Dissecting the functional contributions of different neuronal subtypes in the dorsal striatum to perseverative behaviour in ephrin-A2/A5$^{-/-}$ mice

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Abstract

Overreliance on habit is linked with disorders such as drug addiction and obsessive-compulsive disorder and there is increasing interest in the use of repetitive transcranial magnetic stimulation (rTMS) to alter neuronal activity in the relevant pathways and reduce relapse or accelerated shift towards habit formation. Here we studied the brains of ephrin-A2A5$^{-/-}$ mice, which previously showed perseverative behaviour in progressive-ratio tasks, associated with low cellular activity in nucleus accumbens. We investigated if rTMS treatment had altered the hierarchical recruitment of brain regions from ventral to dorsal striatum associated with abnormal habit formation in these mice.

Brain sections of mice that underwent progressive-ratio tasks with and without LI-rTMS were taken from a previous study. Striatal regions were stained for neuronal activation with c-Fos and for medium spiny neurons with DARPP32. Qualitative analysis was carried out for other neuronal subtypes in the striatum - GABAergic, parvalbumin-expressing and cholinergic interneurons.

Contrary to our hypothesis, we found neuronal activity in ephrin-A2A5$^{-/-}$ mice still reflected goal-directed behaviour. However, we saw that the dorsolateral striatum contributed more to total striatal activity in untreated ephrin-A2/A5$^{-/-}$ mice. This supported our hypothesis that ephrin-A2/A5$^{-/-}$ mice have greater c-Fos activity in habit-associated striatal regions. LI-rTMS in ephrin-A2A5$^{-/-}$ mice also appeared to delay the shift from goal-directed to habitual behaviour as suggested by increased activation in dorsomedial striatum and nucleus accumbens.

Introduction

Habits form over a period of time and a shift from goal-directed behaviour to habitual behaviour is crucial to habit formation. However, sometimes a faulty shift can contribute to disorders such as drug addiction [1] and obsessive-compulsive disorder (OCD) [2]. The formation of habit is generally defined as a shift from response-outcome associations, where actions are primarily mediated by the value associated with the outcome, to stimulus-response associations where, over a period of extended training and repetition, there is a pattern of outcome devaluation and the behaviour becomes more automatic, independent from the outcome of the action [3]. This behavioural shift from goal-directed to habitual behaviour involves dopaminergic pathways and a hierarchical recruitment of brain regions from ventral to dorsal striatum [4,5].

A potential tool to manipulate this hierarchical recruitment is repetitive transcranial magnetic stimulation (rTMS), a non-invasive brain stimulation technique that is able to modulate neuronal activity [6] and various neurotransmitters, including dopamine [7,8]. rTMS is approved for treatment of depression [9] and OCD [10]. Understanding more about the mechanisms of rTMS, could be useful in refining rTMS use.

Although there is broad evidence for an effect of rTMS on dopamine levels in the brain [8], the effect of rTMS on dopamine-related behaviour is relatively unknown. Ephrin A2/A5$^{-/-}$ mice are a strain of mice that have demonstrated abnormal behavioural patterns [11,12] which could be due to abnormal dopaminergic innervation in the striatum [13]. For example, ephrin A2/A5$^{-/-}$ mice that completed progressive ratio (PR) tasks showed perseverative behaviour, with greater number of responses in the PR task compared to wildtype mice irrespective of the increasing task difficulty [12]. The ephrin-A2A5$^{-/-}$ mice also showed reduced cellular activity in the nucleus accumbens (NAc) after two weeks of the PR task,

compared to wildtype mice [12], which may reflect an accelerated dorsal shift in neuronal c-Fos activity characteristic of habitual behaviour [4]. Interestingly, chronic excitatory low intensity rTMS (LI-rTMS) delivered to ephrin-A2/A5$^{-/-}$ mice during the PR task ameliorated the reduced c-Fos expression in the ventral striatum of ephrin-A2/A5$^{-/-}$ mice, resulting in activity more similar to wildtype levels [12]. Therefore, LI-rTMS may mitigate altered activity along the mesolimbic pathway in ephrin-A2/A5$^{-/-}$ mice that resulted in behavioural inflexibility by delaying an accelerated shift in neuronal activity toward the dorsal striatum.

To understand if altered dorsal striatum activity in ephrin-A2/A5$^{-/-}$ mice was responsible for perseverative behaviour in these mice and if rTMS could delay an accelerated shift from goal-directed behaviour to habit, we used tissue from the previous study [12] to identify and compare activation of neuronal populations across the entire striatum, investigating the dorsomedial striatum (DMS), dorsolateral striatum (DLS) and NAc (core and shell) using immunohistochemistry. We compared the neuronal activation in different regions of the striatum for wildtype and ephrin-A2A5$^{-/-}$ mice that either received active LI-rTMS or sham stimulation in order to confirm whether LI-rTMS may have delayed the accelerated dorsal shift of neuronal activity in ephrin-A2/A5$^{-/-}$ mice. If LI-rTMS does reduce this abnormal shift in habit formation, it could indicate wider implications for disorders such as addiction and OCD where the shift of habit formation is faulty. Lastly, since a low percentage of c-Fos expression in medium spiny neurons (MSNs) in the NAc was found [12], we also identified c-Fos expression in other neuronal subtypes of the striatum such as GABAergic and cholinergic interneurons. This was done to better characterise cell activation in the striatum and explore whether strain or treatment differences differentially affected specific neuronal subtypes.

Materials and methods

Animal tissue

All experiments were approved by The University of Western Australia Animal Ethics Committee (AEC 100/1639). Sagittal mouse brain sections (40 µm) from 11 adult (8-24-week-old) wildtype C57BL6/J mice (sham: 6; rTMS: 5) and 10 ephrin-A2/A5$^{-/-}$ mice (sham: 6; rTMS: 4) (series three) [12] were used. Briefly, mice had completed one week of an exponential PR task (required responses increase under an exponential equation) followed by one week of PR7. Mice received 14 days of biomimetic high frequency stimulation (BHFS) LI-rTMS or sham during the first 10 minutes of the daily PR task and were euthanized 90 minutes after the beginning of the final PR task to correspond with the peak of c-Fos expression [12].

