



The role of matrix metalloproteinase-9 in negative reinforcement learning and plasticity in alcohol dependence.

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Abstract

A role for matrix metalloproteinases (MMPs) in plasticity-dependent learning has been established. MMPs degrade the extracellular matrix (ECM) when synaptic reorganization is warranted. Previously, we showed that escalation of alcohol self-administration is a learned plasticity-dependent process that requires an intact MMP system. To identify the MMP subtypes within specific brain regions that are associated with plasticity underlying the negative reinforcing effects of alcohol (as measured by escalated alcohol self-administration) during acute withdrawal in alcohol dependence, male Wistar rats were trained to self-administer alcohol in an operant paradigm, subjected to one month of intermittent alcohol vapor exposure to induce alcohol dependence, and then allowed to self-administer alcohol during repeated acute withdrawal self-administration sessions. Subsequently, rat brains were extracted after initial or stable escalated alcohol self-administration phases of acute withdrawal and analyzed by immunoblot to detect MMP-2, -3 and -9 levels in the anterior cingulate cortex (ACC), bed nucleus of the stria terminalis, central amygdala (CeA), hippocampus and nucleus accumbens (NAc). The results showed that MMP-9 expression in the CeA and NAc of alcohol-dependent rats was increased, however, MMP-9 expression in the ACC was decreased during negative reinforcement learning. Subsequently, the importance of plasticity mediated by MMP-9 in escalated alcohol self-administration during acute withdrawal was functionally assessed through site-specific intra-CeA MMP-9 inhibition during repeated acute withdrawal self-administration sessions. MMP-9 inhibition prevented acute withdrawal-induced escalation of alcohol self-administration in a manner that was not confounded by locomotor effects or a permanent inability to learn about the negative reinforcing effects of alcohol.

Introduction

Alcohol dependence-induced neuroadaptations result in the emergence of aversive negative emotional / affective states, such as depressive-like and anxiety-like behavior, during withdrawal (Williams *et al.* 2012) that can be ameliorated by alcohol consumption making alcohol a negative reinforcer (i.e., the removal of the aversive withdrawal symptoms is

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reinforcing). This ‘self-medication’ can perpetuate alcohol use disorders (Markou *et al.* 1998) as an organism learns that negative affective states can be removed through escalation of alcohol consumption (Koob 2003). The negative reinforcing effects of alcohol become a significant motivational factor during the transition to alcohol dependence (Roberts *et al.* 2000; Walker 2012). Since reinforcement is a learned response and learning is a plasticity-dependent process, the negative reinforcing properties of alcohol in dependence (i.e., escalation of alcohol consumption during acute withdrawal) were hypothesized to be a plasticity-dependent process. Indeed, we showed for the first time that escalation of alcohol self-administration was a plasticity-dependent behavior (Smith *et al.* 2011) with an acquisition pattern that exhibits a standard learning curve evidenced by a progressive increase in alcohol self-administration during repeated acute withdrawal test sessions until escalated alcohol self-administration reaches a plateau and stabilizes (Smith *et al.* 2011).

Matrix metalloproteinases (MMPs) are a family of proteases that degrade components of the extracellular matrix (ECM) to allow for central nervous system reorganization and are important regulators of synaptic plasticity required for multiple forms of hippocampal long-term potentiation (LTP)-mediated learning (Wright and Harding 2009; Huntley 2012). In our initial demonstration of MMP involvement in negative reinforcement learning, we showed that broad-spectrum central MMP inhibition suppressed escalated alcohol self-administration during acute withdrawal in alcohol-dependent rats during the *initial stage of escalation* when negative reinforcement-related neuroplasticity was hypothesized to be necessary. In contrast, in alcohol-dependent animals displaying *stable escalated responding* indicative of completed learning and neuroplasticity, MMP inhibition had no effect (Smith *et al.* 2011; Walker 2012); confirming that once operant alcohol self-administration stabilizes, the plasticity-related MMP-mediated period of learning had concluded.

Of the more than 20 MMP subtypes (Huntley 2012), the MMP-2, -3, and -9 subtypes have been implicated in LTP-dependent dorsal hippocampal-based learning tasks including spatial and associative learning (Wright and Harding 2009), as well as plasticity associated with cocaine extinction and reinstatement (Smith *et al.* 2014). MMP-2 and MMP-9 belong to gelatinase subfamily of MMPs (Sternlicht and Werb 2001). Although MMP-2/-3 have been associated with MMP-9 activation (Ogata *et al.* 1992; Fridman *et al.* 1995; Toth *et al.* 2003), such associations are not consistently observed (Wright *et al.* 2003; Meighan *et al.* 2006). In addition, although MMP-2, -3 and -9 have been implicated in dorsal hippocampal-based learning tasks (Wright and Harding 2009), it could be that selective MMP subtypes are involved with different forms of learning, such as negative reinforcement learning. Specific to the hypothesis under evaluation, MMP-9 has been implicated in appetitive, but not aversive, learning-related plasticity in the central nucleus of the amygdala (CeA) of mice (Knapska *et al.* 2013) and impairments in alcohol seeking in MMP-9 KO mice during alcohol withdrawal were normalized by overexpression of exogenous MMP-9 in CeA (Stefaniuk *et al.* 2017). In human alcoholics, a C/T polymorphism of the MMP-9 gene (that can produce higher MMP-9 gene transcriptional activity) is associated with alcohol dependence (Samochowiec *et al.* 2010) and enhanced motivation to drink alcohol (Stefaniuk *et al.* 2017). Therefore, MMP-mediated plasticity has been identified as an emerging important contributor to factors related to alcohol use disorders (AUDs) and the converging

evidence suggests that MMP-9 activity in the CeA could be important for understanding negative reinforcement learning in alcohol dependence.

The CeA, in conjunction with the nucleus accumbens shell (NAc shell) and bed nucleus of the stria terminalis (BNST), form a functionally-interconnected network that comprise the extended amygdala (Alheid and Heimer 1988) that is important for integrating emotion and motivation and has been championed as a critical network involved in alcohol abuse and dependence (Koob 2013). Nuclei within the extended amygdala have been heavily implicated in the positive and negative reinforcing properties of alcohol (Koob and Volkow 2010) and other limbic nuclei, such as the anterior cingulate cortex (ACC) and dorsal hippocampus (HPC), have been implicated in aversive and avoidance learning (Johansen and Fields 2004; Nagy *et al.* 2007), have AUD-related importance (Rezayof *et al.* 2008; Gremel *et al.* 2011), and are sites of potential neuroplasticity associated with negative reinforcement in alcohol dependence (Sirohi *et al.* 2012).

