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1 **Bumetanide induces post-traumatic microglia–interneuron** 2 **contact to promote neurogenesis and recovery**

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5 A. Hübner,⁷ Christophe Pellegrino¹ and Claudio Rivera^{1,2}

6 **Abstract**

7 Although the Na-K-Cl cotransporter (NKCC1) inhibitor bumetanide has prominent positive effects
8 on the pathophysiology of many neurological disorders, the mechanism of action is obscure.
9 Attention for elucidating the role of Nkcc1 has been mainly focused on neurons. Recent single cell
10 mRNA sequencing analysis has demonstrated that the major cellular populations expressing
11 *NKCC1* in the cortex are non-neuronal.

12 We used a combination of conditional transgenic animals, *in vivo* electrophysiology, two-photon
13 imaging, cognitive behavioral tests and flow cytometry to investigate the role of Nkcc1 inhibition
14 by bumetanide in a mouse model of controlled cortical impact (CCI).

15 Here, we found that bumetanide rescues parvalbumin-positive interneurons by increasing
16 interneuron-microglia contacts shortly after injury. The longitudinal phenotypic changes of
17 microglia were significantly modified by bumetanide, including an increase in the expression of
18 microglial-derived Bdnf. These effects were accompanied by the prevention of CCI-induced
19 decrease in hippocampal neurogenesis. Treatment with bumetanide during the first week post-CCI
20 resulted in significant recovery of working and episodic memory as well as changes in theta band
21 oscillations one month later.

22 These results disclose a novel mechanism for the neuroprotective action of bumetanide mediated
23 by an acceleration of microglial activation dynamics that leads to an increase of parvalbumin
24 interneuron survival following CCI, possibly resulting from increased microglial Bdnf expression
25 and contact with interneurons. Salvage of interneurons may normalize ambient gamma-
26 aminobutyric acid (GABA), resulting in the preservation of adult neurogenesis processes as well
27 as contributing to bumetanide-mediated improvement of cognitive performance.

- 1
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11

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16

17 **Running title:** Bumetanide acts on inflammation post-TBI

18 **Keywords:** traumatic brain injury; microglia; chloride homeostasis; neuroinflammation;

19 GABAergic transmission

20 **Abbreviations:** BDNF = Brain-derived neurotrophic factor; CCI = Controlled Cortical Injury;

21 DCX = Doublecortin; DG = Dentate Gyrus; GFAP = Glial Fibrillary Acidic protein; LPS =

22 Lipopolysaccharide; NKCC1 = Sodium Potassium Chloride Cotransporter 1; NOR = Novel Object

23 Recognition; ODT = Object Displacement Task; RGL = Radial Glial-Like cells; PV =

24 Parvalbumin; TBI = Traumatic Brain injury

25

1 Introduction

2 Traumatic brain injury (TBI) is one of the most prevalent pathologies worldwide with more than
3 20 million people affected each year¹. However, possibilities for intervention to prevent
4 comorbidities and long-lasting sequelae that frequently follow TBI are limited. Understanding the
5 changes and key players involved in the pathophysiology of the latent phase may help to prevent
6 or reduce some of the comorbidities that accompany TBI.

7 TBI can be frequently associated with complications such as epileptic seizures, depression and
8 memory impairment². This may partly result from the alteration of hippocampal information
9 processing and neurogenesis³⁻⁵. However, the mechanisms regulating neurogenesis following TBI
10 remain elusive because of contradictory reports^{3,6}.

11 Adult neurogenesis is controlled by local GABAergic interneurons and GABA_A receptors. In
12 particular, the release of GABA by parvalbumin (PV)-expressing interneurons controls the
13 quiescent state of radial glia-like cells (RGL) in the dentate gyrus (DG)⁷. Studies in humans and
14 mouse models of TBI have demonstrated a progressive loss of PV-expressing interneurons in both
15 the ipsi- and the contralesional hippocampus that can persist over months⁸⁻¹⁰. We have previously
16 shown that the inhibition of chloride uptake by bumetanide, an inhibitor of Na-K-Cl (NKCC1) co-
17 transport, significantly decreased PV interneuron loss with a positive effect on both depressive-like
18 behavior and cognitive performance⁶. Despite the potential clinical relevance of these results, the
19 underlying mechanisms remain unknown.

20 Previous studies on chloride uptake in the brain mainly focused on neuron-intrinsic mechanisms,
21 although the expression of the principal chloride cellular uptake transporter *Nkcc1* is mainly
22 expressed in non-neuronal cells¹¹.

23 Glial cells are involved early on in the temporal sequence of the neuroinflammatory processes
24 following TBI¹². Indeed, quiescent glial cells are rapidly activated by a process called "reactive
25 gliosis". The recruitment of peripheral neutrophils is also observed, which is followed by
26 infiltration of lymphocytes and monocyte-derived macrophages. Simultaneously, the release of
27 pro-inflammatory and anti-inflammatory cytokines promotes and/or inhibits the post-traumatic
28 neuroinflammatory response¹³. Activated microglia trigger and maintain astrocytic activation
29 through the release of cytokines, which in turn act on surrounding glial cells and neurons¹⁴. In

1 addition, recent reports showed an important pro-survival function of physical contact between
2 activated microglia and interneurons, especially PV-positive interneurons¹⁵.

3 In this study, we identified a novel mechanism involving cellular chloride uptake in microglia,
4 interneuron survival and neurogenesis that is linked to TBI-induced cognitive decline.

5

6 **Material and methods**

7 For full description of experimental procedures please see supplementary material and methods.

8

9 **Experimental Animals**

10 Wild-type mice had a C57bl6-J background. Three transgenic lines were used : Nestin-GFP¹⁶,
11 hGFAP-Cre¹⁷ (line 77.6 mice Stock No. 024098; The Jackson Laboratory) x Nkcc1 flox¹⁸ and
12 B6.129P-Cx3cr1tm1Litt/j¹⁹ and were maintained on a mixed genetic background. All animal
13 experiments complied with the French and Finnish ethical committee approved all experimental
14 procedures (N°: APAFIS#2797 and ESAVI/3183/2022), the ARRIVE guidelines and were carried
15 out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated
16 guidelines and EU Directive 2010/63/EU for animal experiments.

17

18 **Controlled-cortical impact (CCI) model**

19 Buprenorphine (0.03 mg/kg) was injected intraperitoneally (IP) into 10-week-old C57bl6-J males
20 30 minutes before surgery. Mice were then anesthetized using 4% isoflurane mixed with air and
21 enriched with oxygen (0.3 %) and positioned in a stereotaxic frame (David Kopf Instruments®).
22 Body temperature was monitored throughout the procedure using a rectal probe and maintained at
23 37±2°C with a heating pad (Harvard Apparatus®). A unilateral craniotomy was performed at the
24 level of the right posterior parietal cortex. CCI was performed using a Leica impactor with
25 standardized parameters (tip diameter 3mm, 6 m/sec speed, 1.5 mm depth and 200 msec duration).
26 Animals were allowed to recover on the heating pad before being transferred to a post-surgical
27 room. Before the experiment, animals were randomly assigned to one of the following groups:

1 sham-vehicle (Sham Veh), sham-bumetanide (Sham Bum), CCI-vehicle (CCI Veh), and CCI-
2 bumetanide (CCI Bum).

3

4 **Drug delivery**

5 A bumetanide stock solution 20 mM (Sigma-Aldrich®, B3023) was prepared by dissolving 36.4
6 mg of powder into 1 ml of absolute ethanol. IP injections were performed twice daily 12h apart
7 either during the first week post-CCI at 2 mg/kg or during one-week preceding CCI.

8 Minocycline (Sigma-Aldrich®, M9511) was injected twice daily, 12h apart (45 mg/kg) for one
9 week. The vehicle solution consisted of the same preparation but lacked the
10 bumetanide/minocycline powder to respect volume and diluent.

11

12 **Immunohistochemistry**

13 Immunohistochemistry was performed on 4% paraformaldehyde fixed free-floating sections.
14 Incubation with primary antibodies diluted in PBS with 5% NGS and 0.3% Triton X-100 was
15 carried out at 4°C overnight using rabbit anti-doublecortin (ab18723, Abcam, 1:1000), mouse anti-
16 parvalbumin (p3088, Sigma, 1:500), rabbit anti-Iba1 (W1W019-19741, Wako, 1:500), mouse anti-
17 GFAP (MAB360, Merck Millipore, 1:500), mouse anti-BDNF antibody (MAB#918), mouse anti-
18 RFP antibody (MA5-15257, ThermoFisher Scientific, 1:500), chicken anti-GFP antibody
19 (AB_2307313, Avès Labs, 1:300) as well as corresponding Alexa Fluor-conjugated secondary
20 antibodies (Thermo Fisher Scientific, 1:500).

21

22 **PV microglia contact quantification**

23 Confocal stack with double immunofluorescent labeling (Parvalbumin and Iba1) were acquired
24 using a LSM-800 Zeiss confocal microscope Plan Flour 40x/1,30 NA oil immersion objective.
25 Optical sections were collected from the ipsi- and contralesional hippocampi of mice treated and
26 non-treated with bumetanide, at 3 and 7 days post-CCI. Images were then reconstructed in 3D with

1 Imaris Software. Then contacts between microglia Parvalbumin interneurons were quantified with
2 imageJ® plugin SynapCountJ.

3

4 **Flow cytometry analysis**

5 Fresh cortical tissues were dissected and gently chopped and minced in 1 mL ice-cold flow
6 cytometry staining buffer (PBS + 1% fetal calf serum) through a 100 µm filter on ice. The
7 homogenates were first blocked in PBS with 10% rat serum with gentle rotation in a cold room and
8 then stained for 1 h protected from light at 4°C with CD206-FITC (cat no. 141704, Biolegend®) +
9 MHCII-PE (cat no. 107608, Biolegend®) + CD11b-PerCP/Cy5.5 (cat no.101228, Biolegend®) +
10 CD45-APC (cat no.103112, Biolegend®). After staining, cells were fixated with 8% PFA for 30
11 mins, centrifuged at 2000 rpm. Samples were acquired with a 2-laser, 6-color cytometer Gallios
12 (Beckman Coulter®). Gating strategy was determined based on the specific staining of markers for
13 microglial populations²⁰.

