Supplemental material

Activity of hippocampal adult-born neurons regulates alcohol withdrawal seizures

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Methods

Cell counting. To examine the effect of alcohol withdrawal (AW) on proliferation of neural stem cells in the hippocampus, 4 doses of BrdU (50mg/kg, 2-hour intervals) were injected into mice at the end of 2 and 4 weeks of AW. Twenty-four hours after the final BrdU injection, brains were isolated and sliced coronally at a thickness 40 μm. The number of $BrdU^+$ cells was quantified in every 6^{th} section of the hippocampus. In addition to the standard procedure described in the Method, additional antigen retrieval procedures, including the treatment with 2 M HCl for 30 min at 37°C and neuralization with 0.1 M boric acid for 10 min, were applied for BrdU antibody staining (Accurate QTB0030, Rat, 1:500). All fluorescence images were collected using laser confocal microscopy (SP5, Leica, Germany) and further processed in Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA).

Coverage of IBA1- and GFAP-positive cells. The effects of AW on microglia and astrocytes were evaluated by immunohistochemistry (IHC) against IBA1 and GFAP, respectively. IHC with IBA1 (monoclonal mouse anti-IBA-1, Chemicon; MABN92; 1:500) and GFAP (polyclonal rabbit anti-GFAP, DakoCytomation; Z0334; 1:500) was performed in three horizontal sections representing the dorsal, intermediate, and ventral hippocampus of pair- and alcohol-fed mice (n=3 mice per group). The coordinates used for the experiments are as follows: Dorsal, Interaural: 5.40 mm, Bregma: -4.60 mm; Intermediate, interaural: 3.90 mm, Bregma: -6.10 mm; and ventral, Interaural: 1.69 mm, Bregma: -8.31 mm. The percentage of area covered by IBA1- or GFAP-positive signals were calculated by using Fiji (ImageJ) software.

Supplementary Figure Legends

Figure S1. Establishment of chronic alcohol exposure mouse model. (**A**) Cartoon depicting the alcohol feeding schedule. (**B**) Amounts of daily consumed alcohol (open circle; 20.19 ± 1.53 *g/kg*, n = 52) and accumulated alcohol over the experimental periods are shown (filled circle, *g/kg*; 0.5 week, 64.77 ± 1.98; 1 week, 186.84 ± 1.37, 2 weeks, 306.29 ± 1.32; 3 weeks, 425.22 ± 1.51; 4 weeks, 545.01 ± 1.53; n =52). (**C**) Alcohol-fed mice show a significantly higher amount of peak BAC when measured 4.5 hours after the onset of the dark cycle (Two-tailed unpaired t test, $P < 0.0001$, alcohol-fed mice, 395.0 ± 8.73 *mg/dL*, n = 25; pair-fed mice, 37.2 ± 4.69 *mg/dL*, n = 19). (**D**) Control and alcohol-fed mice gained comparable

amounts of weight over the course of the feeding schedule (Two-way repeated measures ANOVA; no significant diet effect $F_{1,51} = 20.71$). Data represent Mean ± S.E.M.

Figure S2. Automatic detection and counting of epileptiform SWDs. (**A**) Four electrodes were implanted into the left and right motor cortex (LC and RC), left and right hippocampus (LH and RH), and one electrode was used for reference (Ref). (**B**) A picture showing the continuous Video-EEG monitoring system. (**C**) Only spikes that passed both initiation threshold (THi) and termination threshold (THt) were counted. For the present analysis, we set THi and THt at 100 μV and 0μV, respectively. SWDs with amplitudes greater than 2 times background EEG activity were scored as positive. (**D**) Representative image of SWD. Red dotted lines indicate the range of average intensity of field potentials. This EEG and analysis method were used throughout the experiments.