Immunofluorescence

Every fifth section of the third series which contained dorsal striatum was identified using the mouse brain atlas [14]. These sections were stained for three different antibodies: c-Fos (rabbit polyclonal c-Fos antibody, 1:5000, Abcam, ab190289), a marker for neuronal activity [15], cAMP-regulated phosphoprotein-32 kD (DARPP32) (purified mouse anti-DARPP32, 1:2000, BD Transduction Laboratories, 611520), a marker for MSNs [16], and glutamate decarboxylase 67 (GAD67) (anti-Goat, 1:750, R&D systems, AF2086-SP), a marker for GABAergic neurons [17]. For confirmatory analysis of active parvalbumin (PV)-expressing GABAergic interneurons and cholinergic interneurons, two sections from one animal belonging to each experimental group were stained for c-Fos, PV (mouse monoclonal anti-PV antibody, 1:5000, Millipore Sigma, p3088) and ChAT (anti-choline acetyltransferase antibody, 1:200, Millipore Sigma, AB144P). Hoescht (1:2000, Invitrogen) was used to stain all nuclei.

Free-floating sections were washed 3x in PBS (5 min each), permeabilised by washing in 0.1% Triton-X in PBS (PBS-T) for 15 min and incubated for 2 h in a blocking buffer of 2% bovine serum albumin (Sigma) and 3% normal donkey serum (Sigma) diluted in 0.1% PBS-T. Then, sections were incubated with primary antibodies in blocking buffer overnight at 4°C. Sections were washed in PBS-T 3x (10 min each) and incubated for 2 h with secondary antibodies diluted in blocking buffer to 1:600 (donkey anti-rabbit IgG Alexa Fluor 488, Invitrogen, Thermo Fisher, A21206; donkey anti-mouse IgG Alexa Fluor 555, Invitrogen, Thermo Fisher, A21202; donkey anti-goat IgG H&L Alexa Fluor 647, ab150131). Sections were then washed in PBS-T 3x (10 min each) and incubated for 10 min in Hoescht in PBS (1:1000, Invitrogen). Lastly, the sections were washed in PBS 3x (10 min each) and mounted on gelatin-subbed slides, coverslipped with mounting medium (Dako, Glostrup, Denmark) and sealed with nail polish. Slides were stored at 4°C in a light-controlled environment until imaging.

Imaging and quantification

Commonly used mouse brain atlases [14,18] do not show detailed segmentation of the dorsal striatum [19]. Therefore, in the present study the two regions of the dorsal striatum (DMS and DLS) were identified by creating a custom sagittal atlas that combined the location information of dorsal striatum sections from a mouse brain atlas [19] annotated onto a 3D magnetic resonance imaging (MRI) data of P56 mouse brain atlas using ITK-SNAP [20] software (Fig 1).

Fig 1. Identification of regions of interest on a mouse brain atlas. Location of DMS and DLS was marked in red and green respectively on a coronal plane in the software, ITK-SNAP which produced corresponding images in the horizontal and sagittal planes. Scale bar = 5 mm. CP, coronal plane; SP, sagittal plane; HP, horizontal plane.

Sections were imaged through z-stacks (2 µm apart) on a Nikon C2 confocal microscope (Nikon, Tokyo, Japan). 10x magnification was used to identify the regions of interest. 8-11 fields of view (FOV) for DLS and 6-10 FOV for DMS across 5-7 sections each animal were used to image at 40x magnification to identify c-Fos, DARPP32 and GAD67 markers. For the confirmatory analysis, 2 FOV for DLS and DMS and 1 FOV for NAc across 2 sections from each experimental group was imaged at 40x magnification.

Cell counts were performed using stereological principles using ImageJ software and were carried out blinded to experimental group after all images were captured. The total number of c-Fos single labelled cells, c-Fos^{+}/DARPP32^{+} double labelled cells, c-Fos^{+}/GAD67^{+}/DARPP32^{-} double labelled cells, c-Fos^{+}/PV^{+} double labelled cells and c-Fos^{+}/ChAT^{+} double labelled cells were identified and counted for each image (Fig 2). The combination of DARPP32^{+} and GAD67^{+} double labelling was not assessed since DARPP32^{+} cells are also GAD67^{+} because they are GABAergic in nature. c-Fos^{+} cells not colocalized with DARPP32 were classified as unspecified c-Fos.

Fig 2. Representative images taken at 40x magnification showing different marker expressions. (A) expression of cFos^{+} cells (green) (B) expression of GAD67^{+} cells (yellow) and DARPP32^{+} cells (red) (C) expression of cFos^{+} (green) /DARPP32^{+} (red) colocalised cells (D) expression of cFos^{+} (green) /GAD67^{+} (yellow) colocalised cells (E) expression of cFos^{+} (green) /ChAT^{+} (yellow) colocalised cells (F) expression of cFos^{+} (green) /PV^{+} (red) colocalised cells. Scale bar = 50 µm. GAD67, Glutamate decarboxylase 67; cAMP-regulated phosphoprotein-32 kD, DARPP32.

155 For confirmatory analysis, c-Fos-labelled cells colocalised with GAD67, PV and
156 ChAT were counted and analysed to explain unspecified c-Fos counts. During imaging,
157 sections from one ephrin-A2/A5$^{-/-}$-rTMS animal was excluded due to poor staining (ephrin-
158 A2/A5$^{-/-}$-rTMS *n = 3*).

159 Statistical analysis

160 For analysis, the total c-Fos cells/mm^3 and percentage of c-Fos cells colocalised with
161 DARPP32, GAD67, PV and ChAT were calculated for each animal. Statistical analysis was
162 not done on counts showing c-Fos colocalization with PV and ChAT due to low sample size
163 but assessed qualitatively.