To identify specific MMPs subtypes in distinct brain regions that are required for negative reinforcement learning during acute withdrawal in alcohol-dependent rats (as evidenced by escalated alcohol self-administration), the current experiments examined the role of MMP-2, -3 and -9 expression in the ACC, BNST, CeA, HPC, and NAc in combination with site-specific MMP inhibition in a manner that dissociates the *acquisition* of escalated alcohol self-administration from *stable escalated* alcohol self-administration during acute withdrawal in alcohol-dependent Wistar rats.

METHODS

Animals

Male Wistar rats (N=56; PND 70) housed 2–3/cage in a temperature- and humidity-controlled vivarium on a reversed light-dark cycle (lights off at 6:00AM) had food and water *ad libitum*. Rats were handled daily 1-wk prior to training. All procedures followed the National Research Council Guide for the Care and Use of Laboratory Animals (National Research Council *et al.* 2011) and were approved by the Washington State University Institutional Animal Care and Use Committee.

Pre-dependence alcohol self-administration training

Rats were trained to self-administer a 10% alcohol (w/v) solution (0.1 ml / lever-press) on a continuous ratio schedule of reinforcement during 30-min limited-access sessions (5 days/ week) in standard operant chambers (Med Associates, St. Albans, VT) containing custom drinking wells (Behavioral Pharma, La Jolla, CA) using a sweetener-fade procedure (Walker and Koob 2007). A sweetened fluid (0.125% saccharin and 3% glucose) that is preferred to sucrose (Valenstein *et al.* 1967) was used as the reinforcer for acquisition of operant self-administration. Then, 10% alcohol (w/v) was added and the sweeteners were removed from the solution over 3 weeks until only 10% alcohol remained. The number of lever-presses were converted to alcohol consumption (g/kg). Once responding for alcohol stabilized (three consecutive sessions with <10% deviation), rats progressed to the next phase of the experiments.

Alcohol dependence induction via intermittent alcohol vapor exposure

Following self-administration training, rats were subjected to one-month of intermittent alcohol vapor exposure (14h-on, 10h-off per day) which induces alcohol dependence (O'Dell *et al.* 2004) as evidenced by dependence-like behavioral phenotypes (O'Dell *et al.* 2004; Walker and Koob 2007; Williams *et al.* 2012) when animals are tested 6–8 h into acute withdrawal. Blood ethanol concentrations (BECs) were measured twice-weekly by tail blood collection prior to daily termination of the alcohol vapor exposure and analyzed using the Analox AM1 (Analox Instruments, Lunenburg, MA). Target BECs of 175–225 mg% were maintained throughout the experiments and confirmed prior to termination of daily alcohol vapor exposure on every acute withdrawal self-administration test day. The 6–8 h acute withdrawal timepoint used during each of the repeated acute withdrawal tests was first adopted (Walker and Koob 2007) because the BECs of alcohol-dependent rats maintained at ~225 mg% drop to zero within 6-h (Gilpin *et al.* 2009) and therefore avoids the confound of alcohol presence at the time of acute withdrawal testing.

Experiment 1:

Post-dependence self-administration during acute withdrawal: Following vapor exposure, rats (N=32) were separated into four groups (n=8/grp) matched for 30-min limited-access baseline alcohol consumption (g/kg): 1) initial escalation (IE; represents the first statistically escalated self-administration session during acute withdrawal, 2) initial escalation pharmacological control (IEC), 3) stable escalation (SE; after escalated alcohol self-administration had stabilized), and 4) stable escalation pharmacological control (SEC). Only the IE and SE groups self-administered alcohol following dependence induction, whereas the IEC and SEC groups did not self-administer alcohol following the dependence induction phase but were instead injected intraperitoneally (IP) with equivalent alcohol (g/kg) to that consumed by the IE and SE groups, respectively. The IEC and SEC groups served as pharmacological controls (essentially yoked-controls) since acute alcohol administration can alter MMP expression (Wright *et al.* 2003). After each test, rats were returned to vapor chambers to re-expose them to alcohol vapor between acute withdrawal self-administration tests so that animals could be repeatedly tested under identical conditions of acute withdrawal.

Immunoblotting: Briefly (see Supplementary Materials for detailed procedures), brains were extracted 3-hr after either initial or stable escalation for the IE and SE groups, respectively, or following equivalent IP injections of alcohol (g/kg) for the IEC and SEC groups. The 3-hr extraction timepoint was based on previous research showing that the 3-hr timepoint could capture both pro and active MMP expression (Meighan *et al.* 2006). Micropunches of the ACC, BNST, CeA, HPC, and NAc were sonicated and lysed with RIPA lysis buffer (Millipore:20–188) containing Halt protease and phosphatase inhibitor cocktail (Thermo:78441) and microcystin-LR (Enzo, ALX-350–431-C010). After centrifugation at 10,000g for 20 min at 4°C, supernatant was removed and tested for protein concentration using Pierce BCA protein assay kit (Thermo:23225). 15 µg of protein was utilized for separation by SDS-polyacrylamide gel electrophoresis (Bio-rad:5671025) and transferred to nitrocellulose membranes. To ensure equal loading and appropriate electrophoresis and transfer, as well as serving as a transfer control between membranes, the nitrocellulose

membranes were stained with MemCode (Thermo:24580), a reversible total protein stain procedure (Fig. S1). Given that most MMPs are synthesized and secreted as an inactive pro-form and are activated following the removal of the pro-domain (Fujioka *et al.* 2012; Huntley 2012), we selected primary antibodies (Ab) that could detect both pro (P)- and active (A)-forms of MMP-2 (P72- and A67-kD), MMP-3 (P55- and A21-kD) and MMP-9 (P98-, A92- and A68-kD). Following MemCode stain removal, membranes were incubated overnight at 4°C with primary Ab against MMP-2 (1:150; Millipore: AB19167), MMP-3 (1:50; Fitzgerald:70R-11887), and MMP-9 (1:125; Millipore:AB19016). Following 1hr of incubation with HRP-conjugated secondary antibody (Millipore:12–348) at RT, the membrane was soaked in an enhanced ECL (Thermo:34080). Band images were captured with the Bio-rad ChemiDoc XRS+ imaging system and optical density of bands were measured using Image Lab software. The target protein to MemCode ratio was utilized to provide a standardized value for all bands and the percent pharmacological control was calculated.

