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Altered Hippocampal Synaptic Function in Transgenic Mice with Increased Astrocyte Expression of CCL2 After Withdrawal from Chronic Alcohol

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Abstract

CNS actions of the chemokine CCL2 are thought to play a role in a variety of conditions that can have detrimental consequences to CNS function, including alcohol use disorders. We used transgenic mice that express elevated levels of CCL2 in the CNS (CCL2-tg) and their non-transgenic (non-tg) littermate control mice to investigate long-term consequences of CCL2/alcohol/withdrawal interactions on hippocampal synaptic function, including excitatory synaptic transmission, somatic excitability, and synaptic plasticity. Two alcohol exposure paradigms were tested, a two-bottle choice alcohol (ethanol) drinking protocol (2BC drinking) and a chronic intermittent alcohol (ethanol) (CIE/2BC) protocol. Electrophysiological measurements of hippocampal function were made *ex vivo*, starting ~ 0.6 months after termination of alcohol exposure. Both alcohol exposure/withdrawal paradigms resulted in CCL2-dependent interactions that altered the effects of alcohol on synaptic function. The synaptic alterations differed for the two alcohol exposure paradigms. The 2BC drinking/withdrawal treatment had no apparent long-term consequences on synaptic responses and long-term potentiation (LTP) in hippocampal slices from non-tg mice, whereas synaptic transmission was reduced but LTP was enhanced in hippocampal slices from CCL2-tg mice. In contrast, the CIE/2BC/withdrawal treatment enhanced synaptic transmission but reduced LTP in the non-tg hippocampus, whereas there were no apparent long-term consequences to synaptic transmission and LTP in hippocampus from CCL2-tg mice, although somatic excitability was enhanced. These results support the idea that alcohol-induced CCL2 production can modulate the effects of alcohol exposure/withdrawal on synaptic function and indicate that CCL2/alcohol interactions can vary depending on the alcohol exposure/withdrawal protocol used.

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Conflict of interest

The authors have no conflict of interest to declare.

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Keywords

Two-bottle choice alcohol drinking; chronic intermittent alcohol exposure; hippocampus; synaptic plasticity; synaptic transmission; memory and learning

1. Introduction

Excessive alcohol consumption can result in neuroadaptive changes in the CNS that significantly impact behavior and can lead to alcohol addiction, a serious health and social issue (Most et al., 2014; Tabakoff and Hoffman, 2013). The neuroadaptive changes produced by alcohol have been intensively investigated and these studies have identified many targets of alcohol in the CNS, such as neurotransmitter receptors and synaptic function (Harrison et al., 2017; Lovinger and Roberto, 2013; Roberto and Varodayan, 2017). These studies have also revealed previously unknown actions of alcohol on the CNS, including the production of neuroimmune factors by CNS cells, primarily astrocytes and microglia (Cui et al., 2014). The chemokine CCL2 (chemokine ligand 2, previously known as monocyte chemoattractant protein-1 or MCP-1) is an important neuroimmune factor that is produced at elevated levels in the CNS by the actions of alcohol. Both single and repeated cycles of alcohol exposure/withdrawal at a variety of doses and exposure protocols have been shown to increase expression of CCL2 mRNA or protein in the CNS, an effect that outlasted the period of alcohol exposure (Baxter-Potter et al., 2017; Chang et al., 2015; Drew et al., 2015; Ehrlich et al., 2012; Freeman et al., 2012; Harper et al., 2015a; Kane et al., 2013; Kane et al., 2014; Pascual et al., 2015; Qin et al., 2008; Whitman et al., 2013). For example, increased levels of CCL2 mRNA or protein were observed in several regions of the rat brain, including a prominent increase in the hippocampus, after withdrawal from a 15-day liquid alcohol diet (Harper et al., 2015a; Knapp et al., 2016). Prolonged alcohol drinking (12 months) also resulted in increased CCL2 levels in the rat brain (Ehrlich et al., 2012). Increased levels of CCL2 mRNA were observed in several regions of the mouse brain, including the hippocampus, after withdrawal from repeated cycles of alcohol exposure/withdrawal at a high dose of alcohol (6 gm/kg, by gavage, for 10 days) (Kane et al., 2013; Kane et al., 2014). In a similar study, the increase in CCL2 mRNA and protein produced by alcohol exposure in the CNS of mice was shown to persist for several days after alcohol withdrawal (Qin et al., 2008). In humans, elevated levels of CCL2 were observed in the cerebral-spinal fluid (CSF) of alcoholics compared with CSF from control subjects (Umhau et al., 2014). Post-mortem human alcoholics showed elevated levels of CCL2 in several brain regions, including the hippocampus and cortex, compared to brains from post-mortem human non-alcoholics (He and Crews, 2008; Lewohl et al., 2000).

Several lines of evidence suggest that increased levels of CCL2 in the CNS can impact alcohol-sensitive behaviors. For example, studies have shown that CCL2 can play a role in alcohol consumption, although the exact role and consequences of the CCL2/alcohol interactions remain to be fully elucidated (Blednov et al., 2005; Chen et al., 2017; Valenta and Gonzales, 2016). In alcohol drinking studies using CCL2 gene knockout mice and/or its receptor, CCR2, gene knockout mice, the null mice showed a decrease in alcohol consumption and alcohol preference when compared to wild-type mice in a two-bottle

choice drinking test (Blednov et al., 2005). Interestingly, transgenic mice that persistently express elevated levels of CCL2 in the CNS (CCL2-tg) also showed reduced alcohol consumption during the 2 hour limited access phase of the two-bottle choice drinking test compared to non-transgenic (non-tg) littermate control mice (Bray et al., 2017). Also, CCL2-tg mice exposed to a chronic intermittent alcohol vapor (CIE) paradigm did not exhibit increased alcohol drinking, whereas non-tg control mice exposed to the same CIE paradigm showed enhanced alcohol consumption (Bray et al., 2017). Intracerebroventricular infusion of CCL2 over several weeks resulted in increased self-administration of sweetened alcohol, but not a sucrose solution in rats, indicating that the increased self-administration of sweetened alcohol was due to alcohol and not the sweet taste (Valenta and Gonzales, 2016). The increased self-administration persisted for several weeks after CCL2 infusion was terminated, indicating long-term effects of CCL2/alcohol interactions (Valenta and Gonzales, 2016). These contrasting results indicate a complex role for CCL2 in alcohol consumption.

Differences between CCL2-tg and non-tg mice were also observed in other alcohol-sensitive behaviors, including tests for hippocampal dependent functions such as spatial learning. CCL2-tg mice withdrawn from a 2BC drinking paradigm (2BC-W) showed alcohol-induced impairment in aspects of a spatial learning test (Barnes Maze) compared to their respective alcohol naïve CCL2-tg control mice, an effect that was not observed in non-tg mice (Bray et al., 2017). CCL2-tg mice withdrawn from a CIE (CIE-W) protocol also showed alcohol-induced impairment in aspects of spatial learning (Y-Maze) compared to their respective alcohol naïve control CCL2-tg mice, an effect that was not observed in non-tg mice. Genotypic differences were also apparent in a test for context (hippocampus-dependent) and cued (amygdala-dependent) fear conditioning. Non-tg mice exposed to the CIE-W showed reduced contextual and cued fear conditioning compared to their respective control non-tg mice, effects that were not observed in CCL2-tg mice (Bray et al., 2017).