164 Assumptions of normality and homogeneity were checked. For DLS counts, the
165 assumptions of normality were not met for percentage c-Fos-GAD67 and total c-Fos
166 cells/mm^3 counts ($p<0.05$). Homogeneity of variance was checked, and it was met for all data
167 sets (Levene's test, $p>0.05$). For DMS counts, the assumptions of normality were not met for
168 percentage c-Fos-DARPP32, percentage c-Fos-GAD67 and total c-Fos cells/mm^3 ($p<0.05$).
169 The assumption of homogeneity of variance was not met for percentage c-Fos-DARPP32
170 data (Levene's test, $p<0.05$) but corrected with logarithmic transformation.

171 To compare whether cell counts changed across groups, multiple two-way analysis of
172 variance (ANOVAs) were used with the independent variables of strain and treatment. Each
173 ANOVA had the dependent variable of either total c-Fos cells/mm^3, percentage c-Fos-
174 DARPP32 or percentage unspecified c-Fos cell count data and were run separately for both
175 DLS and DMS. ANOVA was also performed for c-Fos$^+$/GAD67$^+$/DARPP32$^-$ cell counts for
176 the DLS region. For the DMS regions, due to small sample size, Ephrin-rTMS group was
177 excluded from statistical analysis for c-Fos$^+$/GAD67$^+$/DARPP32$^-$ data set and student t-tests
178 were performed to compare remaining experimental groups.

Additional data for percentage of c-Fos^{+}/GAD67^{+}/DARPP32^{-} cell counts were also obtained for NAc core and shell if the regions were present in the stained tissue (NAc core, Group: wildtype-sham *n=5,* wildtype-rTMS *n=4,* ephrin-A2/A5$^{-/-}$-sham *n=4,* ephrin-A2/A5$^{-/-}$-rTMS *n*=2; NAc shell, Group: wildtype-sham *n=6,* wildtype-rTMS *n=5,* ephrin-A2/A5$^{-/-}$-sham *n=4,* ephrin-A2/A5$^{-/-}$-rTMS *n*=3). This was combined with total c-Fos cells/mm^3 and percentage c-Fos-DARPP32 cell counts for NAc core and shell from the previous study [12]. The percentage of c-Fos-GAD67 data set in NAc core and shell assumptions of normality and heterogeneity. Therefore, non-parametric Mann-Whitney U tests were performed to compare the percentage of c-Fos^{+}/GAD67^{+}/DARPP32^{-} cell counts between sham and treatment for both wildtype and ephrin-A2A5$^{-/-}$ groups.

Results

Total c-Fos cells/mm^3

In DLS, there was no overall effect of strain ($F(1,16)=1.040$, $p=0.323$, $\eta^2=0.058$) or treatment ($F(1,16)=0.973$, $p=0.339$, $\eta^2=0.054$) on c-Fos cells/mm^3. The strain*treatment interaction was also not significant ($F(1,16)=0.0007$, $p=0.979$, $\eta^2=0.00$) (Fig 3). Similarly, in DMS there was no significant difference in cell counts between strain ($F(1,16)=0.902$, $p=0.356$, $\eta^2=0.046$) and no overall effect of treatment was observed ($F(1,16)=1.342$, $p=0.264$, $\eta^2=0.068$). No significant strain*treatment interaction was observed ($F(1,16)=1.378$, $p=0.258$, $\eta^2=0.070$) (Fig 3). Qualitatively, DMS showed more c-Fos activity as compared to DLS (Fig 3).

Fig 3. Graphs showing total c-Fos^{+} cells/mm^3 counts for each group in DLS and DMS. No significant difference in total c-Fos^{+} cells/mm^3 cells was found between groups in both

202 DLS and DMS ($p>0.05$). Points represent individual animal counts. Error bars represent ±
203 SEM.

204

205 ## c-Fos colocalization in MSNs

206 In DLS, there was no overall effect of strain ($F(1,16)=0.100$, $p=0.756$, $\eta^2=0.006$) or
207 treatment ($F(1,16)=0.001$, $p=0.976$, $\eta^2=0$) on percentage of c-Fos-DARPP32 colocalization.
208 The strain*treatment interaction was also not significant ($F(1,16)=0.376$, $p=0.548$, $\eta^2=0.023$)
209 (Fig 4). Similarly, in DMS there was no significant difference in percentage of colocalization
210 between strains ($F(1,16)=0.0461$, $p=0.833$, $\eta^2=0.002$) and no overall effect of treatment was
211 observed ($F(1,16)=1.2556$, $p=0.279$, $\eta^2=0.056$). However, there was a significant
212 strain*treatment interaction ($F(1,16)=5.1062$, $p=0.038$, $\eta^2=0.228$). To understand the
213 interaction, we followed up with pairwise post-hoc comparisons using Tukey's test. There
214 was an increase in percentage of c-Fos-DARPP32 colocalization after treatment in ephrin-
215 A2/A5$^{-/-}$ mice compared to ephrin-sham group (Fig 4), however the effect did not survive
216 multiple comparison Tukey correction (uncorrected $p=0.041$, $t(16)=-2.225$, Tukey corrected
217 $p=0.159$) despite the significant omnibus interaction. All other groups were non-significantly
218 different from each other.

219

220 **Fig 4. Graphs showing percentage of c-Fos$^+$ cells colocalized with DARPP32$^+$ cells for**
221 **each group in DLS and DMS.** Following stimulation, a significant increase was only seen in
222 c-Fos-DARPP32 colocalization in DMS in ephrin-A2/A5$^{-/-}$ mice in the omnibus test but not
223 in pairwise post-hoc comparisons. Points represent individual animal counts. Error bars
224 represent ± SEM.

Percentage of unspecified c-Fos cells

In DLS, there was no overall effect of strain ($F(1,16)=1.9052$, $p=0.186$, $\eta^2=0.105$) or treatment ($F(1,16)=0.0309$, $p=0.863$, $\eta^2=0.002$) on the percentage of unspecified c-Fos cells. The strain*treatment interaction was also not significant ($F(1,16)=0.2026$, $p=0.659$, $\eta^2=0.011$) (Fig 5). Similarly in DMS, there was no overall effect of strain ($F(1,16)=0.482$, $p=0.497$, $\eta^2=0.026$) or treatment ($F(1,16)=0.270$, $p=0.611$, $\eta^2=0.015$) on the percentage of unspecified c-Fos cells. The strain*treatment interaction was also not significant ($F(1,16)=1.738$, $p=0.206$, $\eta^2=0.094$) (Fig 5).