Figure S3. Inhibitory DREADD-mediated inducible and reversible decrease of epileptiform SWDs. Vehicle (0.5% DMSO in saline) and CNO were administrated on Day 1 (D1) and Day 2 (D2), respectively, and EEG was monitored for 14 hours on D1 and D2. The frequency of epileptiform SWDs during the first (7h) and second (14h) seven-hour periods are shown at the given days. CNO administration decreased the frequency of epileptic SWDs in the first 7 hours compared to vehicle injection, which returned to basal levels during the second 7 hours in NCE:M4$^{f/f}$ mice. However, CNO injection did not affect the induction of epileptiform SWDs in control-fed naïve or NCE:M4$^{f/f}$ mice, nor alcohol-fed naïve mice, indicating that CNO-mediated induction of epileptic SWDs was dependent upon both alcohol withdrawal and the presence of hM4Dq receptors in newborn DGCs. The effects of CNO on the expression of epileptiform SWDs were determined by two-way repeated measures ANOVAs; $^{**}P < 0.01$ and $^{***}P < 0.001$ were determined by Bonferroni *post hoc* tests.

Figure S4. An inducible and reversible induction of epileptic SWDs by excitatory DREADDs. Vehicle (0.5% DMSO in saline) and CNO were administered on Day 1 (D1) and Day 2 (D2), respectively, and EEG was monitored for 14 hours on D1 and D2. The frequency of epileptiform SWDs during the first and second seven-hour periods is presented at given days. CNO administration increased the frequency of epileptic SWDs compared to vehicle injection,

and this returned to basal levels during the second seven hours in NCE:M3$^{f/f}$ mice. However, CNO injection did not affect the induction of epileptiform SWDs in control-fed naïve or NCE:M3$^{f/f}$ mice, nor alcohol-fed naïve mice, indicating that CNO-mediated induction of epileptic SWDs is dependent upon both alcohol withdrawal and the presence of hM3Dq receptors in newborn DGCs. The effect of CNO on the expression of epileptiform SWDs was determined by two-way repeated measures ANOVAs; $^{**}P < 0.01$ and $^{***}P < 0.001$ were determined by Bonferroni *post hoc* tests.

Figure S5. Alcohol withdrawal decreases hippocampal neurogenesis. (**A**) Diagram showing the schedule of BrdU injections at 2 and 4 weeks of abstinence. (**B**) BrdU^{+} cells were increased at 2 weeks of abstinence, but not at 4 weeks (Two-tailed unpaired t test, $P < 0.01$, 2 weeks of abstinence, control: n = 3 mice, alcohol: n = 4 at 2 weeks of abstinence; control: n = 5, alcohol: n = 6 at 4 weeks of abstinence). A representative image illustrates newborn DGCs positive for BrdU (red). **P<0.01.

Figure S6. Alcohol withdrawal does not significantly impact glial cells. (A-B) AW did not change the coverage of IBA- and GFAP-positive cells, indicating that AW did not significantly impact gliogenesis at 2, 4, or 8 weeks of abstinence (n=3 per group). The effects of AW on IBA- and GFAP-positive microglia and astrocytes were determined by two-tailed unpaired t-tests.

