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Withdrawal from chronic alcohol induces a unique CCL2 mRNA increase in adolescent but not adult brain—relationship to blood alcohol levels and seizures

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Abstract

Background—The role of neuroimmune activation in withdrawal from chronic alcohol (ethanol) has been established in both adolescent and adult models, but direct comparisons across age are sparse. Studies need to elucidate age-dependent neuroimmune effects of alcohol and to focus research attention on age-dependent mechanisms and outcomes.

Materials & Methods—Adult and adolescent rats from two commonly used strains, Wistar and Sprague-Dawley (SD), were maintained on continuous 7%, 5.35%, 4.5% or cycled 7% w/v chronic alcohol diet (CAD) for 15 days. Cortical tissue was collected at 0, 8, 16, and 24 hours post-withdrawal followed by measurement of CCL2, TNFα, and IL-1β mRNA with quantitative real time PCR.

Results—Both age groups and strains showed a strong cytokine mRNA response at 7% CAD. Further, a greater increase in CCL2 mRNA was observed in the cortex of adolescents at 7% CAD, which correlated with higher blood alcohol levels (BALs). Adolescents exposed to 5.35% CAD exhibited similar blood levels and cytokine responses as adults exposed to 7% CAD. Substantial variability in CCL2 mRNA responses was found only in adolescent rats exposed to 7% CAD. In this group, data could be segregated into high responding and low responding groups. Moreover, the data from the high responding group were associated with seizures.

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Conclusions—Relative to other cytokine mRNAs, CCL2 exhibits a unique response profile during withdrawal from CAD. This profile is shown in adolescents, where CCL2 is uniquely influenced by the effects of seizures. Additionally this profile is shown by the fact that only CCL2 expression correlated with blood alcohol level that transcended age groups. These data emphasize the importance of BALs and treatment regimen on developmental neuroimmune responses, and suggest that select components of the neuroimmune system are more responsive to CAD withdrawal and that neurobiological mechanisms differentiating these responses should be further explored.

Keywords

CCL2; adolescent; adult; ethanol; seizures

Introduction

Adult rodents can react differently to alcohol exposure than adolescents (Acheson et al., 1999, Crews et al., 2000, Spear and Varlinskaya, 2005, Wills et al., 2009). Humans who abuse alcohol also can react to alcohol differently as a function of age (Acheson et al., 1998). Evidence is accumulating that abuse of alcohol during adolescence may be partly predictive of development of alcoholism later in life (Grant and Dawson, 1997, Maimaris and McCambridge, 2014). Recent data indicate that cytokines are elevated in the brains of alcoholics (He and Crews, 2008, Crews et al., 2013) as well as the brains of animals during withdrawal (Freeman et al., 2012, Vetreno et al., 2013, Kane et al., 2014, Pascual et al., 2014). Moreover, studies have shown that the chronic continuous alcohol diet (CAD) protocol that has been shown to elevate cytokines in the brain also causes anxiety-like behavioral changes during withdrawal in both adolescents and adults (Wills et al., 2009). Thus, elucidating age-dependent effects of alcohol on cytokines in the brain may be important in furthering our understanding of the risk of alcohol abuse disorders in adulthood following abuse in adolescence.

Alcohol effects on cytokines in research conducted to date in adults or adolescents vary across studies and cytokines measured. For example, in an adolescent binge model used by Pascual et al. (2014), elevations in adolescent prefrontal cortical mRNA of interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNFα), and toll-like receptor 4 (TLR4) were not observed in the adult rat. Conversely, a study in mice using a chronic alcohol paradigm showed that adults compared with adolescents were more vulnerable to the effects of alcohol on chemokine (C-C motif) ligand 2 (CCL2) expression, but not interleukin 6 or TNFα, in the hippocampus, the cortex and the cerebellum (Kane et al., 2014). Interestingly, other studies have shown that adolescent rats exposed to an alcohol binge model exhibit a long-term increase in the frontal cortical TNFα and CCL2, but not IL-1β, into adulthood (Vetreno et al., 2013). In our laboratory's hands, adult rats exposed to the CAD protocol consistently show increases in cytokine mRNAs 24 hours after withdrawal in whole cortex (Whitman et al., 2013). Whether this CAD protocol is associated with similar changes in cytokine mRNAs in the adolescent is not known. Thus, in the research described herein, specific effects of alcohol withdrawal on cytokine mRNA levels in cortical tissue of adolescents and adults were investigated.

Using a similar CAD protocol in both adolescent and adult Wistar and Sprague Dawley rats, the present work provided a means to assess directly the effect of age and strain on cytokine mRNAs in brain. This strategy overcomes the limitations of comparing results across studies that used different species, strains, and alcohol exposure paradigms. In the present investigation, adolescent and adult rats from these two commonly used rat strains were employed to investigate changes in neuroimmune function following withdrawal from CAD exposure. Elucidation of neuroimmune responses to withdrawal from CAD in adults and adolescents could help advance understanding of the mechanism(s) by which alcohol abuse during adolescence is proposed to lead to life-long tendencies toward alcohol abuse (Grant and Dawson, 1997, Brown and Tapert, 2004, Maimaris and McCambridge, 2014).

Materials & Methods

Animals

All protocols were approved by the Institutional Animal Use and Care Committee at UNC Chapel Hill. Male Wistar and Sprague-Dawley (SD) rats (Charles Rivers, Raleigh, NC) arrived at 21 days of age for adolescent experiments or between 45–50 days (180–200g) for adult experiments. One to two days later, rats were placed on a nutritionally complete and calorically balanced liquid diet, control diet (CD), for five days (Frye et al., 1983, Overstreet et al., 2002, Wills et al., 2008, Whitman et al., 2013). For the next 15 days, rats were either placed on an isocalorically matched chronic continuous alcohol liquid diet (CAD) or were kept on CD in a modified pair-feeding paradigm in which volumes consumed by alcohol-exposed animals were matched to the volume given to controls the following day. The 4.5% or 7% (w/v) CAD was used for both adult and adolescent experiments. For each diet concentration and age group used at the 24 hour time point, six rats were CAD and six were control, with the exception of the adolescents at 4.5% CAD where seven rats were exposed to CAD and eight were control. Not all rats were used for analysis (i.e., n = 5–6). Some rats were excluded for technical reasons such as 260/280 ratio less than 1.9 after RNA extraction or lack of space on the microplate. Additionally, a 5.35% CAD (n=8 CAD; n=8 control) was administered to one adolescent group and an additional group of adults was run at 7% CAD (n=8 CAD; n=8 control) for comparison (see Figure 5). At the end of 15 days, all rats were placed on control diet to initiate alcohol withdrawal. A group of Wistar adolescents was subdivided into CAD (n=8), cycled 7% alcohol (CyAD) (n=7) and control (n=8) groups. Those in the CyAD group were exposed to the alcohol diet for 5 days in three cycles separated by 2 days of CD. Rats from both alcohol groups were switched to a liquid diet at the same age, received a total of 15 days of alcohol exposure, and were the same age at withdrawal. There were no significant differences between groups in alcohol consumption (on the day of withdrawal 14.9±.5 g/kg/day continuous and 15.1±.6 g/kg/day cycled) nor in blood alcohol levels (on the day of withdrawal 236±21 mg% continuous and 291±25 mg% cycled). Rats were rapidly decapitated at 0 (n=5–6), 8 (n=5–6), 16 (n=5–6) or 24 hours post-withdrawal (for n values see above). It should be noted that SD rats were only used for 24 hour post-withdrawal experiments at the 4.5% and 7% CADs due to the fact that there was no statistical differences between the strains on most measures (see Figures 1 and 2), so only one strain was used for all subsequent experiments. However, since the remainder of the experiments would focus on CCL2 mRNA response and Wistar rats seemed more sensitive