Fig 5. Graphs showing percentage of unspecified c-Fos$^+$ cells for each group in DLS and DMS. No significant difference in percentage of unspecified c-Fos$^+$ cells was found between groups in both DLS and DMS ($p>0.05$). Points represent individual animal counts. Error bars represent ± SEM.

Confirmatory analysis for unspecified c-Fos-labelled cells

Across all experimental groups, approximately 60% and 40% of c-Fos labelled cells in the ventral and dorsal striatum, respectively were unidentified (i.e., not MSNs). Therefore, the sections were first stained for GABAergic interneurons to identify unspecified c-Fos labelled cells. Qualitative observation suggests that in DMS there were more active GABAergic interneurons as compared to DLS (Fig 6) and NAc where the percentage of colocalization across all groups were less than 1% and 2%, respectively.

Fig 6. Graphs showing percentage of c-Fos$^+$ cells colocalized with GAD67$^+$-DARPP32$^-$ cells for each group in DLS and DMS. No significant difference in percentage of c-Fos$^+$ cells colocalised with GAD67$^+$-DARPP32$^-$ cells were found between groups in both DLS and DMS ($p>0.05$). Points represent individual animal counts. Error bars represent ± SEM.

For percentage of c-Fos$^+$/GAD67$^+$/DARPP32$^-$ colocalization in DLS, no overall effect of strain ($F(1,14)=0.058$, $p=0.814$, $\eta^2=0.004$) or treatment ($F(1,14)=0.061$, $p=0.809$, $\eta^2=0.004$) was observed. The strain*treatment interaction was also not significant ($F(1,14)=0.088$, $p=0.771$, $\eta^2=0.006$) (Fig 6). In the DMS, there was no significant change in c-Fos$^+$/GAD67$^+$/DARPP32$^-$ colocalization between Wildtype-Sham and Wildtype-rTMS mice ($t(9)=-0.864$, $p=0.410$), and there was no significant difference between strains, Wildtype-Sham and Ephrin-Sham ($t(8)=-1.32$, $p=0.222$). Qualitatively, c-Fos$^+$ that is colocalised with GAD67$^+$decreased following LI-rTMS in ephrin-A2A5$^{-/-}$ mice as seen in Fig 6.

In NAc core, there was no significant difference in the percentage of c-Fos$^+$/GAD67$^+$/DARPP32$^-$ colocalization following treatment in both wildtype ($U=8.5$, $p=0.771$) and ephrin-A2A5$^{-/-}$ mice ($U=2$, $p=0.289$). For the same data set, there was no significant difference between strains, Wildtype-Sham and Ephrin-Sham ($U=6$, $P=0.240$). Similarly, in NAc shell there was no significant difference in the percentage of c-Fos$^+$/GAD67$^+$/DARPP32$^-$ colocalization following treatment in both wildtype ($U=12$, $p=0.597$) and ephrin-A2A5$^{-/-}$ mice ($U=5$, $p=0.825$). There was also no significant strain difference, Wildtype-Sham and Ephrin-Sham ($U=11$, $p=0.896$).

As the percentage of c-Fos colocalised with GAD67-labelled cells were also low in both dorsal and ventral striatum, additional confirmatory tests were done for PV-expressing GABAergic interneurons and cholinergic interneurons. Qualitative analysis suggests there were more active PV-expressing GABAergic interneurons in DMS as compared to DLS and NAc. In DMS, a slight increase in active PV-expressing GABAergic interneurons was observed in ephrin-A2A5$^{-/-}$ mice following treatment, whereas a slight decrease was seen in wildtype mice following treatment. No active cholinergic interneurons were observed in both DMS and NAc across all groups. In DLS, only about 2% c-Fos^{+} cells were colocalised with cholinergic interneurons in the wildtype-sham group, in all other groups no active cholinergic interneurons were observed.

Comparison of c-Fos activity across regions and groups

To further explore a potential change in activity distribution across regions in the overall striatum, we examined whether the proportion of c-Fos activity per regions differed between groups. We ran a 4*4 contingency χ^2 test with c-Fos counts per region (NAc shell, NAc core, DMS and DLS) compared across experimental groups. The initial 4*4 contingency χ^2 test was significant (χ^2 (9, N=666524) =8030, $p<0.001$, V=0.0634). We then ran follow up 2*4 contingency χ^2 test to understand where the difference was, comparing the proportion of striatal c-Fos activity per region for each group with the Wildtype-Sham group. Proportions of striatal c-Fos activity per region differed in comparison with Wildtype-Sham for all groups, although the test may be overly sensitive due to the large sample size (Wildtype-rTMS: χ^2 (3, N=436329) =642, $p<0.001$, V = .0384); Ephrin-Sham: χ^2 (3, N=335837) = 2156, $p<0.001$, V = .0801; Ephrin-rTMS: χ^2 (3, N=384288) =2446, $p<0.001$, V = .0798). Although there are significant differences, the effect sizes for each comparison are small.

293 To further understand where the difference in striatal activity per region occurred, we
294 performed region-by-region comparison for the percentage total striatal c-Fos activity present
295 for each region across groups (Fig 7). Comparison between each experimental group and
296 Wildtype-Sham used multiple Mann-Whitney U tests. Statistics are presented in Table 1.
297 Despite significant comparisons on the group level, no regions appeared to be statistically
298 different, however some effect sizes when comparing ephrin-A2A5$^{-/-}$ groups with Wildtype-
299 Sham were moderate (0.3-0.5) suggesting that the proportion of c-Fos in several regions for
300 ephrin-A2/A5$^{-/-}$ mice tended to be larger than Wildtype-Sham.