Figure S1

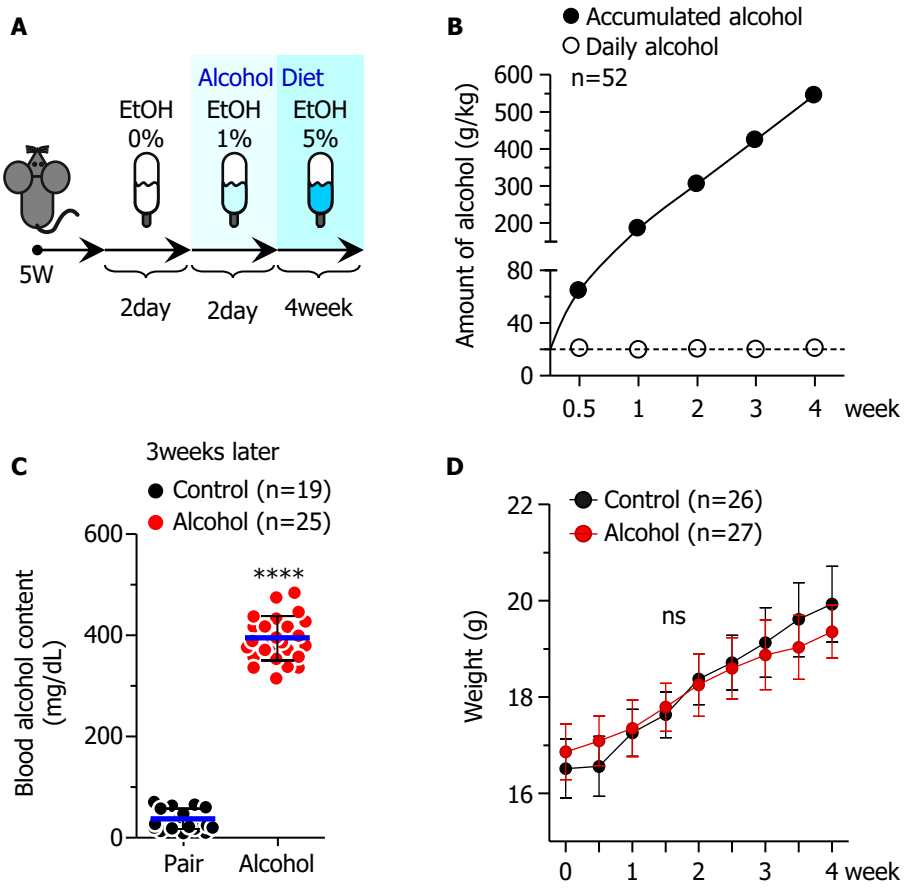
A
Alcohol
Diet
EtOH
0%
EtOH
1%
EtOH
5%
5W
2day
2day
4week
B
Accumulated alcohol
Daily alcohol
n=52
Amount of alcohol (g/kg)
600
500
400
300
200
80
40
0
0.5
1
2
3
4
week
C
3weeks later
Control (n=19)
Alcohol (n=25)
Blood alcohol content (mg/dL)
600
400
200
0
****
Pair
Alcohol
D
Control (n=26)
Alcohol (n=27)
Weight (g)
22
20
18
16
ns
0
1
2
3
4 week

Figure S2

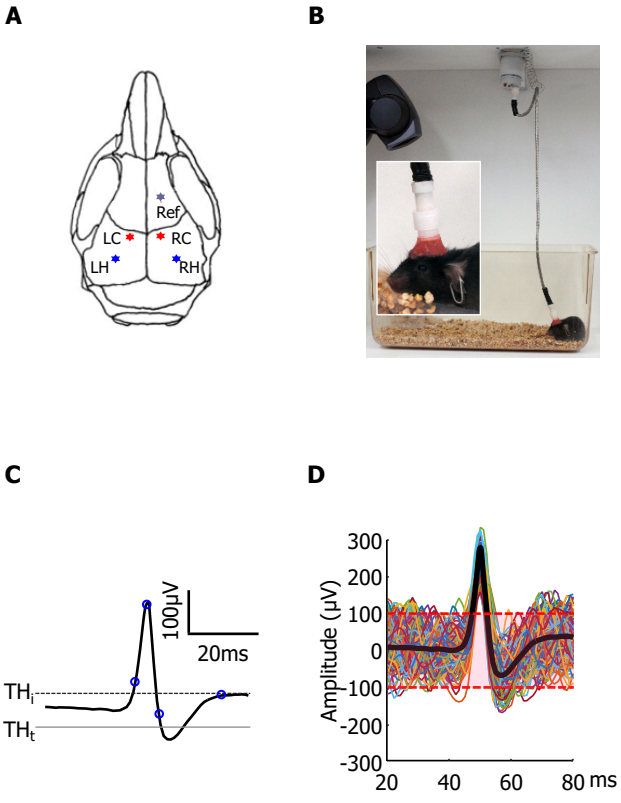
A
B
Ref
LC
RC
LH
RH
C
D
100μV
20ms
THi
THt
300
200
100
0
-100
-200
-300
Amplitude (μV)
20
40
60
80 ms

