

Research Article: New Research | Cognition and Behavior

Inhibition of ASIC1a improves behavioral recovery after stroke

<https://doi.org/10.1523/ENEURO.0341-23.2023>

Received: 5 September 2023

Revised: 19 October 2023

Accepted: 26 October 2023

Copyright © 2024 Armstrong et al.

This is an open-access article distributed under the terms of the [Creative Commons Attribution 4.0 International license](#), which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed.

This Early Release article has been peer reviewed and accepted, but has not been through the composition and copyediting processes. The final version may differ slightly in style or formatting and will contain links to any extended data.

Alerts: Sign up at www.eneuro.org/alerts to receive customized email alerts when the fully formatted version of this article is published.

1 **Title Page**

2

3 **1. Manuscript Title (50 word maximum)**

4 Inhibition of ASIC1a improves behavioral recovery after stroke

5

6 **2. Abbreviated Title (50 character maximum)**

7 Inhibition of ASIC1a improves stroke recovery

8

9 **3. List all Author Names and Affiliations in order as they would appear in the published**
10 **article**

11 Ariel Armstrong, Tao Yang, Tiandong Leng, and Zhi-Gang Xiong

12 Department of Neurobiology, Neuroscience Institute, Morehouse School of Medicine

13

14 **4. Author Contributions: Each author must be identified with at least one of the following:**
15 **designed research, performed research, contributed unpublished reagents/ analytic tools,**
16 **analyzed data, wrote the paper. Example: CS and JS Designed Research; MG and GT**
17 **Performed Research; JS Wrote the paper**

18 Ariel Armstrong – designed research, performed research, analyzed data, wrote the paper

19 Tao Yang – performed research

20 Tiandong Leng – wrote the paper

21 Zhi-Gang Xiong – designed research, wrote the paper

22

23 **5. Correspondence should be addressed to (include email address)**

24 Dr. Zhigang Xiong (zxiong@msm.edu)

25

26 **6. Number of Figures**

27 7

28

29 **7. Number of Tables**

30 1

31

32 **8. Number of Multimedia**

33 0

34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

9. Number of words for Abstract

233

10. Number of words for Significance Statement

110

11. Number of words for Introduction

451

12. Number of words for Discussion

1395

13. Acknowledgements

Research Training Initiative for Scientific Enhancement, T32 Grant

14. Conflict of Interest

The authors declare no competing financial interests.

15. Funding sources

Research Training Initiative for Scientific Enhancement, T32 Grant, and NIH R01NS128018.

61 **Inhibition of ASIC1a improves behavioral recovery after stroke**

62 **Abstract**

63 Stroke continues to be a leading cause of death and long-term disabilities worldwide, despite
64 extensive research efforts. The failure of multiple clinical trials raises the need for continued
65 study of brain injury mechanisms and novel therapeutic strategies for ischemic stroke. The
66 contribution of acid-sensing ion channel 1a (ASIC1a) to neuronal injury during the acute phase
67 of stroke has been well studied, however, the long-term impact of ASIC1a inhibition on stroke
68 recovery has not been established. The present study sought to bridge part of the translational
69 gap by focusing on long-term behavioral recovery after a 30-minute stroke in mice that had
70 ASIC1a knocked out or inhibited by PcTX1. The neurological consequences of stroke in mice
71 were evaluated before and after the stroke using neurological deficit score, open field, and corner
72 turn test over a 28-day period. ASIC1a knocked out and inhibited mice showed improved
73 neurological scores more quickly than wild-type control and vehicle-injected mice after the
74 stroke. ASIC1a knockout mice also recovered from mobility deficits in the open field test more
75 quickly than wild-type mice, while PcTX1-injected mice did not experience significant mobility
76 deficits at all after the stroke. In contrast to vehicle-injected mice which showed clear sidedness
77 bias in corner turn test after stroke, PcTX1-injected mice never experienced significant sidedness
78 bias at all. This study supports and extends previous work demonstrating ASIC1a as a potential
79 therapeutic target for the treatment of ischemic stroke.

80

81

82 **Significance Statement**

83 The contribution of acid-sensing ion channels to neuronal injury is well studied; however, most
84 work focuses on acute-phase molecular and histological endpoints. Here we begin to bridge the
85 bench-to-bedside translational gap by using clinically relevant endpoints in our preclinical
86 model. We show for the first time that both genetic knockout and acute pharmacological
87 inhibition of ASIC1a improve long-term behavioral recovery after stroke. By performing
88 neurological and behavioral tests over 28 days we found that deletion or inhibition of ASIC1a
89 resulted in faster recovery of focal and general neurological deficits and mobility. This work is
90 an important translational step in the identification of ASIC1a as a potential therapeutic target for
91 stroke.

92 **Introduction**

93 Globally, stroke is the second leading cause of death and the third leading cause of disability.
94 Moreover, the incidence of stroke is rapidly growing (Feigin et al., 2021). Projections show that
95 by 2030 approximately 4% of U.S. adults will have had a stroke (Tsao et al., 2022). Among
96 patients recovering from stroke, age-related functional decline is accelerated, with the annual
97 increase in disability increasing considerably after stroke. A year after the stroke, survivors
98 report increased dependence on caregivers for daily living and activities like toileting, walking,
99 transportation, shopping, and laundry (Tsao et al., 2022).

100 Ischemic stroke is the most common type of stroke and occurs when there is a loss of blood
101 circulation to an area of the brain. The only FDA-approved therapeutic for the treatment of
102 ischemic stroke is tissue plasminogen activator (tPA). The number of patients who can be treated
103 with tPA is limited as there is a 4.5-hour time constraint from the onset of symptoms for

104 administration. Unfortunately, even when tPA is administered within the 4.5-hour time limit
105 there is a four-fold increased risk of intracerebral hemorrhage (leading to an almost 50%
106 mortality) when compared to patients given a placebo. This, along with the continued failure of
107 clinical trials testing various therapeutics (e.g., glutamate antagonists), raises the need for
108 continued study of brain injury mechanisms to unveil novel therapeutic strategies for ischemic
109 stroke.

110 Brain acidosis occurs after ischemia due to an increase in anaerobic glycolysis leading to an
111 accumulation of lactic acid. Brain pH is also decreased by the release of protons from the
112 presynaptic terminal and ATP hydrolysis (Nedergaard, Kraig, Tanabe, & Pulsinelli, 1991).
113 During ischemia, extracellular pH commonly falls to 6.5 and in severe instances can get lower
114 than 6.0. It has been well established that acidosis exacerbates ischemic brain damage; however,
115 the detailed mechanism remains unknown. Acid-sensing ion channels (ASICs) are a family of
116 cation channels that are activated by acidosis (Krishtal & Pidoplichko, 1980, 1981). ASIC1a
117 channels are permeable to both Na^+ and Ca^{2+} and mediate an inward current in response to acidic
118 extracellular pH resulting in membrane depolarization. Previous work has shown that the
119 ischemic injury caused by acidosis is mediated by activation of Ca^{2+} -permeable ASICs,
120 independent of glutamate receptors and voltage-gated Ca^{2+} channels (Xiong et al., 2004).
121 Additionally, it has been shown that ASIC1a inhibition has a ~5 hour therapeutic time window
122 (Pignataro et al., 2007). This work has presented substantial evidence that ASICs play an
123 important role in ischemic brain injury, providing a novel therapeutic target for stroke
124 intervention. However, this work has mostly focused on short-term effects of ASIC1a inhibition.
125 The goal of the current work is to determine if deletion or inhibition of ASIC1a results in long
126 term behavioral improvements after stroke.