to the CCL2 mRNA response based upon the 4.5% CAD responses (though this strain difference never reached significance (strain $F(1,22)=1.5$, n.s., strain × diet $F(1,22)=1.2$, n.s.)), Wistars were chosen for subsequent experiments. Whole cortex was immediately dissected before being frozen on dry ice and then stored at −80° C until use. Whole cortex was chosen because our previous work in adults demonstrated that this chronic alcohol diet elevated cytokine mRNAs in this region (Whitman et al., 2013) and previous work comparing adult and adolescent neuroimmune responses to alcohol was done in whole cortex (Kane et al., 2014) or prefrontal cortex (Pascual et al., 2014).

Quantitative real time PCR

Tissue was sonicated immediately before RNA was extracted in Trizol reagent (Life Technologies, Grand Island, NY) followed by use of the SV total RNA isolation system (Promega, Madison, WI). RNA was converted to cDNA using the Superscript III First Strand Synthesis Super mix (Life Technologies, Grand Island, NY). The following TaqMan expression assays were used: CCL2 (Rn00580555_m1), TNFα (Rn01525859_g1), IL-1β (Rn00580432_m1), and β-actin (Rn00667869_m1). 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 $2^{-\Delta\Delta Ct}$.

Video analysis for seizure behavior

At withdrawal, a subset of the SD (n=4) and Wistar (n=4) adolescent rats on 7% CAD from the groups above were placed into new cages that were equipped with cameras enabled for dark cycle recording (Weinberg et al., 2013). An additional 8 adolescent Wistar rats were exposed to the 7% CAD and subsequently used only for video analysis. Rats were recorded for 24 hours following withdrawal until tissue collection. Video recordings were subsequently viewed by two investigators independently to confirm the presence or absence of a seizure. In the case that the two investigators did not agree, a suspected seizure was not classified as such. Limbic seizures were operationally defined as immobility with staring for a prolonged period of time accompanied by head bobs or chewing like movements of the jaw. Tonic-clonic seizures were operationally defined as freezing followed by tonic limb extension and limb or body clonus (Weinberg et al., 2013). In almost all cases, running fits did not precede seizures.

Electroencephalography (EEG) analysis

At postnatal day 23, surgical screws were inserted into the skull over the frontal lobe and the contralateral occipital lobe (Weinberg et al., 2013). Subsequently, wires from a pin connector strip were attached to the screws, and the connector strip was secured to the skull with cranioplastic cement. One to two days later, 2 rats were switched to control liquid diet for two days followed by alcohol diet for the next 15 days while 2 control rats remained on CD throughout the experiment. On the day of withdrawal, EPOCH wireless transmitters (Ripple, Salt Lake City, UT) were attached to the pin connector strip. Rats were placed in new cages on an EPOCH wireless receiver tray (Ripple, Salt Lake City, UT) attached to Powerlab 8/30 (ADInstruments, Colorado Springs, CO). EEG data was recorded and analyzed using Chart5 software (ADInstruments, Colorado Springs, CO).

Blood sampling

Blood was taken between 6 and 7 am either from trunk during brain tissue collection or from the tail if the rat continued in a study. Alcohol levels were measured in the blood samples via gas chromatography as previously described (Overstreet et al., 2002, Knapp and Breese, 2012).

Statistical Analysis

All data were analyzed within responses of the same cytokine; no data were analyzed between cytokine responses. Data were analyzed by 3-way (age, strain, and diet) ANOVA on the experiments that included both strains. All other data were analyzed with Student's t-tests, one-way ANOVA or three-way repeated (age, diet, and time) ANOVA. When appropriate, Fishers Least Significant Difference (LSD) was used for individual post-hoc comparisons. Since no statistically significant difference was observed between strains on the adolescent 7% CAD data (see Figure 2) and only 50% or less of adolescents exposed to 7% CAD seized, data was combined across strains (Table 2) to increase statistical power. With the adolescent 7% CAD data, inequality of variance in CCL2 mRNA data (and in the TNFα data from Figure 4) was found in the alcohol group only along with a non-normal distribution of the alcohol group compared with the control group. When adolescent 7% CAD data was compared with adult 7% CAD data, all CCL2 data was transformed using a $\log_{10}$ X transformation to control for the non-normal distribution in the adolescent data. For within adolescent 7% CAD CCL2 (Figure 4 only for TNFα), the Mann-Whitney U or the Kruskal-Wallis test followed by Dunn's multiple comparison test were used to control for the non-normal distribution of this data. The chi-square test was used to test for the presence or absence of seizures in the two populations of rats in Table 2. Pearson correlation analysis was used for all correlations. All data are displayed as mean±SEM. P-values <0.05 were considered significantly different.

Results

Cortical cytokine mRNAs in adult and adolescent Wistar and Sprague-Dawley (SD) rats following withdrawal from continuous exposure to 4.5%- and 7.0%-alcohol diets

Continuous exposure to the 4.5% CAD did not change interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNFα), or chemokine (C-C motif) ligand 2 (CCL2) mRNAs in cortex of adult SD or Wistar rats 24 hours into withdrawal (Figure 1). The 4.5% CAD in adolescent rats also induced no change in IL-1β, TNFα, and CCL2 mRNAs in SD rats. On the other hand, adolescent Wistar rats exposed to the 4.5% CAD exhibited a small, but significant increase in CCL2 mRNA 24 hours into withdrawal from this alcohol diet ($p<0.01$) without an alteration in IL-1β and TNFα mRNAs (Figure 1).