Figure S3

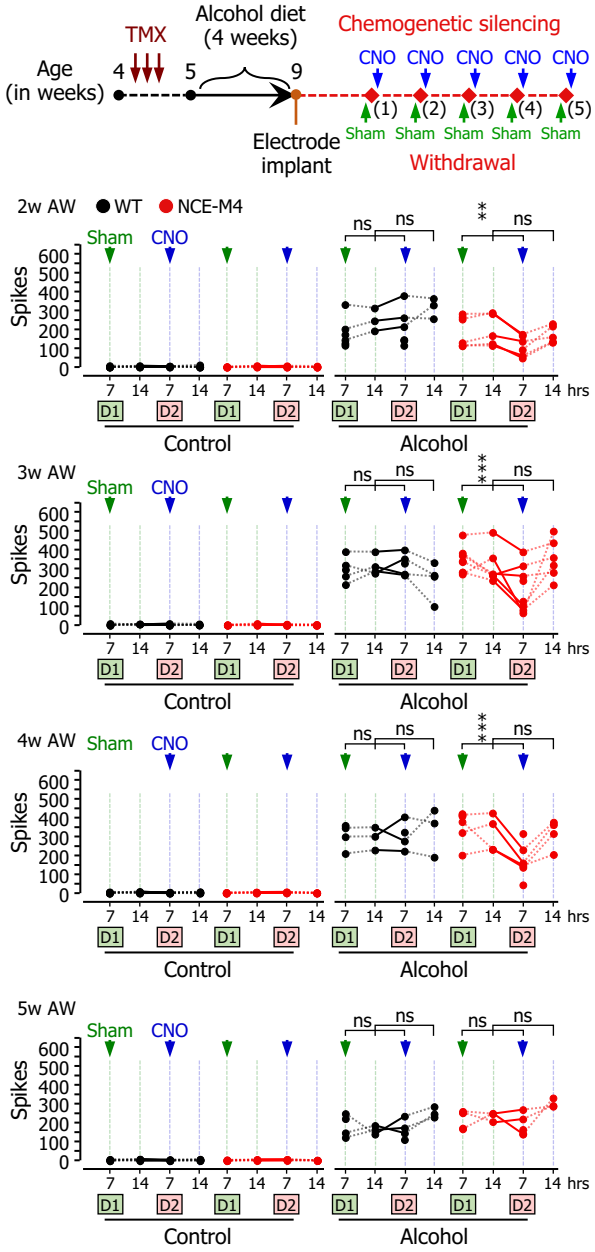
TMX
Alcohol diet
(4 weeks)
Chemogenetic silencing
Age
(in weeks)
4
5
9
CNO CNO CNO CNO CNO
(1) (2) (3) (4) (5)
Sham Sham Sham Sham Sham
Electrode
implant
Withdrawal
2w AW
WT
NCE-M4
3w AW
4w AW
5w AW
Sham
CNO
Spikes
600
500
400
300
200
100
0
7 14 7 14 7 14 7 14 7 14 7 14 7 14 hrs
D1 D2 D1 D2 D1 D2 D1 D2
Control
Alcohol
ns
**
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Figure S4

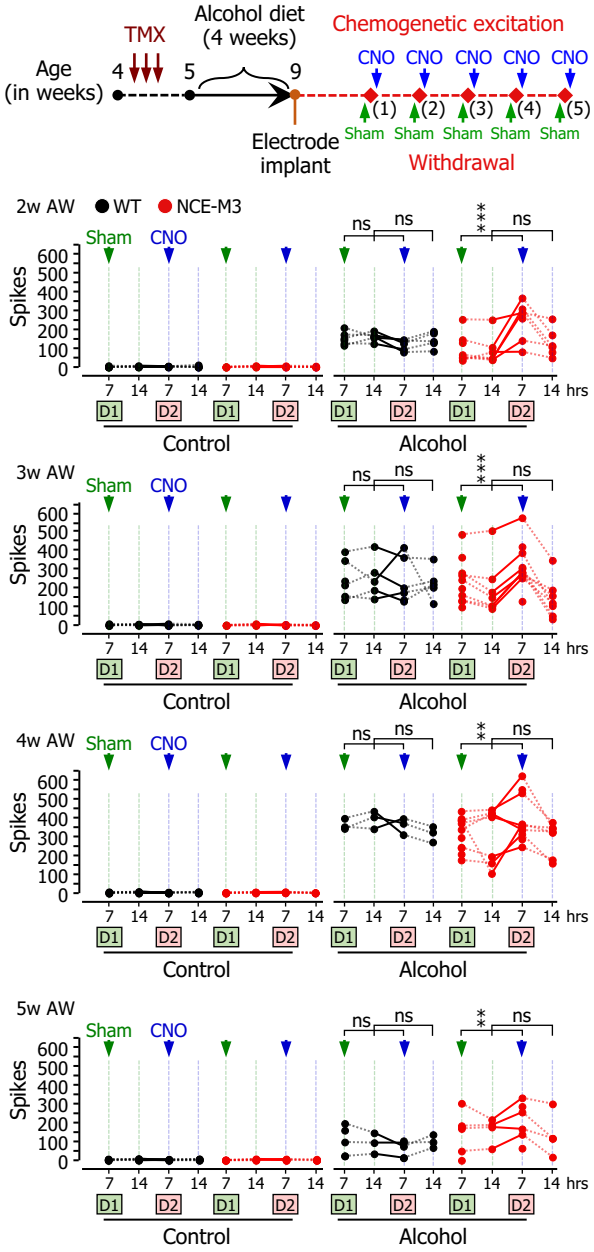
TMX
Alcohol diet
(4 weeks)
Chemogenetic excitation
Age
(in weeks)
4
5
9
CNO CNO CNO CNO CNO
(1) (2) (3) (4) (5)
Sham Sham Sham Sham Sham
Electrode
implant
Withdrawal
2w AW
WT
NCE-M3
3w AW
4w AW
5w AW
Sham
CNO
Spikes
0
100
200
300
400
500
600
7 14 7 14 7 14 7 14 7 14 7 14 7 14 hrs
D1 D2 D1 D2 D1 D2 D1 D2
Control
Alcohol
ns
***
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Figure S5

