by 2 days of withdrawal. Consistent with research implicating stress in the anxiety arising after CIA (Breese et al. 2005; Overstreet et al. 2004), substitution of stress for the initial two alcohol withdrawals from the CIA exposure in adults and adolescents enhanced anxiety-like behavior during the final alcohol withdrawal (Wills et al. 2010). In agreement with research demonstrating that cytokines are increased in brain by stress (Madrigal et al. 2010; Vecchiarelli et al. 2016), substitution of the two initial withdrawals of the CIA protocol with intracerebroventricular (ICV) (1 week apart) of the chemokine (C-C motif) ligand (CCL2) before the final 5 days of the alcohol diet enhanced anxiety-like behavior during withdrawal from the single final 5-day alcohol exposure versus 5 days of ethanol diet (Breese et al. 2008; Knapp et al. 2011). In accord with this finding, elevation of CCL2 alone altered hippocampal synaptic function (Nelson et al. 2011) and elevation of CCL2 in combination with alcohol altered hippocampal synaptic structure (Gruol et al. 2014). CCL2 and CCR2 knockout mice were shown to have altered alcohol drinking behavior (Blednov et al. 2005), a finding that further supports the idea that CCL2 influences neural function underlying behavior. Relatedly, ICV injections of CCL2 also increased drinking behavior (Valenta and Gonzales 2016). If CCL2 plays a role in age-related differences in alcohol-related behaviors such as anxiety, then it might be expected that differences in CCL2 induction accompany these behavioral differences. In fact, 10 days of daily gavage in adult, but not adolescent, mice increased CCL2 messenger RNA (mRNA) in hippocampus, cortex, and cerebellum (Kane et al. 2014). In another study, both adolescent and adult rats exhibited increases in cortical CCL2 mRNA after 15 continuous days of liquid ethanol diet (Harper et al. 2015). However, age-related studies have not been reported for the amygdala, a region shown to have a strong cytokine reponse to alcohol (Freeman et al. 2012; He and Crews 2008).

The central amygdala (CeA) supports the withdrawal-induced anxiety-like behavior that follows CIA exposure (Huang et al. 2010). Additionally, knock down of CCL2 with siRNA in either the CeA or the ventral tegmental area blocked drinking behavior (June et al. 2015). Less is known about CCL2 protein in this context, but this chemokine has been found to co-localize with neurons, astrocytes, and microglia in healthy adult, alcohol naive, Wistar, and alcohol-preferring rats (Banisadr et al. 2005a; June et al. 2015). Both astrocytes and microglia undergo phenotypic changes with age including morphological changes and changes in expression of neuroimmune molecules (Bushong et al. 2004; Crain et al. 2013; Robillard et al. 2016; Schwarz et al. 2012; Yang et al. 2013). Therefore, it would be

important to determine the potential changes across age in cell-type distribution of CCL2 protein in response to alcohol and other challenges such as stress.

Despite the demonstrated roles of stress, the amygdala, and CCL2 in the adaptive changes following CIA withdrawal (e.g., Breese et al. 2008; Knapp et al. 2011), the influence of CIA exposure alone or of stress after CIA in adolescents and adults on withdrawal induced anxiety-like behavior or the induction of CCL2 in the amygdala has not been studied. Thus, in the present investigation, the first experiment sought to confirm that adolescents and adults differ in anxiety-like behavior in response to stress alone, CIA or stress after CIA, even with comparable blood alcohol levels during treatment. Following this experiment, the effects of stress, CIA, and stress after CIA on CCL2 mRNA as a function of age were assessed in the amygdala. The level of co-localization of CCL2 protein with markers of various cellular subtypes in the CeA in adolescents and adults was determined after stress alone, CIA, and stress after CIA. The results were expected to extend our understanding of the interaction of alcohol withdrawal, stress, and CCL2 in behavior and in the relevant adaptations accompanying these challenges.

Materials and methods

Animals

The Institutional Animal Use and Care Committee at UNC Chapel Hill approved all protocols. Male Wistar rats (Charles Rivers, Raleigh, NC) arrived at 21 days of age for adolescents or between 45 and 48 days (180–200 g) for adults. A 12-h light-dark cycle was used, and water was always available ab libitum. One to 3 days after arrival, rats were individually housed and chow was removed for the remainder of the study. A nutritionally complete liquid control diet (CD) was made available on the first day, but on the following day, half the rats were switched to an isocalorically equivalent ethanol diet (ED). Both diets were designed by UNC researchers to meet the National Research Council (1972) nutrient requirements and have been used by this lab for several decades to establish the effects of alcohol withdrawal on anxiety-like behavior (Frye et al. 1983). A pair-feeding paradigm was used to determine the amount of CD given based on the amount of diet consumed the previous day by the rats on ED (Harper et al. 2015). Adolescent and adult rats were exposed to 5.4 and 7 % w/v ethanol concentrations, respectively, because these concentrations were previously shown to create equivalent blood alcohol levels in both age groups using a continuous alcohol dietary paradigm, and 7 % ethanol has been shown to lead to withdrawal induced seizures in adolescents (Harper et al. 2015). In this case, blood alcohol levels were not significantly different across age on the last day of each 5-day cycle; however, total alcohol consumption was higher in the adolescents (Table 1). CD was used for all rats during withdrawal. Both adult and adolescent rats continue to gain weight throughout the protocol (data not shown). Twenty-four hours after the conclusion of the third 5-day cycle, half the rats were exposed to 1-h restraint stress in plastic rat decapicones (DC-200, Braintree Scientific Inc., Braintree, MA) (Knapp et al. 2007). Four hours after the completion of stress, rats were used for either behavioral analysis or tissue collection (Fig. 1). Four hours post-restraint stress was chosen as it has been shown to have significant effects on the neuroimmune system (Knapp et al. 2016; Vecchiarelli et al. 2016).