Without a major change in cytokine mRNAs following the 4.5% CAD, a new cohort of animals composed of both strains of adults and adolescents were then exposed to 7% CAD (Figure 2). Adult SD rats 24 hours into withdrawal from this diet exhibited increases in IL-1β, TNFα, and CCL2 mRNAs in cortical tissue ($p<0.05$; Figure 2B, F, J). Adult Wistar rats exhibited increases in both TNFα and CCL2 mRNAs ($p<0.05$) without a significant change in IL-1β mRNA level (Figure 2A, E, I). Adolescent rats exposed to this diet

exhibited significant increases in IL-1β, TNFα, and CCL2 mRNAs in cortical tissue of both Wistar ($p<0.05$, Figure 2C, G, K) and SD strains ($p<0.01$; Figure 2D, H, L). Across strains, IL-1β and TNFα mRNA levels were similar between adult and adolescent rats; however, a marked increase in CCL2 mRNA was observed in adolescents in responses to 7% CAD (both strains; Figure 2K, L), but not adults (Figure 2I, J).

The time course of CCL2 mRNA in adolescents at 7% CAD was compared with that after 4.5% CAD to assess for concentration-specific effect on peak response. Adult Wistar rats on 7% CAD were included in the analysis to identify possible age-related time course differences. As shown in Figure 3, at time zero when rats were receiving alcohol diet, CCL2 mRNA was not significantly lower in alcohol-exposed adolescents relative to control adolescents. Overall, the time course suggests CCL2 mRNA starts to increase in both adults (from 1.1 ± 0.1 to 1.5 ± 0.2 fold change) and adolescents (4.5% from 0.8 ± 0.1 to 1.1 ± 0.1 and 7% from 0.8 ± .01 to 9.3 ± 2.4) by 8 hours, but this increase at the 8 hour time point is only significant in the adolescent 7% group. Within the 24 hours measured, all groups showed their maximal response at 24 hours with adolescent 7% CAD rats exhibiting the most dramatic increase.

Effect of withdrawal from cycled 7% chronic alcohol exposure on cytokine mRNA increase in adolescent Wistar rats

Previous studies demonstrated that cycling of chronic alcohol exposure enhanced (sensitized) anxiety-like and seizure responses following withdrawal that were not observed with continuous alcohol exposure (McCown and Breese, 1990, Overstreet et al., 2002). To probe for a potential parallel cyclic dietary effect on cytokines, subgroups of adolescent Wistar rats were exposed to a cyclic 7% alcohol diet (CyAD) or continuous 7% alcohol diet (CAD) and assessed for cortical cytokine mRNAs 24 hours into withdrawal. Both dietary strategies elicited significant increases in all three cytokines. Although the cycled strategy was associated with the highest mRNA levels ($p<0.01$ for cycled and $p<0.05$ for continuous versus controls) for IL-1β (Figure 4A) and TNFα (Figure 4B), the difference between these alcohol diets was not significant ($p>0.05$). Further, both the CAD and CyAD rats exhibited an exaggerated CCL2 mRNA response (Figure 4C) comparable to that observed during withdrawal of both strains from 7% CAD in Figure 2. As noted earlier, this increase in cortical CCL2 mRNA (Figure 4C) was far beyond the level of the mRNA increases for the IL-1β and TNFα mRNAs (Figure 4A, B)—further confirmation of the unique change in CCL2 mRNA observed following alcohol withdrawal from 7% CAD in the adolescent rats.

Exploration of the potential basis for the exaggerated increase in CCL2 mRNA in some, but not all adolescent rats following withdrawal from the 7% CAD

Table 1 shows the individual data points for CyAD or CAD rats from Figure 4. The majority of values for IL-1β and TNFα mRNAs fell below a 4 fold change for both treatment protocols (Table 1). In contrast, CCL2 mRNA values ranged from 3.4 fold change to 337.2 fold change (Table 1). In an attempt to discern potential factors associated with this extraordinary CCL2 response, correlations were performed among blood alcohol levels on the morning of withdrawal, on body weight at the end of the study, and on the amount of alcohol consumed (the g/kg/day on the day of withdrawal, the g/kg/day average across the

last five days of the drinking protocol, and the total g/kg/day across all drinking days) (supplemental Table 1). None of these factors were found to explain the wide range in the CCL2 mRNA values.

Seizure activity can induce cytokines in brain (Foresti et al., 2009, Hung et al., 2013), and chronic alcohol can lead to seizure activity (Majchrowicz, 1975, Frye et al., 1983, Knapp et al., 1993). The marked elevation of CCL2 in some, but not all, adolescent rats, after withdrawal from the 7% CAD might be explained by a seizure threshold being exceeded in some adolescents, but not others, during withdrawal. To test this possibility, video recordings were analyzed for seizures during 24 hrs of withdrawal from the 7% CAD in adolescent animals. This analysis revealed that 6 rats displayed a form of seizure activity during the 24 hrs of withdrawal (Table 2). These 6 rats were subsequently documented to have unusually high elevations of brain CCL2 mRNAs ranging from 20.5 to 163.7 fold change (Table 2). The rats designated “high” CCL2 group in Table 2 have CCL2 levels that relate significantly to seizure activity ($p<0.01$). In addition, rats in the high group had TNFα and IL-1β mRNA levels that were significantly higher than observed in those rats with low CCL2 levels. Some of these rats were also assessed for electroencephalographic (EEG) activity. This comparison revealed that the two rats experiencing withdrawal from the 7% alcohol exposure had EEG and behavioral changes during the withdrawal consistent with seizures (Supplemental video 1). Further, the CCL2 mRNAs in these two animals 24 hrs later were increased by 58.1 and 95.8 fold change, respectively (Rats K and M in Table 2). In rats that received only control diet, no indication of EEG waveform or behavioral activity consistent with a seizure was observed (data not shown).

Blood alcohol levels and cytokine responses in adolescents versus adults

Blood alcohol levels were determined an hour or less before the beginning of the light cycle (6–7am) on the final day of alcohol consumption—a period of peak alcohol consumption (Supplemental Figure 1). The adolescents showed higher alcohol consumption of both the 4.5% and 7% CADs than did the adults ($p<0.01$; Table 3). This elevated consumption was accompanied by significantly higher blood alcohol levels in adolescents than in adults that received the comparable alcohol diet ($p<0.05$; Table 3). This effect is consistent with higher CCL2 mRNA levels in the adolescents.