Experiment 2:

Surgery: Following the establishment of stable 30-min limited-access alcohol self-administration and prior to intermittent alcohol vapor exposure (as described above), rats (N = 24) were anesthetized with isoflurane gas (5% for induction / 2% for maintenance). Bilateral guide cannulae (22 gauge; Plastics One, Roanoke) were implanted 2mm above the CeA (AP -2.3, ML ± 4.2, DV -6.2 from bregma; (Paxinos and Watson 2007), secured in place using dental acrylic cement anchored to four stainless-steel machine screws and obturators maintained cannulae patency. Rats received 5-d of post-operative care with an analgesic (flunixin, 2.5 mg/kg), antibiotic (Baytril, 5 mg/kg), 0.9% saline administered subcutaneously and allowed 2-wks of recovery prior to intermittent alcohol vapor exposure (described above).

Post-dependence self-administration during acute withdrawal: Following alcohol dependence induction (see above), rats (N = 24) were divided into two groups (n = 12/grp) matched for pre-dependence 30-min limited-access alcohol consumption as well as BECs on the initial post-dependence induction test day that occurred during acute withdrawal. Rats were repeatedly tested twice weekly during acute withdrawal (6–8 h into withdrawal) for 30-min limited-access operant alcohol self-administration. After each acute withdrawal test, rats were returned to alcohol vapor chambers to re-expose them to alcohol vapor until the next acute withdrawal 30-min limited-access alcohol self-administration test.

Assessment of Intra-CeA MMP-9 Inhibition: To test the hypothesis that CeA MMP-9 activity was necessary for acute withdrawal-induced escalation of alcohol self-administration, a MMP-9 inhibitor (MMP-9i; 0.1 nmol/side; Millipore:444278) or vehicle (0.6% DMSO in aCSF) were infused (0.5ul/side over 2 min) 15-min prior to, and 45-min following, post-dependence acute withdrawal alcohol self-administration sessions via bilateral 28-gauge injectors (extending 2-mm below the guide cannulae; Plastics One, Roanoke) targeting the CeA. Testing during acute withdrawal was repeated for six sessions until the vehicle-treated group displayed statistically significant escalation of alcohol self-administration. The timing and dosing of MMP-9i infusions were selected based on previous

studies (Smith *et al.* 2011; Smith *et al.* 2014). ***Control Conditions:*** Two control conditions were included in this experiment to confirm the primary hypothesis under evaluation, as well as guard against potential confounds: 1) To address the possibility that intra-CeA MMP-9i administration might induce a permanent loss of an organism's capacity to acquire escalated alcohol self-administration, once the MMP-9i infusions were terminated, the animals then received vehicle infusions under the same timing conditions as the original MMP-9i treatments to evaluate whether the animals would show normal negative reinforcement learning (i.e., escalated alcohol self-administration). 2) Implied in the hypothesis that MMP activity is necessary for the acquisition of escalated alcohol self-administration is that once stability of escalated alcohol self-administration occurs, MMPs are no longer required because learning/plasticity has concluded. Therefore, the prediction would be that in alcohol-dependent animals demonstrating stable escalated alcohol self-administration during acute withdrawal, intra-CeA MMP-9i would have no effect on their self-administration performance. In addition, because the MMP-9i was, in part, administered prior to the self-administration sessions, the MMP-9i pretreatment could have rate-decreasing effects and a failure to observe escalated alcohol self-administration could be a result of non-specific MMP-9i effects. To rule out this possibility, once the animals originally receiving vehicle treatments displayed stable escalated alcohol self-administration, an acute intra-CeA MMP-9i infusion was administered 15-min prior to a final self-administration session during acute withdrawal.

Histology: After the completion of Experiment 2 (19/24 animals successfully completed the dependence induction period and subsequent testing), rat brains were removed, sectioned and cannula placement was verified.

Statistics

Experiment 1—*Baseline alcohol self-administration and dependence-induced escalation:*

A univariate analysis of variance (ANOVA) compared baseline alcohol consumption (g/kg) for the four conditions (IE, IEC, SE and SEC). Baseline and escalated alcohol consumption for the IE and SE conditions were compared using a two-way mixed-model ANOVA with condition as the between-group factor and session as the within-subject factor. Post-hoc repeated-measures tests were conducted if main effects or interactions were identified. Lastly, a univariate ANOVA was used to compare escalated alcohol consumption for the IE and SE groups prior to immunoblotting. ***Immunoblotting:*** Within the five brain regions and three different MMP subtypes analyzed (that included both pro and active MMPs), two-way ANOVAs were utilized to assess protein to MemCode ratios with escalation group (initial vs stable) and condition (self-administering vs pharmacological control) as the between-group factors. If significant interactions were identified, post-hoc comparisons were conducted to identify the specific group differences for each MMP pro and active form(s) in each of the brain regions.

Experiment 2—*Baseline alcohol self-administration:* A univariate ANOVA compared baseline alcohol consumption between the vehicle and MMP-9i groups. ***Intra-CeA MMP-9i effects on dependence-induced escalation:*** A two-way mixed-model ANOVA compared vehicle- and MMP-9i treatments during acute withdrawal with treatment (vehicle or

MMP-9i) as the between-group factor and session (initial vs escalated) as the within-subject factor. If main effects or interactions were identified, the appropriate post-hoc comparisons (i.e., between-group and/or repeated-measures tests) were conducted. *Control Conditions:* In those animals that initially received intra-CeA MMP-9i infusions, a repeated-measures ANOVA compared the effects of intra-CeA vehicle infusions over a final three acute withdrawal self-administration sessions, with post-hoc comparisons conducted if a main effect was found. During acute withdrawal in the group of animals that were originally vehicle-treated and subsequently received acute MMP-9i in the CeA, a repeated-measures ANOVA was used to compare alcohol consumption between their final vehicle-treated stable escalated consumption session and the subsequent acute MMP-9i infusion session.

Results

Experiment 1

Baseline alcohol self-administration and dependence-induced escalation (Fig. 1): The univariate ANOVA confirmed that there were no differences in baseline alcohol consumption for the IE, IEC, SE and SEC groups ($F(1,28)=0.002$, $p>0.05$). The two-way mixed-model ANOVA conducted on baseline and escalated alcohol consumption for the IE and SE conditions identified a main effect of session ($F(1,14)=175.929$, $p<0.001$). Post-hoc comparisons showed that both the IE and SE group's alcohol consumption was escalated compared to baseline consumption ($F(1,7)=44.114$, $p<0.001$ and $F(1,7)=295.77$, $p<0.001$). Lastly, the univariate ANOVA comparing post-dependent escalated alcohol consumption for the IE and SE groups showed that there were no differences ($F(1,14)=2.089$, $p>0.05$) between the escalated IE and SE group consumption levels.