The mechanisms underlying the alcohol/CCL2 interactions that affect alcohol-sensitive behaviors have yet to be identified but are likely to involve effects on synaptic function associated with the behavior. In our previous studies of acute alcohol exposure in CCL2-tg and non-tg mice, alcohol-induced impairments in contextual learning were observed in non-tg control mice but not in the CCL2-tg mice (Bray et al., 2013). Consistent with the behavioral results, in hippocampal slices from non-tg mice long-term synaptic plasticity (long-term potentiation, LTP), an important synaptic mechanism of learning and memory, was depressed by exposure to acute alcohol (60 mM), whereas in hippocampal slices from CCL2-tg mice LTP was resistant to this effect of acute alcohol exposure (Bray et al., 2013).

The effects of both acute and chronic alcohol exposure on neuronal and synaptic function have been extensively documented in the hippocampus and other CNS regions (reviewed in (Harrison et al., 2017; Kyzar and Pandey, 2015; Little, 1999; Lovinger and Roberto, 2013; Roberto and Varodayan, 2017; Zorumski et al., 2014). Less is known about the actions of CCL2. Pharmacological studies indicate that CCL2 can have neuronal and synaptic actions (Banisadr et al., 2005; Cho and Gruol, 2008; Guyon et al., 2009; Nelson et al., 2011; van Gassen et al., 2005; Zhou et al., 2011; Zhou et al., 2016). For example, acute application of CCL2 to rat hippocampus slices increased excitatory synaptic responses mediated by AMPA

and NMDA receptors at the Schaffer Collateral to CA1 pyramidal neuron synapse and increased CA1 pyramidal neuron excitability (Zhou et al., 2011; Zhou et al., 2016). Similarly, acute application of CCL2 altered synaptic network activity in primary cultures of rat hippocampus (Cho and Gruol, 2008), and enhanced excitability of dopaminergic neurons in slices of substantia nigra (Guyon et al., 2009). GABA_A receptor-mediated currents were enhanced by acute CCL2 application in cultured cortical neurons (Caioli et al., 2013), but were inhibited in cultured spinal neurons (Gosselin et al., 2005). In cultured cerebellar Purkinje neurons, acute application of CCL2 increased resting Ca²⁺ levels, enhanced Ca²⁺ responses evoked by activation of metabotropic glutamate receptor 1 and depressed action potential generation (van Gassen et al., 2005). Thus, both alcohol and CCL2 can alter neuronal and synaptic function, providing a likely substrate for CCL2/alcohol interactions.

Our studies of CCL2-tg mice that persistently express elevated levels of CCL2 in the CNS have helped to identify potential CCL2/alcohol interactions that could emerge as a consequence of prolonged alcohol-induced production of CCL2 (Bray et al., 2013; Bray et al., 2017; Gruol et al., 2014). The elevated levels of CCL2 in the CCL2-tg mice result from increased astrocyte expression. Astrocytes are a primary source of CCL2 in the CNS under both physiological and pathophysiological conditions, including in response to alcohol exposure (Choi et al., 2014; Conductier et al., 2010; Farina et al., 2007; Szabo and Lippai, 2014). Astrocytes are in close contact with synapses and play an important role in synaptic transmission (Ota et al., 2013).

In the current studies, we used the CCL2-tg mouse model and extracellular field potential recordings to investigate consequences of CCL2/alcohol/withdrawal interactions that affect synaptic function, including excitatory synaptic transmission, somatic excitability, and synaptic plasticity. Two alcohol exposure paradigms were tested, a two-bottle choice alcohol (ethanol) drinking protocol (2BC drinking) and a chronic intermittent alcohol (ethanol) (CIE/2BC) protocol. The CIE/2BC paradigm leads to an increase in alcohol drinking and other behavioral changes associated with alcohol dependence, whereas the mice subjected to 2BC drinking are considered to be non-dependent (Becker and Lopez, 2016). Measurements of synaptic function were made during withdrawal over a several month period, with the earliest recordings starting ~0.6 months after termination of alcohol exposure. Thus, results reflect long-term consequences of CCL2/alcohol/withdrawal interactions. Results show that both alcohol exposure-withdrawal paradigms result in CCL2-dependent interactions that alter synaptic function, and that the synaptic alterations differed for the two alcohol exposure paradigms. These results are consistent with the hypothesis that alcohol-induced CCL2 production modulates the effects of alcohol exposure/withdrawal.

2. Materials and Methods

2.1. Animals

The generation of the transgenic mice with astrocyte-targeted enhanced expression of CCL2 has been described previously (Huang et al., 2002). The initial line was backcrossed onto a C57BL/6J background and has been maintained for several years. Heterozygous CCL2-tg mice and their non-tg littermates were used for all experiments and were obtained by breeding heterozygous CCL2-tg mice with wild-type C57BL/6J mice. Genotyping was

carried out from cut tail tips of individual mice at weaning (21–28 days old), as previously described (Bray et al., 2017). All animal procedures were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Animal facilities and experimental protocols were in accordance with the Association for the Assessment and Accreditation of Laboratory Animal Care.

2.2. Alcohol exposure

Alcohol exposed and the respective control CCL2-tg and non-tg mice (female and male) previously examined in behavioral studies (results reported in (Bray et al., 2017) were used for these studies. The objective was to identify physiological changes at the synaptic level that could contribute to differences observed in the behavioral studies. The animals were subjected to one of two alcohol exposure paradigms, a two-bottle choice alcohol (ethanol) drinking (2BC drinking) protocol followed by withdrawal or a chronic intermittent alcohol (ethanol) (CIE/2BC) protocol followed by withdrawal.

The 2BC drinking protocol was adapted from that of Blednov and colleagues (Blednov et al., 2005) and has been described previously (Bray et al., 2017). Ten non-tg and 11 CCL2-tg were subjected to 2BC drinking. Ten non-tg and 16 CCL2-tg alcohol naïve mice served as controls. Briefly, the animals were singly housed in cages with two drinking tubes continuously available Monday – Friday. One bottle of water was available across weekends. After 4 days of water consumption (on Monday, off Friday; water in both tubes), mice were offered 3% alcohol (v/v) in one type versus water in the 2ND tube on the following Monday-Friday (24 h access). Fluid consumption was measured daily. Tube positions were changed every day to control for position preferences. Food was available ad libitum and mice were weighed weekly. Over the following 4 weeks mice received 6, 9, 12, and 15% alcohol in this same manner. Average (mean $\pm$ SEM) alcohol consumption over the 4 week period showed a trend ($0.10 > p > 0.05$) for higher drinking in the non-tg mice compared to the CCL2-tg mice ($F(1,19) = 3.7$, $p = 0.06$, ANOVA). Mean values were 14.2 ± 0.7 gm/kg ($n=10$) for non-tg mice and 11.9 ± 0.9 gm/kg ($n=11$) for the CCL2-tg mice. Following the 24-hour access drinking, mice were subjected to limited access drinking of 15% alcohol for 2 hr per day for 5 days. Average alcohol consumption over the 5 days was greater ($F(1,19) = 11.3$, $p = 0.003$, ANOVA) for non-tg (6.6 ± 0.2 gm/kg, $n=10$) mice than for CCL2-tg mice (5.5 ± 0.3 gm/kg, $n=11$).