To further examine whether blood levels could be a basis for the differential CCL2 mRNAs across adolescents and adults, a 5.35% CAD was administered to the adolescent rats in an attempt to elicit blood alcohol levels more comparable to those in adults receiving 7% CAD. This strategy resulted in comparable blood alcohol levels as well as cytokine mRNA levels at the 24 hour withdrawal time point that were not different between age groups ($p>0.05$, Figure 5A, B). In addition, blood alcohol levels correlated strongly with CCL2 levels across all age/concentration combinations ($R=.6$, $p<.01$, Figure 5C), but this relationship broke down when animals in the high CCL2 group were included in the data ($R=.3$, $p=.03$, data not shown).

Discussion

In the present studies, the effects of alcohol diet concentration, strain, and age on expression of cortical cytokine mRNA changes in rats were assessed. The 4.5% CAD did not increase cytokine mRNA levels in the adults, but did increase CCL2 mRNA in the adolescents following alcohol withdrawal (Figure 1). Cytokine mRNA responses following withdrawal were similar between strains in adults except for a lack of IL-1β response in only Wistar rats withdrawn from 7% CAD (Figure 2A). No major difference in the changes in cytokine mRNAs were observed as a function of age at CAD exposure except that adolescent-exposed rats had a much larger but variable increase in CCL2 mRNA following the 7% CAD. While the mRNAs for IL-1β and TNFα were increased following withdrawal from the 7% CAD in both ages and strains, these other changes were not as dramatic as with CCL2 mRNA.

Higher blood alcohol levels (BALs) in adolescents that did not seize appears to be an important factor in the CCL2 response, despite both ages receiving the same 7% CAD (adolescent BALs being 232±20 mg% while the adult BALs were only 173±15 mg%) (Table 3). However when considering blood alcohol level it is important to note that blood was only sampled at one time point. This time was chosen because it likely reflected peak alcohol consumption in both ages (Supplemental Figure 1); however, if the temporal pattern of alcohol consumption was different between ages on the day of withdrawal then differences in the blood levels measured between these two groups could occur. Even taking this caveat into consideration, blood alcohol levels correlated with the CCL2 mRNA change across ages and treatments (Figure 5C). IL-1β and TNFα mRNAs were responsive to the 7% CAD, but these responses did not show the relationship to BALs (Figure 5) or the dramatic increase observed with CCL2 mRNA (Tables 1& 2).

In both ages, CCL2 followed the same pattern of changes across the first 24 hours of withdrawal. Basically a rise in CCL2 between withdrawal and the first 8 hours was observed, but usually did not reach significance. These responses were followed by a slight dip in CCL2 mRNA before rising to maximum levels in that 24 hour window. Freeman et al. (2012) showed that at 4 hours post-withdrawal from 35 days chronic dietary alcohol there was no significant increase in CCL2 levels. Perhaps levels of CCL2 begin to rise between 4 and 8 hours post-withdrawal. Importantly, it is during this time window when rats on this chronic dietary alcohol model reach zero BALs (Wills et al., 2008). There is potential for a link between the loss of alcohol in the blood and the rise in CCL2 mRNA that would be worth exploring. Both Freeman et al. (2012) and Whitman et al. (2013) have shown that CCL2 was still significantly elevated at 48 hours and 72 hours post-withdrawal, respectively. Thus, it is possible that CCL2 can have effects on the nervous system for several days post-withdrawal.

When blood alcohol levels were equated between adolescents (5.35% CAD) and adults (7% CAD), no age effect on cytokines was observed. In contrast, earlier reports suggest an adolescent vulnerability or resistance to neuroimmune effects of alcohol withdrawal from chronic alcohol (Kane et al., 2014, Pascual et al., 2014). That research employed gavage and intraperitoneal injections, both of which cause peaks with rapid rises and declines in blood

alcohol level (Livy et al., 2003, Kane et al., 2014, Pascual et al., 2014). With dietary alcohol, in contrast, rats drink and maintain BALs throughout the night; therefore, they are not as likely as rats in the previous research to experience bouts of withdrawal throughout the night (Supplemental Figure 1). While previous work demonstrated that cycling of chronic alcohol induced anxiety (Overstreet et al., 2002), no differences between adolescent continuous and cycled 7% rats were found in cytokine mRNAs levels (Figure 4) in the current study.

Clinical and basic science evidence demonstrates that alcohol withdrawal symptoms, including anxiety and seizures, sensitize with repeated chronic alcohol bouts or cycles and withdrawals whereas a similar amount of alcohol consumed continuously does not sensitize these behavioral responses (Ballenger and Post, 1978, Brown et al., 1988, Overstreet et al., 2002, Breese et al., 2008, Knapp et al., 2011). A comparison of the effect of cycled (CyAD) and continuous alcohol diet (CAD) exposures on cytokine mRNA induction was conducted to see if an increase in responsiveness of cytokine mRNAs in the CyAD protocol compared to the CAD would be noted in adolescents. This effort was motivated in part by previous behavioral results showing that withdrawal-induced social interaction deficits occur only in the CyAD protocol (Overstreet et al., 2002). The finding that the adolescent 7% CyAD rats exhibited the same profile of cytokine mRNAs as the adolescent 7% CAD (including the marked increase in CCL2 mRNA) suggests that the CCL2 mRNA changes did not relate to the withdrawal-induced social interaction deficits. These interpretations should be tempered with the knowledge that behavioral assessments were made 6 hrs after withdrawal from a 4.5% cycled alcohol withdrawal protocol rather than 24 hrs after withdrawal when the cytokine mRNAs were determined. It is also notable that this cycled paradigm contains only 3 cycles while some earlier studies testing the effects of kindling on alcohol withdrawal seizures used up to 10 cycles (e.g., McCown and Breese, 1990). Thus the present work does not preclude the possibility that cycling may be important in cytokine expression profiles.

The increase in CCL2 mRNA in adolescents exposed to 7% CAD was associated with a large standard error reflecting a wide difference in values across this group. With closer analysis, the data points for these CCL2 mRNA values were found to be distributed into two groups—one with “high” CCL2 mRNA values and the other having “low” values (Table 2). This exaggerated increase in the CCL2 mRNA level in only a portion of the adolescent rats exposed to the 7% CAD protocol suggested that some factor within the group was responsible. The failure to observe different BALs between the CCL2 mRNAs with “high” and “low” values suggests that some other factor was responsible for this difference in the CCL2 mRNA values in the adolescents.