14

15 **Live Imaging**

16 Imaging was performed on 5-month-old male mice (B6.129P-Cx3cr1tm1Litt/j, n=5) carrying a
17 cranial window surrounded by a metallic head plate. One month after surgery, trained awake
18 animals were head-fixed on a Mobile HomeCage® platform (MHC V5, Neurotar®) under a
19 custom-built two-photon microscope (Femtonics®) equipped with Mai Tai® Ti:Sapphire Ultrafast
20 Laser (Spectra-Physics) and imaged longitudinally using a Nikon Apo LWD 25X objective with
21 1.10 numerical aperture for 5 days. An image stack with a 5 µm step size covering 300 µm was
22 acquired. After a 30 min baseline recording, injury was then achieved by focal excitation of 1 cell
23 for 20s at 75 µs/pixel at 100% laser intensity. After the injury, animals were treated
24 (Vehicle/Bumetanide) twice per day and imaged, and z-stacks were collected with 5 min intervals
25 for 1 h.

26 To compute the microglial cell dynamics, we designed a semi-automated procedure using a using
27 Python. Sub-volumes were cropped around each cell before undergoing spatial registrations using
28 pystackreg (G. Lichtner, pystackreg, (2022), <https://github.com/glichtner/pystackreg>). Cell

1 dynamics were evaluated from the segmented image by calculating the percentage of binary pixels
2 changing in value from one time point to another ²¹.

3

4 **Behavioral tests**

5 One month post-CCI, mice were tested on different tasks using the object recognition paradigm to
6 test the individual components of episodic-like memory, namely the novel object recognition
7 (NOR) and the object displacement task (ODT)²⁵. For NOR, mice were placed in the center of a
8 box (Noldus apparatus®, 38.5 cm x 38.5 cm) and allowed to freely explore the space for 10 mins,
9 then two identical objects were added. After a 3 min retention time, one of the objects was replaced
10 and the time of exploration was measured for a 3 min period. The same experiment was also
11 performed with 1 h retention time. A similar protocol was used for ODT with the difference that
12 after the 3 min retention time, one of the objects was moved and the time of exploration of each
13 object was recorded.

14

15 Mice were also tested in the Barnes maze (BM) test. One-month post-CCI, mice were trained twice
16 daily for 4 days. On trial probe day (day 5), the target box was replaced with a false escape. The
17 distance travelled, the number of errors, the time spent in the target zone and the latency to reach
18 the target hole were measured.

19

20 Finally, mice were tested for anxiety using the elevated plus maze (EPM) test (Ugo Basile
21 company®). For each test, mice were placed in the center square, facing an open arm, and allowed
22 to move freely for 5 min. Entries and time spent in one of the two open or closed arms and center
23 were monitored. Recording and analyses were done using the Ethovision software (Noldus®).

24

25 ***In vivo* electrophysiological recordings**

26 Scalp electroencephalographic (EEG) recordings were performed in freely moving mice 3 weeks
27 post-CCI. Telemetric recording electrodes were implanted at the level of the posterior parietal
28 cortex (2 mm posterior to and 1.5 mm left of the bregma). A reference electrode was placed rostral

1 in the cerebellum. After a 72-hour recovery, EEG (amplified 31,000, filtered at 0.1–120Hz pass,
2 acquired at 1000Hz) was monitored using a telemetric system (Emka Technologies S.A.S) for 3
3 days, 24 hours per day.

4 Multisite intrahippocampal recordings were performed by implanting a one shank 16 channels
5 linear probe (A1x16-3mm-100-177, Neuronexus) through a 1mm diameter craniotomy at the
6 posterior parietal level (2 mm posterior, 1.5 mm left of the bregma and 1mm ventral) on head
7 restrained animals trained on Mobile HomeCage® (MHC V5, Neurotar®). Animals were recorded
8 3 times 10 minutes. Recordings were acquired using the Allego software (SmartBox Pro™,
9 Neuronexus) and a sampling rate of 30K Hz per channel.

10 For EEG recordings Animal temperature as well as movement were used to determine active and
11 non-active time windows. Delta (1-4 Hz), Theta (4-12 Hz) and Gamma (30-80 Hz) bands²⁶ were
12 normalized using the ratio of power in the first to the last minute within each recording session.
13 The mean normalized power of active time windows was subtracted from non-active for each
14 recording.

15 From intrahippocampal recordings individual units were isolated from multisite LFP recordings
16 using Spyking-Circus²⁷. The Single-unit activity were further classified into wide-spiking (WS)
17 and narrow-spiking (NS) units²⁸ based on the spike waveform, autocorrelogram, and cross-
18 correlogram (CCG). NS and WS presumably correspond to putative inhibitory and excitatory units,
19 respectively^{29,30}.

20

21 **Statistical Analysis**

22 All mean values are given with the standard error mean (SEM). Normality was tested using a
23 D'Agostino-Pearson test. Two-tailed Student's t tests were used for testing statistical significance
24 between two groups while Mann-Whitney tests were used when the normality test failed. Ordinary
25 one-way ANOVA tests were used to compare three or more groups. In the event that the normality
26 assumption was violated here, the Kruskal-Wallis test was used. When variances were unequal, the
27 Brown-Forsythe ANOVA was used.

28

1 Statistics were done using Prism Version 8.3.0 (GraphPad software, Inc., La Jolla, CA, USA) and
2 are represented as follows: * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$; all results and statistical
3 significance are reported in Appendix table S1.

4

5 **Data availability**

6 The data that support the findings of this study are available from the corresponding author, upon
7 reasonable request.

8

9 **Results**

10 **Bumetanide ameliorates CCI-induced memory impairment and** 11 **pathological neuronal activity**

12 To investigate the effects of bumetanide on post-TBI cognitive behavior, we performed four
13 cognitive performance tests: the Novel Object Recognition (NOR) and the Object Displacement
14 Task (ODT). CCI induced a significant decrease in the recognition ratio of the tasks examined after
15 normalization to Sham conditions unless otherwise stated (ODT: CCI Veh 0.38 ± 0.06 ; NOR 5
16 mins: CCI Veh 0.44 ± 0.20 ; NOR 1 h Sham 1.17 ± 0.58 vs CCI Veh 0.507 ± 0.31 , Fig 1B, C and D).
17 Interestingly, the strongest effect of bumetanide was found in NOR with 1 h retention (CCI Bum
18 0.86 ± 0.40 , Fig 1D). We then used a spatial memory test, the Barnes Maze (BM), which showed
19 that CCI induced a significant increase of the latency in reaching the target hole (Sham 8.00 ± 0.58
20 vs CCI Veh 44.00 ± 7.61 , Fig 1E). Similarly, bumetanide treatment resulted in a better performance
21 compared to vehicle (CCI Bum 13.00 ± 1.95 , Fig 1E). Finally, as NOR and ODT are strongly
22 influenced by anxiety, we used the Elevated Plus Maze (EPM) to test this parameter and did not
23 find significant differences in the number of entries that animals made into each zone (Center:
24 Sham 40.00 ± 0.58 vs CCI Veh 44.00 ± 4.02 vs CCI Bum 41.40 ± 3.11 ; Open arms: Sham 35.33
25 ± 2.186 vs CCI Veh 35.00 ± 6.18 vs CCI Bum 46.00 ± 1.86 ; Closed arms: Sham 65.33 ± 10.11 vs
26 CCI Veh 79.75 ± 10.82 vs CCI Bum 58.00 ± 12.03) (Fig 1F). There were also no differences in the
27 time that animals spent in the center (Sham 51.18 ± 1.657 vs CCI Veh 35.00 ± 5.343 vs CCI Bum
28 43.048 ± 5.306), nor in the open arms (Sham 82.44 ± 18.79 vs CCI Veh 100.70 ± 23.10 vs CCI Bum

1 71.98 \pm 12.39), nor in the closed arms (Sham 145.72 \pm 9.97 vs CCI Veh 151.96 \pm 18.27 vs CCI Bum
2 176.264 \pm 8.27) (Fig 1G). These results show that CCI has a negative impact on learning
3 performance and spatial memory, that it is not linked with anxiety.

4
5 Memory impairment following TBI are characterized by changes in theta rhythms^{31,32,33}. Using
6 telemetric EEG, we found that at 1-month post-CCI, the power of theta band oscillations in vehicle
7 treated animals was significantly lower compared to sham (Sham 6.50 % \pm 0.18 vs CCI Veh 3.42
8 % \pm 0.41, Fig 1I). Bumetanide treatment normalized the theta band power and was significantly
9 different from vehicle treated animals (CCI Bum 6.14 % \pm 0.29, Fig 1I). The power of the delta
10 band was lower in CCI animals and was not affected by bumetanide treatment (Sham 5.44 % \pm 0.18
11 vs CCI Veh 1,14 % \pm 0,17 vs CCI Bum 1.82 % \pm 0.53, Fig 1H). Similarly, the power of the gamma
12 band was significantly higher in vehicle-treated animals compared to sham but not changed in
13 bumetanide animals (Sham 0.39 % \pm 0,03 vs CCI Veh 0.89 % \pm 0,13 vs CCI Bum 0,70% \pm 0.08, Fig
14 1J). To assess the impact on single-unit activity we isolated putative interneuron and principal
15 neuron single-units from intrahippocampal multisite recordings. Here we found that the spike rate
16 of putative interneurons was significantly decreased in vehicle compared to Sham animals (Sham
17 23,38 \pm 2,42 Hz vs CCI Veh 3,26 \pm 0,6824 Hz) but not in bumetanide animals (Bum 15,08 \pm 2,274
18 Hz; Fig 1K). Interestingly, putative principal cell unit spike rate was not affected in all groups (Fig
19 1L). The number of total recorded putative interneurons was much lower in Vehicle (5) animals
20 than Sham (10) and Bumetanide (12) treated whereas principal cell number was constant in all
21 conditions (Sham: 47 cells; Veh:48 cells; Bum:48 cells; Fig 1M). These results suggest that
22 bumetanide ameliorates CCI-induced changes in interneuron function.