The CIE/2BC model, which involves intermittent vapor exposure (CIE) and 2BC limited access drinking, was used for chronic alcohol exposure as described previously and summarized in Figure 1. (Bray et al., 2017). The specific methodology used is available to the public on the INIA-West website <http://www.scripps.edu/cnad/inia/under> “Methodology”, “SOP Chronic Intermittent Alcohol – Two-Bottle Choice Mouse Model PDF” (Becker and Lopez, 2004; Finn et al., 2007; Griffin et al., 2009a; Griffin et al., 2009b; Lopez and Becker, 2005). To determine baseline drinking, CCL2-tg and non-tg male and female mice were singly housed for two hours (starting 30 min before the lights turned off) with access to two drinking tubes, one containing 15% alcohol and the other containing water (i.e. 2BC drinking) for 5 days per week for 3 weeks. Alcohol and water consumption during the 2-hour periods was recorded. Mice were then divided into two groups that

showed equal alcohol and water consumptions for each genotype/sex. One group was designated the vapor group and was exposed to intermittent alcohol vapor (CIE) in a vapor chamber, and the second group was designated the control group and was exposed to air in an identical chamber. The vapor group was injected with 1.75 g/kg alcohol plus 68.1 mg/kg pyrazole (alcohol dehydrogenase inhibitor; used to stabilize blood alcohol levels) and placed in the chambers to receive intermittent alcohol vapor for 3 days (16 hours vapor on, 8 hr off). Following each 16 hr bout of alcohol vapor, mice were removed and tail blood was sampled for blood alcohol determination. Target blood alcohol levels (BALs) were 150–225 mg%. Following the third day of exposure, mice were allowed 72 hours of alcohol-free undisturbed time. The mice were then given 5 days of 2-hour access to bottles containing 15% alcohol or water. The control group was injected with 68.1 mg/kg pyrazole in saline and placed in chambers delivering air for the same periods as the alcohol vapor group and then given 5 days of 2-hour access to two drinking bottles containing 15% alcohol or water. The vapor/air exposure and 5 days of two-bottle choice drinking was repeated another two times for a total of three rounds of alcohol vapor and two-bottle choice drinking.

Under baseline conditions, average alcohol consumption values for mice targeted for the CIE/2BC paradigm were 3.7 ± 0.3 (n=24) g/kg and 4.2 ± 0.2 (n=27) g/kg for CCL2-tg and non-tg mice, respectively, and were not significantly different ($F_{1,49} = 2.05$, $p = 0.16$). Average values for the 2BC drinking phase of the CIE/2BC paradigm did not increase significantly from baseline for CCL2-tg mice but did increase significantly (to ~5 gm/kg) for the non-tg mice (Bray et al., 2017). BALs were measured at the end of each round of alcohol vapor. Average BALs (mean $\pm$ SEM) for the three alcohol vapor rounds were similar ($F_{1,15} = 2.14$, $p = 0.16$, ANOVA) for non-tg (162 ± 6 mg/dl, n=8) and CCL2-tg mice (178 ± 9 mg/dl, n=9). BALs were not measured during 2BC drinking phase for either the 2BC-W or CIE-W treatments. Because non-tg mice drank more than CCL2-tg mice, the possibility that this difference in alcohol drinking contributed to results cannot be eliminated. In addition, in these studies all animals were behaviorally tested and it is unknown if results would be the same for non-behaviorally tested animals, although results for non-tg mice are consistent with known actions of alcohol in non-behaviorally tested animals (e.g., (Molleman and Little, 1995; Roberto et al., 2002; Rogers and Hunter, 1992; Tremwel and Hunter, 1994).

2.3. Extracellular Field Potential Recordings

Mice were 7–9 months of age at the time of sacrifice for electrophysiological studies. Alcohol treated and control mice were studied on alternating days. Females and males were used in approximately the same numbers (for some studies, successful recordings from the same number of females and males was not achieved). Statistical analysis in the behavioral studies (ANOVA) showed that there were no significant sex differences or sex x genotype interaction for the behavior tests and results were collapsed across sex for presentation of data (Bray et al., 2017). For consistency and to facilitate comparisons, data were also collapsed across sex for electrophysiological studies. There were no obvious sex differences in results from the electrophysiological studies. Samples size was not sufficient to determine differences in post-alcohol time-dependent effects over the timespan of the studies.

To prepare hippocampal slices for electrophysiological recordings, mice were decapitated under isoflurane anesthesia and the brains rapidly removed and immersed in chilled artificial cerebrospinal fluid (ACSF). The composition of the ACSF was (in mM) 130.0 NaCl, 3.5 KCl, 1.25 NaH₂PO₄, 24.0 NaHCO₃, 2.0 CaCl₂, 1.3 MgSO₄, and 10.0 glucose (all chemicals were purchased from Sigma-Aldrich, St. Louis, MO). The ACSF was bubbled continuously with 95% O₂/5% CO₂ (pH 7.2–7.4). The hippocampi were isolated from the brain and hippocampal slices (400 µm) were cut from one hippocampus using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Surrey, UK). Slices were allowed to recover in a gas-fluid interface chamber maintained at 33°C with an ACSF perfusion rate of 0.55 ml/min for at least one hour prior to the onset of recording. The second hippocampus from each mouse was snap frozen on dry ice and stored at -80 °C for later studies of protein levels.

For recordings, hippocampal slices were transferred into a second gas-fluid recording chamber and continuously superfused with ACSF at a rate of 1 ml/min (33°C). Extracellular field potential recordings of synaptic responses at the Schaffer collateral to CA1 pyramidal neuron synapse were made with glass electrodes filled with ACSF (3–5 MΩ resistance). A concentric bipolar stimulating electrode (Rhodes Medical Instruments Inc., Summerland, CA) was placed at the border of the CA2 and CA1 regions. Square wave pulses (50 µs duration) generated by a Neurodata PG4000 digital stimulator (Neuro Data Instruments Corp., New York, NY) and a S88 Square Pulse Stimulator (Grass Technologies, West Warwick, RI) were used to activate the Schaffer collateral-commissural afferent pathway. One recording electrode was positioned in the stratum radiatum to record the dendritic field excitatory postsynaptic responses (fEPSPs), and a second recording electrode was positioned in the stratum pyramidale to record somatic population spike (PS) elicited by the fEPSP. Responses to electrical stimulation were recorded using an Axoclamp-2B amplifier combined with a Digidata data acquisition system and pCLAMP software (all from Molecular Devices, Sunnyvale, CA).

Data were analyzed off-line using AxoGraph software (AxoGraph Scientific, Sydney, Australia). Measurements were made of the fEPSP slope and PS amplitude. The slope of the fEPSP was determined over a range (typically 40–60%) that allowed reliable measurements without interference from the preceding presynaptic volley or the reflection from the somatic PS that occurs on the falling phase the fEPSP. The magnitude of the PS was measured as the peak amplitude of the negative deflection from a baseline value. Baseline value was estimated from a tangent line fitted across the two positive peaks of the synaptic response recorded in the somatic region.

2.6. Assessment of synaptic function

To determine characteristics of synaptic function at the Schaffer collateral to CA1 pyramidal neuron synapse in alcohol naïve and alcohol-exposed/withdrawn mice, input-output (I/O) relationships and short-term synaptic plasticity were determined for the fEPSP and PS in each slice. I/O relationships were assessed by applying a series of stimuli of increasing intensities to the Schaffer collaterals at a rate of 2 Hz with stimulation intensities starting at subthreshold stimulus intensity (i.e., no synaptic response) and increasing from 50 µA up to

300 μ A in increments of 10–20 μ A until the maximum PS was reached. To construct I/O graphs of the data, mean values for fEPSP slope or PS amplitude were plotted as a function of stimulus intensity. The threshold stimulus intensity for eliciting a PS and fEPSP varied across slices from both CCL2-tg and non-tg hippocampus. To standardize for this variability in I/O graphs of mean values, for each slice the stimulus intensity was expressed relative to the intensity that elicited a threshold fEPSP (e.g., ~0.15 mV/ms) or PS (e.g., ~0.70 mV) and was incremented (e.g. 10 or 20 μ A intervals) from the threshold stimulus intensity according to the increments used during the experiments. With this experimental design, potential differences in threshold values for excitability (either presynaptic or postsynaptic) could not be not examined.