Blood alcohol levels

At 6 am, 2 h before withdrawal at the end of each 5-day cycle, tail blood was collected. ED was available before and after the blood collection period until withdrawal, then CD was available, but no chow was available. This time point has previously been shown to be at peak alcohol consumption for both adolescent and adult rats (Harper et al. 2015). Blood data were taken from a random sample of rats of each age. Given the size of adolescents, blood draw did not always result in sufficient volume of blood to run samples. Blood was analyzed using gas chromatography as previously described (Harper et al. 2015; Knapp and Breese 2012).

Behavioral analysis

The social interaction test was used as a measure of anxiety-like behavior, as per previous reports (File and Seth 2003; Knapp et al. 2011; Overstreet et al. 2002). Rats were paired by age and approximately similar weight, and the data from individual rats were used as prior reports demonstrated that the behavior across CD and ED groups is independent within pairs (Overstreet et al. 2002). Rats were placed simultaneously in an unfamiliar black Plexiglas arena (60 × 60 cm) under 340 lx lighting. Social interaction was recorded for 5 min. An experienced observer blind to treatment scored recordings for the time each rat engaged in social behavior (conspecific grooming, sniffing, following, crawling over/under its partner). A reduction in social interaction was taken to indicate an elevated anxiety-like state.

Tissue collection and qPCR

Rats were sacrificed by rapid decapitation, the brain chilled to 4 °C in phosphate-buffered saline (PBS), and the amygdala immediately dissected on an ice-chilled surface. The amygdala was gross dissected as a block of tissue with the following landmarks as guides. Following a coronal cut of the brain at the anterior end of the hypothalamus, cuts were made sagitally though the lateral edges of the left and right sides of the hypothalamus with a final cut extending laterally from the posterior ends of the sagittal cuts to the rhinal fissure so as to collect tissue ventral to the rhinal fissure. The amygdala was frozen on dry ice then stored at −80 °C until use. Tissue was sonicated in TE buffer containing 1 % SDS. TRIzol reagent (Life Technologies, Grand Island, NY) was then used to extract the RNA followed by use of the SV total RNA isolation system (Promega, Madison, WI). To convert RNA to cDNA, the Superscript III First Strand Synthesis Super mix (Life Technologies, Grand Island, NY) was used. The CCL2 (Rn00580555_m1) Taqman assay with the β-actin (Rn00667869_m1) assay were used to assay mRNA levels. Samples were run in duplicate on the StepOnePlus real-time PCR machine (Life Technologies, Grand Island, NY). Cycle times were used to calculate fold change using the formula $2^{-\Delta\Delta Ct}$.

Tissue collection and immunohistochemistry

Rats were given intraperitoneal injections of pentobarbital 100 mg/kg. Using cardiac perfusion, 0.1 M cold PBS was perfused through the brain followed by 4 % paraformaldehyde in 0.1 M PB. Tissue was sliced coronally at 40 μm using a VT 1000S vibratome (Leica Biosystems, Buffalo Grove, IL). All tissue was blocked in 10 % normal serum (choice of serum was dependent on secondary antibodies) and stained for CCL2

1:200 (Santa Cruz Biotechnology, Inc., Dallas, TX) with a rabbit anti-goat Alexa Fluor 594 secondary 1:1000 (Thermo Fisher Scientific, Waltham, MA). The specificity of this antibody for CCL2 was confirmed previously (Banisadr et al. 2005a). CCL2 staining was combined serially with one of three cell-type-specific markers. Tissue was placed in all primary antibodies overnight at 4 °C on a rocker, and tissue was placed in all secondary antibodies for 45 min at 4 °C on a rocker. For neurons, the neuronal nuclei marker, NeuN 1:500 (EMD Millipore, Billerica, MA) was used followed by secondary goat anti-mouse Alexa Fluor 488 1:500 (Thermo Fisher Scientific). For astrocytes, GFAP 1:2 K (Dako, Carpinteria, CA) followed by secondary chicken anti-rabbit Alexa Fluor 488 1:500 (Thermo Fisher Scientific). For microglia, Iba1 1:1 K (Wako, Richmond, VA) followed by secondary chicken anti-rabbit Alexa Fluor 488 1:500 (Thermo Fisher Scientific). All images were captured with a LSM 780 Confocal Microscope (Carl Zeiss AG, Oberkochen, Germany) and then analyzed using ImageJ. Two biological replicates were run. To control for differences in staining, a corrected table was used to equalize the means within each age/experimental group. Images used in the figures were brightened by 25 % and cropped, but only unaltered images were used for analysis. Particle analysis on Fiji ImageJ software (NIH, Bethesda, MD) was used to unbiasedly count the number of cells in the cell-type-specific marker images (NeuN, GFAP, or Iba1 images). CCL2 images were then added to these images using the add function in Fiji image calculator. This function of image calculator creates a final combined image containing only cells that appeared in both the CCL2 image and the cell-type-specific marker image. Lastly, the final combined images underwent particle analysis to determine the number of cells that contain the cell-type-specific marker and CCL2. The percentage co-localization was determined using the number of cells determined by particle analysis in the combined image over the number of cells determined by particle analysis from the cell-type-specific marker images.

