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

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Supplementary Methods

Western Blot Procedure: For immunoblotting of MMPs, brains were extracted 3-h (Meighan *et al.* 2006) after either initial or stable escalation (for the IE and SE groups) or following equivalent IP injections of alcohol (for the IEC and SEC groups, respectively). Micropunches of brain tissue from both hemispheres of the ACC, BNST, CeA, HPC, and NAc, were sonicated and lysed with RIPA lysis buffer (Millipore: 20-188) containing a 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, the supernatant was removed and tested for protein concentrations using Pierce BCA protein assay kit (Thermo: 23225). Proteins were mixed with Laemmli sample buffer (Bio-rad: 1610737) containing 5% β-mercaptoalcohol and then heated at 95°C for 5 min. The lysate was separated by SDS-polyacrylamide gel electrophoresis (Bio-rad: 5671025), in duplicate, and transferred to nitrocellulose membranes (Bio-rad: 1704159). To ensure that each sample had equal loading and confirm 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 protein stain procedure (see Figure S1), and optical densities for individual lanes were compared to ensure that there were no differences in protein loading between lanes. Subsequently, the membranes were placed in MemCode Stain Eraser (Thermo: 24580) for two minutes to remove the stain. This technique has been shown to be fully reversible before antibody incubation and does not interfere with chemiluminescence substrates (Antharavally *et al.* 2004) and serves as a loading and transfer control within, and between, membranes given the use of a primary antibody/Memcode ratio. MMP-9 and MMP-2 belong to gelatinase subfamily of MMPs (Sternlicht and Werb 2001) and MMP-2 is associated with MMP-9 activation (Fridman *et al.* 1995; Toth *et al.* 2003). MMP-3 is also known to mediate MMP-9 processes (Ogata *et al.* 1992). 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) and 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). After blocking in 5% non-fat milk/TBST for 1hr at room temperature (RT), the membrane was incubated overnight at 4°C with primary Ab against MMP-2 (Millipore: AB19167;

1:150), MMP-3 (Fitzgerald Industries International: 70R-11887; 1:50), and MMP-9 (Millipore: AB19016; 1:125). 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 (see Figure S2 and S3 for MMP-2, MMP-3 and MMP-9 images) were captured with the Bio-rad ChemiDoc XRS+ imaging system and optical density of bands were measured using NIH 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 for the IE and SE groups relative to the IEC and SEC groups, respectively.

Supplementary References

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Toth M, Chvyrkova I, Bernardo MM, Hernandez-Barrantes S, Fridman R (2003) Pro-MMP-9 activation by the MT1-MMP/MMP-2 axis and MMP-3: role of TIMP-2 and plasma membranes. *Biochem. Biophys. Res. Commun.* 308: 386-395.

Supplementary Figure Captions

Figure S1. Representative Memcode Images for Immunoblotting Data. MemCode protein staining for the anterior cingulate cortex (ACC), central amygdala (AMYG) and nucleus accumbens (NAc) indicating equivalent loading in each lane for each of the four conditions within each brain region (univariate ANOVAs showed no differences in AMYG ($F(3,28) = 0.895$, $p = 0.456$), NAc ($F(3,28) = 0.237$, $p = 0.87$) or ACC ($F(3,28) = 0.822$, $p = 0.493$). MC = MemCode; IE= Initial escalation; IEC= Initial escalation pharmacological control; SE=stable escalation; SEC=stable escalation pharmacological control.

Figure S2. Statistically Significant Western Blot Images for MMP-9 in the Anterior Cingulate Cortex, Central Amygdala and Nucleus Accumbens. Western blot images showing 98-, 92- and 68-kD MMP-9 immunoreactivity. Three gels were loaded in a counterbalanced fashion according to group (N=32, n=8/grp), in duplicate. Group 1 = initial escalation (IE), Group 2 = initial escalation pharmacological control (IEC), Group 3 = stable escalation (SE), Group 4 = stable escalation pharmacological control (SEC). ACC = anterior cingulate cortex; AMYG = central amygdala; NAc = nucleus accumbens.

Figure S3. Statistically Non-significant Western Blot Images for MMP-2, -3 and -9. Western blot images showing MMP-9 immunoreactivity. Three gels were loaded in a counterbalanced fashion according to group (N=32, n=8/grp), in duplicate. Group 1 = initial escalation (IE), Group 2 = initial escalation pharmacological control (IEC), Group 3 = stable escalation (SE), Group 4 = stable escalation pharmacological control (SEC). ACC = anterior cingulate cortex;

AMYG =central amygdala; BNST =bed nucleus of the stria terminalis; Hipp =hippocampus; NAc = nucleus accumbens.

Figure S1.