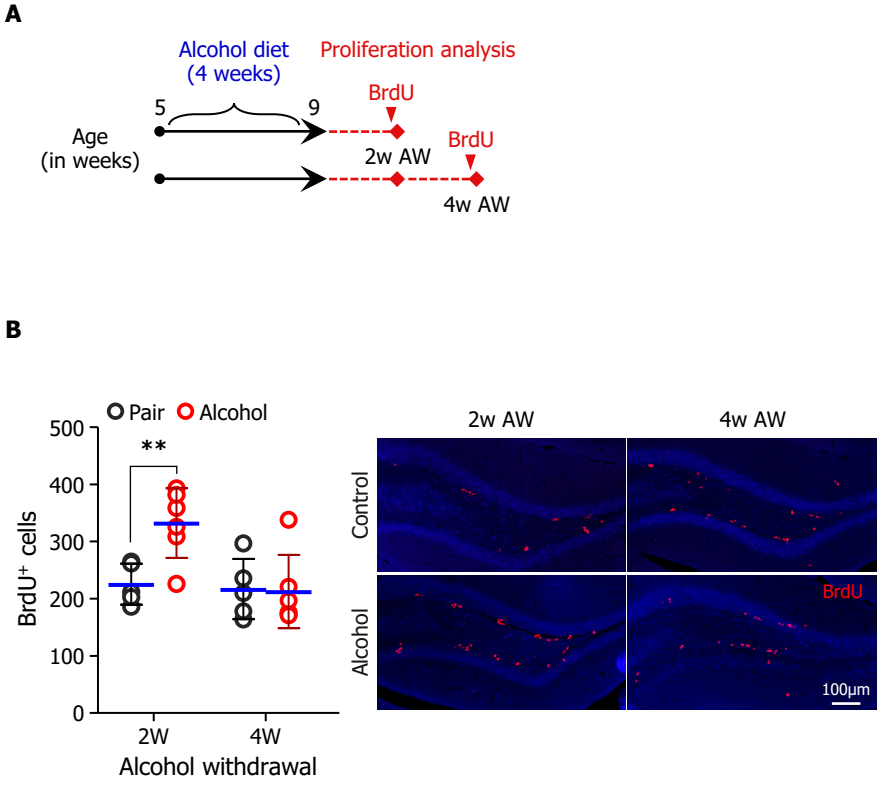
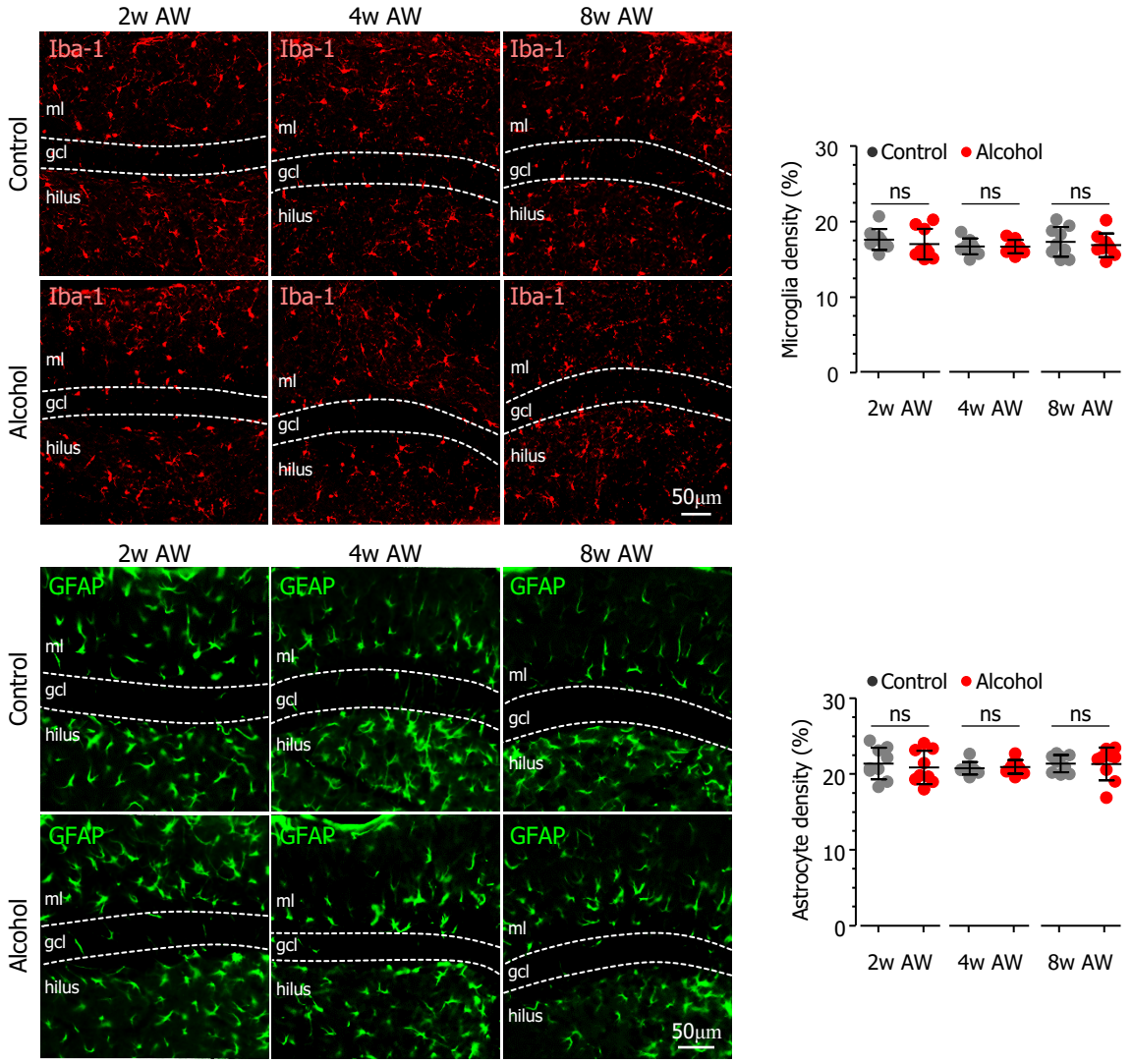

A
Alcohol diet
(4 weeks)
Proliferation analysis
BrdU
5
9
Age
(in weeks)
BrdU
2w AW
4w AW
B
Pair
Alcohol
500
400
300
200
100
0
**
BrdU+ cells
2W
4W
Alcohol withdrawal
2w AW
4w AW
Control
Alcohol
BrdU
100μm

Figure S6


2w AW
4w AW
8w AW
Control
Alcohol
Iba-1
GFAP
ml
gcl
hilus
50μm
Microglia density (%)
Astrocyte density (%)
Control
Alcohol
ns
0
10
20
30