301

302 **Fig 7. Average region-by-region proportion of total striatal activity in the experimental**
303 **groups.**

304

305 **Table 1. Region by region Mann-Whitney U comparisons for proportion of striatal c-**
306 **Fos**

Group comparison (Wildtype-Sham vs.)	**Mann-Whitney U**	**p**	**Effect Size (Rank biserial correlation)**	**Mean Difference**	**95% Confidence Interval**	
					Lower	**Upper**
Wildtype-rTMS						
Region						
DLS	14	0.926	0.0667	0.271	-2	4
DMS	11	0.537	0.267	-5.5	-22	21
NAc Core	14.5	1	0.0333	0.00368	-18	14
NAc Shell	12	0.662	0.200	5.5	-11	17

Ephrin-Sham						
Region						
DLS	11.5	0.333	0.361	-3.88	-21	3
DMS	15	0.699	0.167	-5	-24	20
NAc Core	11	0.31	0.389	9.5	-9	27
NAc Shell	17	0.936	0.056	3	-13	13
Ephrin-rTMS						
Region						
DLS	4.5	0.276	0.500	2	-2	6
DMS	7	0.714	0.222	4.5	-20	42
NAc Core	9	1	0	-0.0727	-23	17
NAc Shell	6	0.548	0.333	-4.5	-21	8

Discussion

It was hypothesized that there is a shift in neuronal activity towards striatal regions involved in habit formation in ephrin-A2/A5$^{-/-}$ mice as they showed a habitual responding pattern in the previous study [12]. However, contrary to the hypothesis, neuronal activation was mostly seen in the regions of the striatum that are involved in goal-directed behaviour in all animals, regardless of strain. We also hypothesized that the LI-rTMS change in c-Fos densities seen previously in ephrin-A2/A5$^{-/-}$ mice NAc would extend to the dorsal striatum. We did not observe an overall change in total c-Fos activation but in the DMS there was an increase in MSN activation following stimulation in ephrin-A2/A5$^{-/-}$ mice. Although the proportion of c-Fos labelling across the striatum was significantly different across all groups

compared to Wildtype-Sham animals, there was not one specific region driving this difference.

Neuronal activation was highest in regions involved in goal-directed behaviour

The neuronal activation in different regions of the striatum was measured by c-Fos expression. The proportion of overall c-Fos activity was significantly different between Wildtype-Sham group and all other groups. However, comparing the difference in proportions across regions did not identify specific regions that were significantly different to the Wildtype-Sham group. Nonetheless, the biggest effect sizes were associated with the difference between Wildtype-Sham and the Ephrin groups, suggesting less activity in the DLS of Ephrin-rTMS while the comparisons with Ephrin-Sham animals suggest less activity in the NAc and greater activity in the DLS compared to Wildtype-Sham animals. Therefore, it appears that there is an increased proportion of dorsal striatum activity in Ephrin-Sham animals compared to Wildtype animals, and this difference is reduced following LI-rTMS. This is in line with our hypothesis that LI-rTMS may delay an accelerated shift from goal-directed behaviour to habit seen in ephrin-A2/A5$^{-/-}$ mice.

While looking at the change in c-Fos activity in specific striatal regions, it was found that in both sham and treatment groups of ephrin-A2/A5$^{-/-}$ mice, DMS had more c-Fos activity than DLS. Although DMS is a part of the nigrostriatal pathway, it is a transition zone and is versatile in its function as it is involved in both stimulus-response and action-outcome learning [21]. Therefore, DMS shows more activity when the behaviour is goal-directed, and this activity slowly decreases with over-training and shifts to DLS for formation of habits [22]. Similarly, a progression of c-Fos activity during training in a fixed-ratio (FR) task, from higher c-Fos activity in NAc shell on the first day of the task, towards the NAc core, DMS

342 and finally to DLS as training progressed has been previously described [4]. In our study, the
343 major proportion of activity across groups remained in the NAc core and DMS suggesting
344 that behaviour in these mice did remain goal-directed (Fig 7). However, the Ephrin-Sham
345 group showed signs of higher relative DLS activity, and less NAc core activity compared to
346 Wildtype-Sham group which supports our hypothesis that behaviour may have shifted
347 towards habit formation in ephrin-A2/A5$^{-/-}$ mice. A caveat is that, unlike previous studies [4],
348 the neuronal activation reported in this study only reflects the final timepoint, after the
349 completion of PR task. Also, in a PR task, unlike in a FR schedule, the response requirement
350 changes over time so mice cannot generally predict how much work is required to obtain a
351 reward based on previous trials. Therefore, although we can assume a similar hierarchical
352 progression of activity within the striatum, the variable nature of the PR task could impact the
353 behavioural and cellular response differently than the FR framework previously suggested
354 [4].

355 The perseverative and inflexible behaviour in ephrin-A2/A5$^{-/-}$ mice [12] is likely not
356 due to habit formation, because neuronal activity in these mice is mostly localised in regions
357 responsible for goal-directed behaviour. However, previous studies have also shown
358 abnormal behavioural patterns in ephrin-A knockout mice that suggest compulsive or
359 perseverative behaviour. Self-injurious compulsive grooming behaviour has been seen in
360 ephrin-A knockout mice which is similar to the behaviours seen in autism spectrum disorder
361 [23]. This behaviour was attributed to abnormalities in the corticostriatal pathway. Although
362 we saw repetitive responding pattern in the ephrin-A2/A5$^{-/-}$ mice in our study, we did not
363 observe self-injurious compulsive grooming. Furthermore, in another study, maintenance of
364 responding pattern in ephrin-A2$^{-/-}$ mice was observed and this behaviour was insensitive to
365 the reward involved in the task [24]. This suggested perseverative behaviour in these mice
366 and was broadly attributed to abnormalities in the orbitofrontal cortex and the circuitry

involving the thalamus, striatum and the prefrontal cortex. Therefore, perturbations in ephrin-A signalling appear to be consistently linked to compulsive and perseverative behaviour. While dopamine abnormalities play a key role in such behaviours, it may be that disrupted and disorganised organization of neural circuits due to the absence of certain ephrin-A ligands may be a contributing mechanism behind perseverative and compulsive behavioural patterns. Neurological disorders often have genetic and developmental factors contributing to their presentation [25]. Therefore, future studies with ephrin-A2/A5$^{-/-}$ mice could give insight into how disruption of neural circuit organization during development have behavioural impacts [26,27]. Additionally, applying LI-rTMS and its history of successful network reorganization in ephrin models [11,28,29] to more established models of neuropsychiatric disorders, could be an interesting avenue to elucidate therapeutic potential and mechanisms of rTMS.