Immunoblotting (Fig. 2): In the CeA and NAc, the two-way ANOVAs conducted on the P98-, A92- and A68-kD forms of MMP-9 identified main effects for Group (CeA: $F(1,28)=169.958$, $p<0.001$; $F(1,28)=18.529$, $p<0.001$ and $F(1,28)=36.166$, $p<0.001$, respectively; NAc: $F(1,28)=17.924$, $p<0.001$; $F(1,28)=7.161$, $p=0.01$ and $F(1,28)=8.790$, $p<0.01$, respectively) and Condition (CeA: $F(1,28)=202.861$, $p<0.001$; $F(1,28)=29.345$, $p<0.001$ and $F(1,28)=47.215$, $p<0.001$, respectively; NAc: $F(1,28)=18.285$, $p<0.001$; $F(1,28)=6.809$, $p=0.01$ and $F(1,28)=8.497$, $p<0.01$, respectively), as well as Group x Condition interactions (CeA: $F(1,28)=169.958$, $p<0.001$; $F(1,28)=18.529$, $p<0.001$ and $F(1,28)=36.166$, $p<0.001$, respectively; NAc: $F(1,28)=17.924$, $p<0.001$; $F(1,28)=7.161$, $p=0.01$ and $F(1,28)=8.790$, $p<0.01$, respectively). Alternatively, in the ACC, only A92-kD MMP-9 showed a main effect of Group ($F(1,28)=8.927$, $p<0.01$), Condition ($F(1,28)=9.104$, $p<0.01$) and a Group x Condition interaction ($F(1,28)=8.927$, $p<0.01$). Post-hoc comparisons indicated that in the CeA and NAc, the P98-, A92- and A68-kD forms of MMP-9 were *elevated* in the IE group when compared to IEC (CeA: $t(14)=15.662$, $p<0.001$; $t(14)=5.781$, $p<0.001$ and $t(14)=7.579$, $p<0.001$, respectively; NAc: $t(14)=4.961$, $p<0.001$; $t(14)=3.128$, $p<0.01$ and $t(14)=3.110$, $p<0.01$, respectively) and SE (CeA: $t(14)=14.645$, $p<0.001$; $t(14)=5.325$, $p<0.001$ and $t(14)=6.621$, $p<0.001$, respectively; NAc: $t(14)=4.855$, $p<0.001$; $t(14)=2.896$, $p=0.01$ and $t(14)=3.201$, $p<0.01$, respectively). Conversely, in the ACC, the A92-kD MMP-9 was *selectively reduced* in the IE group when compared to IEC ($t(14)=-4.623$, $p<0.001$) and SE ($t(14)=-3.906$, $p<0.01$). See Figure S2 for ACC, CeA and NAc

MMP-9 western blot images. No changes were identified for MMP-2 or MMP-3 expression in the CeA, NAc and ACC or MMP-2, -3 or -9 expression in the BNST or HPC (see Figure S3 for western blot images).

Experiment 2

Histology: Only animals with verified CeA MMP-9i infusion sites were included in the statistical analysis (Fig. 3). The final number of animals included in the Experiment 2 analysis was n=10 for the MMP-9i group and n=9 for the vehicle-treated group. **Baseline alcohol self-administration** (Fig. 4B): The univariate ANOVA confirmed no differences in baseline alcohol consumption between the treatment (vehicle or MMP-9i) groups ($F(1,17)=0.635$, $p>0.05$). **Intra-CeA MMP-9i effects on dependence-induced escalation** (Fig. 4B): The two-way mixed-model ANOVA identified a main effect of session ($F(1,17)=22.579$, $p<0.001$) and a treatment x session interaction ($F(1,17)=14.090$, $p<0.01$), with post-hoc comparisons identifying that MMP-9i treatment group had significantly reduced alcohol consumption when compared to the vehicle-treated condition ($t(17)=2.554$, $p<0.05$). **Control Conditions** (Fig. 4C): In animals that were originally treated with intra-CeA MMP-9i and did not escalate, a repeated-measures ANOVA identified that the animals showed a significant escalation of alcohol consumption over three sessions when vehicle-treated ($F(2,18)=42.596$, $p<0.001$) with post-hoc comparisons identifying that both the 2nd and 3rd acute withdrawal alcohol self-administration were significantly escalated when compared to the 1st session ($p<0.001$ in both cases). Lastly, a repeated-measures ANOVA confirmed that there were no differences between the vehicle-treated group's escalated responding when compared to the acute intra-CeA MMP-9i treatment condition ($F(1,8)=0.944$, $p>0.05$).

Discussion

The current experiments tested the hypothesis that MMP-mediated plasticity within limbic circuitry is required for negative reinforcement-related learning in alcohol-dependent rats during acute withdrawal. The results of the first experiment showed that in alcohol-dependent rats, the *initial escalation* phase of alcohol self-administration during acute withdrawal was specifically associated with increased P98-, A92- and A68-kD MMP-9 (but not MMP-2 or MMP-3) in the CeA and NAc (Fig. 1), with no changes in MMP-2, -3 or -9 expression in the BNST or HPC. Once escalated alcohol self-administration had stabilized, when learning / plasticity is posited to have completed, MMP-9 expression returned to pharmacological control levels in CeA and NAc (Fig. 2). Intriguingly, concomitant with increased MMP-9 expression in the CeA and NAc, a *selective decrease* in A92-kD MMP-9 expression was observed in the ACC (Fig. 2). Given the previously-observed relationships between MMP-2/3 and MMP-9 (Ogata *et al.* 1992; Fridman *et al.* 1995; Toth *et al.* 2003), an important caveat in relation to the timing of brain extractions is that MMP-2 or MMP-3 expression and activity could occur before MMP-9 expression. Therefore, it could be possible that MMP-2/3 expression was altered before we harvested brain tissue for analysis and would not have been detectable, but it should be noted that previous research has observed alterations in MMP-3 and MMP-9 under learning-related conditions using the same brain extraction timing (Meighan *et al.* 2006). In rat dorsal hippocampus, 92- and 68-

kD MMP-9 have been identified as active forms of MMP-9 (Meighan *et al.* 2006), although a 86-kD form of MMP-9 detected in human serum has also been posited as active (Demestre *et al.* 2005) with these differences potentially attributable to species and substrate variation. The primary MMP-9 Ab used in the present study was able to consistently detect P98-, A92- and A68-kD MMP-9 in all brain regions assessed, whereas an 86-kD band was inconsistently observed and therefore, not included in our assessment (see Figure S2 and S3 for MMP-9 blots).