127 **Materials and methods**

128 **Animals**

129 Male wild-type (ASIC1a^{+/+}) and ASIC1a knocked out (ASIC1a^{-/-}) mice with congenic C57BL/6
130 background aged 2-4 months were used to assess behavioral and histological outcomes 1-28 days
131 following the ischemic stroke induction. The animals were single housed with standard food and
132 water ad libitum on a 12 h light/dark cycle. All animal procedures were reviewed and approved
133 by the Institutional Animal Care and Use Committee (IACUC) of Morehouse School of
134 Medicine.

135 **Stroke Induction**

136 Transient focal ischemia was induced by suture occlusion of the middle cerebral artery (MCAO)
137 for 30-60 minutes, as described in previous studies (Xiong et al., 2004). Animals were
138 anesthetized using 1.5% isoflurane, 70% N₂O, and 28.5% O₂ with intubation and ventilation.
139 Rectal and temporalis muscle temperatures were maintained at 37°C ± 0.5°C with a
140 thermostatically controlled heating pad and lamp. Cerebral blood flow was monitored by
141 transcranial LASER doppler. Animals with blood flow above 20% during ischemia were
142 excluded (Xiong et al., 2004). Sham animals were subjected to the same procedure, but the
143 suture was not tightened around the middle cerebral artery.

144 **Intranasal Psalmotoxin 1 administration**

145 ASIC1a^{+/+} mice were assigned to treatment groups in a blinded fashion. All mice received a
146 single dose of either vehicle (lactated ringers; 50 µl) or psalmotoxin 1 (PcTX1) solution (50 µl, 1
147 µM) administered intranasally 30 minutes after MCAO. A total volume of 50 µl was delivered

148 with an Eppendorf pipette in 10-12 μ l drops to alternating nostrils. Individuals blinded to
149 treatment groups performed all manipulations and analyses.

150 **Mortality and weight**

151 Mortality was classified as death occurring in the acute (2-48 h post-MCAO), subacute (3-7 days
152 post-MCAO), or chronic (>7 days post-MCAO) phase of stroke recovery. Animals in both
153 groups were provided soft food and injected subcutaneously with 1 ml of lactated ringers for
154 three days following MCAO. Body weight (BW) was measured prior to surgery (i.e., baseline)
155 and daily thereafter until day 7 and then measured weekly.

156 **Infarct quantification**

157 Twenty-four hours after MCAO, animals were euthanized and brains were sectioned coronally at
158 1 mm intervals and stained by vital dye immersion with 2% 2,3,5-triphenyltetrazolium
159 hydrochloride (TTC) in paraformaldehyde solution. Infarction area was calculated by subtracting
160 the area stained with TTC in the ischemic hemisphere from the area of the non-ischemic
161 hemisphere. Surgery and analyses were performed by individuals blinded to experimental
162 groups.

163 **Behavioral tests**

164 All tests were performed by the same experimenter that was blinded to the genotype and
165 treatment group of mice from post-operative day 1-28 between 12:00 h and 14:00 h. All animals
166 were evaluated before ischemia and then on post-ischemic days 1, 3, 5, 7, 14, 21, and 28. The
167 order of test descriptions below corresponds to the chronological order that tests were performed
168 on test days. The neurological deficit tests were performed and scored in real-time. The open

169 field tests were video-recorded and then automatically scored using Noldus Ethovision XT 14
170 (Noldus IT, Netherlands). The corner test was video-recorded and then scored manually.

171 **Neurological deficit**

172 Neurobehavioral deficit was assessed using a modified version of the methods described by
173 Lourbopoulos et al. to score both focal neurological deficits and systemic general deficits
174 (Lourbopoulos et al., 2017). Focal deficits, functional outcomes that are directly related to the
175 brain injury and affect a specific region/part of the body, were evaluated by body symmetry, gait,
176 circling behavior, forelimb symmetry, hindlimb symmetry, compulsory circling, whisker
177 response, and trunk. General deficits, systemic processes that are affected by stroke severity but
178 not specific to a certain area of the brain, were evaluated by examining hair, ears, eyes,
179 spontaneous activity, and anxiety behavior. These tests were chosen as a quick and simple
180 assessment of neurological deficit after stroke.

181 **Open field**

182 To evaluate whether the presence of ASIC1a affects mobility behaviors after stroke, locomotion
183 was recorded using an open field test via an electronic imaging system (Noldus Ethovision XT).
184 The arena contained 2 circular zones: a center (diameter 30 cm) and a peripheral zone next to the
185 wall (diameter 55 cm). Mice were placed in the center of the arena and allowed to explore for 4
186 mins. The results of the open field test were expressed as total distance moved and time in zones.

187 **Corner test**

188 To evaluate differences in sidedness bias after MCAO, mice were placed in a corner with a 30°
189 angle. A small opening was cut into the corner to motivate the animals to explore the corner.

190 Once placed, the mice were left to explore the corner freely. After entering the corner, animals
191 typically rear and turn to the left or right side (Balkaya, Kröber, Rex, & Endres, 2013; Zhang et
192 al., 2002). There was no time limit to record the moves. The first 5 successful turns after
193 approaching the corner were recorded and noted as “right turn” or “left turn”. The directional
194 results of the corner test were expressed as % of the total number of turns.

195 **Statistical Analysis**

196 Survival data are represented by percentage surviving and were analyzed via log rank test. All
197 other values are expressed as means \pm SEM and statistical analysis was performed via t-tests or
198 ANOVA followed by the Dunnett’s test. Statistical significance was accepted at the 95%
199 confidence level ($P < 0.05$).

200 **Results**

201 **Knockout of ASIC1a did not impact mortality or weight after 30 min MCAO**

202 To determine if knockout of ASIC1a impacts mortality and weight after 30 min MCAO,
203 ASIC1a^{+/+} and ASIC1a^{-/-} mice were monitored for 28 days following MCAO. A total of 10
204 ASIC1a^{+/+} mice were used for the survival experiment; one animal was sacrificed during surgery
205 due to complications, so it is excluded from this analysis. 89% of ASIC1a^{+/+} mice survived the
206 first 24hrs after MCAO and 67% survived the entire 28 days of the experiment. A total of 11
207 ASIC1a^{-/-} mice were used for the survival experiment. 100% of ASIC1a^{-/-} mice survived the first
208 24hrs after MCAO and 73% survived the entire 28 days of the experiment. There was no
209 significant difference in survival between ASIC1a^{+/+} and ASIC-1a^{-/-} mice after 30 min MCAO.