Statistical analysis

All graphs were made in GraphPad Prism (GraphPad Software Inc., La Jolla, CA), and data were analyzed in Statview (SAS, Cary, NC). Data were analyzed by three-way (age, diet, and stress) ANOVA or in the case of blood alcohol levels and alcohol consumed two-way repeated ANOVA (age and time) was used. Following a significant ANOVA, Fisher's least significant difference (LSD) was used for post hoc comparisons or an exploratory Student's *t* test was done. All data are displayed as mean ± SEM. *P* values <0.05 were considered significantly different.

Results

Stress after CIA withdrawal in adolescents and adults induces opposing effects on anxiety-like behavior

A significant main effect of age ($F(1,72) = 42.03$, $p < 0.01$), diet ($F(1,72) = 12.52$, $p < 0.01$) and an interaction between the two factors (age × diet $F(1,72) = 6.3$, $p < 0.05$) on anxiety-like behavior was observed (Fig. 2). Among adolescents, the CIA withdrawal group (ED-no stress) had higher anxiety-like behavior than controls (CD-no stress) ($p < 0.05$) (Fig. 2a). No other significant differences were observed among adolescents. Among adults, only stress

following CIA withdrawal (ED-stress) showed a significant increase in anxiety-like behavior compared with controls ($p < 0.05$) (Fig. 2b).

The pattern of CCL2 mRNA changes in amygdala after withdrawal from CIA exposure and after stress following withdrawal from CIA in adults and adolescents mimics the age-specific pattern of changes in anxiety-like behavior

A significant main effect of age ($F(1,44) = 5.2$, $p < 0.05$), diet ($F(1,44) = 23.42$, $p < 0.01$) and an interaction between age and stress ($F(1,44) = 5.03$, $p < 0.05$) on CCL2 mRNA levels was observed (Fig. 3). Among adolescents, only the CIA withdrawal group had elevated CCL2 mRNA relative to control ($p < 0.05$) (Fig. 3a). However, stress following CIA withdrawal in adolescents significantly decreased the elevated levels in the CIA withdrawal group ($p < 0.05$) (Fig. 3a). Among adults, both the CIA withdrawal group ($p < 0.05$) and the stress following CIA withdrawal group ($p < 0.01$) had elevated CCL2 mRNA levels above control (Fig. 3b). No other comparisons within the adult age group were significant.

Withdrawal from CIA decreased CCL2 co-localization with NeuN in central nucleus of the amygdala (CeA) neurons in adolescents, but only stress following CIA decreased this co-localization in adults

In controls of both adolescents and adults (CD-no stress, Fig. 4), CCL2 protein was found in the cell bodies of CeA neurons as confirmed by the co-localization of CCL2 and NeuN (a neuronal nuclei marker) in the same cells. An interaction between age and stress ($F(1,64) = 4.94$, $p < 0.05$) was observed, as was an interaction between all three factors (age × diet × stress $F(1,64) = 6.93$, $p < 0.05$) on CCL2 co-localization with NeuN (Fig. 4). Among adolescents, the CIA withdrawal subgroup had a decreased percentage of NeuN-positive neurons co-localizing with CCL2 relative to control ($p < 0.05$) (Fig. 4a–g). No other significant differences from controls were observed among the adolescent groups. Among adults, stress following CIA withdrawal significantly decreased CCL2 co-localization with NeuN ($p < 0.05$) (Fig. 4h–n). The adult group that received stress following CIA withdrawal also had significantly reduced CCL2 co-localization with NeuN compared with rats that received only CIA withdrawal ($p < 0.05$) (Fig. 4h–n).

Withdrawal from CIA and stress following CIA alters CCL2 co-localization with GFAP in CeA astrocytes of adolescents only

In controls of both adolescents and adults (CD-no stress, Fig. 5), CCL2 was found in astrocytes of the CeA as shown by the co-localization of CCL2 and GFAP in the same cells. A significant main effect of age ($F(1,69) = 116.03$, $p < 0.01$) and an interaction between age and diet ($F(1,69) = 4.14$, $p < 0.05$) on CCL2 co-localization with GFAP was observed. Among adolescents, both rats undergoing CIA withdrawal and those that underwent stress following CIA withdrawal had significantly decreased CCL2 co-localization with GFAP ($p < 0.05$) (Fig. 5a–g). No significant differences were noted between experimental groups within the adult age group (Fig. 5h–n).

Stress alters CCL2 co-localization with Iba1 in CeA microglia of adolescents and adults

In controls of both adolescents and adults (CD-no stress, Fig. 6), CCL2 in the CeA was co-localized with microglial Iba1. Significant main effects of age ($F(1,65) = 11.72$, $p < 0.01$) and stress $F(1,65) = 9.71$, $p < 0.01$) on CCL2 co-localization with Iba1 were observed. Among adolescents, only rats that underwent stress alone (CD-stress) had significantly reduced CCL2 co-localization with Iba1 compared with controls ($p < 0.05$) (Fig. 6a–g). Among adults, stress following CIA withdrawal significantly decreased CCL2 co-localization with Iba1 ($p < 0.05$) (Fig. 6h–n). The adults that received stress following CIA withdrawal also had significantly reduced CCL2 co-localization with Iba1 compared with rats that received only CIA withdrawal ($p < 0.05$).