Alterations in MMP-9 expression in the present study are consistent with results showing that MMP-9 activity in the CeA is elevated in appetitive, but not aversive, learning and is localized to postsynaptic glutamatergic synapses (Knapska *et al.* 2013). Also consistent with these results, MMP-9 knock-out mice displayed disrupted LTP in the basal and central amygdala (Gorkiewicz *et al.* 2015) and disruptions in alcohol-seeking were ameliorated once MMP levels were normalized through exogenous overexpression of MMP-9 in the CeA (Stefaniuk *et al.* 2017). Given that the CeA results in the present study were accompanied by a parallel increase in MMP-9 expression in the NAc, but not in the BNST, is interesting given the functional interconnectivity of these extended amygdala regions and evidence implicating these regions in the motivational and reinforcing effects of alcohol (Koob *et al.* 1993; Koob 2013; Roberto and Varodayan 2017; Vranjkovic *et al.* 2017). The concomitant increase in NAc and CeA MMP-9 expression suggests that plasticity in both the NAc and CeA contribute to the negative reinforcing effects of alcohol during acute withdrawal. Neuroadaptations in both the NAc and CeA have been shown to mediate escalated operant alcohol self-administration during acute withdrawal in alcohol-dependence (e.g., Nealey *et al.* 2011; Kissler *et al.* 2014) and in the CeA, MMP-9 KO mice did not show silent synapse formation consistent with the plasticity-related changes associated with alcohol withdrawal (Stefaniuk *et al.* 2017). Within the NAc, plasticity-related adaptations such as silent synapse formation have been implicated in incubation of craving and an amygdala / accumbens circuit, consistent with the present results, has been shown to be important in drug relapse (Lee *et al.* 2013). Although we did not observe alterations in BNST MMP expression under the conditions tested, it should be noted that BNST dysregulation does contribute to escalated alcohol self-administration during acute withdrawal (Erikson *et al.* 2018). Future research could delineate BNST contributions to the formation or removal of negative affective states that appear to be required for escalated alcohol self-administration in dependence (Walker 2012). Although the dorsal HPC has been repeatedly implicated in MMP-mediated learning and plasticity in a variety of learning paradigms (Wright and Harding 2009; Huntley 2012), dorsal HPC MMP-2, -3 or -9 expression was not altered during the *initial phase of escalation* when compared to stable escalation or when compared to the alcohol pharmacological control during acute withdrawal in alcohol-dependent rats.

In contrast to increased MMP-9 in the CeA and NAc, the ACC showed a *selective reduction* in the A92-kD MMP-9 during the *initial phase of escalation* when compared to stable escalation and when compared to the alcohol pharmacological control (Fig. 2). Since the ACC is important for avoidance learning (Johansen and Fields 2004) and the CeA/NAc important for reinforcement (Koob 1992; Koob and Le Moal 2001), the disparity in MMP-9 activity between these brain regions could be indicative of the coordinated actions that underlie progressive escalation of alcohol self-administration during acute withdrawal.

Given that the up-regulation of MMP-9 in the CeA/NAc of the IE group comprised both pro- and active-forms of MMP-9, whereas there was a specific reduction in A92-kD MMP-9, but not the pro-form, in the ACC suggests that this decrease in ACC MMP-9 activity occurs in a manner distinct from the traditional MMP synthetic pathway. The selective reduction in the A92-kD MMP-9 expression in the ACC could be mediated by increased expression and/or activity of tissue inhibitors of metalloproteinases (TIMPs), endogenous inhibitors of matrix metalloproteinases that can terminate MMP activity (Huntley 2012; Smith *et al.* 2015), with TIMP-1 identified as a potent inhibitor of MMP-9 (Okulski *et al.* 2007).

Based on the results of Experiment 1 showing specific increases in MMP-9 expression in the CeA and NAc as well as the existing literature (Knapska *et al.* 2013; Stefaniuk *et al.* 2017) related MMP-9 in the CeA, Experiment 2 directly evaluated the role of CeA MMP-9 in escalated alcohol self-administration during acute withdrawal, using our previously validated model for investigating the negative reinforcement learning in alcohol dependence and withdrawal (Smith *et al.* 2011; Walker 2012) in conjunction with site-specific intra-CeA MMP-9i infusions. In Experiment 2, we identified that inhibition of MMP-9 in the CeA during acute withdrawal suppressed the development of dependence-induced escalation of alcohol self-administration (Fig. 4), confirming that MMP-9 activity in the CeA is necessary for acute withdrawal-induced escalation of alcohol self-administration in alcohol dependence. The possibility that the intra-CeA MMP-9i infusions could spread to nuclei adjacent to the CeA and influence withdrawal-induced escalation should be noted, however we have shown that micro-injections of ligands in the CeA specifically impact escalated alcohol self-administration, while off-target infusions do not (Kissler and Walker 2016). These results are consistent with our previously published data using a broad spectrum MMP-inhibitor that prevented the development of dependence-induced escalation of alcohol self-administration (Smith *et al.* 2011). It is important to recognize that in those animals receiving the MMP-9i in the CeA, MMP-9 inhibition did not alter alcohol self-administration levels from baseline levels that are putatively indicative of the positive reinforcing effects of alcohol (Walker 2012). Indeed, MMP-9 inhibition specifically prevented the *acquisition of escalated alcohol self-administration* but did not impact *stable escalated alcohol self-administration*. Collectively, these data are indicative of the fact that once stable responding for alcohol has been achieved, MMP activity in the CeA is no longer relevant since learning-related plasticity has concluded.

The control conditions in the second experiment confirmed that the escalation-preventing effects of MMP-9 inhibition in the CeA were not related to learning deficits or response inhibition following intra-CeA MMP-9i infusions (Fig. 5). Namely, animals originally treated with the MMP-9i in the CeA that did not display escalated alcohol self-administration, showed normal escalation of alcohol self-administration during acute withdrawal once intra-CeA MMP-9i infusions were replaced with intra-CeA vehicle infusions showing that the MMP-9i infusion-mediated prevention of escalated alcohol self-administration was not permanent. Once dependence-induced escalation of alcohol self-administration stabilized, subsequent infusion of the MMP-9i had no effect on dependence-induced escalation of alcohol self-administration during acute withdrawal, suggesting that MMP-9 activity in the CeA was no longer relevant once synaptic plasticity associated with negative reinforcement learning had occurred. Furthermore, the lack of an acute MMP-9i

effect on stable escalated alcohol self-administration showed that acute intra-CeA MMP-9i had no rate-decreasing effects. The MMP-9i used in the present study is reported to be a reversible ligand that has selectivity for the hypothesis under test. Although the MMP-9i used in the present study has been shown to inhibit MMP subtypes other than MMP-9, namely MMP-1 and MMP-13 (MMP-9, $K_i = 5$ nM; MMP-1, $K_i = 1.05$ mM; MMP-13, $K_i = 113$ nM; Levin *et al.* 2001), neither MMP-1 or MMP-13 have been implicated in learning-related neuronal plasticity (Huntley 2012) and therefore the specificity of the MMP-9i was assured when evaluating the plasticity associated with escalated alcohol self-administration during acute withdrawal in alcohol-dependence.