Short-term synaptic plasticity was determined from paired-pulse ratios (PPR), which were assessed by standard paired-pulse stimulation protocols. PPRs were determined from the ratio of the response to the second stimulation divided by the magnitude of the response to the first stimulation for each paired-pulse interval. Three paired-pulse responses (1 minute between acquisitions) were averaged. For the fEPSP, the PPR was determined by a stimulation protocol applied at paired-pulse intervals of 40, 100, and 200 ms at a stimulus intensity that elicited a fEPSP slope equal to 50% of the maximal fEPSP (determined from I/O protocols). For the PS, the PPR was determined by a stimulation protocol applied at short paired-pulse intervals (10 & 20 ms) at a test stimulus intensity that elicited a PS equal to 50% of the maximal PS amplitude (determined from I/O protocols).

Several forms of long-term synaptic plasticity induced by high-frequency stimulation were also assessed in each slice including post-tetanic potentiation (PTP), short-term potentiation (STP), and long-term potentiation (LTP) of the fEPSP slope. The high-frequency stimulation, a theta burst (TBS), consisted of 15 bursts of four pulses each delivered at a frequency of 100 HZ with a 200 ms interburst interval. A stimulus intensity that elicited a half maximum response of the fEPSP as determined from the I/O protocols was used. Prior to and following TBS, the same stimulus intensity applied at 0.017 Hz was used to elicit a synaptic response for the assessment of the impact of the TBS on the synaptic responses. For LTP, PTP, and STP, the slope of the fEPSP was expressed as a percentage of the average of five baseline (pre-TBS) test responses in each slice. Compiled data are expressed as the mean $\pm$ SEM. For electrophysiological studies, n = the number of slices measured. Statistical significance was determined by ANOVA or unpaired t-test with significance set at $p < 0.05$; $0.10 > p > 0.05$ was considered a trend in the data.

2.7. CCL2 measurements

CCL2 levels in hippocampus from CCL2-tg and non-tg mice were determined by ELISA using the Mouse CCL2 ELISA Ready-SET-Go! Kit (eBioscience, Inc., San Diego, CA) following manufacturer's instructions. Protein samples for ELISA were prepared using standard protocols as described previously (Gruol et al., 2014). Briefly, proteins were extracted from each hippocampus by sonication in cold lysis buffer containing 50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5% NP-40, a Complete Protease Inhibitor Cocktail Tablet (Roche Diagnostics, Mannheim, Germany), and a cocktail of phosphatase inhibitors (Na^+ pyrophosphate, β -glycerophosphate, NaF, Na^+

orthovanadate; all from Sigma-Aldrich). The samples were incubated on ice for 30 minutes, centrifuged at 13,860g for 30 minutes at 4°C, and the supernatants were collected. Protein concentration in the supernatants was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA). Aliquots were stored at -80°C.

3. Results

Effects of 2BC drinking/withdrawal (2BC-W) or CIE/2BC/withdrawal (CIE-W) on synaptic function were assessed *ex vivo* in hippocampal slices obtained from alcohol exposed/withdrawn CCL2-tg and non-tg mice and their appropriate control mice. The mice were previously subjected to behavioral testing, which was carried out at the end of the alcohol exposure period. The electrophysiological studies were started after the completion of behavioral studies, which have been published (Bray et al., 2017). The earliest recordings starting ~ 0.6 months after termination of alcohol exposure and took approximately two months to complete. Thus, differences in synaptic function between CCL2-tg and non-tg mice subjected to the alcohol exposure/withdrawal paradigms reflect long-term consequences of CCL2/alcohol/withdrawal interactions on synaptic function.

3.1. CCL2-tg mice show altered effects of alcohol on hippocampal synaptic transmission

Potential long-term effects of CCL2/alcohol/withdrawal interactions on synaptic transmission were investigated in the CA1 pyramidal neurons of the hippocampus by a comparison of I/O relationships for synaptic responses (fEPSP slope and the PS amplitude) elicited by Schaffer collateral stimulation. In hippocampal slices from alcohol naïve mice (not shown), there was no significant difference in the I/O relationships for the fEPSP slope ($F(1,25) = 0.66, p = 0.42$) or PS amplitude ($F(1,24) = 0.66, p = 0.42$) between CCL2-tg and non-tg slices, as reported previously (Bray et al., 2013). Measurements of CCL2 in the hippocampus at the time of animal sacrifice showed markedly higher levels of CCL2 in the CCL2-tg hippocampus compared to non-tg hippocampus for all treatment groups (CCL2-tg vs. non-tg: naïve, $F(1,48) = 284.35, p < 0.0001$; 2BC, $F(1,22) = 284.35, p < 0.0001$; CIE cont $F(1,18) = 218.02, p < 0.0001$; CIE, $F(1,16) = 46.62, p < 0.0001$) (Fig. 2A). However, there were no treatment ($F(3,104) = 1.30, p = 0.28$) or genotype x treatment ($F(3,104) = 1.58, p = 0.20$) interactions for CCL2 levels, perhaps due to the length of time between the termination of alcohol exposure and sacrifice of the animals for electrophysiological studies.

Non-dependent animals—There were no significant differences in the I/O relationship for the fEPSP slope or PS amplitude between hippocampal slices from 2BC-W treated non-tg mice and alcohol naïve non-tg mice (fEPSP slope: $F(1,24) = 0.02, p = 0.89$; PS: $F(1,24) = 0.04, p = 0.85$, Repeated Measures ANOVA) (Fig. 2B1,B2). In contrast, in hippocampal slices from 2BC-W treated CCL2-tg mice fEPSP slope and PS amplitude were significantly smaller than in hippocampal slices from alcohol naïve CCL2-tg mice (fEPSP slope: $F(1,25) = 5.28, p = 0.03$; PS: $F(1,24) = 5.22, p = 0.03$, Repeated Measures ANOVA) (Fig. 2C1,C2). Analysis of fEPSP-spike (E-S) coupling for the CCL2-tg hippocampus, where the PS amplitude was normalized to the fEPSP slope at each stimulus intensity for each slice, revealed no difference in the I/O relationships for the normalized PS between hippocampus from 2BC-W treated and alcohol naïve CCL2-tg mice (Fig. 2C3). This result indicates that

the reduction of the PS in the hippocampus of the 2BC-W treated CCL2-tg mice can be attributed to the reduced fEPSP. Thus, an interaction between CCL2 and alcohol drinking/withdrawal in mice exposed to 2BC-W resulted in a prolonged reduction in excitatory synaptic transmission in the CCL2-tg hippocampus, an effect that also reduced somatic excitability as measured by the PS amplitude.

Dependent animals—Hippocampal slices from CIE-W treated non-tg mice showed a significant increase in the I/O relationships for the fEPSP slope compared with hippocampal slices from the CIE-W control non-tg mice ($F(1,25) = 13.16$, $p = 0.001$, Repeated Measures ANOVA) (Fig. 3A1). There was no significant difference for the PS amplitude ($F(1,26) = 1.60$, $p = 0.22$, Repeated Measures ANOVA) (Fig. 3A2). In contrast, there was no significant difference between the I/O relationships for fEPSP slope of hippocampal slices from CIE-W treated CCL2-tg mice compared with hippocampal slices from the CIE-W control CCL2-tg mice ($F(1,27) = 1.72$, $p = 0.20$, Repeated Measures ANOVA) (Fig. 3B1). However, there was a significant increase in I/O relationships for the PS amplitude in hippocampal slices from CIE-W CCL2-tg mice compared to hippocampal slices from CIE-W control CCL2-tg mice ($F(1,24) = 10.34$, $p = 0.004$, Repeated Measures ANOVA) (Fig. 3B2). Thus, an interaction between CCL2 and CIE-W resulted in an increase in excitability in the CCL2-tg hippocampus as measured by the PS amplitude.