210 To determine if there were differences in weight loss and gain between ASIC1a^{+/+} and
211 ASIC1a^{-/-} mice, all animals were weighed on the day of surgery, daily for the first seven days
212 post-MCAO, and then weekly thereafter. There were no significant differences in weight loss or
213 gain between ASIC1^{+/+} and ASIC1a^{-/-} mice when compared to each other at any day post-
214 MCAO, however there was some differences in the timing of weight loss when compared to
215 baseline weight post-MCAO. ASIC1a^{+/+} and ASIC1a^{-/-} mice had a similar drop in weight one
216 day after MCAO (-12.6±2.1 and -11.8±1.6 percent difference from baseline, respectively).
217 ASIC1a^{+/+} mice had a quicker weight loss, with weight becoming significantly different from
218 baseline starting at day two post-MCAO (-15.1±2.6, p=0.01) (**Figure 1b**). The weight of
219 ASIC1a^{-/-} mice did not become significantly different from baseline until day three post-MCAO
220 (-14.09±3.0, p=0.02). Both ASIC1a^{+/+} and ASIC1a^{-/-} mice hit their lowest weight on day six (-
221 21.4±4.2 and -19.0±4.2, respectively) post-MCAO. ASIC1a^{+/+} and ASIC1a^{-/-} mice also both
222 began regaining weight on day 7, ASIC1a^{+/+} mice going from a -21.4% weight loss from
223 baseline on day six to a -18.5% weight loss on day 7 and ASIC1a^{-/-} mice going from a -19.0%
224 weight loss from baseline on day six to a -17.0% weight loss on day 7. By day 28 post-MCAO
225 both ASIC1a^{+/+} and ASIC1a^{-/-} mice had regained most of their baseline weight (-4.8±2.4 and -
226 3.7±2.4, respectively).

227 **Reduced infarct volume in ASIC1a^{-/-} mice**

228 It has been shown previously that ASIC1a knock out reduced the infarct volume after 60 min
229 MCAO (Xiong et al., 2004). To determine if infarct volume is also reduced in ASIC1a^{-/-} mice
230 after 30 min MCAO, brains were removed at 24h post-MCAO and stained with TTC. As
231 expected, ASIC1a^{-/-} mice had significantly reduced infarct volume (18.3 ± 2.9%) when compared
232 to ASIC1a^{+/+} mice (36.9 ± 2.3%, **p<0.01) (**Figure 2**).

233 **Neurological functions recovered more rapidly in ASIC1a^{-/-} mice**

234 To evaluate the neurological deficits following MCAO we used a stroke scale that was
235 previously validated to measure focal and general deficits (Lourbopoulos et al., 2017). There
236 were no significant differences in focal or general neurological deficits in ASIC1a^{+/+} versus
237 ASIC1a^{-/-} mice at any of the timepoints post-MCAO. However, there were differences in the
238 speed of recovery between ASIC1a^{+/+} and ASIC1a^{-/-} mice when post-MCAO day one deficit
239 scores were compared to the following recovery days. We found that both ASIC1a^{+/+} and
240 ASIC1a^{-/-} mice had focal deficits that lasted for 28 days. However, ASIC1a^{-/-} mice significantly
241 recovered from day 1 post-MCAO focal deficit 7 days sooner than ASIC1a^{+/+} mice (p<0.05 at
242 day 14 and p<0.05 at day 21, respectively) (**Figure 3a**). We found that both ASIC1a^{+/+} and
243 ASIC1a^{-/-} mice had general deficits that lasted for 5 days; however, ASIC1a^{-/-} mice significantly
244 recovered 14 days sooner than ASIC1a^{+/+} mice (p<0.05 at day 14 and p<0.05 at day 28,
245 respectively) when general deficit at day 1 post-MCAO was compared to later recovery days
246 (**Figure 3b**).

247 **Mobility recovery is improved in ASIC1a^{-/-} mice**

248 To evaluate differences in mobility after 30 min MCAO we used the open field test. At baseline
249 ASIC1a^{-/-} mice were more mobile than ASIC1a^{+/+} mice but there were no significant differences
250 in mobility between the two groups on any post-MCAO day. Mobility was significantly
251 decreased from baseline at one day post-MCAO and remained significantly lower for the entire
252 28-days for both ASIC1a^{+/+} and ASIC1a^{-/-} mice. However, the rate at which mobility was
253 recovered after MCAO differed between ASIC1a^{+/+} and ASIC1a^{-/-} mice. ASIC1a^{-/-} mice had
254 significantly improved mobility from day one post-MCAO to post-MCAO day 14 and that

255 improvement was maintained on days 21 and 28 ($p < 0.05$) (**Figure 4a-b**). In contrast, the
256 mobility in ASIC1a^{+/+} mice did not improve significantly from one day post-MCAO.

257 **Corner turn test**

258 To evaluate differences in sidedness bias after MCAO, we used the corner test. Both ASIC1a^{+/+}
259 and ASIC1a^{-/-} mice significantly increased their preference for the right side on day five post-
260 MCAO. Interestingly, ASIC1a^{+/+} mice were significantly more biased towards the right side on
261 days five and seven post-MCAO ($p < 0.0001$ and $p < 0.005$, respectively), while ASIC1a^{-/-} mice are
262 significantly more biased towards the right side on days 5 and 28 post-MCAO (both $p < 0.05$)
263 (**Figure 4c**). Neither ASIC1a^{+/+} nor ASIC1a^{-/-} significantly recovered from the right sidedness
264 bias caused by MCAO during the 28 days of measurement when comparing day 5 post-MCAO
265 bias to the later recovery days.

266 **Acute inhibition of ASIC1a using PcTX1 reduced infarct volume after MCAO**

267 To determine if acute inhibition of ASIC1a would also result in improved behavioral recovery
268 we administered PcTX1 intranasally to wild-type mice after 30 min MCAO. PcTX1 was
269 administered at 30 min post-MCAO and brains were removed at 24h post-MCAO and stained
270 with TTC. As expected, mice given PcTX1 had significantly reduced infarct volume ($8.28 \pm$
271 2.7%) when compared to Vehicle mice ($20.94 \pm 3.3\%$, $p < 0.05$) (**Figure 5**).

272 **Neurological deficits were reduced after MCAO in PcTX1 treated mice**

273 We evaluated neurological deficits as described above. There were no significant differences in
274 focal or general neurological deficits in Vehicle versus PcTX1 mice at any timepoints post-
275 MCAO. However, Vehicle mice had a significant increase in focal deficit for 7 days post-MCAO
276 when compared to baseline, whereas PcTX1 mice only had significant focal deficit for 3 days

277 post-MCAO. Vehicle mice also had a significantly increased general deficit for the first 7 days
278 post-MCAO and began to be significantly recovered from those deficits on day 14 post-MCAO.
279 PcTX1 mice only had significant general deficit on one day (day 5) post-MCAO and
280 significantly recovered from those deficits on day 21 post-MCAO.

281 **Mobility was slightly impaired after MCAO in PcTX1 mice**

282 To evaluate differences in mobility after MCAO we used the open field test as described above.
283 Vehicle mice had significantly impaired mobility when compared to baseline on all days post-
284 MCAO, with no significant improvement. PcTX1 mice only incurred a significant decrease in
285 mobility on one day (day 14) post-MCAO (**Figure 7a-b**).

286 We evaluated differences in sidedness bias recovery in Vehicle and PcTX1 mice as described
287 above. Vehicle mice significantly increased their preference for the right side on day 5 post-
288 MCAO and significantly recovered by day 14 post-MCAO. PcTX1 mice did not incur a
289 significant increase in sidedness bias at all for the full 28 days of measurement post-MCAO
290 (**Figure 7c**).