Adolescent rats exposed to 7% CAD are more sensitized to audiogenic seizures than adults exposed to the 7% CAD (Wills et al., 2008) and CCL2 levels increase in other seizure models (Foresti et al., 2009, Hung et al., 2013). Therefore, the possibility that seizures in some 7% CAD exposed animals might be involved was considered. This analysis revealed that the levels of CCL2 mRNAs in the cortex after withdrawal from the 7% CAD were statistically related to the presence or absence of seizure activity. Because evidence for seizures was not observed in two rats with high levels of CCL2 mRNA during evaluation for seizures, the high CCL2 mRNA levels in these animals may reflect minor seizure activity that was not readily apparent (i.e., missed) during the video analysis. Overall, these results

suggest that seizure activity may be a key factor in contributing to the marked increases in CCL2 mRNAs observed in a subset of the adolescent rats withdrawn from the 7% CAD. While this assessment is a plausible explanation for the unique increase in the CCL2 mRNAs in some adolescents, attempts to determine why only some adolescents show seizures will require further study.

The level of CCL2 expression during alcohol withdrawal has the potential to be a marker for alcohol withdrawal seizures and, possibly, heightened neural activity in general, in alcohol withdrawal models. The percent increase in CCL2 mRNA levels measured here are lower than the percent increase in CCL2 mRNA, but higher than the ratio of the protein levels in the seizure group versus the control group observed in pilocarpine-induced seizure models (Foresti et al., 2009, Hung et al., 2013). The reason for the higher mRNA levels found in the pilocarpine model might be due to the fact that those papers examined the hippocampus instead of the cortex (Foresti et al., 2009, Hung et al., 2013) or due to the length of seizure (more than 5 minutes in Hung et al. 2013 versus 43.8 sec in present work). Additionally, the differences between mRNA and protein in the pilocarpine model suggests that not all of the mRNA generated by the alcohol withdrawal induced seizures will be converted into measureable protein. Alcohol-exposed rats also exhibited higher CCL2 levels than controls in cases where there was no evidence for seizure activity.

To a lesser extent TNFα was found to be elevated in response to seizures. This result is consistent with findings that TNFα might have a role in seizure induction (Vezzani et al., 2011, Weinberg et al., 2013). On the other hand, IL-1β has also been implicated in seizure induction (Vezzani et al., 2011), but there was only a limited difference in IL-1β across seizure versus non-seizure groups. However, it is unknown in these animals what the IL-1β levels were before the seizure. The possibility exists that the IL-1β mRNA levels were elevated pre-seizure and returned to a baseline more consistent with the nonseizure level seen with alcohol withdrawal. In a model of febrile status epilepticus, only those rats who underwent epileptogenesis showed chronically elevated IL-1β (Dube et al., 2010) and this effect is consistent with the observation that elevated IL-1β levels are found in humans with conditions that cause repeated seizures (Choi et al., 2009). The rats in this protocol would therefore not be likely to become epileptic.

Neuroimmune factors have long been known to be associated with glial cell activities. The traditional view holds that glia activation is initiated in response to the release of these neuroimmune factors then once activated glia release neuroimmune factors in a positive loop that likely includes CCL2 (John et al., 2003, Crews, 2012). However, with the discovery of the presence of CCL2 in neurons (Banisadr et al., 2005), and electrophysiological evidence suggesting that neurons respond to CCL2 (van Gassen et al., 2005), it is likely that neurons play a role or are affected by this feedback loop. Consistent with this new view, the present work demonstrates a potential relationship between CCL2 mRNA and seizures that is consistent with the possibility that CCL2 mRNA can be altered---directly or indirectly---by neural activity. These observations suggest that CCL2 may play multiple roles in neuronal function, a possibility that warrants further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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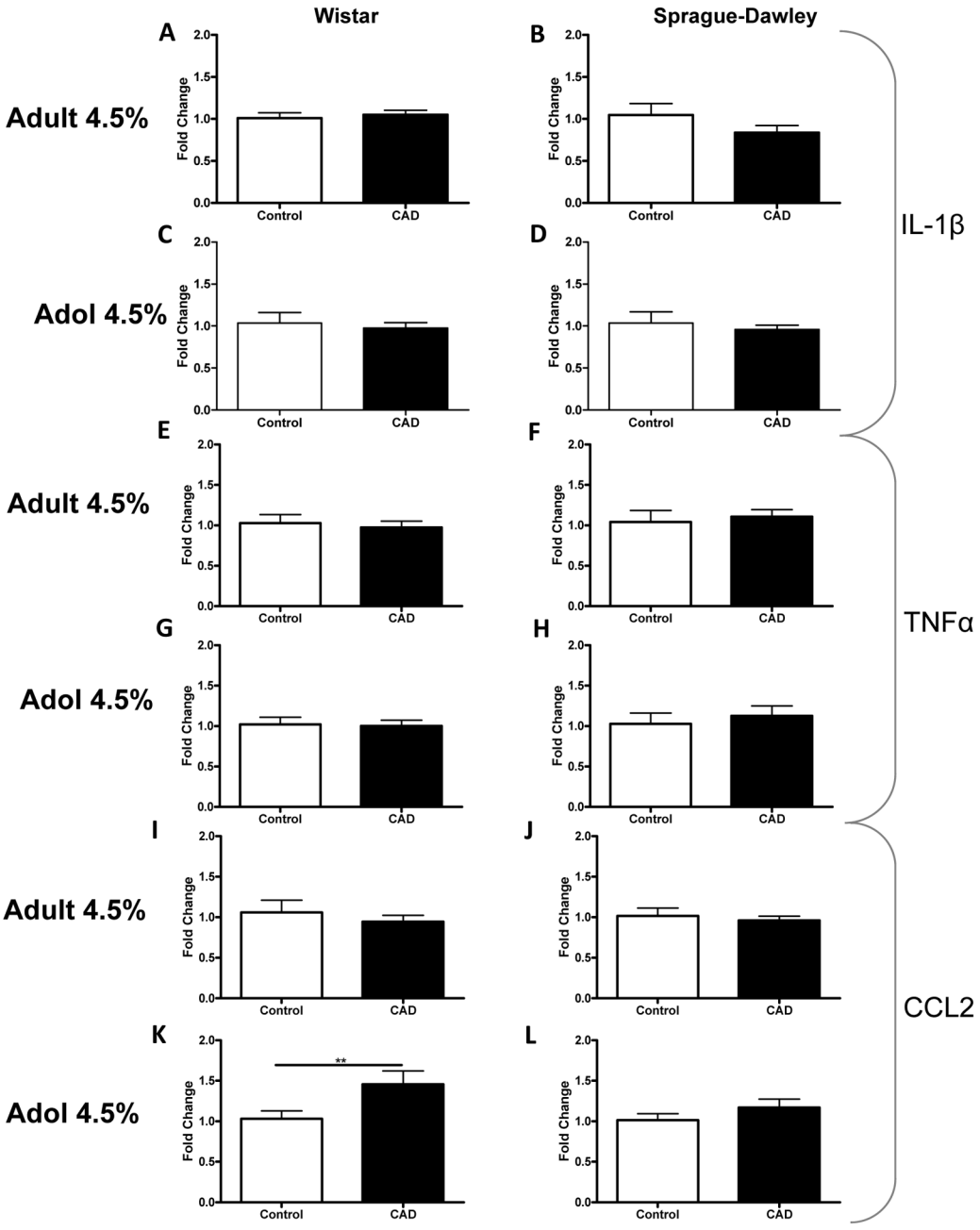