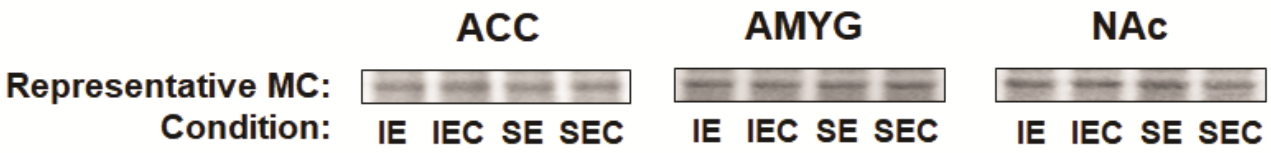

ACC
AMYG
NAc
Representative MC:
Condition:
IE IEC SE SEC
IE IEC SE SEC
IE IEC SE SEC

Figure S2.

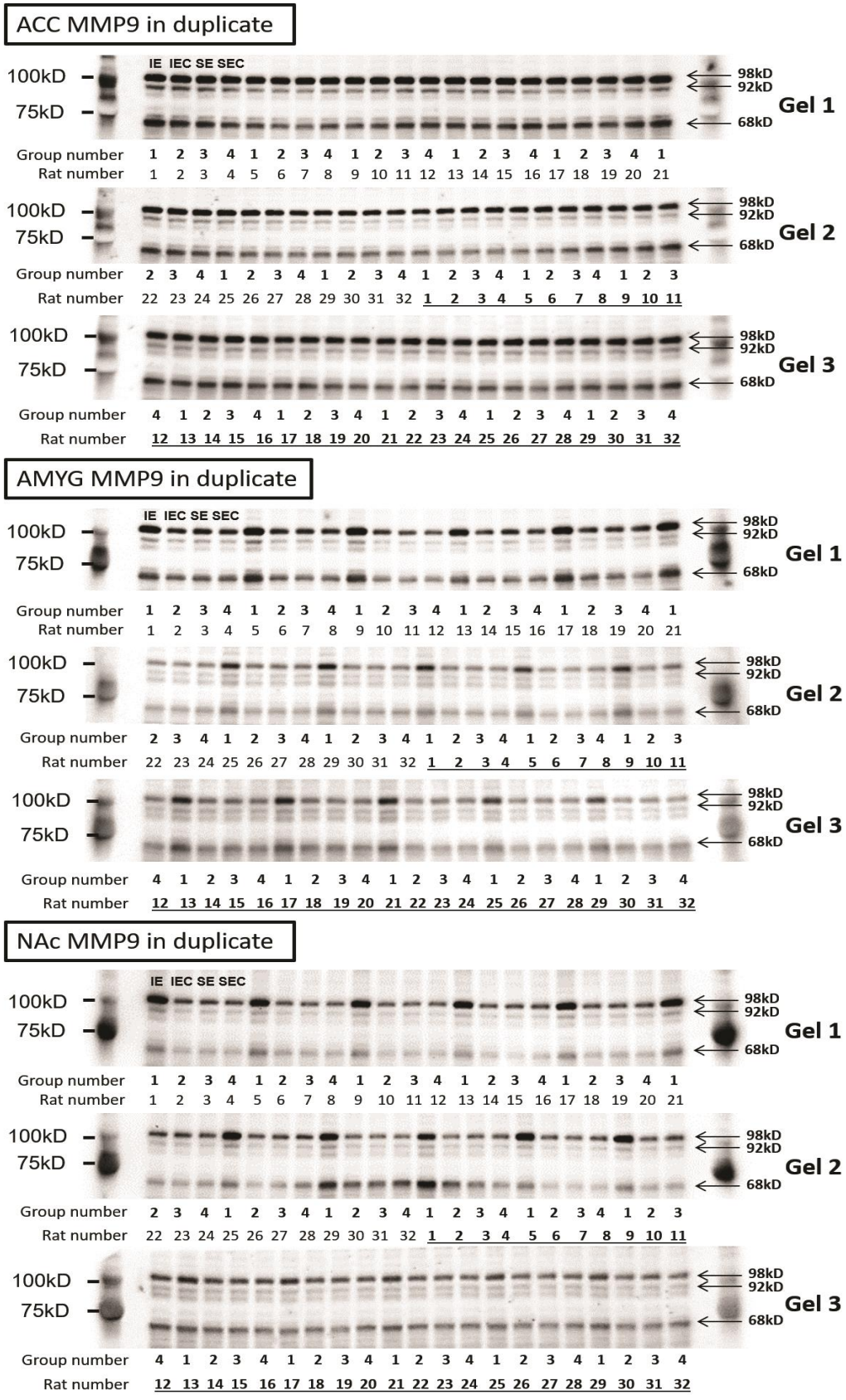
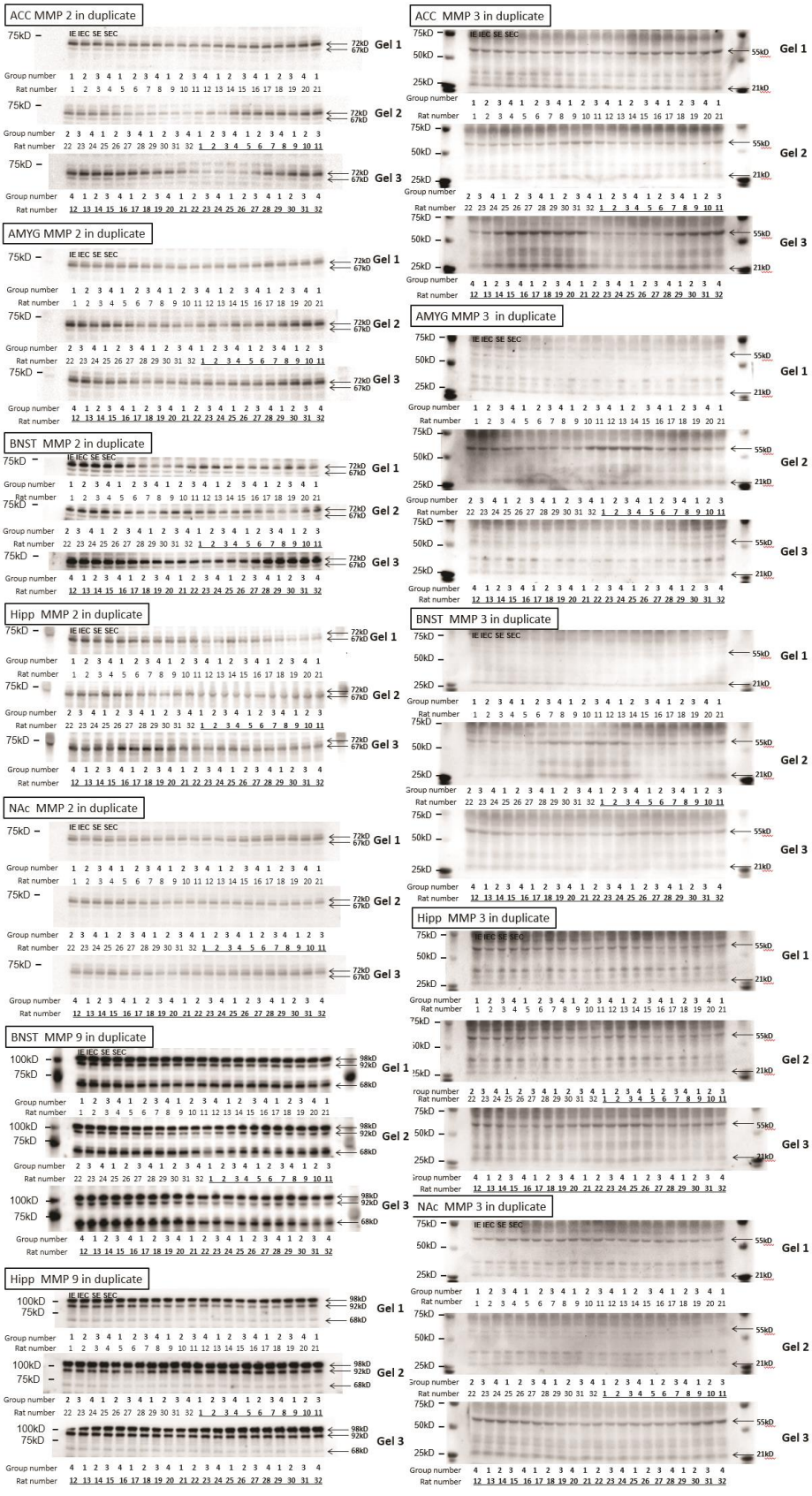