291 **Discussion**

292 Stroke continues to be a leading cause of death and disability worldwide, despite extensive
293 research efforts. The failure of translating preclinical treatments to clinical practice in humans is
294 often attributed to the lack of similarities between preclinical and clinical research endpoints
295 (Endres et al., 2008; Gannon, 2014; Lourbopoulos et al., 2021). One factor that may contribute to
296 the eventual clinical failure of stroke treatments that were effective in preclinical studies is the
297 focus on acute molecular or histological endpoints like infarct size rather than clinically relevant
298 endpoints. In clinical studies for stroke, the core target endpoint is clinical improvement which is

300 measured as clinical-neurological improvement at later time points using the modified Rankin
301 Scale (Broderick, Adeoye, & Elm, 2017). Additionally, since much of the focus of pre-clinical
302 trials is on differences in infarct volume (measured within 24-72 hours), improvements in long-
303 term behavioral outcomes are rarely considered as the primary endpoint. The typical clinical
304 stroke patient is monitored for neurological improvement for weeks or months following the
305 stroke, rather than hours or days. Consequently, there is an increasing need to assess long-term
306 functional and behavioral outcomes in pre-clinical research to reduce the “translational
307 roadblock” to the development of stroke therapies. The contribution of ASICs to neuronal injury
308 during the acute phase of stroke is well studied. The current work sought to bridge the
309 translational gap by investigating the effect of ASIC1a on long-term behavioral outcomes after
310 stroke.

311 Neurological scoring was developed for human clinical trials to measure patient’s performance
312 in activities of daily life (D’Olhaberriague, Litvan, Mitsias, & Mansbach, 1996). The most
313 widely used neurological scoring scale is the modified Rankin Scale because of its sensitivity,
314 validity, simplicity of use and interpretability (Banks & Marotta, 2007; de Haan, Limburg,
315 Bossuyt, van der Meulen, & Aaronson, 1995; Wolfe, Taub, Woodrow, & Burney, 1991).
316 Though, there are various scoring systems that have been validated in humans including the
317 Stroke Impact Scale and the Barthel Index (Duncan et al., 1999). Rodents and humans exhibit
318 multiple similarities in sensorimotor and neurological deficits after stroke (Grabowski, Brundin,
319 & Johansson, 1993; Zausinger, Hungerhuber, Baethmann, Reulen, & Schmid-Elsaesser, 2000).
320 However, due to anatomical and functional differences between rodents and humans, accurate
321 assessment of neurological function after stroke is dependent on the use of appropriate
322 behavioral tests. Like in humans, several scoring systems have also been developed to evaluate

322 stroke outcomes in rodents. We used a modified version of a previously validated experimental
323 stroke scale (ESS) that was developed by Loubopoulos et al (Loubopoulos et al., 2017). The
324 ESS includes two components, a focal component which evaluates focal neurological deficits
325 and a general component which evaluates systemic behavioral deficits. These two components
326 are comparable to clinical measurements of weakness/degree of disability and ability to perform
327 activities of daily living (ADLs) respectively (Narayanaswami, 2017).

328 The primary and secondary endpoints in ischemic stroke clinical trials are often measured as
329 improvements in modified Rankin Score over time. We chose to evaluate recovery in our
330 behavioral tests with a similar approach. First, we analyzed the impact of the MCAO by
331 comparing the baseline measurement to the measurements taken after MCAO during recovery.
332 Then, we analyzed the improvement post-MCAO by comparing day 1 post-MCAO
333 measurements to the rest of the recovery days. At baseline, ASIC1a^{+/+} and ASIC1a^{-/-} mice
334 performed similarly in most behavioral tests, except for the open field test where ASIC1a^{-/-} mice
335 are more active at baseline. This is consistent with a previous study which demonstrated that
336 ASIC1a^{-/-} mice show less signs of fear and anxiety in the open field (Coryell et al., 2007). Like
337 vehicle-treated ASIC1a^{+/+} mice, mice that had ASIC1a either knocked out or pharmacologically
338 inhibited showed significant focal and general neurological deficits following stroke. However,
339 ASIC1a knocked out or inhibited mice showed improvement in both the focal and general
340 neurological scores more quickly than vehicle-treated ASIC1a^{+/+} mice. This pattern of similar
341 initial impact on both groups but increased speed of recovery amongst ASIC1a^{-/-} mice was also
342 shown in the open field test, where ASIC1a^{-/-} mice displayed significant improvement in
343 mobility by day 14, while ASIC1a^{+/+} mice did not show significant improvement for the duration
344 of the study. The pattern of increased speed of recovery did not show in the corner test, however,

345 this could be a limitation of the 28-day timescale used, as neither ASIC1a^{+/+} nor ASIC1a^{-/-} mice
346 significantly recovered within the 28 days of testing. Mice that had ASIC1a acutely inhibited via
347 PcTX1 did not incur significant impairments in mobility for most of the test days. Additionally,
348 PcTX1-treated mice did not incur any significant increase in right-sidedness bias for the duration
349 of the experiment, likely due to the smaller infarct size.

350 Inflammation plays a key role in the pathogenesis of stroke and has become a target for
351 therapeutic intervention. However, inflammatory cells are multifunctional, playing both
352 beneficial and detrimental roles where inhibiting a pathway at the wrong time could lead to an
353 exaggerated pathogenesis (Jayaraj, Azimullah, Beiram, Jalal, & Rosenberg, 2019). The CNS
354 response to ischemic insult happens in three broadly defined overlapping phases: 1) cell death
355 and inflammation, 2) cell proliferation for tissue replacement, and 3) tissue remodeling (Burda &
356 Sofroniew, 2014). The first phase, cell death and inflammation, begins rapidly over a time scale
357 of seconds to hours, with more gradually progressing events developing over days. Brain
358 acidosis occurs during the early parts of this phase, causing the activation of ASICs leading to
359 cell death. The dead and dying cells release “danger signals” that activate the innate immune
360 response. There has been evidence suggesting that ASIC1a mediates these “danger signals” by
361 activating voltage-activated K⁺ channels which in turn activate inflammasomes (Rajamäki et al.,
362 2013; Wang et al., 2015). Inflammasomes are an assembly of pattern-recognition receptors
363 (PRRs) that control maturation and secretion of potent proinflammatory interleukins (IL-1 β and
364 IL-18). The inflammation process in the first phase of stroke recovery is intrinsically bounded to
365 the processes that follow in the second and third phases which mark the beginning of recovery
366 via cell proliferation for tissue replacement and tissue remodeling. There is little literature on the
367 role of ASIC1a in inflammatory cascades, and that is especially true for long-term mechanisms.

368 Thus, the role that ASIC1a plays in long-term stroke recovery remains unclear. The exact reason
369 why PcTX1 injected mice performed better after stroke than the ASIC1a^{-/-} mice is unclear. One
370 possibility is that normal ASIC1a function might play a role in the recovery phase of the stroke.
371 PcTX1 applied in the acute phase of stroke reduces the infarct volume without interfering with
372 the potential beneficial function of the channel in the recovery phases. In contrast, although
373 knockout of the channel can reduce the infarct volume in the acute phase, it also eliminates the
374 potential beneficial function of the channel in the recovery phases. Future work focusing on
375 elucidating the role of ASIC1a during post-stroke recovery phases could help provide better
376 explanations.