Discussion

The present investigation is consistent with previous work showing that induction of anxiety-like behavior in adult rats after CIA exposure was absent by 24 h post-alcohol withdrawal but persisted for up to a week after withdrawal in adolescents (Wills et al. 2009). This age-specific behavioral response to CIA might be attributed to more marked alterations in the brain of the adolescents than in adults. The CIA withdrawal followed by stress increased anxiety-like behavior in adults, with the stress/withdrawal combination having the unexpected outcome of returning behavior to control levels in the adolescents. Overall, these behavioral effects were moderate in magnitude, and additional studies to replicate these findings and to identify optimal conditions are warranted.

Results of this study also confirm previous data showing that CCL2 is expressed in neurons, astrocytes, and microglia in brain (Banisadr et al. 2005a; June et al. 2015). The present report is the first to show the occurrence of CCL2 cell-type-specific responses based on age and experimental exposure. While astrocytes exhibited a decrease in CCL2 co-localization with GFAP in response to CIA with or without stress in adolescents, no such change in adults was observed to the CIA withdrawal with or without stress. Whether this difference in CCL2 responsiveness is wholly attributed to age is unknown. As this outcome does not correlate with the behavioral changes, astrocytes may not be the source of CCL2 that affects behavior in the adult. Microglia appear to be the only cell type that is responsive to stress—an outcome once again that is inconsistent with the behavioral pattern observed. CCL2 co-localization with NeuN in CeA neurons decreased with CIA in adolescents and to CIA withdrawal followed by stress in adults. This latter pattern is consistent with that seen with behavior changes in these age groups.

Altogether, these data from various cell types suggest that neurons are a probable source of the CCL2 that regulates anxiety-like behavior deficits induced by withdrawal from chronic alcohol. However, neurons might not be the sole source of CCL2. CCL2 release from multiple sources might be necessary for extracellular CCL2 to reach levels sufficient to induce behavioral effects. For example, in the adolescent, CIA withdrawal decreases CCL2 co-localization with both astrocytic GFAP and neuronal NeuN while in the adult, stress following CIA decreases CCL2 co-localization with both microglia Iba1 and neuronal NeuN. It is notable that there is no relationship between behavior and the reduced co-localization of CCL2 with GFAP in adolescent rats exposed to stress following CIA

withdrawal. It is also notable that there is no relationship between behavior and the reduced co-localization of CCL2 with Iba1 in microglia of adolescent rats exposed to stress. Thus, it is likely that CCL2 release from only astrocytes or microglia is insufficient to impact behavior in this model. Altogether, this data suggests that it also likely that blocking neuronal CCL2 selectively should be sufficient to reduce the behavior even if CCL2 from other sources contribute to extracellular CCL2 levels. This interpretation is consistent with the finding that neuronal CCL2 was specifically involved with drinking behavior (June et al. 2015). Overall, the approach of blocking CeA neural activity seems useful in further evaluating the conditions in which induction of CCL2 contributes to the anxiety-like behavioral associated with CIA withdrawal and stress after CIA withdrawal.

Collectively, the experiments suggest that CIA withdrawal decreases the number of CeA neurons containing CCL2 protein, a phenomenon that could influence the expression of anxiety-like behavior during withdrawal. Potentially, this latter alteration could lead to the subsequent increase in CCL2 mRNA activity that could replace CCL2 protein in CeA neurons. However, it was interesting that CIA withdrawal alone was sufficient to elicit these effects in the adolescent whereas the combination of CIA withdrawal followed by stress was required to cause the same effects in adults. Thus, the CIA withdrawal appears to prime the adult amygdala such that subsequent stressors create a greater neuroimmune response. Surprisingly, the stress after CIA caused CCL2 levels to return to control levels in the adolescent rats. Potentially, the prolonged response to adolescent CIA withdrawal fosters an adaptation that desensitizes the amygdala to a subsequent stress.

The cause of the decrease in the number of neurons containing CCL2 protein after the alcohol and stress challenges in adults and adolescents is unknown but could be explained by CCL2 degradation within these neurons; shuttling of CCL2 from the cell bodies to the terminals for vesicular release at that site; and/or CCL2 release from the cell bodies in the CeA itself. Previous work from the spinal cord has shown vesicular release of CCL2 from nerve terminals (Dansereau et al. 2008; Van Steenwinckel et al. 2011)—a finding consistent with the idea that chemokines like CCL2 can act as neurotransmitters. CCL2 receptors (CCR2) are located on neurons in many brain regions including the amygdala (Banisadr et al. 2005b). Such receptor localization on CeA neurons would allow for CCL2 release to regulate behavior, a possibility that should be evaluated in future research.

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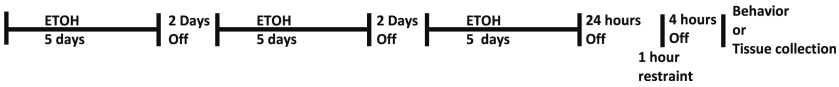

Fig. 1.