c-Fos colocalization with MSNs and interneurons in the striatum

MSNs form the major population of striatal neurons as they constitute 95% of striatal neurons and the remaining 5% are interneurons [30]. These interneurons are also quite diverse and have been classified into two major classes, cholinergic interneurons and GABAergic interneurons [31]. In our study, we saw an increase in active MSNs as marked by c-Fos-DARPP32 colocalization in DMS of rTMS-stimulated ephrin-A2/A5$^{-/-}$ mice and not in sham stimulated ephrin-A2/A5$^{-/-}$ mice. This suggests that rTMS specifically enhances DMS MSN activity, which may contribute to delaying the shift in striatal activity to DLS. If LI-rTMS can potentially delay an accelerated shift to habitual behaviour, it would also be interesting to investigate whether higher dose or more prolonged rTMS treatment could reverse the behaviour from DLS to regions that are involved in early acquisition of a behaviour, perhaps through activation of DMS MSN. Such an effect would be relevant to

391 reversing DLS activity associated with overreliance on habits as seen in drug seeking
392 behaviours [32].

393 Although MSNs account for the majority of striatal neurons, in our study qualitative
394 analysis suggests that about 60% and 40% of c-Fos single-labelled cells were not MSNs in
395 the NAc and dorsal striatum, respectively in all experimental groups. This indicates activation
396 of other neuronal populations in the striatum. Therefore, as an exploratory analysis we
397 stained for GABAergic and cholinergic interneurons to identify the nature of the unspecified
398 c-Fos single-labelled cells. In all groups, DMS had more c-Fos^{+}-GAD67^{+}/DARPP32^{-}
399 colocalization and c-Fos^{+}-PV-expressing GABAergic interneurons compared to DLS which
400 could mean more interneuron-related inhibitory activity in the DMS. However, we saw no
401 significant changes in GAD67 activity between groups. In NAc core and shell, across all
402 experimental groups we found that less than 2% of the c-Fos^{+} cells that were colocalised with
403 GAD67^{+}/DARPP32^{-} cells. Therefore, although there were no major group differences with
404 GABAergic interneuron activity, the modulatory role of GABAergic interneurons is likely
405 most influential in the DMS due to the higher proportion of active neurons that are
406 GABAergic interneurons. Lastly, in our qualitative analysis we did not find evidence to
407 support activation of cholinergic interneurons in both dorsal and ventral striatum.

408 As we tested for all neuronal populations in the striatum, the identity of the high
409 number of unspecified c-Fos^{+} cells is still not clear, as together the staining accounted for
410 only around 40% and 50% of c-Fos^{+} cells in the ventral and dorsal striatum, respectively.
411 There is a possibility that these unspecified c-Fos^{+} cells could be glial cells, however, a
412 confirmatory test in the ventral striatum found that most c-Fos^{+} cells were colocalised with
413 neurons [12].

414

415 # Relevance

416 Although the small sample size of the study limits our conclusions, the possibility of
417 rTMS delaying habit formation is exciting. It shines light on potential mechanisms of rTMS
418 as a therapy for disorders such as drug addiction and OCD. rTMS is already used to treat
419 OCD, and there is evidence for anti-craving effects for cocaine [33] and nicotine [34]. The
420 anti-craving effects of rTMS have been thought to be linked to the ability of rTMS to
421 modulate reward circuitry in the brain. For example, rTMS may counteract addiction-related
422 plasticity by increasing dopamine release and dopamine receptor density, as well as
423 modulating glutamatergic receptor expression [8]. Ephrin-A2/A5$^{-/-}$ mice have low dopamine
424 levels in the striatum possibly due to reduced dopaminergic innervation [35,36]. Future
425 studies could look at whether rTMS can alter these abnormal dopaminergic innervations in
426 ephrin-A2/A5$^{-/-}$ mice to normalise dopamine levels in the striatum. Linking this to changes in
427 perseverative behaviour in ephrin-A2/A5$^{-/-}$ mice following rTMS could add to evidence that
428 can support rTMS as a therapy for disorders with overreliance on habits.

429 # Conclusion

430 The present study did not fully support the assumptions made by our previous study
431 [12] in which it was hypothesized that neuronal activity in ephrin-A2/A5$^{-/-}$ mice with
432 repetitive and perseverative responding behaviour would have shifted to the striatal region
433 involved in habit formation. Rather, in this study, it was found that activity remained
434 dominant in regions involved in goal-directed behaviours. However, the relative proportion
435 of c-Fos activity in the striatum slightly shifted towards DLS activation in untreated ephrin-
436 A2/A5$^{-/-}$ mice when compared to other experimental groups. This was in line with our
437 hypothesis that ephrin-A2/A5$^{-/-}$ mice would have greater c-Fos activity in striatal regions

438 associated with habitual behaviour and that LI-rTMS would reduce this difference. As a
439 whole, rTMS seems to partly delay the shift from goal-directed behaviour to habit in ephrin-
440 A2/A5$^{-/-}$ mice, and this appears to be mostly due to rTMS-induced changes in MSN activity
441 in the DMS. Overall, LI-rTMS shows some promise for delaying the shift from goal-directed
442 behaviour to habit and/or compulsive behaviour, on a cellular level. This delay could be
443 further investigated in more clinically relevant animal models as a mechanism behind clinical
444 rTMS effects seen in disorders like drug addiction and OCD.