377 Our study had some limitations. In this study we used 30 min MCAO. ASIC1a inhibition may be
378 less protective in circumstances where more severe damage is caused by a 60 min or longer
379 MCAO. Also, the ASIC1a^{-/-} mice used were full body genetic knockouts, and as such they could
380 give insight into the global role of ASIC1a in long-term stroke recovery. However, this limited
381 our ability to draw brain mechanism specific conclusions on its role in behavioral recovery.

382 Another limitation of our study is that PcTX1 was administered once at 30 mins post-MCAO.
383 Acute cell death after ischemic stroke can last for up to 48 hrs. Administration of PcTX1 for at
384 least the first 48 hrs may reduce the negative impact of stroke on behavior even more.

385 Additionally, administering PcTX1 so quickly after MCAO is not a realistic representation of
386 how an ASIC1a inhibitor would be administered in a clinical setting. Past work (Pignataro et al.,
387 2007) found that intranasal administration of PcTX1 to be effective at reducing infarct size even
388 when administered up to five hours after MCAO. Future work will apply PcTX1 at later
389 timepoints that are more typical for a clinical setting.

390 The major goal of this study was to determine if inhibition of ASIC1a channels would result in
391 improved long-term behavioral recovery after stroke. The contribution of ASIC1a to neuronal
392 injury during the acute phase of stroke is well studied, and we sought to bridge part of the
393 translational gap by focusing on long-term behavior recovery. We show for the first time in an *in*
394 *vivo* model that both genetic knockout and acute inhibition of ASIC1a channels improve long
395 term recovery after stroke. This study expands on previous work by demonstrating that the
396 reduction in tissue damage caused by inhibiting ASIC1a translates into significant improvements
397 in speed of recovery in focal deficits, general deficits, and mobility. This study also supports and
398 extends previous work demonstrating ASIC1a as a potential therapeutic target for the treatment
399 of ischemic stroke.

400 **References**

- 401 Balkaya, M., Kröber, J. M., Rex, A., & Endres, M. (2013). Assessing post-stroke behavior in mouse models
402 of focal ischemia. *J Cereb Blood Flow Metab*, *33*(3), 330-338. doi:10.1038/jcbfm.2012.185
- 403 Banks, J. L., & Marotta, C. A. (2007). Outcomes Validity and Reliability of the Modified Rankin Scale:
404 Implications for Stroke Clinical Trials. *Stroke*, *38*(3), 1091-1096.
405 doi:doi:10.1161/01.STR.0000258355.23810.c6
- 406 Broderick, J. P., Adeoye, O., & Elm, J. (2017). Evolution of the Modified Rankin Scale and Its Use in Future
407 Stroke Trials. *Stroke*, *48*(7), 2007-2012. doi:10.1161/strokeaha.117.017866
- 408 Burda, J. E., & Sofroniew, M. V. (2014). Reactive gliosis and the multicellular response to CNS damage
409 and disease. *Neuron*, *81*(2), 229-248. doi:10.1016/j.neuron.2013.12.034
- 410 Coryell, M. W., Ziemann, A. E., Westmoreland, P. J., Haenfler, J. M., Kurjakovic, Z., Zha, X.-m., . . .
411 Wemmie, J. A. (2007). Targeting ASIC1a Reduces Innate Fear and Alters Neuronal Activity in the
412 Fear Circuit. *Biological Psychiatry*, *62*(10), 1140-1148. doi:10.1016/j.biopsych.2007.05.008

413 D'Olhaberriague, L., Litvan, I., Mitsias, P., & Mansbach, H. H. (1996). A reappraisal of reliability and
414 validity studies in stroke. *Stroke*, *27*(12), 2331-2336. doi:10.1161/01.str.27.12.2331

415 de Haan, R., Limburg, M., Bossuyt, P., van der Meulen, J., & Aaronson, N. (1995). The clinical meaning of
416 Rankin 'handicap' grades after stroke. *Stroke*, *26*(11), 2027-2030. doi:10.1161/01.str.26.11.2027

417 Duncan, P. W., Wallace, D., Lai, S. M., Johnson, D., Embretson, S., & Laster, L. J. (1999). The stroke
418 impact scale version 2.0. Evaluation of reliability, validity, and sensitivity to change. *Stroke*,
419 *30*(10), 2131-2140. doi:10.1161/01.str.30.10.2131

420 Endres, M., Engelhardt, B., Koistinaho, J., Lindvall, O., Meairs, S., Mohr, J. P., . . . Dirnagl, U. (2008).
421 Improving outcome after stroke: overcoming the translational roadblock. *Cerebrovasc Dis*, *25*(3),
422 268-278. doi:10.1159/000118039

423 Feigin, V. L., Stark, B. A., Johnson, C. O., Roth, G. A., Bisignano, C., Abady, G. G., . . . Murray, C. J. L.
424 (2021). Global, regional, and national burden of stroke and its risk factors, 1990-2019: a
425 systematic analysis for the Global Burden of Disease Study 2019. *The Lancet Neurology*, *20*(10),
426 795-820. doi:10.1016/S1474-4422(21)00252-0

427 Gannon, F. (2014). The steps from translatable to translational research. *EMBO Rep*, *15*(11), 1107-1108.
428 doi:10.15252/embr.201439587

429 Grabowski, M., Brundin, P., & Johansson, B. B. (1993). Paw-reaching, sensorimotor, and rotational
430 behavior after brain infarction in rats. *Stroke*, *24*(6), 889-895. doi:10.1161/01.str.24.6.889

431 Jayaraj, R. L., Azimullah, S., Beiram, R., Jalal, F. Y., & Rosenberg, G. A. (2019). Neuroinflammation: friend
432 and foe for ischemic stroke. *Journal of neuroinflammation*, *16*(1), 142-142. doi:10.1186/s12974-
433 019-1516-2

434 Krishtal, O. A., & Pidoplichko, V. I. (1980). A receptor for protons in the nerve cell membrane.
435 *Neuroscience*, *5*(12), 2325-2327. doi:10.1016/0306-4522(80)90149-9

436 Krishtal, O. A., & Pidoplichko, V. I. (1981). A receptor for protons in the membrane of sensory neurons
437 may participate in nociception. *Neuroscience*, 6(12), 2599-2601. doi:10.1016/0306-
438 4522(81)90105-6

439 Loubopoulos, A., Mamrak, U., Roth, S., Balbi, M., Shrouder, J., Liesz, A., . . . Plesnila, N. (2017).
440 Inadequate food and water intake determine mortality following stroke in mice. *J Cereb Blood*
441 *Flow Metab*, 37(6), 2084-2097. doi:10.1177/0271678x16660986

442 Loubopoulos, A., Mourouzis, I., Xinaris, C., Zerva, N., Filippakis, K., Pavlopoulos, A., & Pantos, C. (2021).
443 Translational Block in Stroke: A Constructive and "Out-of-the-Box" Reappraisal. *Front Neurosci*,
444 15, 652403. doi:10.3389/fnins.2021.652403

445 Narayanaswami, P. (2017). The Spectrum of Functional Rating Scales in Neurology Clinical Trials.
446 *Neurotherapeutics*, 14(1), 161-175. doi:10.1007/s13311-016-0488-5