Diagram of the chronic intermittent alcohol/stress paradigm used

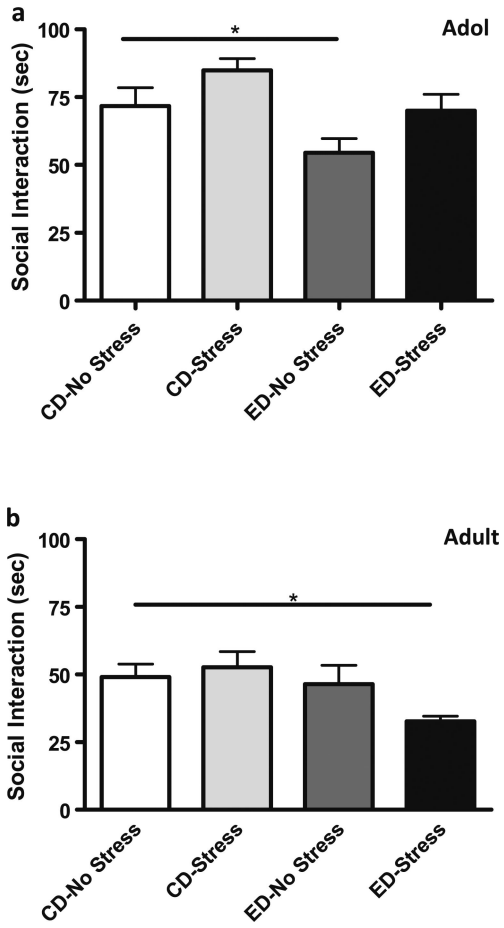

Fig. 2.

Adolescent and adult rats show different anxiety-like responses to CIA withdrawal combined with stress. **a** Adolescent rats had increased anxiety-like behavior (reduced social interaction) following CIA withdrawal which was reversed by combining CIA withdrawal with stress (CD-no stress vs ED-no stress $p < 0.05$). **b** In adult rats combining CIA withdrawal with stress resulted in greater anxiety-like behavior (CD-no stress vs ED-stress $p < 0.05$). CD-no stress = control rats that received control liquid diet and no stress (*white bar*), CD-stress = rats that received control liquid diet and 1 -h restraint stress (*light gray bar*), ED-no stress = rats that received ethanol liquid diet (*dark gray bar*), ED-stress = rats that received ethanol liquid diet and at 24 h into withdrawal had 1-h restraint stress (*black bar*). Data presented as mean ± SEM. $N = 9–12$ per group. Post hoc versus CD-no stress $*p < 0.05$

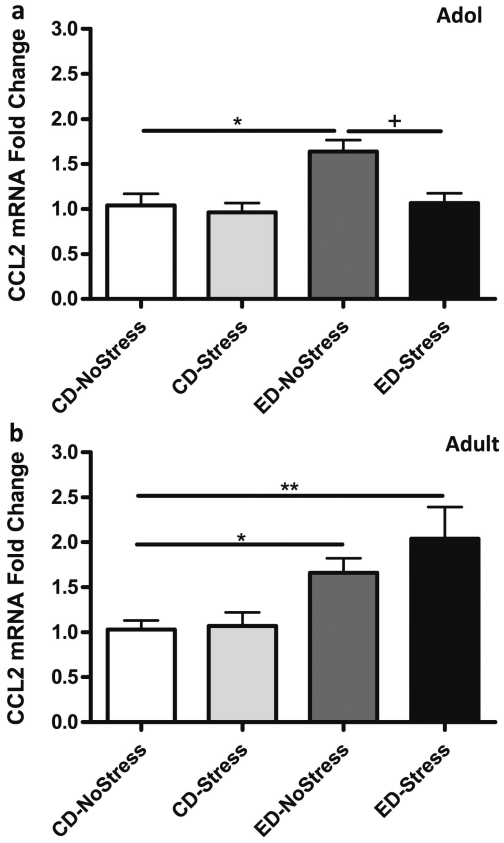

Fig. 3.

Adolescent and adult rats show different amygdalar CCL2 mRNA responses to stress following CIA withdrawal. **a** Adolescent rats had increased CCL2 mRNA following CIA withdrawal (CD-no stress vs ED-no stress $p < 0.05$) that was reversed by combining CIA withdrawal with stress (ED-no stress vs ED-stress $p < 0.05$). **b** In adult rats stress following CIA withdrawal resulted in the greatest CCL2 mRNA response (CD-no stress vs ED-stress $p < 0.01$). CD-no stress = control rats that received control liquid diet and no stress (*white bar*), CD-stress = rats that received control liquid diet and 1-h restraint stress (*light gray bar*), ED-no stress = rats that received ethanol liquid diet (*dark gray bar*), ED-stress = rats that received ethanol liquid diet and at 24 h into withdrawal had 1-h restraint stress (*black bar*). Data presented as mean ± SEM. $N = 6–7$ per group. Post hoc versus CD-no stress $^{*}p < 0.05$, $^{**}p < 0.01$, post hoc ED-no stress versus ED-stress $^{+}p < 0.05$