In summary, results from studies of alcohol naïve non-tg and CCL2-tg mice showed that chronically elevated levels of CCL2 in the CNS of CCL2-tg mice did not alter basic I/O properties of hippocampal synaptic transmission. However, alcohol exposure/withdrawal revealed neuroadaptive changes in the CCL2-tg mice that altered the effects of alcohol exposure/withdrawal on hippocampal synaptic transmission. Moreover, the consequences of the neuroadaptive changes varied depending on alcohol exposure paradigm used.

3.2. CCL2-tg mice show altered effects of alcohol on hippocampal synaptic plasticity

Repetitive stimulation of the Schaffer collaterals can result in an enhancement of the fEPSP, a plastic process thought to be an important mechanism of hippocampal memory and learning. Depending on the pattern of repetitive stimulation, the enhancement can be short-term (e.g., short-term potentiation) or persistent (e.g., long-term potentiation). Alcohol is known to significantly affect both short-term and long-term forms of synaptic plasticity (Lovinger and Roberto, 2013; McCool, 2011; Zorumski et al., 2014). To determine if CCL2 interacted with alcohol to alter the effects of alcohol on synaptic plasticity, we carried out experiments to assess short- and long-term forms of synaptic plasticity in hippocampal slices from non-dependent and dependent CCL2-tg and non-tg mice.

Short-term synaptic plasticity of the fEPSP—Stimulation of the Schaffer collaterals using a paired-pulse stimulation protocol produces a short-term enhancement of the fEPSP slope, referred to as paired-pulse facilitation (PPF). This form of short-term synaptic plasticity lasts for seconds to minutes and primarily results from presynaptic mechanisms involving Ca^{2+} homeostasis and probability of transmitter release (Jackman et al., 2016; Zucker and Regehr, 2002). PPF was assessed in CCL2-tg and non-tg hippocampal slices using paired-pulse stimulation at intervals of 40, 100, and 200 ms. PPF was determined from

the ratio of the slope of the fEPSP elicited by the second stimulation of the paired-pulses to the slope of the fEPSP elicited by the first stimulation of the paired-pulses.

The paired pulse ratio was greater than 1 and of similar value for all treatment groups, indicating no genotype or treatment effect on PPF. For example, PPF was ~1.6, ~1.5 and ~1.2, respectively for the 40, 100 and 200 ms paired-pulse intervals for hippocampal slices from 2BC-W treated and alcohol naïve CCL2-tg mice (40 ms, $F(1,27) = 0.27$, $p = 0.61$; 100 ms, $F(1,275) = 1.25$, $p = 0.27$; 200 ms, $F(1,27) = 0.08$, $p = 0.78$), and 2BC-W treated and alcohol naïve non-tg mice (40 ms, $F(1,23) = 0.12$, $p = 0.73$; 100 ms, $F(1,23) = 0.70$, $p = 0.41$; 200 ms, $F(1,23) = 0.65$, $p = 0.43$) (not shown).

Similarly, PPF was ~1.5, ~1.4 and ~1.2, respectively for the 40, 100 and 200 ms paired-pulse intervals for hippocampal slices from CIE-W treated CCL2-tg and their respective control CCL2-tg mice (40 ms, $F(1,27) = 0.10$, $p = 0.75$; 100 ms, $F(1,27) = 0.12$, $p = 0.73$; 200 ms, $F(1,27) = 0.77$, $p = 0.39$), and CIE-W treated non-tg mice and their respective control non-tg mice (40 ms, $F(1,27) = 1.75$, $p = 0.20$; 100 ms, $F(1,27) = 0.48$, $p = 0.50$; 200 ms, $F(1,27) = 0.07$, $p = 0.79$) (not shown). Thus, neither 2BC-W nor CIE-W treatment altered transmitter release at this synapse under low frequency stimulation conditions. These results indicate that the alcohol-induced depression of the fEPSP in CCL2-tg hippocampus is not due to reduced transmitter release and suggest that postsynaptic mechanisms are likely to be involved.

Short-term synaptic plasticity of the PS—Paired-pulse stimulation can also result in short-term synaptic plasticity in the somatic region resulting from the impact of synaptic responses on somatic excitability. Somatic excitability is a function of the interactions between the excitatory synaptic response and somatic/dendritic ion channels that mediate excitability. In addition, effects of recurrent GABAergic inhibitory synaptic transmission in the vicinity of the CA1 pyramidal cell layer play a prominent role. Somatic plasticity can be assessed by the PPR for the PS (amplitude of the second PS divided by the amplitude of the first PS), and is elicited by paired-pulse stimulation of the Schaffer collaterals at short intervals (10 or 20 ms). In our studies, PPR of the PS was greater than 1 for hippocampal slices from alcohol naïve CCL2-tg and non-tg mice, indicating a facilitation of the PS by the paired-pulse stimulation. This facilitation implies that inhibitory influences, which would produce inhibition of the second PS of the pair, were not prominent in the CCL2-tg and non-tg slices.

PPR of the PS in hippocampal slices from 2BC-W treated non-tg mice was not significantly different from PPR of the PS in hippocampal slices from alcohol naïve non-tg mice (10 ms interval: $t(24) = -0.33$, $p = 0.74$; 20 ms intervals: $t(24) = -0.75$, $p = 0.46$; unpaired t -test) (Fig. 4A). PPR of the PS in hippocampal slices from 2BC-W treated CCL2-tg mice was significantly increased relative to PPR of the PS in hippocampal slices from alcohol naïve CCL2-tg mice, but only for the 10 ms paired-pulse interval (10 ms interval: $t(24) = 0.003$, $p = 0.04$; 20 ms intervals: $t(24) = 1.61$, $p = 0.12$, unpaired t -test) (Fig. 4A).

PPR of the PS in hippocampal slices from CIE-W treated non-tg mice was significantly larger than in hippocampal slices from CIE-W control non-tg mice at both 10 ms ($t(30) =$

–2.15, $p = 0.04$, unpaired t -test) and 20 ms ($t(30) = -2.19$, $p = 0.04$, unpaired t -test) paired-pulse intervals (Fig. 4B). In contrast, in hippocampal slices from CIE-W treated CCL2-tg mice PPR of the PS was significantly reduced at both 10 ms and 20 ms paired-pulse intervals compared with PPR of the PS in hippocampal slices from CIE-W control CCL2-tg mice (10 ms interval: $t(27) = 2.88$, $p = 0.008$; 20 ms intervals: $t(24) = 2.14$, $p = 0.04$, unpaired t -test) (Fig. 4B). These results show that the elevated expression of CCL2 in the hippocampus of CCL2-tg mice interacts with the effects of alcohol on inhibitory influences in the somatic region of CA1 neurons. In particular, CCL2/CIE-W interactions result in a reduction of inhibitory influence in the somatic region of CA1 neurons of non-tg mice but an increase in inhibitory influences in the somatic region of CA1 neurons of CCL2-tg mice.

Long-term synaptic plasticity of the fEPSP—Several persistent forms of synaptic plasticity of the fEPSP are also sensitive to alcohol exposure. These forms of synaptic plasticity are induced by high frequency synaptic activity, which in our experiments was produced by TBS applied to the Schaffer collaterals. TBS induced three forms of synaptic plasticity of the fEPSP at the CA1 pyramidal neuron synapse, post-tetanic potentiation (PTP), short-term potentiation (STP), and long-term potentiation (LTP). All three forms of synaptic plasticity are characterized by an increase in synaptic efficacy and are thought to be cellular mechanisms involved in learning and memory (Lynch, 2004; Martin et al., 2000). PTP underlies the initial increase in the magnitude of the fEPSP slope following TBS. The subsequent phase of declining fEPSP slope reflects STP and lasts ~30 minutes, whereas the long lasting, stable enhancement of the fEPSP slope that occurs ~50–60 minutes after the induction protocol reflects LTP. Mean values for PTP, STP, and LTP were calculated for each slice and compared statistically to identify treatment effects. Mean PTP was determined for each slice by averaging the first three measurements after TBS. Mean STP was determined for each slice by averaging the measurements from 15–25 min after TBS. Mean LTP was determined for each slice by averaging measurements from 50–60 min after TBS.