447 Nedergaard, M., Kraig, R. P., Tanabe, J., & Pulsinelli, W. A. (1991). Dynamics of interstitial and
448 intracellular pH in evolving brain infarct. *Am J Physiol*, 260(3 Pt 2), R581-588.
449 doi:10.1152/ajpregu.1991.260.3.R581

450 Pignataro, G., Simon, R. P., & Xiong, Z. G. (2007). Prolonged activation of ASIC1a and the time window
451 for neuroprotection in cerebral ischaemia. *Brain*, 130(Pt 1), 151-158. doi:10.1093/brain/awl325

452 Rajamäki, K., Nordström, T., Nurmi, K., Åkerman, K. E. O., Kovanen, P. T., Öörni, K., & Eklund, K. K.
453 (2013). Extracellular acidosis is a novel danger signal alerting innate immunity via the NLRP3
454 inflammasome. *The Journal of Biological Chemistry*, 288(19), 13410-13419.
455 doi:10.1074/jbc.M112.426254

456 Tsao, C. W., Aday, A. W., Almarzooq, Z. I., Alonso, A., Beaton, A. Z., Bittencourt, M. S., . . . Martin, S. S.
457 (2022). Heart Disease and Stroke Statistics—2022 Update: A Report From the American Heart
458 Association. *Circulation*, 145(8), e153-e639. doi:doi:10.1161/CIR.0000000000001052

459 Wang, Y.-C., Li, W.-Z., Wu, Y., Yin, Y.-Y., Dong, L.-Y., Chen, Z.-W., & Wu, W.-N. (2015). Acid-sensing ion
460 channel 1a contributes to the effect of extracellular acidosis on NLRP1 inflammasome activation
461 in cortical neurons. *Journal of neuroinflammation*, *12*, 246-246. doi:10.1186/s12974-015-0465-7
462 Wolfe, C. D., Taub, N. A., Woodrow, E. J., & Burney, P. G. (1991). Assessment of scales of disability and
463 handicap for stroke patients. *Stroke*, *22*(10), 1242-1244. doi:doi:10.1161/01.STR.22.10.1242
464 Xiong, Z.-G., Zhu, X.-M., Chu, X.-P., Minami, M., Hey, J., Wei, W.-L., . . . Simon, R. P. (2004).
465 Neuroprotection in Ischemia: Blocking Calcium-Permeable Acid-Sensing Ion Channels. *Cell*,
466 *118*(6), 687-698. doi:10.1016/j.cell.2004.08.026
467 Zausinger, S., Hungerhuber, E., Baethmann, A., Reulen, H., & Schmid-Elsaesser, R. (2000). Neurological
468 impairment in rats after transient middle cerebral artery occlusion: a comparative study under
469 various treatment paradigms. *Brain Res*, *863*(1-2), 94-105. doi:10.1016/s0006-8993(00)02100-4
470 Zhang, L., Schallert, T., Zhang, Z. G., Jiang, Q., Arniago, P., Li, Q., . . . Chopp, M. (2002). A test for
471 detecting long-term sensorimotor dysfunction in the mouse after focal cerebral ischemia. *J*
472 *Neurosci Methods*, *117*(2), 207-214. doi:10.1016/s0165-0270(02)00114-0

473

474 **Figure Legends**

475 **Figure 1.** ASIC1a deletion does not impact mortality or weight after MCAO. **(a)** ASIC1a^{+/+} and
476 ASIC1a^{-/-} mice did not significantly differ in survival for up to 28 days after MCAO. Survival
477 data are presented as percentage surviving and analyzed by log rank test. **(b)** ASIC1a^{+/+} and
478 ASIC1a^{-/-} mice also did not have significant differences in weight changes for up to 28 days after
479 MCAO. Weight data are presented as mean±SEM and analyzed by ANOVA (n=9-11 for each
480 group). Asterisks represent significant changes in weight as compared to baseline measurement
481 *p<0.05, **p<0.01, ***p<0.001.

482 **Figure 2.** Protective effect of ASIC1a knockout on infarct volume after MCAO. (a) Twenty-four
483 hours after 30 min MCAO, mice were sacrificed, and infarct volumes were determined using
484 TTC staining. (b) Knockout of ASIC1a significantly reduced brain infarct volume. Data
485 presented as mean±SEM and analyzed by t-test (n=4-5 for each group). *p<0.05 when compared
486 with ASIC1a^{+/+}.

487 **Figure 3.** Knockout of ASIC1a accelerates neurological deficit recovery. Neurological scoring
488 was done prior to MCAO for baseline measurement and at the specified timepoints post-MCAO.
489 Both ASIC1a^{+/+} and ASIC1a^{-/-} mice had significant focal deficits over a 28-day period following
490 MCAO. (a) The focal deficit of ASIC1a^{-/-} mice significantly improved from day 1 deficit a week
491 earlier than ASIC1a^{+/+} mice. (b) ASIC1a^{-/-} mice had general deficits for fewer days and
492 significantly improved from day 1 deficit 14 days earlier than ASIC1a^{+/+} mice. Data presented as
493 mean±SEM and analyzed by two-way ANOVA (n=11-13 for each group). Asterisks represent
494 significant differences as compared to day 0 measurement. *p<0.05, **p<0.01, ***p<0.001,
495 ****p<0.0001. Pound signs represent significant differences as compared to day 1 measurement
496 #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001.

497 **Figure 4.** Knockout of ASIC1a improved mobility recovery but not sidedness bias after stroke.
498 (a) Representative visualization of open field test. (b) Both ASIC1a^{+/+} and ASIC1a^{-/-} mice had
499 reduced mobility after MCAO. Only ASIC1a^{-/-} mice significantly recovered mobility. (c) Both
500 ASIC1a^{+/+} and ASIC1a^{-/-} mice had significantly right sidedness bias after MCAO and neither
501 group significantly recovered within 28 days. Data presented as mean±SEM and analyzed by
502 two-way ANOVA (open field n=13-18 for each group, corner n=7-11 for each group). Asterisks
503 represent significant differences as compared to baseline measurement *p<0.05, **p<0.01,

504 ***p<0.001, ****p<0.0001. Pound signs represent significant differences as compared to day 1
505 measurement #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001.

506 **Figure 5.** Protective effect of ASIC1a inhibition on infarct volume after focal cerebral ischemia.
507 (a) Representative images of TTC stained brains 24h after 30 min MCAO. (b) PcTX1 treated
508 mice had a significantly reduced infarct volume when compared to Vehicle mice. Data are
509 presented as mean±SEM and analyzed by t-test (n=5 for each group). *p<0.05 when compared
510 with vehicle.