Figure 1. Adolescent rats show an increased sensitivity to withdrawal from 4.5% CAD compared to adults at 24 hours into withdrawal

Adult (A & B) and adolescent (C & D) rats of both strains show no IL-1β mRNA response to withdrawal. Similarly, there was no TNFα response of adults (E & F) and adolescents (G & H) of both strains to withdrawal. Adolescents (K & L) of both strains show a CCL2 mRNA response to withdrawal, but adults (I & J) of both strains show no response (age $F(1,39)=3.4$, $p=.07$, age × diet $F(1,39)=6.2$, $p<.05$). Data presented as mean±SEM. N=5–8 per group. Post-hoc CAD versus control **=$p<.01$.

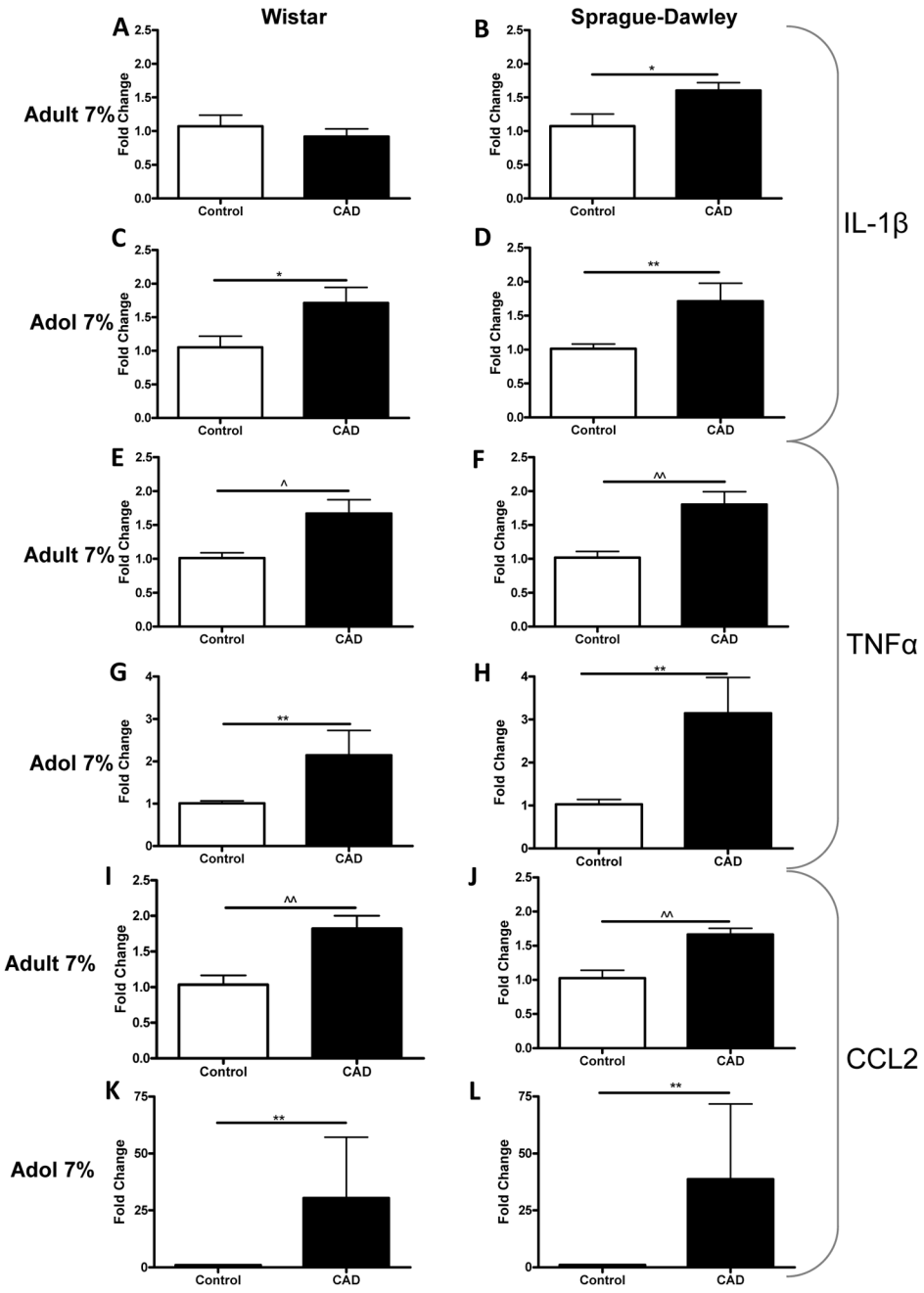


Figure 2. Adolescent rats compared to adults show an increased cytokine mRNA response to withdrawal from 7% CAD at 24 hours into withdrawal

Adult SD (B), but not Wistar (A) show an increase in IL-1β response to withdrawal while both Wistar (C) and SD (D) adolescents show increased IL-1β response (diet $F(1,36)=11.9$, $p<.01$, age × diet $F(1,36)=3.8$, $p=.06$). Adult (E & F) and adolescents (G & H) of both strains show increased TNFα in response to withdrawal (diet $F(1,37)=18.5$, $p<.01$). Adolescents from both strains (K & L) show an exaggerated response to CCL2 compared to adults (I & J) of both strains (age $F(1,36)=4.3$, $p<.05$, diet $F(1,36)=14.9$, $p<.01$, age × diet

F(1,36)=4.3, p<.05). Data presented as mean±SEM. N=5–6 per group. Post-hoc CAD versus control *=p<0.05, **=p<0.01. T-test CAD versus control ^=p<0.05, ^^=p<0.01.