ACC MMP9 in duplicate
IE IEC SE SEC
100kD
75kD
98kD
92kD
68kD
Gel 1
Group number 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1
Rat number 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Gel 2
Group number 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3
Rat number 22 23 24 25 26 27 28 29 30 31 32 1 2 3 4 5 6 7 8 9 10 11
Gel 3
Group number 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
Rat number 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
AMYG MMP9 in duplicate
IE IEC SE SEC
Gel 1
Group number 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1
Rat number 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Gel 2
Group number 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3
Rat number 22 23 24 25 26 27 28 29 30 31 32 1 2 3 4 5 6 7 8 9 10 11
Gel 3
Group number 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
Rat number 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
NAc MMP9 in duplicate
IE IEC SE SEC
Gel 1
Group number 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1
Rat number 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Gel 2
Group number 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3
Rat number 22 23 24 25 26 27 28 29 30 31 32 1 2 3 4 5 6 7 8 9 10 11
Gel 3
Group number 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
Rat number 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32

Figure S3.


ACC MMP 2 in duplicate
AMYG MMP 2 in duplicate
BNST MMP 2 in duplicate
Hipp MMP 2 in duplicate
NAc MMP 2 in duplicate
BNST MMP 9 in duplicate
Hipp MMP 9 in duplicate
ACC MMP 3 in duplicate
AMYG MMP 3 in duplicate
BNST MMP 3 in duplicate
Hipp MMP 3 in duplicate
NAc MMP 3 in duplicate
IE IEC SE SEC
Gel 1
Gel 2
Gel 3
Group number
Rat number
75kD
100kD
72kD
67kD
98kD
92kD
68kD
55kD
50kD
25kD
21kD