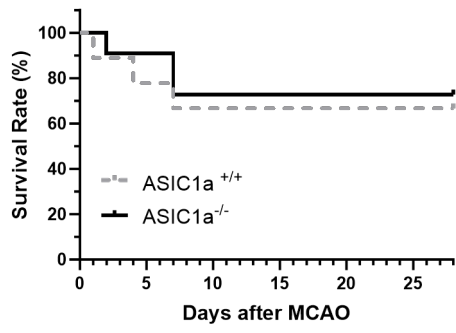
511 **Figure 6.** Acute inhibition of ASIC1a improves neurological deficit recovery. Neurological
512 scoring was done prior to MCAO for baseline measurement and at the specified timepoints post-
513 MCAO using the previously described stroke scale. (a) PcTX1 mice have significantly increased
514 focal deficits for the first 3 days and vehicle mice had significantly increased deficits for 7 days
515 post-MCAO. (b) PcTX1 mice only had significantly increased general sickness on day 5 post-
516 MCAO and significantly recovered from that increase on day 21 post-MCAO. Vehicle mice had
517 significantly increased general sickness on all days up to 7 days post-MCAO and significantly
518 recovered from that increase on day 21 post-MCAO. Data presented as mean±SEM and analyzed
519 by two-way ANOVA (n=9-11 for each group). Asterisks represent significant differences as
520 compared to baseline measurement *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Pound signs
521 represent significant differences as compared to day 1 measurement #p<0.05, ##p<0.01,
522 ###p<0.001, ####p<0.0001. Crosses represent significant differences as compared to day 5
523 measurement +p<0.05, ++p<0.01, +++p<0.001, ++++p<0.0001.

524 **Figure 7.** Acute inhibition of ASIC1a improved mobility recovery and sidedness bias after
525 stroke. (a) Representative visualization of open field test (b) Vehicle mice had reduced mobility

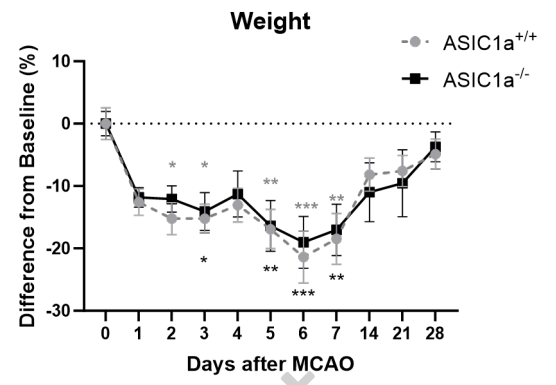
526 for all days post-MCAO and did not significantly recover within 28 days post-MCAO. PcTX1
527 mice only had significantly reduced mobility on day 14 post-MCAO. (c) Vehicle mice had
528 severe right-sidedness bias on day 5 post-MCAO and significantly recovered by day 14 post-
529 MCAO. PcTX1 mice did not have a significant increase in sidedness bias. Data presented as
530 mean±SEM and analyzed by two-way ANOVA (n= 11-9 for each group). Asterisks represent
531 significant differences as compared to baseline measurement. *p<0.05, **p<0.01, ***p<0.001,
532 ****p<0.0001. Pound signs represent significant differences as compared to day 1 measurement.
533 #<≤0.05, ##p<0.01, ###p<0.001, ####p<0.0001.

eNeuro Accepted Manuscript

(a)

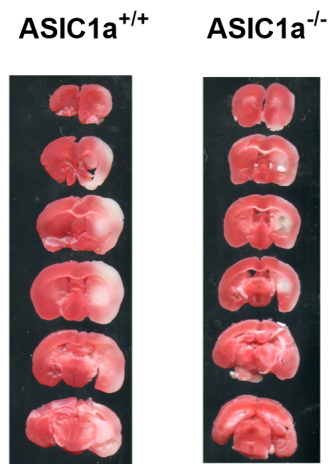


(b)

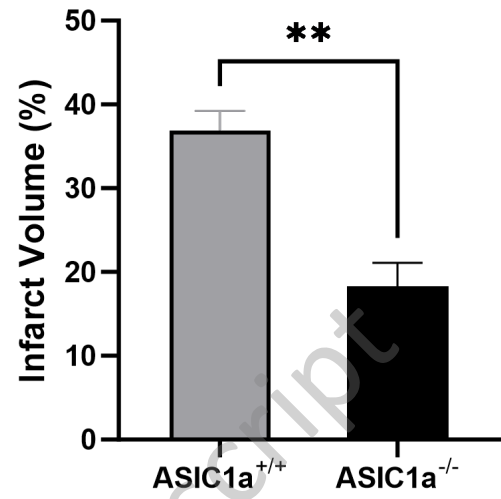


eNeuro Accepted Manuscript

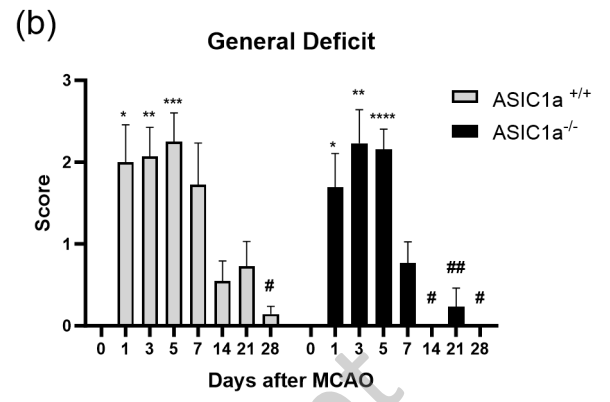
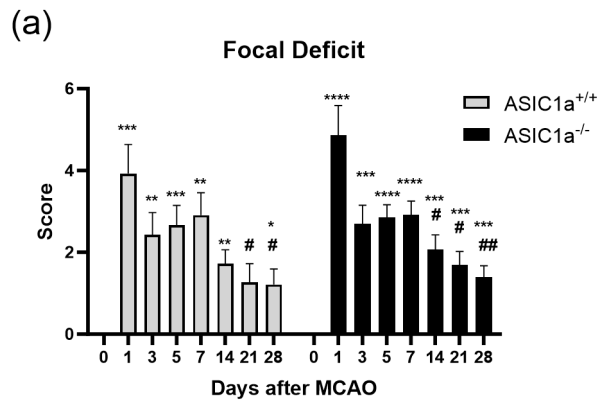
(a)