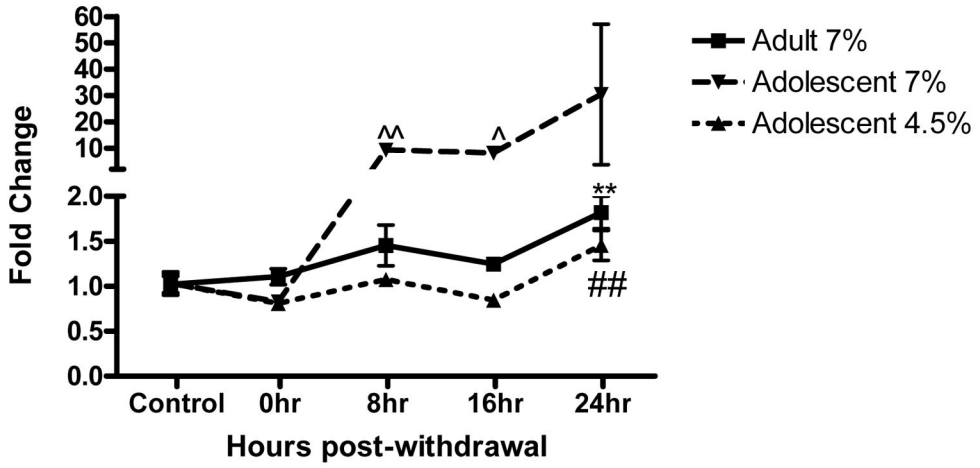


Figure 3. Cyclic cytokine time course of cortical CCL2 expression in both adult and adolescent Wistar rats following CAD

Adult and adolescent rats show a similar CCL2 time course throughout the first 24 hours post-withdrawal, but the adolescents had different levels of CCL2 transcripts (Adult 7% CAD effect of time $F(4,24)=4.8$, $p<.01$; Adol 7% CAD effect of time $H=19.6$, $p<.01$; Adol 4.5% CAD effect of time $F(4,24)=7.5$, $p<.01$). For ease of comparison the 24hr time point from Figure 1 and Figure 2 were replotted. Data presented as mean±SEM. N=5–6 per group. Post-hoc adult 7% CAD time point comparisons with their 0hr time point **=$p<.01$; adolescent 7% CAD time point comparisons with their 0hr time point ^=$p<.05$, ^^=$p<.01$; adolescent 4.5% CAD time point comparisons with their 0hr time point ##=$p<.01$.

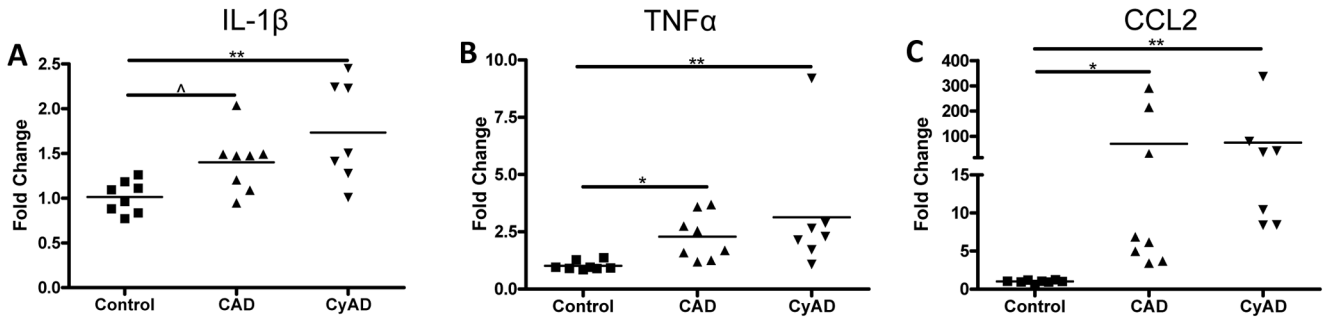
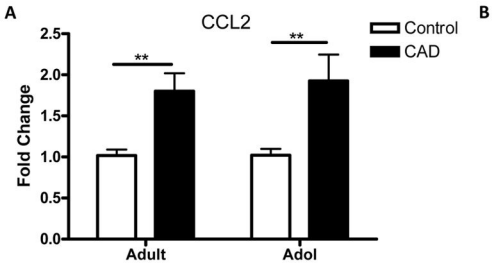


Figure 4. Some adolescent rats show an exaggerated CCL2 mRNA response to withdrawal from 7% continuous (CAD) and cycled alcohol (CyAD) at 24 hours into withdrawal

During withdrawal from continuous CAD or CyAD, Wistar adolescents show elevated IL-1β (A; ANOVA $F(2,20)=6.7, p<.01$), TNFα (B; Kruskal-Wallis $H=12.2$, 2 df, $p<.01$) and CCL2 (C; Kruskal-Wallis $H=16.0$, 2 df, $p<.01$). Note different y-axis scales used on A, B, and C. N=7–8 per group. Data presented as mean±SEM. Post-hoc CAD or CyAD versus control *=p<.05, **=p<.01. T-test CAD versus control ^=p<.05.



B

Group	BAL (mg%)	Ave CCL2 (Fold Change)	Ave TNFα (Fold Change)	Ave IL-1β (Fold Change)
Adult 4.5%	42.6±7.5	.95±.05	1.0±.1	.95±.1
Adol 4.5%	65.1±13.3	1.3±.1	1.1±.1	.97±.04
Adult 7%	173.4±16.2	1.8±.1	1.7±.1	1.6±.1#
Adol 5.35%	196.7±11.0	1.9±.3	1.9±.1	1.5±.1
Adol 7%	231.5±20.4	2.2±.2	1.7±.4	1.4±.1

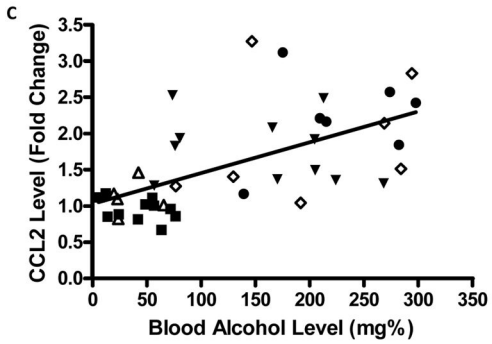


Figure 5. Blood alcohol level is more important than age when determining CCL2 mRNA levels

Adult rats exposed to 7% CAD have the same CCL2 levels as adolescent rats exposed to 5.35% CAD at 24 hours into withdrawal (A; age $F(1,27)=.1$, n.s.; diet $F(1,27)=19.3$, $p<.01$; diet × age $F(1,27)=.1$, n.s.). Summary of the average blood alcohol levels and cytokine mRNA from all strains tested at that age/alcohol contration (B). # = Note for adult 7% CAD, data only include SD rats since there is no IL-1β responses in Wistar rats at this concentration. Correlation between the age/concentation group's blood alcohol level and their CCL2 mRNA levels (C; $R=.6$, $p<.01$). Line was drawn in to assist in showing correlation. Data presented as mean±SEM. N=8–18 per group for A & B and N=42 for C. Post-hoc CAD versus control **=$p<.01$. No data were included from adolescent rats from the high CCL2 group (see text for more details).