23 24 **Bumetanide rescues CCI-induced changes in secondary neurogenesis** 25 **and PV interneuron loss**

26 Interneurons are important contributors to brain oscillations³⁴. PV-positive interneurons are a
27 particularly vulnerable subpopulation following CCI⁷. In addition, the impact of CCI on
28 interneuron activity and reduction in numbers suggest changes in the interneuron population of the
29 hippocampus following CCI. We quantified PV-positive interneurons in the granular layer of the

1 ipsi- and contralesional DG during the first post-traumatic week at 3 and 7 days post-CCI (dpCCI).
2 Both sides showed a significant reduction in the number of PV interneurons at 3 dpCCI (normalized
3 value on sham: sham contra 1.00 ± 0.20 vs contra Veh 0.52 ± 0.18 and sham ipsi 1.00 ± 0.22 vs ipsi
4 Veh 0.22 ± 0.18 , Figs 2A and B). This loss was significantly reduced by bumetanide treatment in
5 the contra- (0.77 ± 0.23) and ipsilesional side (0.51 ± 0.14 , Fig 2A and B). We also found a
6 significant reduction in the PV-containing interneurons survival at 7 dpCCI in both sides
7 (normalized value on sham: sham contra 1.0 ± 0.27 vs contra Veh 0.59 ± 0.18 and sham ipsi 1.00
8 ± 0.27 vs ipsi Veh 0.14 ± 0.12 , Fig 2C and D). This loss was significantly reduced at the contra-
9 (1.02 ± 0.34) and ipsilesional sides (0.71 ± 0.24 , Fig 2C and D) by bumetanide treatment.
10 Bumetanide-induced interneuron survival was not restricted to the DG, as we found a similar effect
11 on other hippocampal regions (in ipsilesional CA3: Sham 1.00 ± 0.12 vs Ipsi Veh 0.52 ± 0.09 ; and
12 in ipsilesional CA1: Sham 1.00 ± 0.25 vs Ipsi Veh 0.37 ± 0.07).

13 Ambient GABA, provided by the ongoing activity of DG interneurons and particularly PV
14 interneurons, plays a role in the proliferation and migration of granular cell progenitors^{6,35}. A loss
15 of PV interneurons may significantly contribute to the TBI-induced changes in stem cell
16 proliferation by reducing GABA release. At 7 dpCCI, we observed a significant reduction in the
17 number of DCX-positive neurons within both the ipsi- and contralesional DG (normalized value
18 on sham: sham ipsi 1.00 ± 0.31 vs. CCI Veh ipsi 0.19 ± 0.09 and sham contra 1.00 ± 0.31 vs. CCI
19 Veh contra 0.78 ± 0.24 , Figs 2E, F, H and I). This coincided with an increase in the number of
20 Nestin-positive cells within the DG (sham ipsi 1.00 ± 0.17 vs. CCI Veh ipsi 2.08 ± 0.29 and sham
21 contra 1.00 ± 0.18 vs. CCI Veh contra 1.37 ± 0.34 Figs 2E, G, H and J). Bumetanide treatment
22 reduced the number of Nestin-positive cells (CCI Bum ipsi 1.4 ± 0.24 and CCI Bum contra 1.0
23 ± 0.15 , Figs 3E, G, H and J) and triggered an increase in the number of DCX-positive cells (CCI
24 Bum ipsi 0.47 ± 0.16 and CCI Bum contra 0.98 ± 0.23 , Figs 2E, F, H and I).

26 **Bumetanide rescues CCI-induced changes in microglia** 27 **morphology/phenotype and increases contacts with PV interneurons**

28 Neuroinflammation is present in the primary (acute) and secondary (chronic) stages of TBI¹² where
29 microglia and astrocyte activation are involved in the mechanisms leading to both adverse and

1 beneficial effects. Under physiological conditions *Nkcc1* is predominantly expressed in astrocytes
2 and microglia compared to neurons³⁶. Therefore, we wondered whether these cell populations
3 contribute to the effect of bumetanide. This was monitored in GFAP-*Nkcc1*-KO mice. Although
4 we observed an abnormal morphology of astrocytes in this transgenic line, we did not find a
5 significant increase in interneuron survival following CCI, suggesting that astrocytic chloride
6 homeostasis is not relevant in CCI-induced apoptosis. (See full description in Supplementary
7 Results and Fig. S1).

8 TBI is known to induce a strong inflammatory reaction³⁷. To see if TBI-induced effects on PV
9 survival and proliferation are sensitive to microglial activation, we used the anti-inflammatory drug
10 minocycline. Interestingly, treatment with this agent resulted in similar effects on both PV survival
11 (Contra Veh 0.59 ± 0.18 vs Contra Mino 1.21 ± 0.34 and ipsilesional side Ipsi Veh 0.14 ± 0.12 vs
12 Ipsi Mino 0.74 ± 0.39 , Fig 2A, B, C and D) and newborn neurons (CCI Mino ipsi 0.63 ± 0.17 and
13 CCI Mino contra 1.12 ± 0.10 , Fig 2E, F, G and H). These results suggest that microglia activation
14 affect post-CCI interneuron survival and adult born neuron production.

15 To investigate whether the effect of bumetanide depends on *Nkcc1* in microglia, we used a
16 transgenic approach for Tamoxifen (Tam) inducible knock-down of *Nkcc1* in CX3CR1-positive
17 microglia. Here, we observed that bumetanide treatment induced a significant increase in the
18 average cell size in the control condition (Tam + Bum $105732 \mu\text{m}^2 \pm 18324$ vs ShNKCC1 + RFP
19 $27650 \mu\text{m}^2 \pm 4322$ vs Tam + RFP $17482 \mu\text{m}^2 \pm 2164$). Interestingly, knockdown of *Nkcc1* also
20 induced an increase in size (Tam + ShNKCC1 + RFP $127090 \mu\text{m}^2 \pm 29570$ vs ShNkcc1 + RFP
21 $27650 \mu\text{m}^2 \pm 4322$ vs Tam + RFP $17482 \mu\text{m}^2 \pm 2164$) (Fig 3A and C). Similar results were found in
22 BV2 cells (Fig S2). Then, we investigated the phagocytosis capacity of the cells in the different
23 conditions. Bumetanide treatment induced a significant increase in the number of engulfed beads
24 (Tam + Bum 28.00 ± 6.66 vs ShNkcc1 + RFP 9.34 ± 3.45 vs Tam + RFP 6.08 ± 3.64). Knockdown
25 of *Nkcc1* had similar effect (Tam + ShNkcc1 + RFP 25.46 ± 3.58 vs ShNkcc1 + RFP 9.34 ± 3.45 vs
26 Tam + RFP 6.08 ± 3.64) (Fig 3B and C). These results suggest that bumetanide can regulate
27 microglia morphology and activation by modulating *Nkcc1* activity.

28 Furthermore, bumetanide treatment *in vivo* induced a significant increase of microglia endpoint
29 numbers (Sham 715.9 ± 81.03 vs CCI Veh 698.8 ± 331.2 vs CCI Bum 1076.0 ± 85.13), number of
30 attachment points (Sham 41.86 ± 12.40 vs CCI Veh 101.9 ± 43.70 vs CCI Bum 140.8 ± 54.96) and

1 soma area (Sham $1458 \mu\text{m}^2 \pm 652.7$ vs CCI Veh $3494 \mu\text{m}^2 \pm 757.6$ vs CCI Bum $4985 \mu\text{m}^2 \pm 850.6$)
2 (Fig 4A and B) in the ipsilesional DG 3 dpCCI.

3 Microglia process motility and their interactions with interneurons play an important role in PV
4 survival³⁸. We found significant differences in the number of contacts between microglial cells and
5 PV interneurons between vehicle and bumetanide-treated animals at 3 dpCCI in both the contra-
6 (CCI Veh 40.00 ± 24.92 vs CCI Bum 103.50 ± 23.55) and ipsilesional side (CCI Veh 2.75 ± 2.48 vs
7 CCI Bum 63.20 ± 9.04) (Fig 4C and D). In addition, there was no significant difference between
8 Sham and CCI Bum in the contralesional side (Sham contra 115.9 ± 13.50 vs CCI Bum contra 93
9 ± 12.48) but a slight difference in the ipsilesional side (Sham ipsi 99.57 ± 13.01 vs CCI Bum ipsi
10 63.20 ± 9.04) (Fig 4C and D).

11 Using flow cytometry, we found that microglia were more likely to express the pro-inflammatory
12 markers CD45, MHCII and CD172a (Veh MHCII⁺ $13.55 \% \pm 4.91$ vs Bum MHCII⁺ $21.59 \% \pm 6.03$;
13 Fig 4E and F) after 3 days of bumetanide treatment. To confirm this, we measured interleukins in
14 brain tissue and found a significant increase in IL-6 in the hippocampus of CCI animals treated
15 with bumetanide (CCI Bum $21.94 \text{ pg/mL} \pm 6.03$ vs Sham $11.49 \text{ pg/mL} \pm 0.91$ and CCI Veh 12.85
16 $\text{pg/mL} \pm 3.89$, Fig 4G). Since TBI is characterized by inflammatory cell infiltration promoted by
17 opening of the blood brain barrier (BBB), we also looked at blood cells. The CD45^{high}-CD11^{low}
18 cells (e.g., possibly lymphocytes) were few in our samples and included not only neutrophils but
19 also other innate immune cells (e.g., monocytes, macrophages and DCs) as reported in the second
20 gating plot of Figure 4E. However, systemic inflammation is not influenced by IP bumetanide
21 treatment, while intracortical inflammation is reduced by the same treatment³⁹. Thus, it is plausible
22 that the effects of bumetanide may result from resident microglial cells and not infiltrating cells.