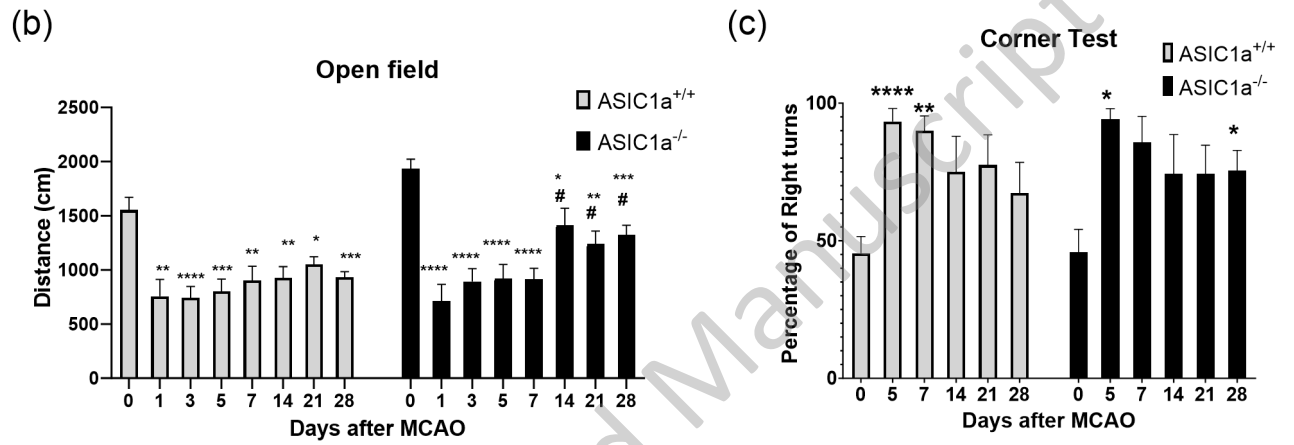
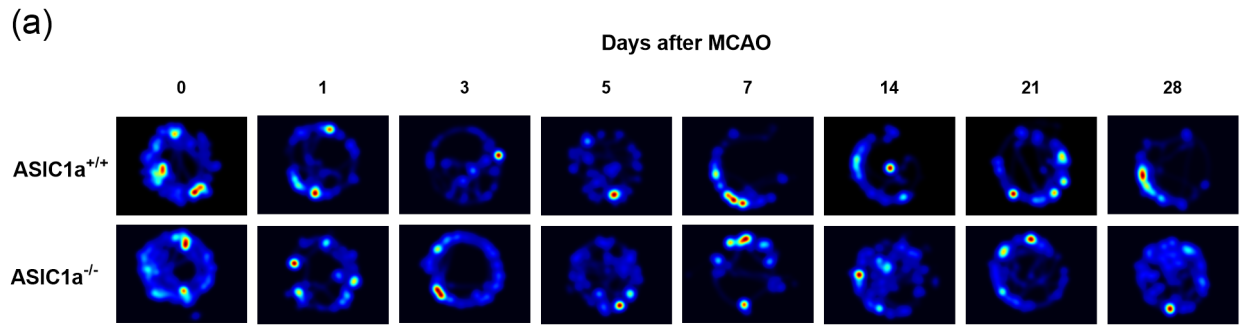
(b)



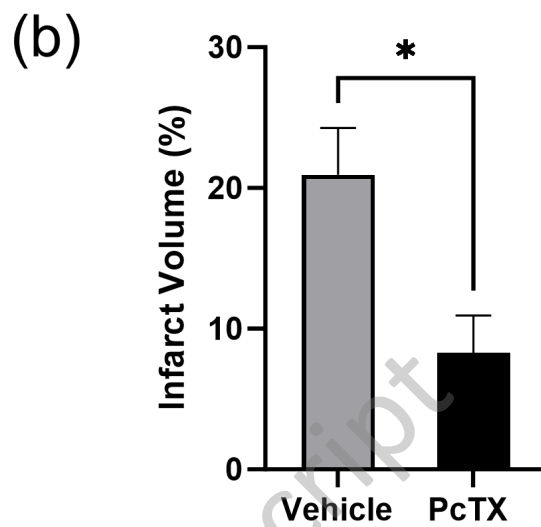
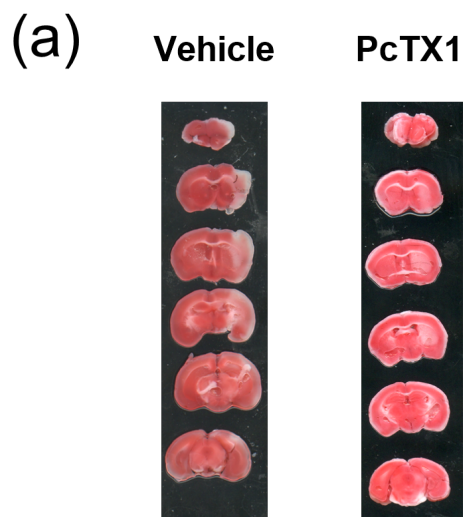
eNeuro Accepted Manuscript



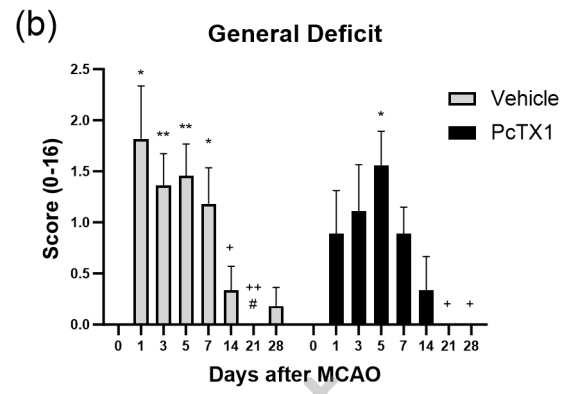
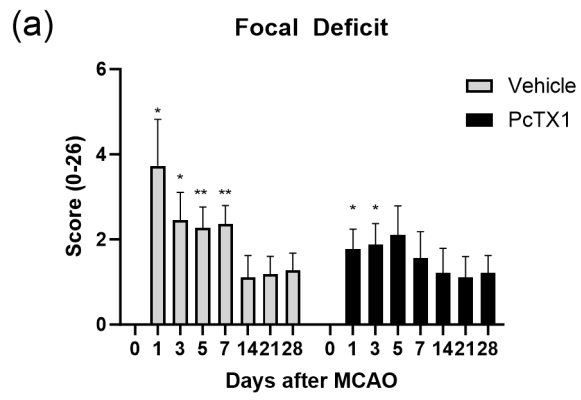
eNeuro Accepted Manuscript



eNeuro Accepted Manuscript

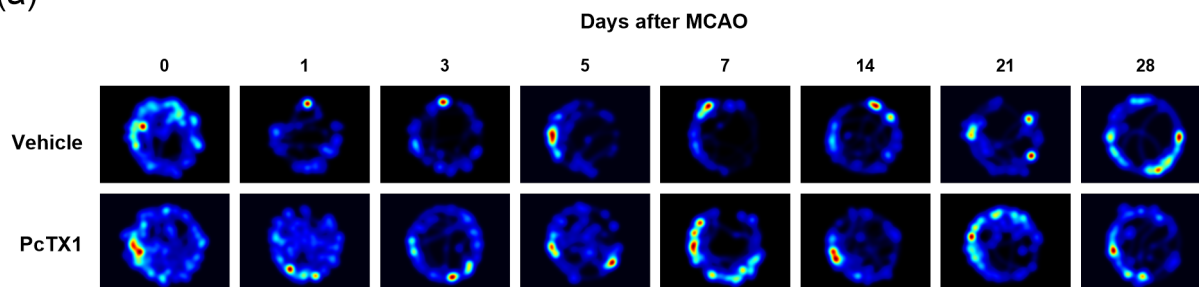


eNeuro Accepted Manuscript

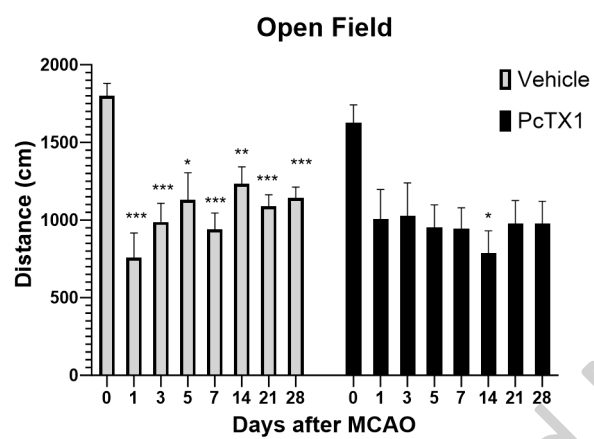


eNeuro Accepted Manuscript

(a)



(b)



(c)