Table 1

Individual data points for 7% continuous (CAD) and cycled (CyAD) in adolescents Wistar rats at 24 hours into withdrawal from Figure 4.

Animal Number	Group	CCL2 (Fold change)	TNFα (Fold Change	IL-1β (Fold Change)	Average Alcohol Consumed (g/kg/ day)	Blood Alcohol Level (mg%)	Body Weight (g)
1	CAD	3.4	1.2	1.5	14.1	276.7	141
2	CAD	3.7	1.7	.9	14.3	153.6	108
3	CAD	5.0	2.7	1.5	15.1	NA	133
4	CAD	6.2	2.5	2.0	14.9	NA	130
5	CAD	6.9	1.6	1.2	14.2	243.9	142
6	CyAD	8.4	1.1	1.3	16.4	285.4	87
7	CyAD	8.5	2.7	2.5	15.7	NA	123
8	CyAD	10.4	1.7	1.0	16.0	344.3	115
9	CAD	30.6	1.3	1.1	14.3	NA	132
10	CyAD	37.2	2.1	2.2	15.1	237.9	120
11	CyAD	42.8	2.9	1.5	16.1	NA	116
12	CyAD	80.5	2.3	1.4	14.3	319.4	103
13	CAD	214.9	3.7	1.5	16.2	243.5	115
14	CAD	291.2	3.6	1.5	14.9	212.3	137
15	CyAD	337.2	9.2	2.2	16.2	267.8	102

NA= not applicable due to the fact that no blood sample was taken from these individuals. Correlation CCL2 vs TNFα R=.81, p<.05; CCL2 vs IL-1β R=.33, n.s.; TNFα vs IL-1β R=.6, n.s.

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Table 2

Summary of seizure activity in rats that underwent video analysis during the first 24 hours of withdrawal and their CCL2 levels at the end of this period.

Rat	Time post withdrawal (hrs)	# of seizures	Total duration all seizures (sec)	Type of seizure	CCL2 levels (Fold Change)	TNFα levels (Fold Change)	IL-1β levels (Fold Change)	Average g/kg/day over all 15 days
A	----	----	----	----	1.2	.9	.75	15.1
B	----	----	----	----	1.8	1.2	.9	14.6
C	----	----	----	----	1.8	1.4	1.4	15.5
D	----	----	----	----	2.2	1.9	1.3	15.6
E	----	----	----	----	2.4	1.5	2.1	15.7
F	----	----	----	----	2.9	1.6	1.0	15.6
G	----	----	----	----	3.1	1.7	1.6	15.2
H	----	----	----	----	5.1	1.9	1.1	17.2
Low Ave	----	----	----	----	2.6±.4	1.5±.1	1.3±.2	15.6±.2
I	----	----	----	----	10.5	2.1	1.7	16.2
J	14–15	1	38	C-T	20.5	4.2	1.8	17.0
K	13–14	1	45	Limbic	58.1	1.8	1.7	23.5
L	9–10	1	46	Limbic	79.5	3.0	1.6	16.7
M	4-5	2	26	C-T	95.8	4.6	1.4	23.0
N	9–10	2	51	C-T	103.4	3.6	1.5	14.8
O	18–19	2	57	C-T	163.7	4.4	2.3	16.6
P	----	----	----	----	203.1	6.8	2.9	13.9
High Ave	11–12	1.5	43.8		91.8±23.4**	3.8±.6**	1.9±.2*	17.7±1.3

The grey row depicts the mean±SEM for low and high groups. Low group versus high group

** =p<.01 Mann-Whitney,

* =p<.05 T-test.

CCL2 vs TNFα R=.88, p<.01. Correlation for presence or absence of seizures in the high CCL2 group ($X^2(1)=9.6$, p<.01). Note if rat I is placed in the low group correlation remains the same ($X^2(1)=12.3$, p<.01).

Table 3

Blood alcohol level is age dependent.

	Blood Alcohol at withdrawal (mg %)	Average alcohol consumed first 5 days of the CAD (g/kg/day)	Average alcohol consumed second 5 days of the CAD (g/kg/day)	Average alcohol consumed last 5 days of the CAD (g/kg/day)
Adult (7% CAD)	173.4±15.2	10.1±0.2	11.0±0.2	12.3±0.3
Adult (4.5% CAD)	42.6±7.5	8.8±0.1	9.4±0.2	9.2±0.2
Adol (7% CAD)	231.5±20.4**	15.0±0.2**	15.8±0.3**	16.0±0.4**
Adol (4.5% CAD)	65.1±13.3^	15.3±0.3**	14.2±0.3**	13.8±0.2**
Adol (5.35% CAD)	196.7±11.0	16.3±0.6	15.5±0.3	15.3±0.2

Data presented as mean±SEM. Age and alcohol concentration determine blood alcohol level (age $F(1,46)=6.4$, $P<.05$, concentration $F(1,46)=86.7$, $p<.01$). There is no significant difference between adult 7% CAD and adolescent 5.35% CAD ($T(17)=-1.1$, n.s.). N=10–18 per group. Age, concentration, and time period effect the amount of alcohol consumed (age $F(1,82)=469.2$, $p<.01$, concentration $F(1,82)=49.4$, $p<.01$, time $F(2,164)=3.3$, $p<.05$, age × time $F(2,164)=8.3$, $p<.01$, concentration × time $F(2,164)=16.0$, $p<.01$). N=12–38 per group. All subjects included in alcohol consumed data, except animals for the time course study because weighing on the day of tissue collection might interfere with other measures. Post-hoc adult vs adolescent at the same concentration

** =p<.01, T-test adult vs adolescent on the same concentration

^ =p=.05.