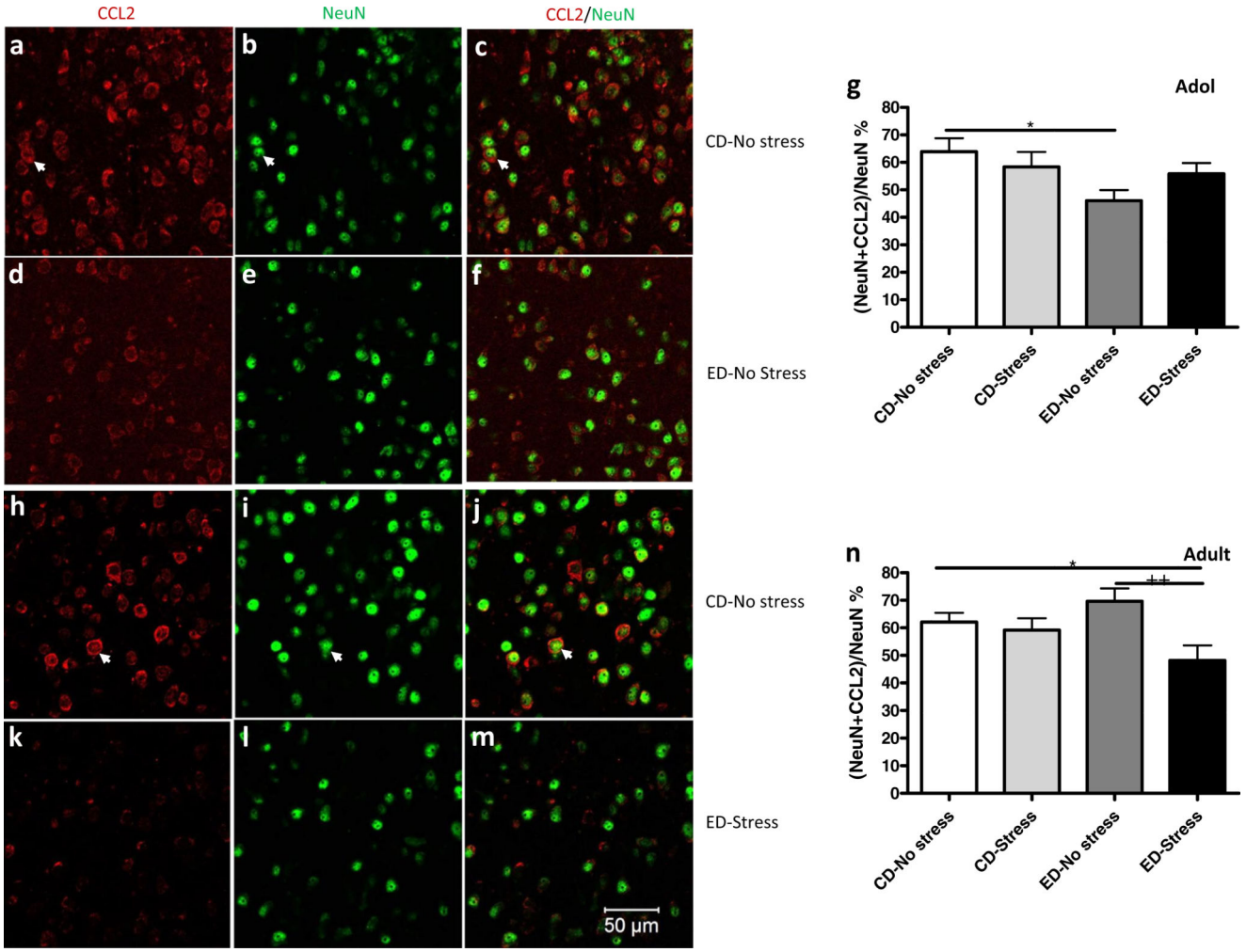

Fig. 4.

Adolescent rats show greater reduction in CCL2 co-localization with NeuN in neurons of the CeA following CIA withdrawal than adult rats. CCL2 is in *red* and NeuN (used as a neuron marker) is in *green*. Representative images of the CeA of adolescent rats exposed only to control diet (**a–c**) or 29 h into withdrawal from CIA (**d–f**). Cell counts demonstrated that in the CeA withdrawal significantly decreased CCL2 protein co-localization with NeuN in neurons in adolescent rats (CD-no stress versus ED-no stress $p < 0.05$) (**g**). Representative images of the CeA of adult rats exposed only to control diet (**h–j**) or rats that were stressed 24 h into withdrawal from CIA (**k–m**). In the CeA withdrawal combined with stress significantly decreased CCL2 protein co-localization with NeuN in adult rats (CD-no stress versus ED-stress $p < 0.05$) (**n**). *White arrows* indicate co-localization. CD-no stress = control rats that received control liquid diet and no stress (*white bar*), CD-stress = rats that received control liquid diet and 1-h restraint stress (*light gray bar*), ED-no stress = rats that received ethanol liquid diet (*dark gray bar*), ED-stress = rats that received ethanol liquid diet and at 24 h into withdrawal had 1-h restraint stress (*black bar*). Data presented as mean ± SEM. N = 8–10 per group. Post hoc versus CD-no stress $^{*}p < 0.05$, $^{**}p < 0.01$, post hoc ED-no stress versus ED-stress $^{++}p < 0.01$ (color figure online)