MMP-9 polymorphisms (i.e., a C/T polymorphism of the MMP-9 gene that can induce higher MMP-9 gene transcriptional activity) have been associated with alcohol dependence in humans (Samochowiec *et al.* 2010) and the serum MMP-9 concentration in alcoholics is higher compared to controls. Furthermore, MMP-9 knockout (KO) mice exhibit impaired synaptic plasticity in CeA following chronic alcohol exposure and display reduced alcohol-seeking that was normalized in the KO mice by exogenous expression of MMP-9 in CeA (Stefaniuk *et al.* 2017). Additionally, in humans, the same C/T MMP-9 gene polymorphisms observed by Samochowiec *et al.* 2010 have been shown to correlate with enhanced motivation to drink in alcoholic patients (Stefaniuk *et al.* 2017).

In addition to a role for MMPs in AUDs, research has also implicated MMPs in other substance use disorders (SUDs). MMP-9 protein levels in medial PFC of rats are upregulated following cocaine-induced reinstatement of cocaine-induced CPP (Brown *et al.* 2008) and MMP-9 activity is reduced in the hippocampus of cocaine abusers (Mash *et al.* 2007). Importantly, MMP-9 activity is transiently up-regulated in NAc following cue-induced cocaine reinstatement and MMP-9 inhibition in NAc suppressed cue-mediated reinstatement of cocaine seeking in rats (Smith *et al.* 2014). Similarly, repeated, but not acute, METH treatment increased MMP-9 activity in the rat frontal cortex and NAc (Mizoguchi *et al.* 2007b) and MMP-9 inhibition in the rat frontal cortex decreased repeated METH-induced behavioral sensitization as well as METH-induced increase in extracellular dopamine levels in the NAc (Mizoguchi *et al.* 2007a).

In summary, the present experiments identified increased MMP-9 expression in an amygdaloaccumbal circuit concomitant with reduced MMP-9 in the anterior cingulate cortex during the initial phase of acute withdrawal-induced escalated alcohol self-administration that is hypothesized to correspond to negative reinforcement-mediated learning and plasticity in alcohol dependence. Importantly, the necessity of MMP-9 activity was functionally assessed through site-specific intra-CeA MMP-9 inhibition with results demonstrating that MMP-9 activity in the CeA is necessary for escalated alcohol self-administration during acute withdrawal. The current results are consistent with investigations into the role of MMPs in multiple forms of learning, as well as AUDs and SUDs. Collectively, the data presented herein, as well as that in the literature, support the emerging conceptual shift away from the importance of the tripartite synapse (i.e., pre- and post-synaptic neurons in addition to glial regulation) towards a tetrapartite synapse that incorporates the extracellular matrix domain as a critical component of synaptic plasticity

(Dityatev and Rusakov 2011; Smith *et al.* 2015) that could be targeted by future therapeutic development efforts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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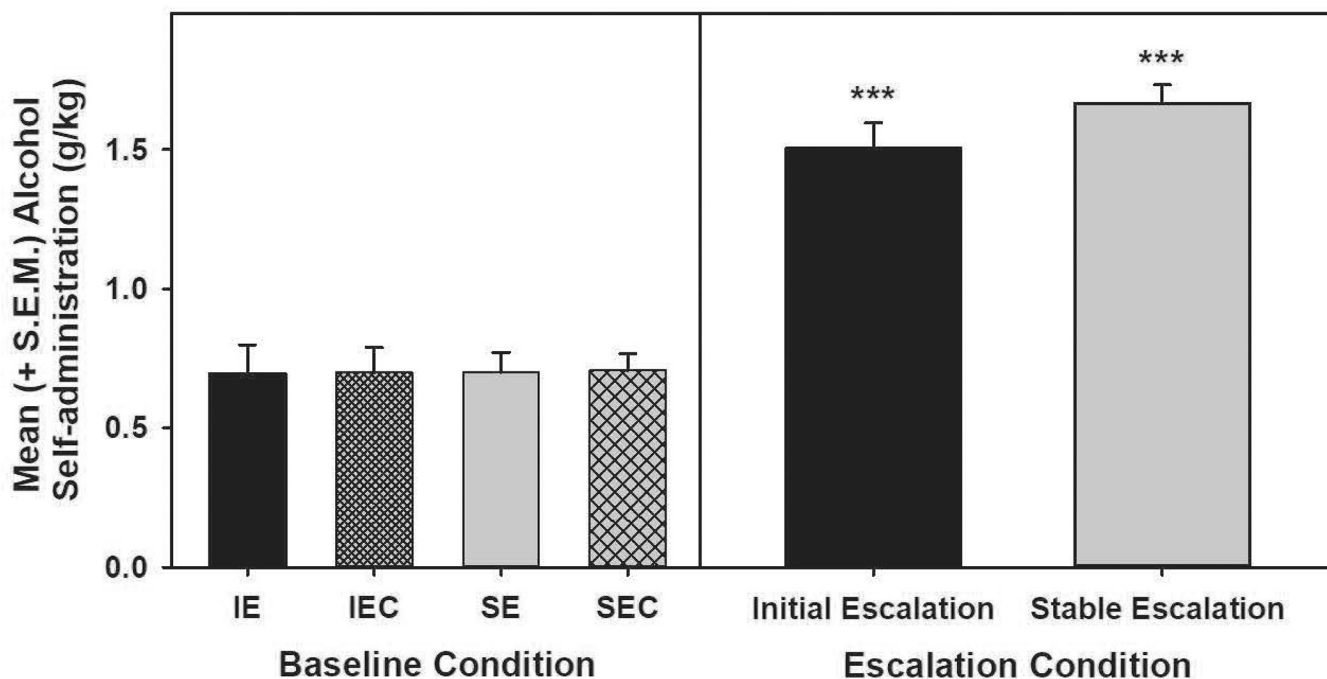


Figure 1. Alcohol consumption during pre-vapor baseline and post-vapor dependence-induced escalation sessions.

Data shown as mean + SEM. Baseline alcohol consumption was similar between groups (n=8/grp). Alcohol consumption for the IE and SE groups was significantly escalated when compared to baseline (***) $p < 0.001$ vs baseline of the same group), but there were no differences in consumption levels between IE and SE when escalated. IE= Initial escalation; IEC= Initial escalation control; SE=stable escalation; SEC=stable escalation control.