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448 Author contributions

449 **Maitri Tomar:** Methodology, Writing - original draft, Writing – review & editing, Formal
450 analysis, Investigation, Visualization. **Jennifer Rodger.** Writing – review & editing,
451 Supervision. **Jessica Moretti.** Conceptualization, Writing – review & editing, Methodology,
452 Formal analysis, Visualization, Supervision.

453 Declaration of competing interests

454 The authors declare no conflict of interest.

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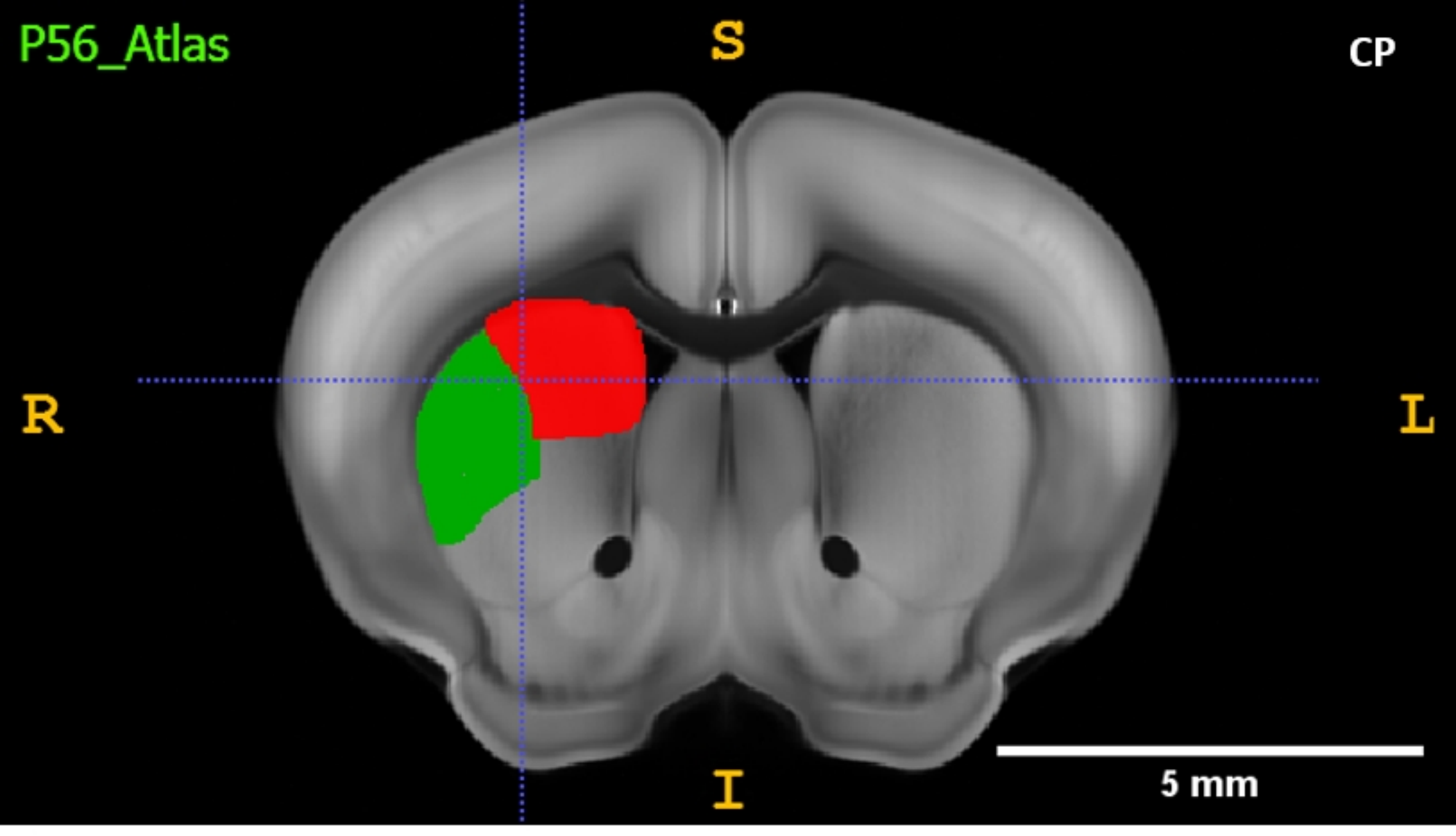