23 At 7 dpCCI, we found less prominent morphological changes in microglia cells with no effect of
24 bumetanide in both the ipsi- and contralateral side: a decrease in the number of endpoints (Sham
25 900.7 ± 167.9 vs CCI Veh 696.5 ± 86.72 vs CCI Bum 659 ± 100.4) and of process length (Sham
26 $256.7 \mu\text{m} \pm 46.75$ vs CCI Veh $197.8 \mu\text{m} \pm 17.17$ vs CCI Bum $192.7 \mu\text{m} \pm 42.21$) (Fig 5A and B).
27 Despite this, the number of contacts between microglial cells and PV interneurons still differed for
28 the ipsilesional side (CCI Veh 25.20 ± 12.30 vs CCI Bum 39.56 ± 5.85), without difference between
29 Sham and CCI Bum (Sham ipsi 57 ± 6.382 vs CCI Bum ipsi 41.23 ± 2.472) (Fig 5C and D).
30 Surprisingly, bumetanide promoted the expression of the pro-phagocytosis marker CD206 by

1 microglia (Veh CD206⁺ 16.79 % \pm 2.957 vs Bum CD206⁺ 27.44 % \pm 8.635, Fig 5E and F). In line
2 with these results, we also found a significant increase of interleukin 10 (IL-10) expression in CCI
3 animals treated with bumetanide compared to control and vehicle-treated animals (CCI Bum 5.32
4 pg/mL \pm 0.86 vs Sham 1.69 pg/mL \pm 0.78 and CCI Veh 2.57 pg/mL \pm 0.65, Fig 5G).

5 These results suggest that bumetanide protects PV interneurons by increasing microglia contacts
6 during the first post-traumatic week as well as by regulation of microglia phenotypes. This allows
7 us to postulate that bumetanide treatment protects PV interneurons through anti-inflammatory and
8 pro-survival mechanisms mediated by microglia.

9

10 **Bumetanide increases microglia Bdnf expression**

11 Microglia are a central source of Bdnf, an important neurotrophin controlling neuronal survival
12 and plasticity⁴⁰⁻⁴². Therefore, we measured the level of BDNF expression in microglial-like BV2
13 cells after 24 and 72h of treatment. 24 hours of bumetanide treatment led to a significant increase
14 of Bdnf levels (Ctrl 0.18 pg/mL \pm 0.02 vs Bum 2.79 pg/mL \pm 0.18, Fig S2H; Fig S2H). This effect
15 was attenuated but still present after 72h of treatment (Ctrl 0.19 pg/mL \pm 0.03 vs Bum 0.31 \pm 0.14,
16 Fig S2I). In addition, we quantified the immuno-like intensity of Bdnf in sham vehicle, sham
17 bumetanide, CCI vehicle and CCI bumetanide treated animals and found a consistent increase in
18 Bdnf expression within Iba1-positive microglia in bumetanide-treated animals either on the
19 contralesional (normalized value on sham vehicle : Sham Veh 1.00 \pm 0.12 vs Sham Bum 3.55 \pm 0.30
20 vs CCI Veh 2.27 \pm 0.41 vs CCI Bum 5.60 \pm 0.75) or the ipsilesional side (normalized value on sham
21 vehicle : Sham Veh 1.00 \pm 0.06 vs Sham Bum 4.28 \pm 0.20 vs CCI Veh 3.28 \pm 0.18 vs CCI Bum
22 12.86 \pm 1.06; Fig 5G, H and I). These results indicate that bumetanide induces Bdnf expression,
23 which may play an important role in the survival of PV interneurons.

24

25 **Bumetanide regulates post-CCI microglia process motility**

26 To further investigate microglia *in vivo*, we performed longitudinal 2-photon imaging in
27 Cx3CR1^{+/GFP} mice following laser-induced injury on the posterior parietal cortex (see
28 supplementary information for a full description of the procedure). Quantification of microglial
29 process length and motility during the first week post-injury revealed that bumetanide induced a

1 significant decrease in process length one day post-laser injury. This difference was not significant
2 5 dpCCI (Veh Day 1 33.58 ± 3.66 vs Bum Day 1 24.43 ± 1.37 ; Veh Day 5 ± 3.69 vs Bum Day 5
3 42.51 ± 2.23 ; Fig 6B, D and C). Interestingly, bumetanide induced a significant decrease in the
4 motility ratio at both 1- and 5-dpCCI (Veh Day 1 0.59 ± 0.1 vs Bum Day 1 0.52 ± 0.01 ; Veh Day 5
5 0.70 ± 0.03 vs Bum Day 5 0.61 ± 0.12 , Fig 6B, D and E). These experiments are in line with the
6 effect of bumetanide on process length in CCI animals, and a decreased motility ratio is consistent
7 with the increased number of contacts between microglia and PV interneurons.

8

9 **The effect of bumetanide is physiological state dependent**

10 Brain injury is paradoxically aggravated in microglia NKCC1-deficient mice³⁹. As under these
11 conditions chloride uptake is impaired prior to the trauma we asked if bumetanide treatment prior
12 to CCI would have a different effect than when administered after CCI. Interestingly,
13 administration of bumetanide during one week before CCI worsened CCI outcomes and, more
14 particularly, PV survival. Indeed, bumetanide-treated animals showed a significant decrease of PV
15 interneurons compared to Sham and vehicle treated animals on the ipsilesional side (normalized
16 value on sham: Sham 1 ± 0.05 vs CCI Veh 0.54 ± 0.09 vs CCI Bum 0.10 ± 0.02) and compared to
17 Sham on the contralesional side (normalized value on sham: Sham 1 ± 0.05 vs CCI Veh 0.90 ± 0.12
18 vs CCI Bum 0.57 ± 0.12) (Fig 7A and B). These results indicate that the impact of bumetanide on
19 PV-interneuron survival is dependent of the physiological state and the mechanism disclosed in
20 this study is mainly relevant under pathological conditions.

21

22 **Discussion**

23 How cognitive deficits observed in experimental models and clinical TBI develop following an
24 initial concussion is not well understood⁵. In this study, we were interested how chloride
25 homeostasis might be involved in post-traumatic changes in working memory⁴³. Theta rhythm is a
26 potential electrophysiological biomarker of altered neuronal activity^{44,45} and is believed to
27 synchronize activity both within local networks and between distal cortical regions involved in
28 cognitive processing^{46,47}. In both humans and rats, the power of theta rhythm increases during the
29 acquisition phase of spatial learning and object recognition. Treatment- or injury-induced inhibition

1 of theta oscillations correlates with cognitive dysfunction^{32,48,49}. In agreement, we found a positive
2 correlation between decreased theta-band power and poor performance in the spatial cognitive test
3 after one-month post-CCI. Interestingly, we found that an early transient treatment with the
4 chloride cellular uptake inhibitor bumetanide was effective to counteract the long-term changes in
5 both behavior and theta oscillations.

6 Several studies link cognitive processes with normal adult neurogenesis in the hippocampus. This
7 corresponds with the generation of functional neurons from adult neural stem cells. TBI can induce
8 significant changes in the proliferation and maturation of granular cells^{50,51}. In the present study
9 we found a significant increase in the number of Nestin-positive cells in both ipsi- and
10 contralesional DG of mice 7 dpCCI. Nestin is a radial glia (RGL) cell marker, and represents a
11 pool of quiescent cells that divide only occasionally¹⁶. The division of these RGLs leads to the
12 generation of intermediate progenitor cells (IPCs) that will undergo a limited number of rapid
13 divisions before entering the neuronal differentiation pathway⁵². IPCs are divided into two
14 subtypes: i) type 2a cells that are positive for Nestin and negative for double-cortin (DCX), an
15 immature neuronal marker, and ii) type 2b cells, positive for Nestin and DCX⁵³. IPCs then give
16 rise to neuroblasts (type 3 cells), which express DCX⁵⁴. The increased number of Nestin-positive
17 cells after TBI might intuitively suggest that there will be an increase in the number of newborn
18 neurons. This is, however, not the case since we found a significant decrease of DCX positive cells
19 in both the contra- and ipsilesional side of the DG. These results indicate that TBI leads to an
20 increase in the quiescent cell pool and a resulting decrease in the number of immature granule cells
21 consistent with previous findings⁶. Furthermore, treatment with bumetanide during the first week
22 post-TBI significantly reduced the CCI-induced decrease in Nestin-positive cells, as well as
23 increased the number of DCX-positive cells. These results suggest that the effects of bumetanide
24 on cognitive performance could be related to positive effects on post-traumatic granule cell
25 production. In addition, they may suggest a common upstream mechanism for the action of
26 bumetanide.

27 GABA, especially released by PV interneurons^{7,55}, appears to play a central role in regulating the
28 activity of RGLs by inhibiting their proliferation. Thus, post-traumatic changes in granule cells
29 could be related to abnormal interneuron function. Indeed, we found loss of PV interneurons in the
30 granular layer of the hippocampus, as early as 3 dpCCI in both the ipsi- and contralesional side that

1 was sustained at 7 dpCCI and one month after trauma⁶. This loss of PV interneurons may also
2 contribute to abnormal brain activity in TBI⁵⁶.

3 The increase of Nestin-positive cells and decrease in DCX cells that we detected after TBI can thus
4 be related to the loss of PV interneurons, which results in less ambient GABA^{57,58}. In accordance
5 with this assumption, treatment with bumetanide significantly counteracted the loss of PV
6 interneurons and normalized the CCI induced change in Nestin and DCX positive cells. As these
7 cell populations were not significantly affected in sham animals by bumetanide a direct effect on
8 progenitors may have less prominent importance.

9 Neuroinflammation is observed in both the acute and chronic stages of TBI¹². It appears to be
10 responsible for both detrimental and beneficial effects by contributing to primary injury and
11 secondary damage, but also facilitating tissue repair⁵⁹. Both astrocytes and microglia are important
12 players in the mechanisms of brain inflammation and, in addition, display one of the highest
13 expression levels of Nkcc1 in the brain. Thus, it is plausible that the post-traumatic effects produced
14 of bumetanide could be related to an influence on the dynamics of inflammatory processes^{59,60}.