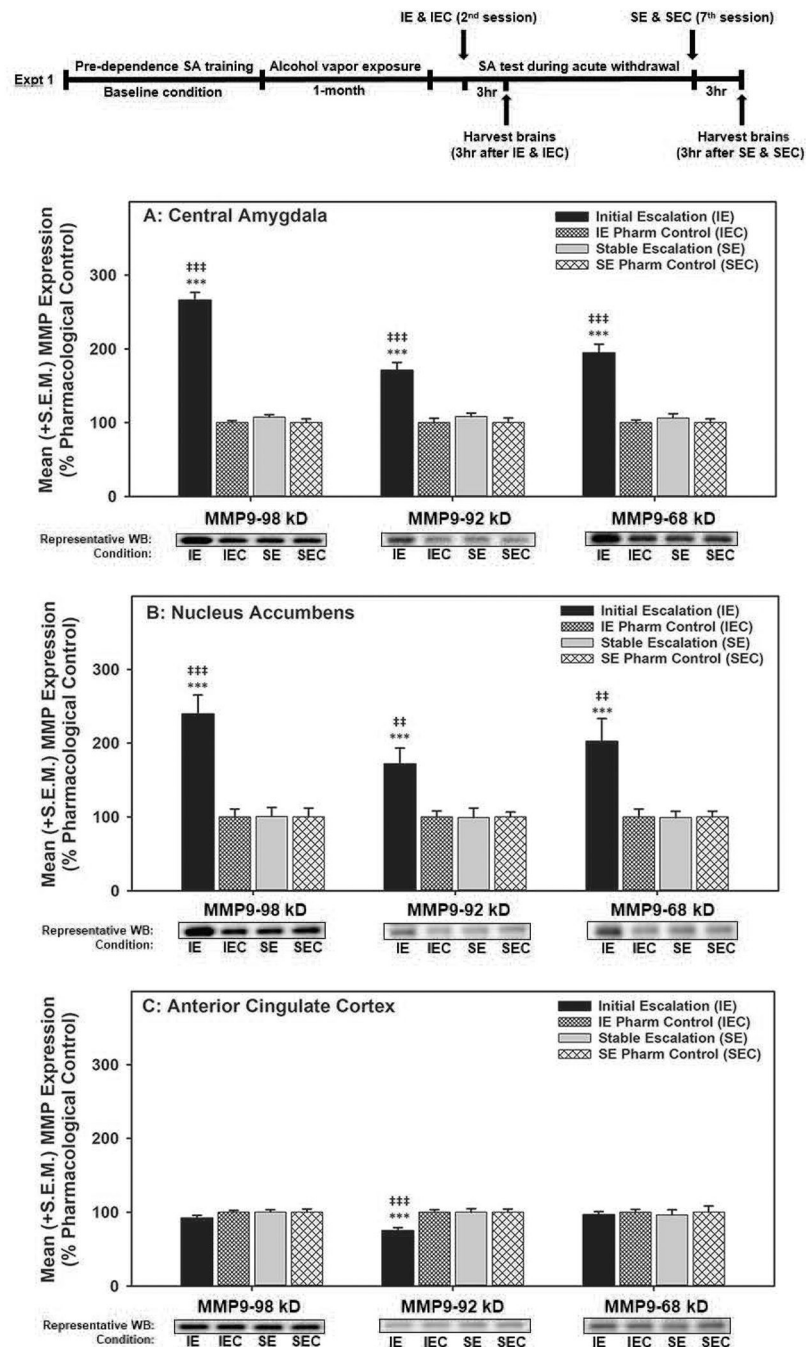


Figure 2. Alcohol dependence-induced MMP-9 immunoreactivity in the central amygdala, nucleus accumbens, and anterior cingulate cortex during acute withdrawal.

Data shown as mean + SEM (n=8/grp). *Top Panel:* Timeline for Experiment 1. *Panel A:* In the CeA, the IE group showed increased expression of pro and active (92- and 68-kD) MMP-9 than the IEC group ($^{\dagger\dagger\dagger} = p < 0.001$ when compared to IEC) and SE group ($^{***} = p < 0.001$). *Panel B:* Similarly, in the NAc, the IE group showed elevated pro and active (92- and 68-kD) MMP-9 expression compared to the IEC group ($^{\dagger\dagger} = p < 0.01$ and $^{\dagger\dagger\dagger} = p < 0.001$) and the SE group ($^{***} = p < 0.001$). *Panel C:* In contrast to the CeA and NAc, the IE group in ACC showed a selective decrease in expression of active 92-kD MMP-9 compared

to IEC and SE ($\dagger\dagger\dagger = p < 0.001$ and $*** = p < 0.001$, respectively). IE = Initial escalation; IEC = Initial escalation pharmacological control; SA = self-administration; SE = stable escalation; SEC = stable escalation pharmacological control; WB = western blot.

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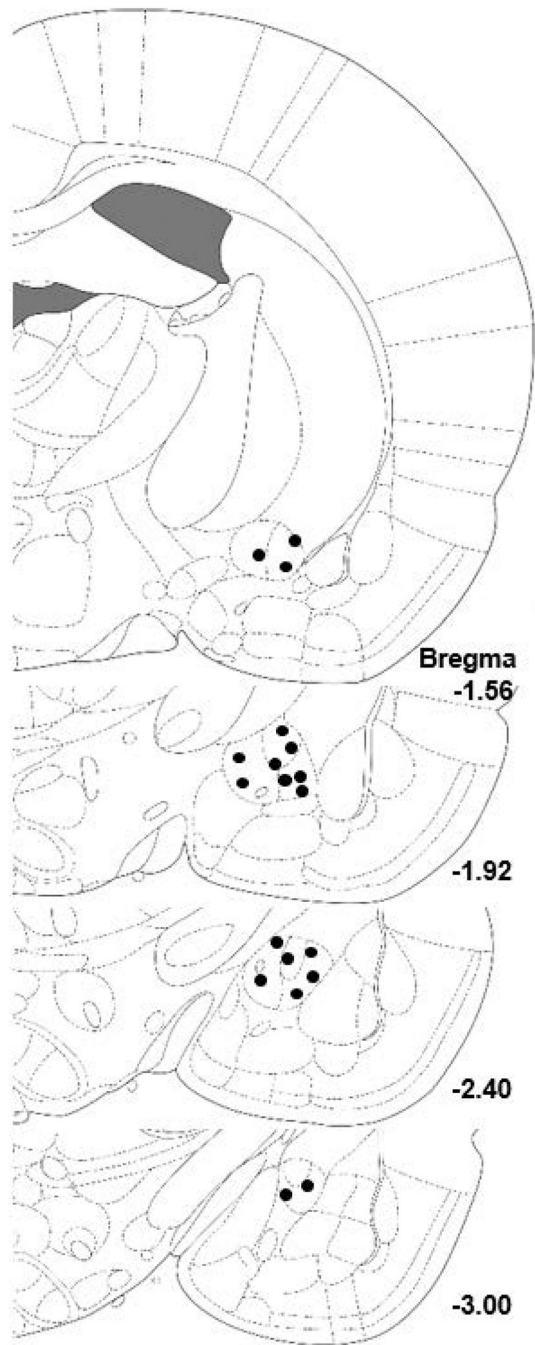