In hippocampal slices from alcohol naïve mice, there was no significant difference in the enhancement of the fEPSP slope during PTP ($t(26) = -1.20$, $p = 0.24$), STP ($t(26) = -1.11$, $p = 0.28$), or LTP ($t(26) = -0.26$, $p = 0.80$) (not shown) between hippocampal slices from CCL2-tg and non-tg mice, consistent with previous studies in the lab showing that elevated levels of CCL2 alone does not affect PTP, STP or LTP (Bray et al., 2013)

In hippocampal slices from 2BC-W treated non-tg mice, there was no significant difference in the enhancement of the fEPSP slope during PTP compared to hippocampal slices from alcohol naïve non-tg mice (PTP: $t(20) = 1.25$, $p = 0.23$; STP: $t(20) = 1.16$, $p = 0.26$) (Fig. 5A1). There was a trend ($0.10 > p > 0.05$) for a greater enhancement of LTP in hippocampal slices from 2BC-W non-tg mice compared with alcohol naïve non-tg mice ($t(20) = 1.99$, $p = 0.06$) (Fig. 5A1). In contrast, in hippocampal slices from 2BC-W treated CCL2-tg mice, the enhancement of PTP ($t(28) = 2.34$, $p = 0.03$), STP ($t(28) = 2.70$, $p = 0.01$), and LTP ($t(28) = 2.46$, $p = 0.02$) were significantly larger than observed in hippocampal slices from alcohol naïve CCL2-tg mice (Fig. 5A2).

In hippocampal slices from CIE-W treated non-tg mice, there was no significant difference in the enhancement of PTP ($t(23) = 1.62$, $p = 0.12$) compared to hippocampal slices from CIE-W control non-tg mice (Fig. 5B1). The enhancement of STP ($t(23) = 2.16$, $p = 0.04$, ANOVA) and LTP ($t(23) = 2.78$, $p = 0.01$, ANOVA) were significantly smaller in hippocampal slices from CIE-W treated non-tg mice compared with hippocampal slices from CIE-W control non-tg mice (Fig. 5B1). In hippocampal slices from CIE-W treated CCL2-tg mice, the enhancements of the fEPSP slope during PTP ($t(18) = 1.57$, $p = 0.14$), STP ($t(18) = 1.38$, $p = 0.19$), and LTP ($t(18) = 1.11$, $p = 0.28$) were similar to the enhancements in hippocampal slices from the CIE-W control CCL2-tg mice (Fig. 5B2). Taken together, these results show that in both non-dependent and dependent mice CCL2/alcohol interactions alter the effects of alcohol on synaptic mechanisms underlying memory and learning. Results from the studies of synaptic function are summarized in Table 1.

4. Discussion

Alcohol exposure/withdrawal has been shown to produce elevated levels of CCL2 in the CNS in both experimental animals and alcoholics (see Introduction). In the current studies, CCL2-tg mice were used to model conditions of increased CCL2 expression in the CNS, such as occurs with alcohol abuse. The goal of the studies was to gain an understanding of potential CCL2/alcohol/withdrawal interactions that affect synaptic function and could play a role in long-term effects of alcohol on the CNS and behavior. The alcohol exposure/withdrawal paradigms used in our studies were 2BC-W, which is not thought to produce alcohol dependence, and CIE-W, which leads to increases in alcohol drinking and other behaviors associated with alcohol dependence (Becker and Lopez, 2016). The mice used in the studies of synaptic function were previously studied in behavioral tests, which showed that CCL2-tg mice differed from non-tg mice in the effects of 2BC-W and CIE-W on behavior (Bray et al., 2017), and the level of cell and synaptic proteins that play a role in hippocampal function (Gruol et al., 2014). Here we show that the CCL2-tg and non-tg mice also differ in the effects of 2BC-W and CIE-W on hippocampal synaptic function, including synaptic transmission and synaptic plasticity. The genotypic differences in synaptic function produced by the alcohol exposure/withdrawal paradigms could contribute to the genotypic differences observed in the behavioral studies of the mice (Bray et al., 2017). These results support the idea that alcohol-induced production of CCL2 can result in CCL2/alcohol interactions and neuroadaptive changes that influence the effects of alcohol exposure/withdrawal on both synaptic function and behavior. The studies also show that alcohol/CCL2 interactions can vary depending on the characteristics of the alcohol exposure/withdrawal paradigm used and levels of CCL2.

4.1. Expression of CCL2 in the 2BC-W and CIE-W mice

A number of studies in rodents have reported that chronic alcohol exposure/withdrawal increased the level of CCL2 mRNA or protein in the brain. For example, Kane et al (Kane et al., 2014) observed ~2.5 fold increase in CCL2 mRNA in the mouse hippocampus 24 hours after termination of a daily exposure to 12 g/kg (by gavage) alcohol for 10 days. Harper et al (Harper et al., 2015b) showed that CCL2 mRNA was increased ~1.8 fold in rat brain 24 h after termination of 15 days of alcohol drinking. These authors also showed a correlation

between the level of CCL2 mRNA and BALs, such that CCL2 mRNA increased with increasing BALs (Harper et al., 2017). Freeman et al (Freeman et al., 2012) showed a ~2 fold increase in CCL2 mRNA in rat central nucleus of amygdala 48 hrs after termination of a 35 day exposure to alcohol drinking. Qin et al (Qin et al., 2008) reported that CCL2 mRNA and protein were increased in the mouse brain (1.38 fold for mRNA: 138% for protein) 8 days after termination 10 days of alcohol treatment (daily 5 g/kg, i.g.). Taken together, these results indicate that increased levels of CCL2 are expressed in the brain for at least 1–8 days following termination of alcohol exposure. In our studies, CCL2 levels were significantly higher in the hippocampus of CCL2-tg mice than in hippocampus of non-tg mice for all treatment groups. However, for both CCL2 and non-tg mice the hippocampus did not show a significantly higher level of CCL2 for either 2BC-W or CIE/2BC treatments. Assuming that CCL2 levels were increased in the hippocampus of both CCL2-tg and non-tg mice by 2BC-W and CIE/2BC treatments, our results showing no effect of 2BC-W and CIE/2BC-W on CCL2 levels several months after termination of alcohol exposure suggests that hippocampal levels of CCL2 can recover during a prolong abstinence period. However, such a recovery does not appear occur when alcohol abuse is excessive, as alcoholics express elevated levels of CCL2 in the brain (He and Crews, 2008)

4.2. Hippocampal synaptic transmission and short-term plasticity

In vitro studies of synaptic function in hippocampus obtained from 2BC-W or CIE-W treated CCL2-tg and non-tg mice showed genotypic differences for both alcohol treatment paradigms. Within a genotype, differences were also observed for 2BC-W vs. CIE-W treatments. The mechanisms mediating these differences remain to be determined. The electrophysiological studies were completed several months after alcohol exposure. Therefore, the changes in synaptic function produced by 2BC-W and CIE-W reflect persistent neuroadaptive changes, whereas no change in a synaptic function was considered an indication of recovery or resistance to the effect of alcohol exposure/withdrawal on that function.