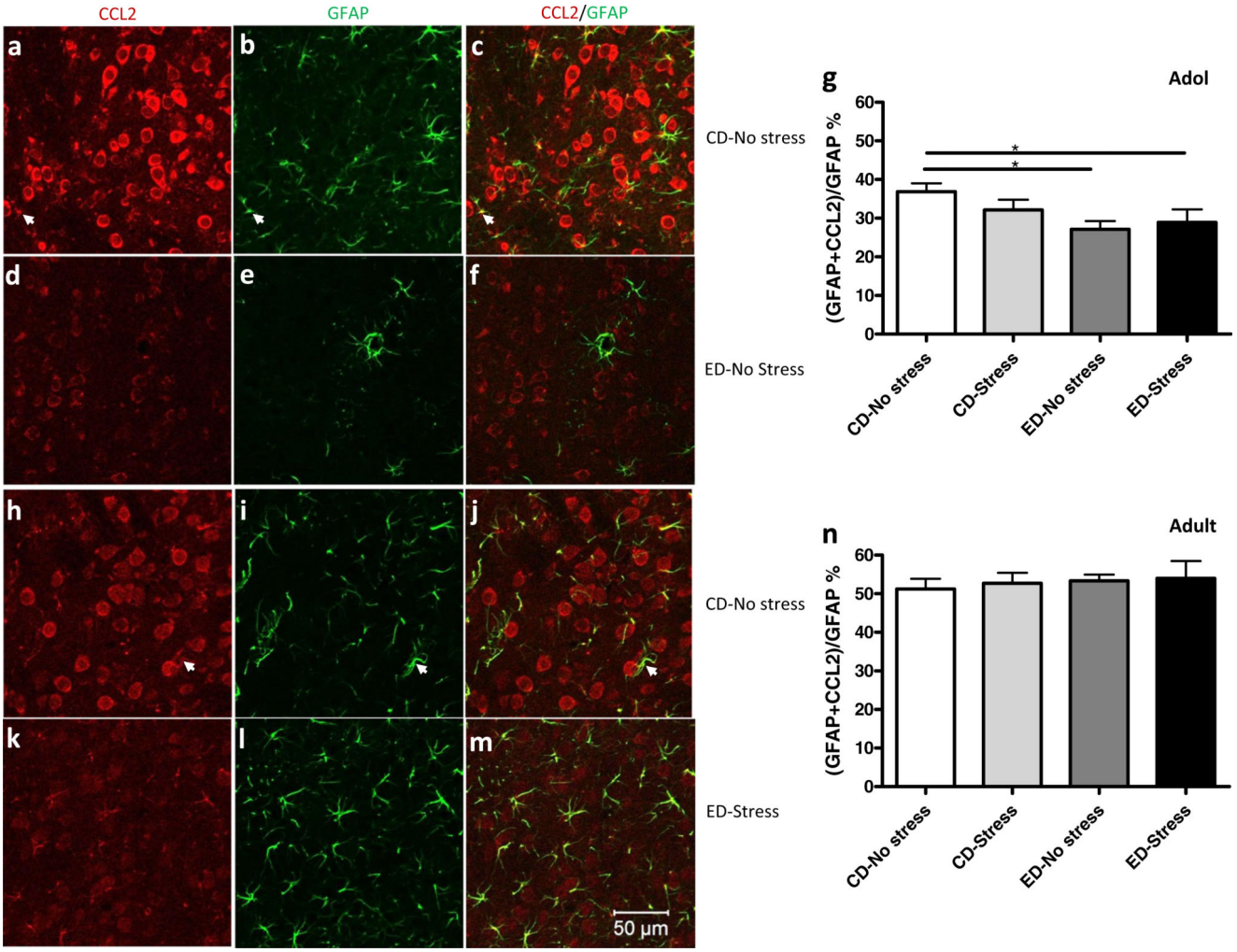

Fig. 5.

Only adolescent rats show a change in CCL2 co-localization with GFAP in astrocytes following withdrawal from CIA or withdrawal combined with stress. CCL2 is in *red* and GFAP (used as an astrocyte marker) is in *green*. Representative images of the CeA of adolescent rats exposed only to control diet (**a–c**) or 29 h into withdrawal from chronic intermittent alcohol (**d–f**). Cell counts demonstrated that in the CeA withdrawal (CD-no stress versus ED-no stress $p < 0.05$) and withdrawal combined with stress (CD-no stress versus ED-stress $p < 0.05$) significantly decreased CCL2 protein co-localization with GFAP in adolescent rats (**g**). Representative images of the CeA of adult rats exposed only to control diet (**h–j**) or rats that were stressed 24 h into withdrawal from CIA (**k–m**). In the CeA, none of these experimental conditions altered CCL2 protein co-localization with GFAP in adult rats (**n**). *White arrows* indicate co-localization. CD-no stress = control rats that received control liquid diet and no stress (*white bar*), CD-stress = rats that received control liquid diet and 1-h restraint stress (*light gray bar*), ED-no stress = rats that received ethanol liquid diet (*dark gray bar*), ED-stress = rats that received ethanol liquid diet and at 24 h into withdrawal had 1 -h restraint stress (*black bar*). Data presented as mean ± SEM. $N = 9–10$ per group. Post hoc versus CD-no stress $*p < 0.05$ (color figure online)