Figure 3: Schematic representation of CeA infusion sites. Black circles indicate cannulae placement within the central amygdala and reflect bilateral infusion sites. Adapted from Paxinos and Watson, 2007.

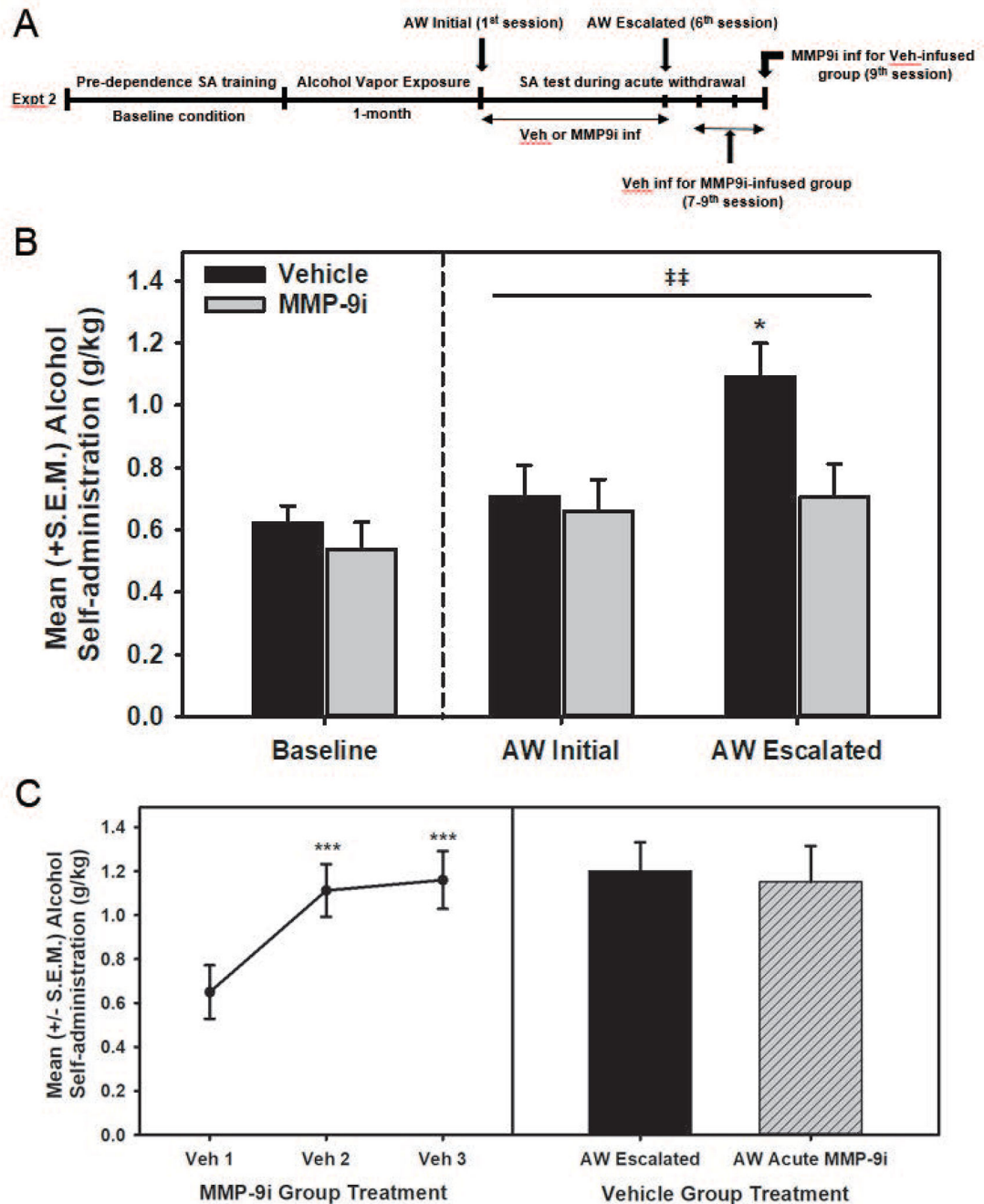


Figure 4.

Panel A: Timeline for Experiment 2. **Panel B:** MMP-9 inhibition in the CeA prevents acute withdrawal-induced escalation of alcohol self-administration in alcohol dependence. Data shown as mean \pm SEM. Baseline alcohol consumption between the treatment groups (vehicle or MMP-9i) was comparable. The MMP-9 inhibitor ($n=10$) or vehicle ($n=9$) were infused into the CeA 15-min prior to and 45-min after acute withdrawal self-administration sessions following the one-month dependence induction phase (denoted by the vertical dotted line). The two treatment conditions displayed differential escalation of

alcohol self-administration behavior (as evidenced by a significant treatment x session interaction, $\ddagger\ddagger = p < 0.01$) with the vehicle-treated group significantly escalated compared to the MMP-9i group ($* = p < 0.05$). ***Panel C: Control conditions for interpreting intra-CeA MMP-9 inhibition of escalated alcohol self-administration during acute withdrawal.*** Data shown as mean \pm SEM. ***Left Panel:*** The MMP-9 inhibitor-treated rats (n=10) that did not show escalated alcohol self-administration received subsequent vehicle infusions in the CeA 15-min prior to and 45-min after acute withdrawal self-administration sessions and displayed escalated alcohol self-administration over three sessions ($*** = p < 0.001$ when compared to Veh 1). ***Right Panel:*** The vehicle-treated rats (n=9) that displayed escalated alcohol self-administration during acute withdrawal were acutely administered the MMP-9 inhibitor in the CeA 15-min prior to a final post-dependence self-administration which did not alter their escalated alcohol self-administration profile. AW = acute withdrawal; inf = infusion; MMP-9i = MMP-9 inhibitor; SA = self-administration; Veh = Vehicle.