15 Following this idea, we first studied the role of Nkcc1-mediated chloride uptake in astrocytes using
16 conditional transgenic animals. Despite bumetanide having substantial effects on the morphology
17 of GFAP-positive cells in both the contra- and ipsilateral sides in sham animals, the ablation of
18 Nkcc1 in these cells did not rescue the loss of PV-positive interneurons. This indicates that
19 astrocytes are not significantly involved in the bumetanide-induced increase in interneuron
20 survival. Nevertheless, in this study we only monitored the first week post-CCI and we cannot
21 exclude effects at more delayed stages of injury.

22 To define if CCI induced neuroinflammation through microglia could influence PV interneuron
23 survival we first treated animals with minocycline, a second-generation tetracycline derivative that
24 has an anti-inflammatory and neuroprotective action. Minocycline is known to have anti-
25 inflammatory effect via the inhibition of microglial cells activation⁶¹ and induce microglial Bdnf
26 expression⁶². Minocycline increased the survival of interneurons and the number of newborn
27 neurons, suggesting that microglia inflammation is involved.

28 Interestingly, bumetanide treatment induced phenotypic changes in microglia and lead to a faster
29 activation of microglial cells into a pro-inflammatory phenotype at early stages, which is required
30 for cell debris removal and phagocytosis of apoptotic cells. At 7 dpCCI, microglial cells of

1 bumetanide-treated animals acquired pro-phagocytotic characteristics. Microglia in control
2 animals acquired pro-inflammatory characteristics slightly later, but importantly it persisted more
3 than 7 dpCCI, resulting in a deleterious inflammatory state. Intriguingly, bumetanide increased the
4 concentration of interleukin 6 (IL-6) 3 dpCCI in line with a recent study using the same trauma
5 model showing that hippocampal granule cells derived release of IL-6 is triggered by renewed
6 microglial cells. This increase in neuronal survival, allows adult neurogenesis to proceed normally
7 and prevents behavioral deficits⁶³. One-week post-CCI, we detected higher levels of IL-10 in
8 bumetanide-treated animals, along with a phenotype switch. Expression of IL-10 can promote
9 neuronal and glial cell survival and dampen inflammatory responses via a number of signaling
10 pathways⁶⁴. To further investigate if chloride uptake is important for microglia function, we
11 knocked down the expression of Nkcc1 in primary microglia. We observed identical morphological
12 changes and the same higher phagocytosis activity. This is interesting as microglia mediated
13 phagocytosis is important for recovery as well as clearance of degenerated cells⁶⁵. Inhibition of
14 phagocytosis can lead to increase neuronal damage and decrease neuronal cell viability⁶⁶.

15 In addition, we found that bumetanide treatment leads to more contact sites between microglia and
16 PV interneurons *in vivo*. This is consistent with previous results showing pro-survival signaling
17 mediated by microglia contacts on interneurons¹⁵. These results imply that the mechanism by which
18 bumetanide rescues PV interneurons can be related to additional effects on microglia morphology.
19 In accordance, bumetanide treatment leads to a significant increase of microglia processes and
20 ramifications 3 dpCCI. Moreover, it also significantly changes microglia process motility *in vivo*.

21 The neurotrophic factor Bdnf is crucial for post-traumatic neuronal survival and plays a central role
22 in microglia sensitive learning paradigms^{67,68} as well as microglia modulation of post-traumatic
23 network function⁴². Interestingly, we found here a significant increase of Bdnf in microglia induced
24 by bumetanide following CCI. There is a complex interplay between neurotrophic signaling and
25 chloride homeostasis in neurons e.g Bdnf can normalize post-traumatic GABA_A mediated
26 responses from depolarizing and hyper excitable to hyperpolarizing but have the opposite effect
27 under physiological conditions. This could contribute to neuronal survival under pathological
28 conditions^{69,70}. Thus, changes in GABAergic transmission in combination with increased Bdnf
29 release from microglia and increased contact between microglia and interneurons could contribute
30 to bumetanide induced increased survival of PV interneurons.

1 The effects observed here suggest that bumetanide directly acts on microglia. Indeed, recent work
2 by Tóth *et al*³⁹ using microglia Nkcc1 deficient mice are in agreement with our results. In addition,
3 they showed that systemic application of bumetanide had positive post-traumatic effects. In
4 contrast, deletion of Nkcc1 in microglia resulted in more severe tissue damage after ischemic
5 stroke. Although these effects are compelling and indicate complicated actions of bumetanide, they
6 do not provide a mechanistic view on the post-traumatic benefits of bumetanide. Moreover, Tóth
7 *et al* disrupted chloride uptake before induction of ischemia. We obtained similar results, when we
8 administered bumetanide already before induction of TBI. Thus, these results show a complex and
9 context dependent effect of bumetanide and indicate that the mechanism disclosed in this study
10 may apply to post-traumatic conditions.

11 Some limitations of this study could be addressed in future research. For example, we only used
12 males⁷¹⁻⁷³ and, as previously described^{74,75}, there are likely sex differences in clinical outcomes
13 after TBI. On the other hand, no significant differences between males and females in lesion
14 volume, neurodegeneration, blood-brain barrier (BBB) alteration and microglia activation have
15 been found in animal models^{76,77}. A second potential limitation is that we did not fully study the
16 infiltrating immune cells due to the limited sample size. However, it would be interesting to address
17 this, either as in Tóth *et al*³⁹ by using specific markers e.g. CD3, CD20 and Ly-6G, or by using
18 transgenic mice like CX3CR1creERT2 x iDTR as in Willis *et al*⁶³.

19 In conclusion, this study shows that a treatment targeting inhibition of the chloride co-transporter
20 Nkcc1 can regulate the activation kinetics of microglia and provides a mechanistic link for the
21 positive effect of bumetanide on post-traumatic cognitive decline. These results also open an
22 additional avenue to better understand chloride uptake dependent pathophysiological mechanisms
23 in the post-traumatic brain as well as to identify the elements involved in the development of long-
24 term sequelae following TBI.

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29

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6

7 **Competing interests**

8 Authors report no conflict of interest.

9

10 **Supplementary material**

11 Supplementary material is available at *Brain* online.

12

ACCEPTED MANUSCRIPT

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1 **Figure Legends**

2 **Figure 1 Effect of bumetanide on CCI-induced behavioral changes.** (A) Schematic
 3 representation of the experimental timeline. (B) The Object Displacement Task after a 5 min
 4 retention time. (C) The Novel Object Recognition task after a 1-hour retention time. (D) The Novel
 5 Object Recognition task after a 5-min retention time. For all experiments, the results are presented
 6 as a ratio of time of new versus familiar. n = 10, 11 and 10 respectively. (E) Time spent finding the
 7 target box in the Barnes Maze. (F) Number of entries in different zones of the elevated plus maze.
 8 (G) Time spent in different zones of the elevated plus maze. n = 3, 5 and 5 respectively. (H)
 9 Normalized spectral density of delta band in mice 1-month post-CCI. (I) Normalized spectral
 10 density of theta band. (J) Normalized spectral density of gamma band. n=6, 9 and 9 respectively.
 11 (K) Spike rate of narrow-spiking (NS) units. (L) Spiking rate of wide-spiking (WS) units. (M)
 12 Total number of isolated cells. Data from B were analyzed using the Kruskal-Wallis test. Data from
 13 C to G and L to M were analyzed using One-way ANOVA with Dunnett's post hoc test. Data from
 14 H to J were analyzed using One-way ANOVA with a Tukey post hoc test *p < 0.05; **p < 0.01;
 15 ***p < 0.001.

16
 17 **Figure 2 Effect of bumetanide and minocycline on CCI-induced changes in adult**
 18 **neurogenesis and PV interneuronal loss in the DG.** (A) Parvalbumin (PV) labeling (white
 19 arrows) at 3 dpCCI in the contralesional (upper pictures) and ipsilesional (lower pictures) DG of
 20 sham, CCI vehicle and CCI bumetanide-treated animals (scale bar = 100 μ m). (B) Quantification
 21 of PV-positive cells 3 dpCCI in the contralesional and ipsilesional DG of sham, CCI vehicle and
 22 CCI bumetanide-treated animals. (C) Same as (A) at 7 dpCCI with minocycline-treated animals
 23 added. (D) Same as (B) at 7 dpCCI. (E) Doublecortin (DCX, upper pictures) and Nestin (lower
 24 pictures) labeling at 7 dpCCI in the ipsilesional DG of sham, CCI vehicle, bumetanide-treated and
 25 minocycline-treated animals (scale bar = 100 μ m). (F) Quantification of DCX-positive cells 7
 26 dpCCI in the ipsilesional DG of sham, CCI vehicle, bumetanide-treated and minocycline-treated
 27 animals. (G) Quantification of Nestin-positive cells 7 dpCCI in the ipsilesional DG of sham, CCI
 28 vehicle, bumetanide-treated and minocycline-treated animals. (H) Same as in (E) in the
 29 contralesional DG. (I) Same as in (F) in the contralesional DG. (J) Same as in (G) in the
 30 contralesional DG. n = 7 animals per condition, 3 slices per animal. Sets of data from B, D, F, G

1 and I were analyzed using a one-way ANOVA test with Dunnett's post hoc test. Data from J were
2 analyzed using a Kruskal-Wallis test * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3
4 **Figure 3 Effect of impaired chloride uptake in microglia morphology and phagocytosis**
5 **capacity.** (A) Average size of primary microglia cells from CX3CR1-Cre ERT2 mice under
6 different conditions: co-transfected with pSico-sh Nkcc1 and pCAG-RFP, treated with bumetanide
7 and tamoxifen, transfected with pCAG-RFP and treated with tamoxifen, co-transfected with pSico-
8 sh Nkcc1 and pCAG-RFP and treated with tamoxifen. $n = 4$ wells per conditions. 5 cells chosen
9 randomly per well. (B) Number of latex beads engulfed by cells in the different conditions. $n = 4$
10 wells per conditions. 12 cells chosen randomly per wells. (C) Immunostainings of the primary
11 microglia cell culture from CX3CR1-Cre ERT2 mice in the different conditions. Anti-Iba1 was
12 used to label microglial cells (purple), anti-RFP to label the pCAG-RFP plasmid (red), GFP to
13 enhance the pSico-sh Nkcc1 fluorescence (green). YFP was expressed when cells were treated with
14 tamoxifen (green) and the beads were labelled with a Rabbit IgG-DyLight 633 Complex (blue).
15 All data set were analyzed using a one-way ANOVA test with Dunnett's post hoc test. * $p < 0.05$;
16 ** $p < 0.01$; *** $p < 0.001$.