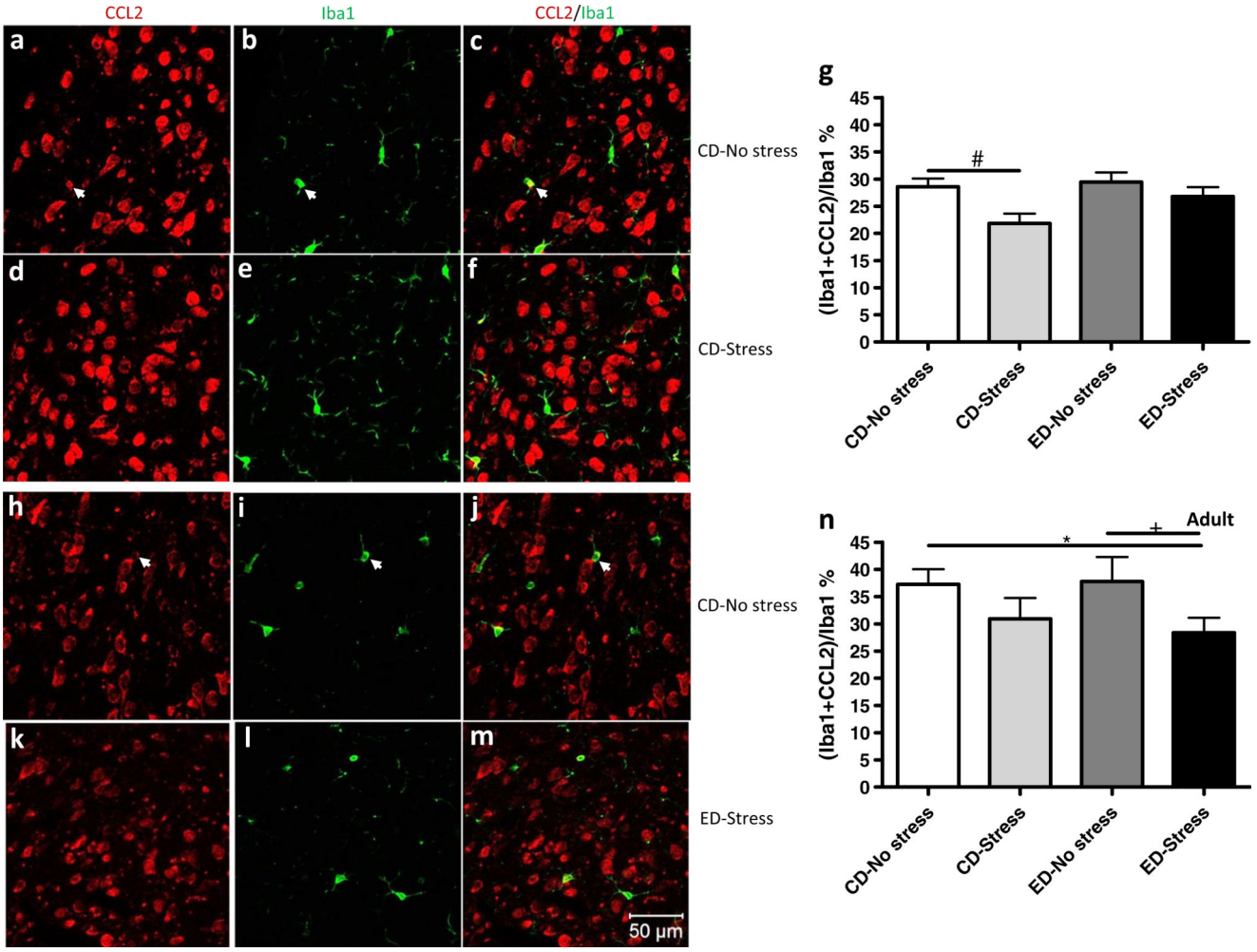

Fig. 6.

Stress alters CCL2 co-localization with Iba1 in microglia. CCL2 is in *red* and Iba1 (used as a microglia marker) is in *green*. Representative images of the CeA of adolescent rats exposed only to control diet (**a–c**) or 4 h after stress (**d–f**). Cell counts demonstrated that in the CeA stress alone, decreased CCL2 protein co-localization with Iba1 in adolescent rats (CD-no stress vs CD-stress $p < 0.05$) (**g**). Representative images of the CeA of adult rats exposed only to control diet (**h–j**) or rats that were stressed 24 h into withdrawal from CIA (**k–m**). In the CeA of adult rats, there was a decrease in CCL2 protein co-localization with Iba1 in rats that received stress following CIA withdrawal (CD-no stress vs ED-stress $p < 0.05$) (**n**). *White arrows* indicate co-localization. CD-no stress = control rats that received control liquid diet and no stress (*white bar*), CD-stress = rats that received control liquid diet and 1-h restraint stress (*light gray bar*), ED-no stress = rats that received ethanol liquid diet (*dark gray bar*), ED-stress = rats that received ethanol liquid diet and at 24 h into withdrawal had 1 -h restraint stress (*black bar*). Data presented as mean ± SEM. $N = 9–10$ per group. Post hoc versus CD-no stress $^{*}p < 0.05$. *t* Test versus CD-no stress $^{\#}p < 0.05$

Table 1

Blood alcohol levels and alcohol consumption across age

	Cycle 1		Cycle 2		Cycle 3	
	Blood alcohol level (mg %)	**Alcohol consumed (g/kg/day)**	**Blood alcohol level (mg %)**	**Alcohol consumed (g/kg/day)**	**Blood alcohol level (mg %)**	**Alcohol consumed (g/kg/day)**
Adolescent (5.4 % *w/v*)	198.1 ± 41.4	17.8 ± 0.3**	214.9 ± 36.6	18.2 ± 0.3**	218 ± 19.2	16.5 ± 0.3**
Adult (7 % *w/v*)	135.6 ± 13.5	10.2 ± 0.1	195.7 ± 18.0	11.8 ± 0.2	186.9 ± 20.8	12.0 ± 0.2

There was no significant effect of age on blood alcohol level with these alcohol concentrations. Both age and cycle affect the amount of alcohol consumed. Adolescent blood $N = 5–8$, consumption $N = 33$; adult blood $N = 8$, consumption $N = 35$. Data are presented as mean ± SEM. Post hoc adolescent versus adult at same cycle

** $p < 0.01$