Basic I/O properties for the fEPSP and PS were similar in hippocampal slices from alcohol naïve CCL2-tg and non-tg mice, as reported previously (Bray et al., 2013). The primary effect of 2BC-W observed in hippocampal slices from the CCL2-tg mice was a depression of the fEPSP, and consequently the PS, whereas there was no apparent effect of 2BC-W on the fEPSP or PS in the hippocampus from the non-tg mice. Our previous studies showed that exposure to acute alcohol in vitro depressed the fEPSP, and consequently PS, to a similar extent in the hippocampus from both the CCL2-tg and non-tg mice (Bray et al., 2013). Thus, the effect of 2BC-W on hippocampus from the CCL2-tg mice was similar to the effect of acute alcohol exposure in vitro on hippocampal slices from the CCL2-tg mice. It is likely that the initial response to alcohol exposure for the 2BC-W paradigm would be similar to that of acute alcohol exposure in vitro, a depression of the fEPSP and PS for both genotypes. Thus, the depression of the fEPSP and PS in the hippocampus of 2BC-W treated CCL2-tg mice may reflect a persistent form of the depressive effects of acute alcohol exposure observed in vitro on the fEPSP. The apparent lack of effect of 2BC-W treatment on the fEPSP, and consequently the PS, in hippocampus slices from 2BC-W treated non-tg mice may reflect recovery from depressive effects of alcohol.

CIE-W treatment produced an enhancement of the fEPSP in hippocampus from non-tg mice, an effect consistent with the known effects of chronic alcohol exposure on postsynaptic aspects of glutamatergic synaptic transmission at the Schaffer collateral to CA1 pyramidal neuron synapse of the hippocampus (Molleman and Little, 1995; Nelson et al., 2005; Whittington et al., 1995). There was no apparent effect of CIE-W treatment on the fEPSP in hippocampus from CCL2-tg mice, which could reflect recovery from or a resistance to the effects of CIE-W.

The mechanisms mediating the effects of 2BC-W or CIE-W treatment on the fEPSP do not appear to involve presynaptic mechanisms, because PPF of the fEPSP was not altered by the alcohol treatments for the hippocampus from either the CCL2-tg or non-tg mice. With respect to postsynaptic mechanisms, our Western blot measurements of glutamate receptor subunit levels, including AMPA (GluA1) and NMDA (GluN1, GluN2B) receptor subunits, showed elevated levels of GluA1 and GluN2B in the hippocampus of CCL2-tg mice for both 2BC-W and CIE-W treatments, but not in the hippocampus of 2BC-W and CIE-W treated non-tg mice (Gruol et al., 2014). GluN1 was also elevated in hippocampus of 2BC-W treated CCL2-tg mice, but not in the hippocampus of CIE-W treated CCL2-tg or non-tg mice (Gruol et al., 2014). The increased expression of these receptor subunits is consistent with studies showing that chronic alcohol exposure/withdrawal increases NMDA and AMPA receptor subunit expression in experimental animals and humans (Carpenter-Hyland et al., 2004; Christian et al., 2012; Enoch et al., 2014; Nelson et al., 2005; Roberto and Varodayan, 2017; Trevisan et al., 1994). These alcohol-induced changes in AMPA and NMDA receptor subunits would likely result in an increase in the fEPSP, which was not observed in our studies. However, the increased NMDA receptor subunit expression in the could contribute to the reduction in the fEPSP observed in the hippocampus from the 2BC-W treated CCL2 tg mice if NMDA receptors, which are known to be highly sensitive to alcohol, made a larger contribution to the fEPSP in 2BC-W treated CCL2 tg mice than in hippocampus from alcohol naive CCL2-tg mice.

Studies show that synaptic receptors increase during chronic alcohol exposure but relocate to extrasynaptic locations during alcohol withdrawal (Clapp et al., 2010). Thus, in addition to genotypic differences in the expression of glutamate receptors, the time course of receptor relocation could contribute to our results. However, there are several caveats to speculation based on our Western blot studies including that whole hippocampus was used rather than just the CA1 region, synaptic levels were not specifically measured, and changes in receptor subunit expression may not reflect changes in functional receptor expression.

CIE-W produced an enhancement of the PS in hippocampus from CCL2-tg mice, an effect consistent with the known effects of chronic alcohol on the PS in CA1 pyramidal neurons (Bailey et al., 1998; Little, 1999). The enhancement of the PS in hippocampus from CIE-W treated CCL2-tg mice could be due to reduced somatic inhibition. Somatic inhibition is a result of inhibitory synaptic influences mediated by GABA_A receptors at the somatic region, and influences from soma/dendritic excitability mediated by membrane ion channels. The functions of both GABA_A synaptic transmission (Kumar et al., 2009; Rogers and Hunter, 1992) and ion channels mediating soma/dendritic excitability (Little, 1999; Mulholland et

al., 2015) are known to be altered by chronic alcohol exposure, effects that could increase excitability and result in a larger PS.

The ability of CCL2/alcohol interactions to impact somatic inhibition was indicated by results from studies of synaptic plasticity of the PS. In hippocampal slices from the 2BC-W treated CCL2-tg mice, PPR (10 ms) of the PS was increased indicating a reduction in inhibitory influences during repetitive stimulation, whereas there was no change in synaptic plasticity of the PS hippocampus from 2BC-W treated non-tg mice. In addition, PPR of the PS was decreased in hippocampus from CIE-W treated CCL2-tg mice indicating an increase in inhibitory influences, whereas a reduction in inhibitory was observed in hippocampus from the CIE-W treated non-tg mice. The mechanisms mediating these genotypic differences and differences in the effects of 2BC-W and CIE-W treatments remain to be identified.

4.3. Long-term hippocampal synaptic plasticity

Our previous studies showed no genotypic difference in hippocampal LTP between alcohol naïve CCL2-tg and non-tg mice (Bray et al., 2013). In the current studies, enhancement of LTP was observed in hippocampus from CCL2-tg mice subjected to 2BC-W treatment but no effect on LTP was observed in hippocampus from CIE-W treated CCL2-tg mice. These effects are similar to effects of acute alcohol exposure in vitro observed in our previous studies of hippocampal slices from CCL2-tg mice. Acute alcohol at 20 mM enhanced LTP in hippocampus from CCL2-tg mice but 60 mM acute alcohol had no effect (Bray et al., 2013). In contrast, acute alcohol exposure in vitro at 20 mM and 60 mM depressed LTP in hippocampus from non-tg mice (Bray et al., 2013). Hippocampus from the non-tg mice subjected to CIE-W also showed a depression of LTP, whereas no effect on LTP was observed in hippocampus from 2BC-W treated non-tg mice. These similarities between the effects of acute alcohol exposure in vitro and CIE-W treatment on hippocampus from CCL2-tg and non-tg mice may reflect similar target sites for acute and chronic alcohol within a genotype.

Our results from hippocampus of CIE-W treated non-tg mice are consistent with previous studies showing that prolonged in vivo treatment of rodents with alcohol results in a reduction in LTP measured in hippocampal slices from the animals and that this reduction can persist for one month or more after the alcohol is withdrawn (Durand and Carlen, 1984; Stephens et al., 2005; Tremwel and Hunter, 1994). The apparent resistance of the hippocampus from the CCL2-tg mice to the depressive effects of 2BC-W and CIE-W on LTP observed in hippocampus from non-tg mice may relate to an increase in the level of NMDA receptors in the CCL2-tg hippocampus, as NMDA receptors are critical to the expression of LTP. In addition, presynaptic factors appeared to be involved for the 2BC-W treated CCL2-tg hippocampus under conditions of high frequency stimulation, because PTP was also increased in the hippocampus of CCL2-tg mice subjected to 2BC-W, whereas no effect on PTP was observed in hippocampus from non-tg mice.