17
18 **Figure 4 Effect of bumetanide on microglia phenotypes and morphological changes after CCI**
19 **in the DG 3 dpCCI.** (A) Total area of Iba1+ cells soma, quantifications of Iba1+ process endpoints,
20 attachment points and length in the ipsilesional DG. $n = 5$ animals, 2 slices per animal. (B) Iba1
21 immunostaining from sham, CCI-vehicle and CCI-bumetanide treated animals. (C) Quantification
22 of the number of contacts between microglia (Iba1 staining) and PV interneurons at 3 dpCCI. $n =$
23 5 animals per condition, 3 slices per animal. (D) Example of PV and Iba1 immunostaining from
24 sham, CCI-vehicle and CCI-bumetanide treated animals in the ipsilesional DG, 3D representations
25 (upper pictures), and stack (lower pictures). (E) Flow cytometry gating plots at 3 dpCCI on the
26 ipsilesional side. (F) Analysis of microglia phenotypes by detection of MHCII⁺ (pro-inflammatory)
27 and CD206⁺ (pro-phagocytosis) markers by flow cytometry on the ipsilesional side at 3 dpCCI. n
28 = 5-6 animals per condition. (G) Quantity of IL-4, IL-10 and IL-6 produced in the ipsilesional
29 hippocampus. Phenotype changes were analyzed by t-test. Number of contacts and morphological
30 analysis (quantified using the ImageJ plugins Neurphology and SynapCountJ) were analyzed using

1 a one-way ANOVA with Dunnett's post hoc test. Interleukin production was analyzed using a
2 Brown-Forsythe ANOVA with Dunnett's post hoc test * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3
4 **Figure 5 Effect of bumetanide on microglia phenotypes and morphological changes after CCI**
5 **in the DG at 7 dpCCI. (A)** Total area of Iba1+ cells soma, quantifications of Iba1+ process
6 endpoints, attachment points and length in the contralesional DG 7 dpCCI. $n = 5$ animals, 2 slices
7 per animal. **(B)** Iba1 immunostaining from sham, CCI-vehicle and CCI-bumetanide treated animals
8 in the contralesional DG. **(C)** Quantification of the number of contacts between microglia (Iba1
9 staining) and PV interneurons at 7 dpCCI. $n = 5$ animals per condition, 3 slices per animal. **(D)**
10 Example of PV and Iba1 immunostaining, from sham, CCI-vehicle and CCI-bumetanide treated
11 animals in the contralesional DG, 3D representations (upper pictures), and stack (lower pictures).
12 **(E)** Analysis of microglia phenotypes by detection of MHCII⁺ (pro-inflammatory) and CD206⁺
13 (pro-phagocytosis) markers by flow cytometry on the contralesional side at 7 dpCCI. $n = 5-6$
14 animals per condition. **(F)** Quantity of IL-4, IL-10 and IL-6 produced in the contralesional
15 hippocampus. **(G)** Quantification of Bdnf signal intensity within microglia of the ipsi- and
16 contralesional brain of Sham Veh, Sham Bum, CCI Veh and CCI Bum treated animals. **(H)**
17 Example of Iba1 and Bdnf staining in DG of Sham and CCI animals treated with bumetanide or
18 vehicle. **(I)** Example of Iba1 and Bdnf staining in a single microglial cell. $N = 3$, 3 slices per animal.
19 Phenotype changes were analyzed by Student's t-test. Number of contacts and morphological
20 analysis (quantified using the ImageJ plugins Neurphology and SynapCountJ) were analyzed using
21 a one-way ANOVA with Dunnett's post hoc test. Interleukin production was analyzed using a
22 Kruskal-Wallis test for IL-10. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

23
24 **Figure 6 Effect of bumetanide on post-injury microglia surveillance monitored by 2-photon**
25 ***in vivo* imaging. (A)** Schematic representation of the experimental timeline. **(B)** Maximal
26 projection of image at 1 and 5 days after laser induced focal injury. Lower panel showing a single
27 microglia cell and the corresponding temporal color-coded projection (recorded time of 1 h at 5
28 min intervals). **(C)** Quantification of process length at 1 and 5 days after laser induced focal injury
29 in the presence and absence of bumetanide. **(D)** Same as **(B)** in bumetanide-treated animals in
30 bumetanide-treated animals. Lower panel showing one single microglia and the corresponding

1 temporal color-coded projection (recorded time of 1 h at 5 min intervals). (E) Quantification of
2 motility ratio at 1 and 5 days in the presence and absences of bumetanide. All data was analyzed
3 using unpaired Student's t-test. * $p < 0.05$.

4
5 **Figure 7 Effect of pre-CCI bumetanide treatment on PV interneuron survival.** (A)
6 Quantification of parvalbumin (PV) positive cells 1 dpCCI in the contralesional and ipsilesional
7 DG of sham, CCI vehicle and CCI bumetanide animals treated for 7 days before CCI, $n = 3, 5$ and
8 5 respectively, 3 slices per animal. (B) PV labeling at 1 dpCCI in the contralesional and ipsilesional
9 DG of sham, CCI vehicle and CCI bumetanide animals, treated for 7 days before CCI (scale bar =
10 $100 \mu\text{m}$). All data set were analyzed using a one-way ANOVA with Dunnett's post hoc test. * $p <$
11 0.05 ; ** $p < 0.01$; *** $p < 0.001$.

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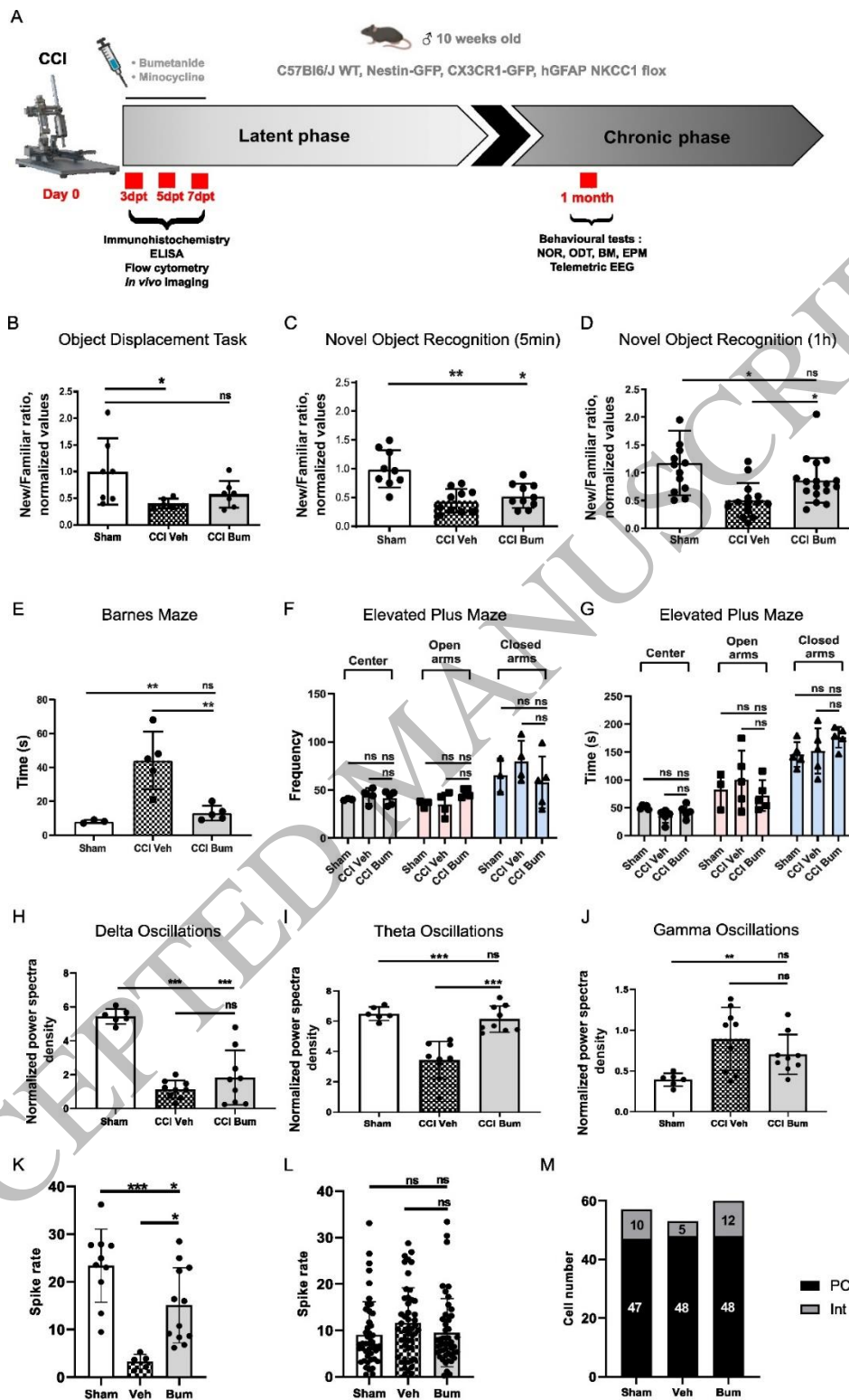


Figure 1
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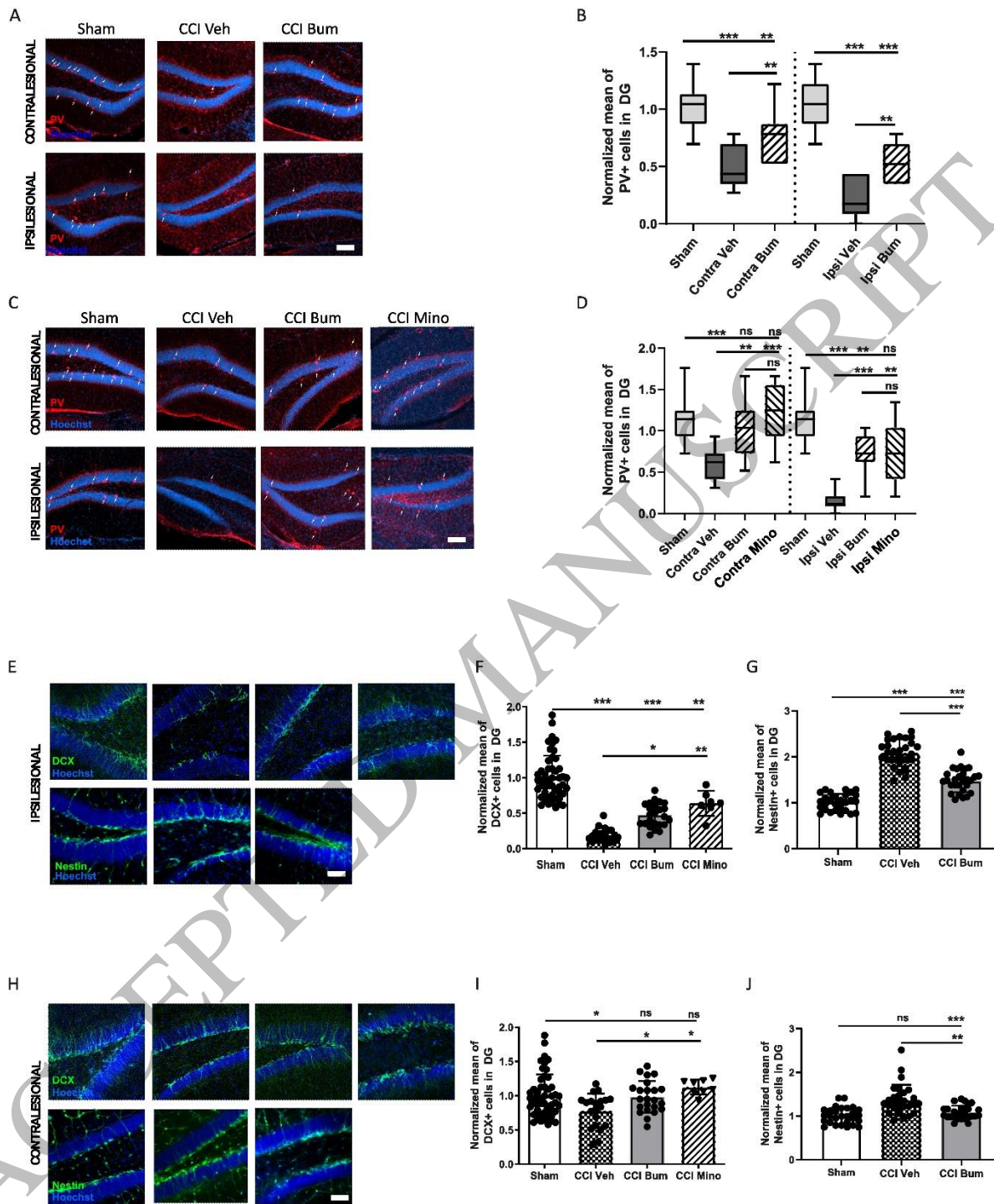


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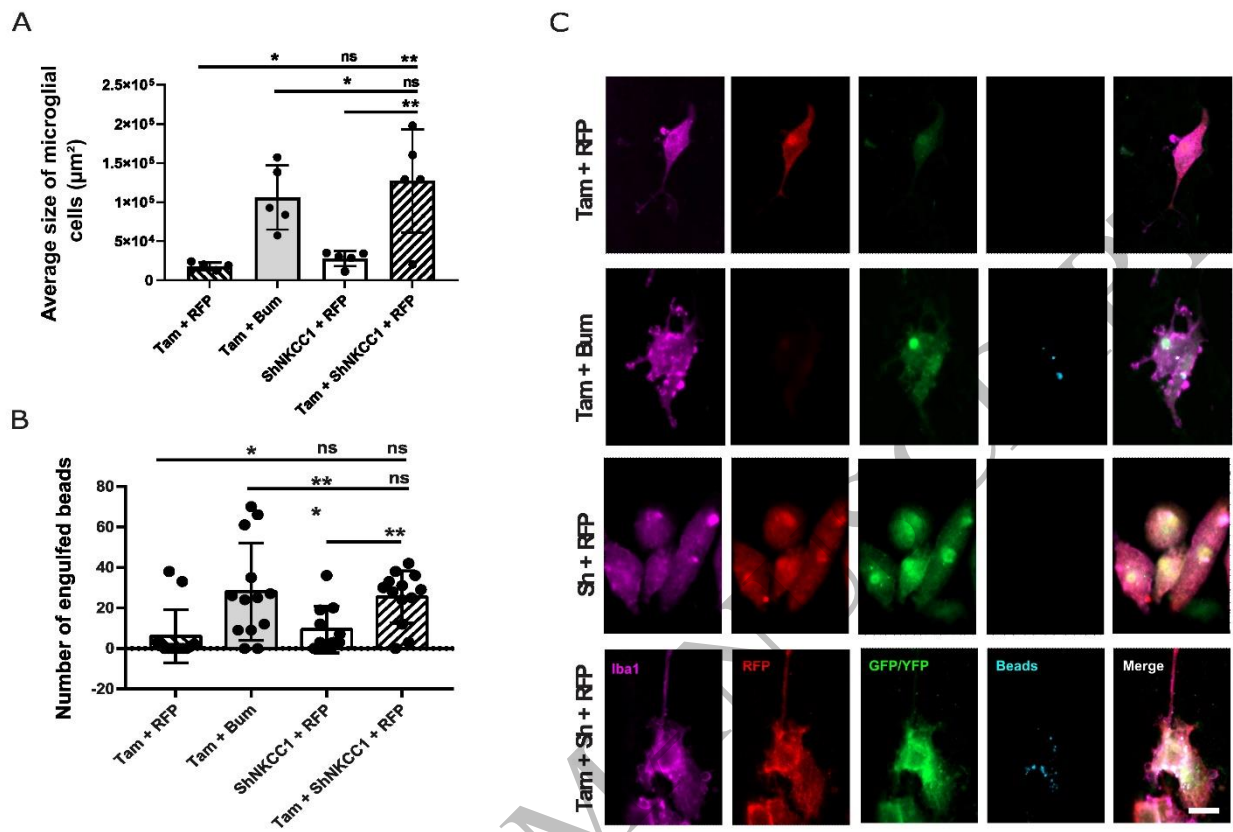


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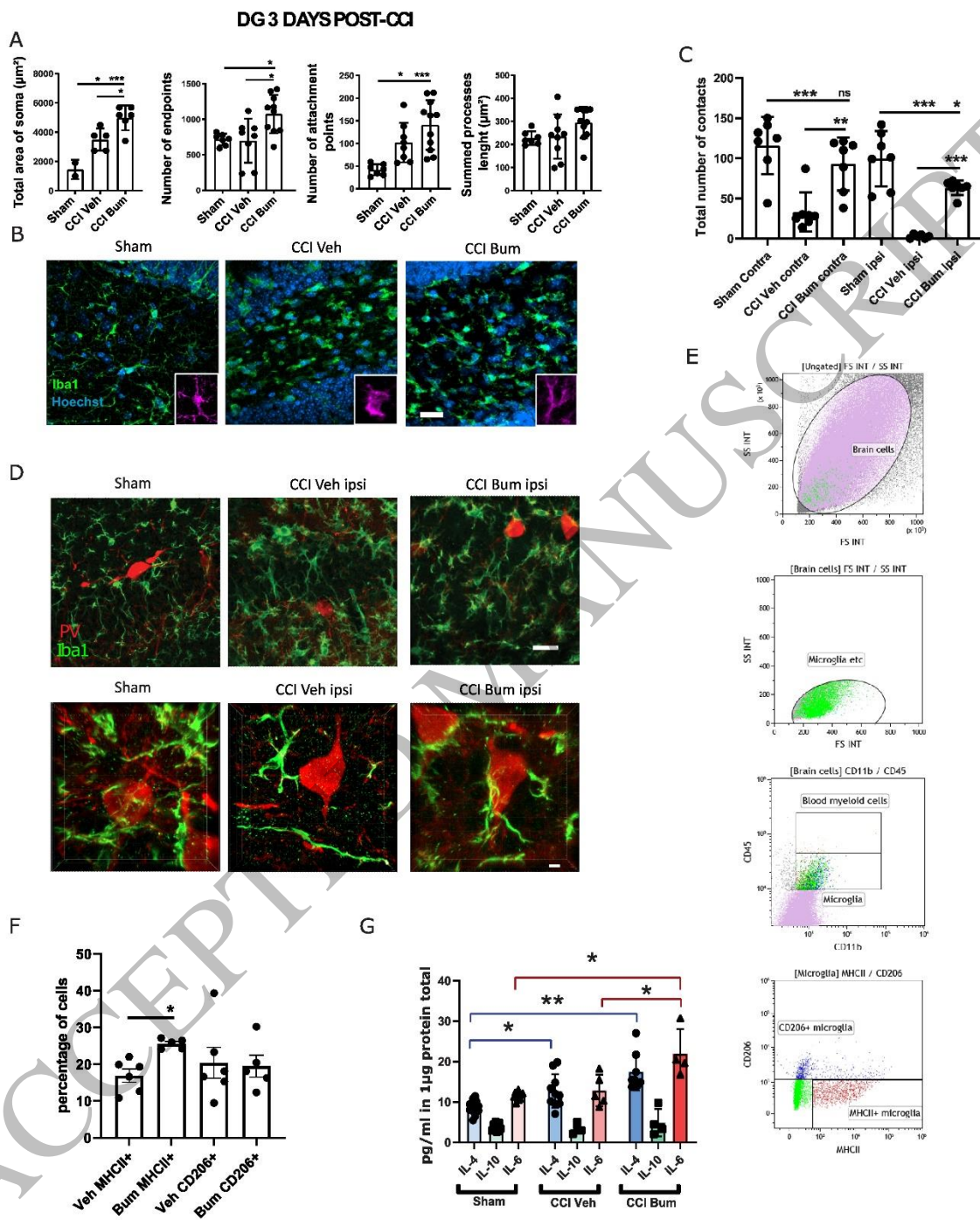