4.4 Comparisons with behavior studies

Our previous studies showed that the performance of alcohol naïve CCL2-tg and non-tg mice was similar in several behavioral tests including Y-maze, fear conditioning, light/dark transfer and forced-swim test (Bray et al., 2017). These results are consistent with the lack of genotypic difference in hippocampal synaptic function (basic I/O properties and LTP) between alcohol naïve CCL2-tg and non-tg mice (Bray et al., 2013). In the 2BC-W treated group, non-tg mice showed no effect of 2BC-W on spatial learning (Barnes maze), consistent with the lack of effect of 2BC-W on synaptic transmission or LTP (Bray et al., 2017). However, in CCL2-tg mice 2BC-W treatment resulted in impairment in spatial learning, although for two aspects of the spatial learning test (latency to escape and number of errors), impairment in the CCL2-tg mice only occurred early in training, with recovery as training progressed (Bray et al., 2017). Analysis of synaptic function in the 2BC-W CCL2-tg mice showed a depression of synaptic transmission but an enhancement of LTP (Table 1). The reduced synaptic transmission in hippocampus from the 2BC-W treated CCL2-tg mice may be related to the impairments in spatial learning, whereas the enhancement of LTP may play a role in the recovery during training.

For mice exposed to CIE-W, non-tg mice did not show impairments in spatial learning (Y-maze) (Bray et al., 2017), but showed an increase in synaptic transmission (fEPSP) and a decrease in LTP (Table 1). CCL2 tg mice exposed to CIE-W showed impairments in spatial learning (Y-maze) (Bray et al., 2017) but no change in synaptic transmission (fEPSP) or LTP (Table 1). Thus, at the time during withdrawal that measurements of synaptic function were made, there was no consistent correlation between results from behavioral tests for spatial learning and electrophysiological tests of synaptic function. This dichotomy may be related to the difference in time during the withdrawal process that the two types of measurements were made. Further studies will be required to resolve this issue.

In contrast to the measurements of spatial learning, measurements of fear conditioning correlated with measurements of hippocampal synaptic function. Thus, non-tg mice exposed to CIE-W showed impairments in cued and contextual fear conditioning (Bray et al., 2017) and depression of hippocampal LTP (Table 1). In the CCL2-tg mice there was no effect of CIE-W on cued and contextual fear conditioning (Bray et al., 2017) or LTP (Table 1). These results suggest that CCL2/alcohol exposure/withdrawal interactions may specifically target certain forms of memory, presumably through actions at underlying mechanisms.

5. Summary

Taken together, our results show that elevated levels of CCL2 in the hippocampus produced by increased astrocyte expression can significantly interact with the effects of alcohol exposure/withdrawal. The interactions are evident at both behavioral (Bray et al., 2017) and synaptic levels and are evidenced by altered consequences of alcohol action. Some of the CCL2/alcohol/withdrawal interactions produced by 2BC drinking or CIE treatment had consequences that were similar to that of acute alcohol exposure of hippocampal slices in vitro (Bray et al., 2013). This similarity may reflect common target sites of alcohol action across the different alcohol treatment paradigms. In these studies, the CCL2-tg mice were used to model persistently increased levels of CCL2 in the CNS produced by prolonged

alcohol use. However, results may also be relevant to effects of alcohol in the CNS of individuals that have neurological conditions that result in persistent increases in levels of CCL2 in the CNS.

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Highlights

- CCL2/alcohol interactions alter hippocampal synaptic responses.
- CCL2/alcohol interactions alter hippocampal synaptic plasticity.
- CCL2/alcohol interactions vary across alcohol exposure paradigms.

Figure 1.
Diagram showing CIE/2BC treatment paradigm.

Figure 2.

Synaptic responses were depressed in hippocampus from non-dependent CCL2-tg mice. (A) CCL2 levels in hippocampus from mice used in behavioral studies measured at the time of sacrifice for electrophysiological studies. (B1,C1) Graphs showing I/O relationships for fEPSP in hippocampus from non-dependent and alcohol naïve non-tg (B1) and CCL2-tg mice (C1). (B2,C2) Graphs showing I/O relationships for PS in hippocampus from non-dependent and alcohol naïve non-tg (B2) and CCL2-tg mice (C2). Inserts show sample recordings. The top recording (black) is from a naïve mouse and the bottom recording (red) is from an alcohol-treated mouse. *significant difference from control values for the same genotype (Repeated Measures ANOVA).

Figure 3.

Synaptic responses were altered in hippocampus from dependent mice. (A1,B1) Graphs showing I/O relationships for fEPSP in hippocampus from dependent (CIE) non-tg (A1) and CCL2-tg mice (B1) and their respective controls (cont). (A2,B2) Graphs showing I/O relationships for PS in hippocampus from dependent non-tg (A1) and CCL2-tg mice (B1) and their respective controls. Inserts show sample recordings. The top recording (black) is from a naïve mouse and the bottom recording (red) is from an alcohol-treated mouse *significant difference from control values for the same genotype (Repeated Measures ANOVA).

Figure 4.

PPR of the PS is differentially altered in hippocampus from non-dependent (2BC drinking) and dependent mice (CIE). Left panels show sample recordings. The black recording is the response to the first stimulation of the paired pulses and the red recording is the response to the second stimulation of the pair. The interpulse interval for the sample recording was 10 ms. Right panels show graphs of mean values. (A) PPR was greater than 1 in hippocampus from non-dependent non-tg and CCL2-tg mice. PPR was increased at the 10 ms timepoint in hippocampus from non-dependent CCL2-tg mice, indicating that 2BC drinking reduced inhibition. (B) PPR was increased in hippocampus from dependent non-tg mice at both time intervals tested, reflecting reduced inhibitory influences. In contrast, PPR was reduced in hippocampus from dependent CCL2-tg mice at both 10 and 20 ms intervals, indicating increased inhibitory influences. *significant difference from control values for the same genotype (unpaired *t*-test).

Figure 5.

Genotypic differences in synaptic plasticity elicited by TBS observed in hippocampus from non-dependent (2BC) and dependent (CIE) mice. Left panels show sample recordings for baseline (base), PTP, and LTP under the various experimental conditions. Right panels show graphs of mean values. (A1,2) Enhancement of the fEPSP by TBS in hippocampus from non-dependent non-tg (A1) and CCL2-tg (A2) mice. Enhancement of the fEPSP was similar in hippocampus from non-dependent and naïve non-tg mice, but was greater in hippocampus from the non-dependent CCL2-tg mice compared to naïve CCL2-tg mice. (B1,2) Enhancement of the fEPSP by TBS in hippocampus from dependent non-tg (B1) and CCL2-tg (B2) mice. STP and LTP were reduced in dependent non-tg mice compared to controls. There was no change observed in dependent CCL2-tg mice compared to 2BC controls. TBS was applied at the 0 time point (arrow) of the graphs. Insert graphs show mean values for PTP, STP, and LTP. *significant difference from the respective control values for the same genotype (unpaired *t*-test).

Table 1

Summary of Effects of Ethanol on Synaptic Function

Measure	naive	2BC-W		CIE-W	
	CCL2-tg vs non-tg	CCL2-tg 2BC vs naive	non-tg 2BC vs naive	CCL2-tg CIE vs cont	non-tg CIE vs cont
Synaptic transmission					
-fEPSP I/O	No	↗	No	No	↗
-PS I/O	No	↗	No	↗	No
P-P synaptic plasticity					
-fEPSP (PPF)	No	No	No	No	No
-PS (PPR)	No	↗	No	↗	↗
Long-term synaptic plasticity					
-PTP	No	↗	No	No	No
-STP	No	↗	No	No	↗
-LTP	No	↗	No	No	↗

↘ = decrease, ↗ = increase, No = no difference