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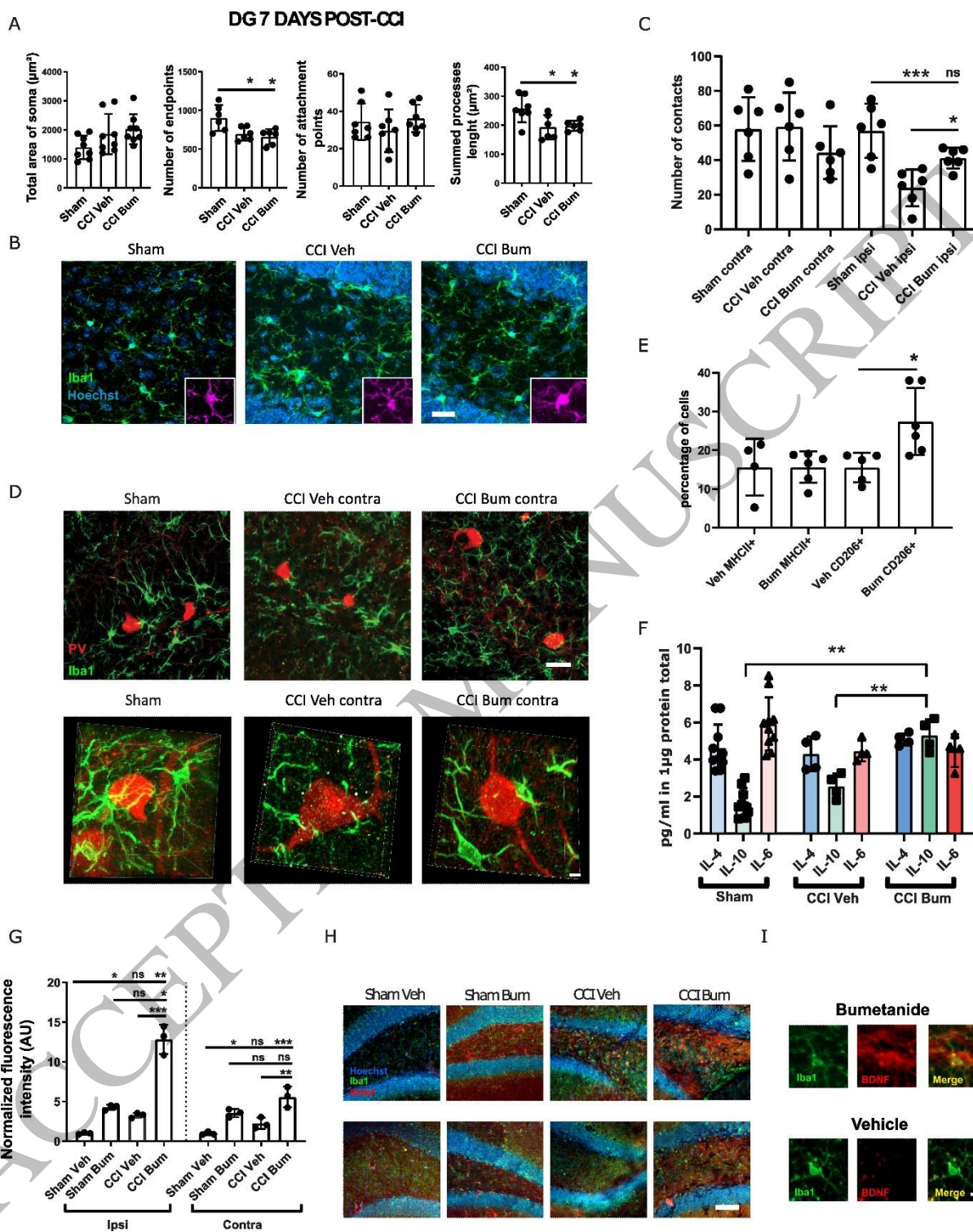
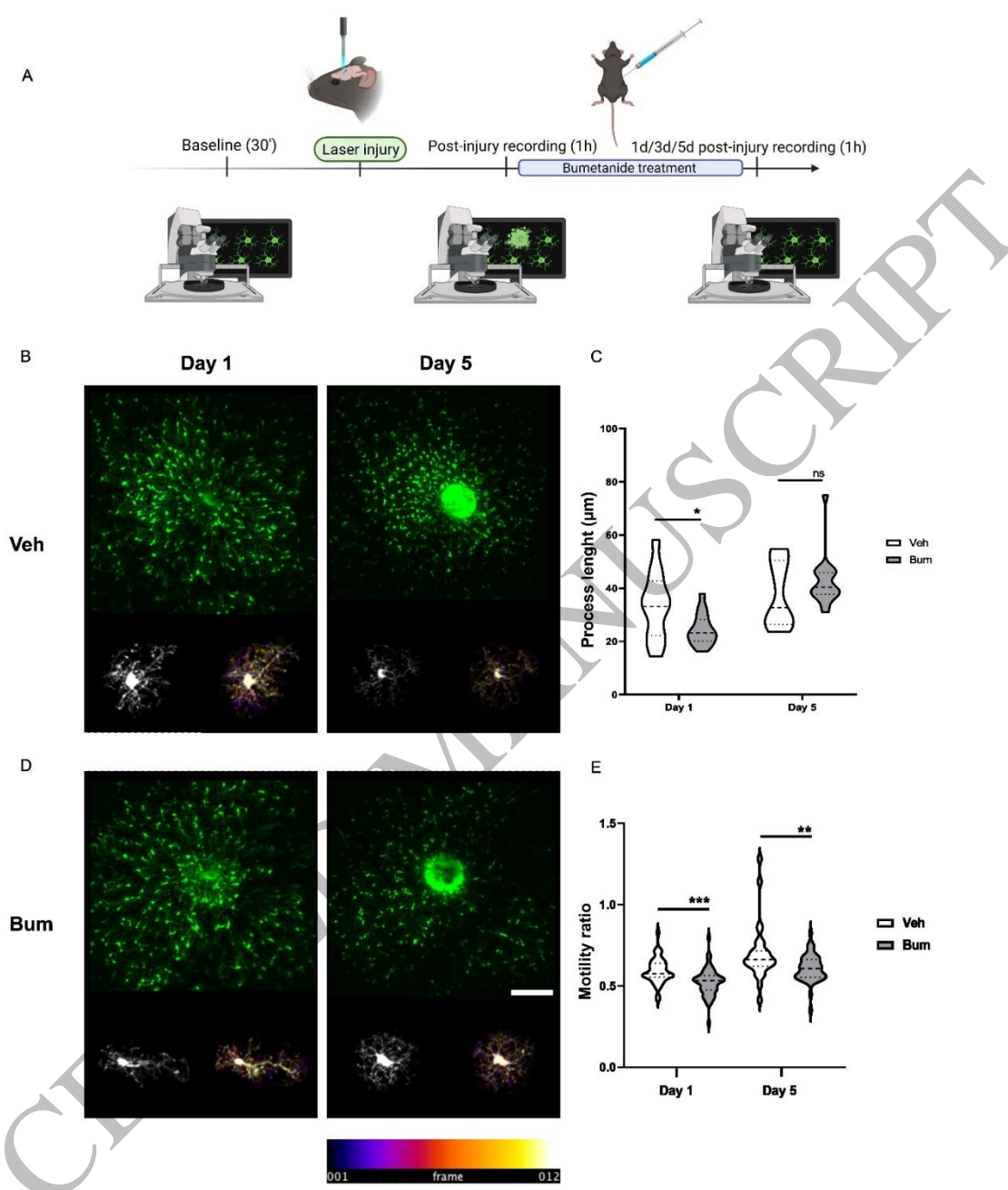


Figure 5
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Figure 6
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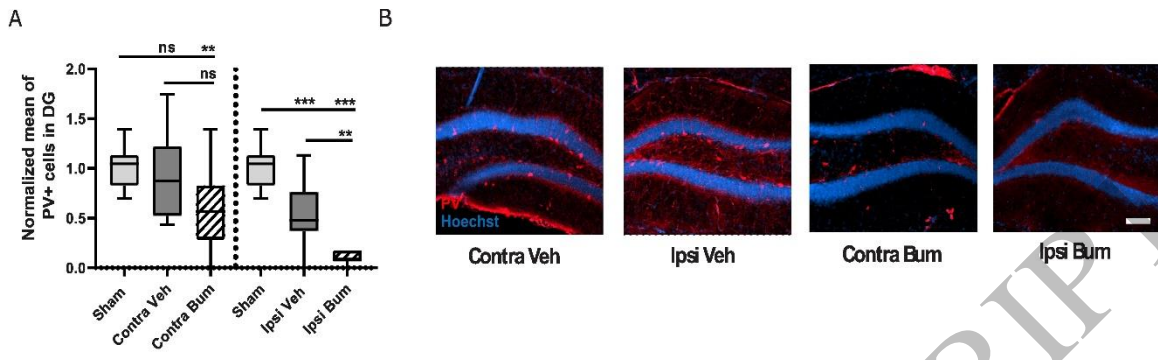


Figure 7
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