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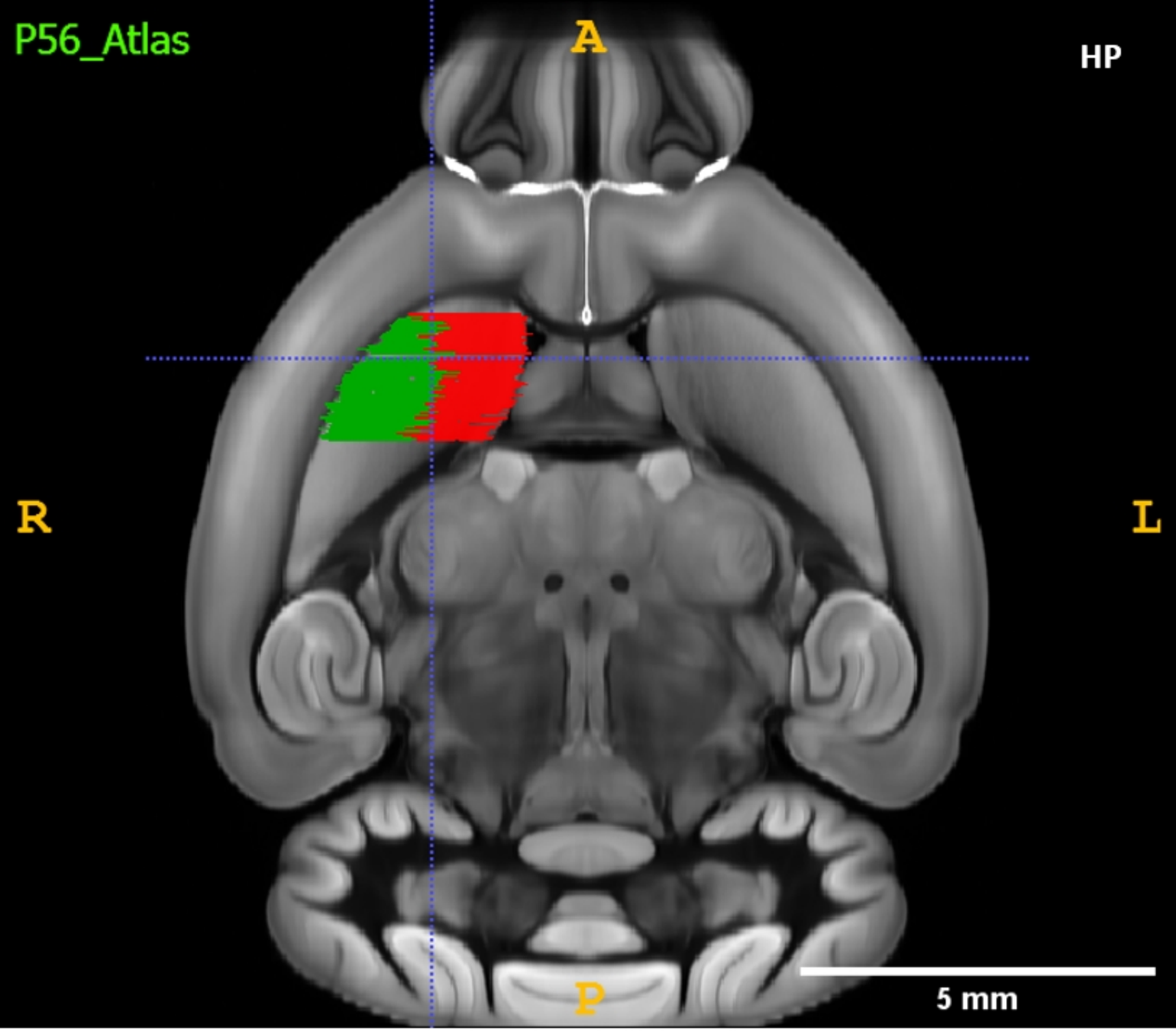

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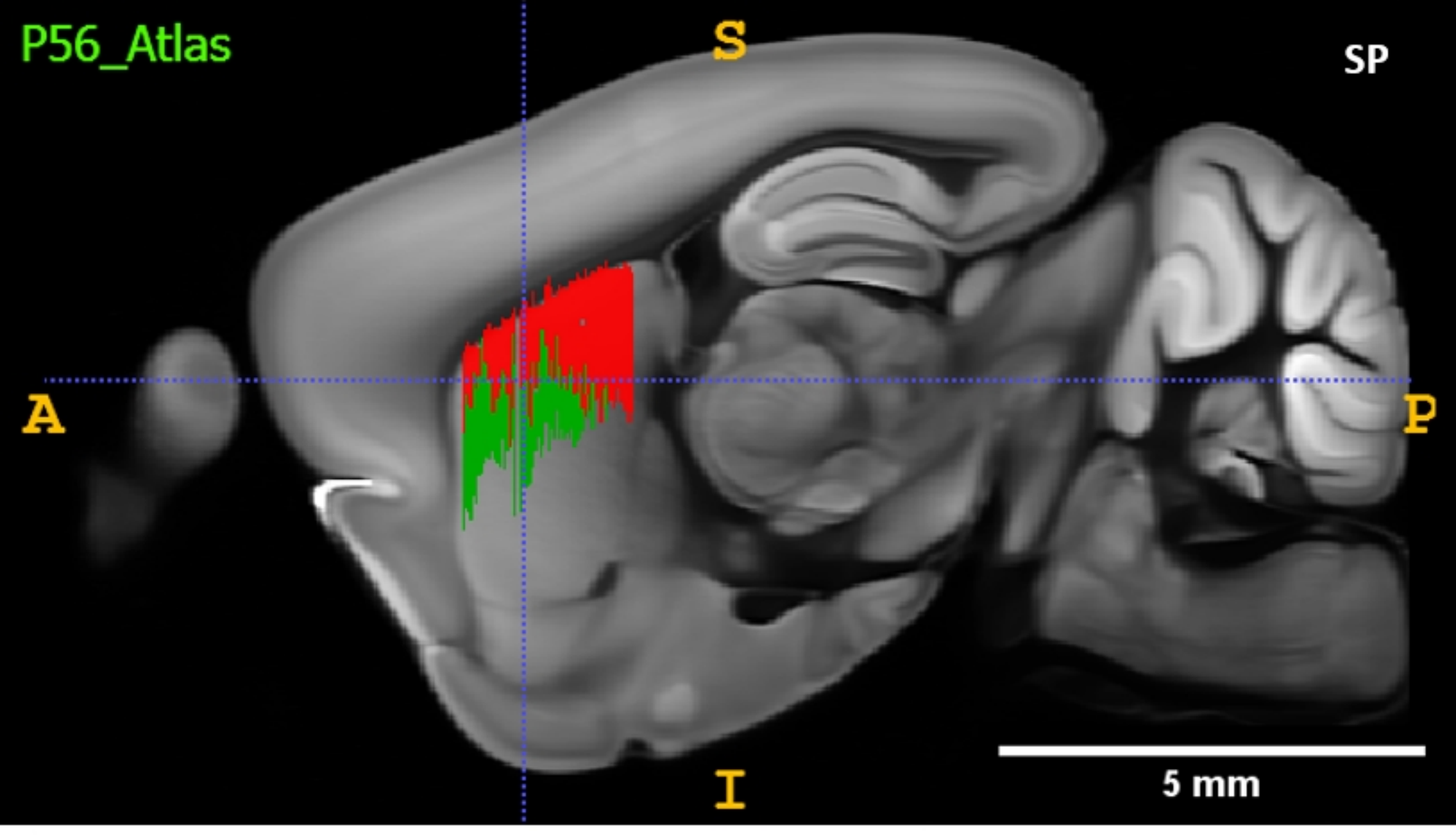

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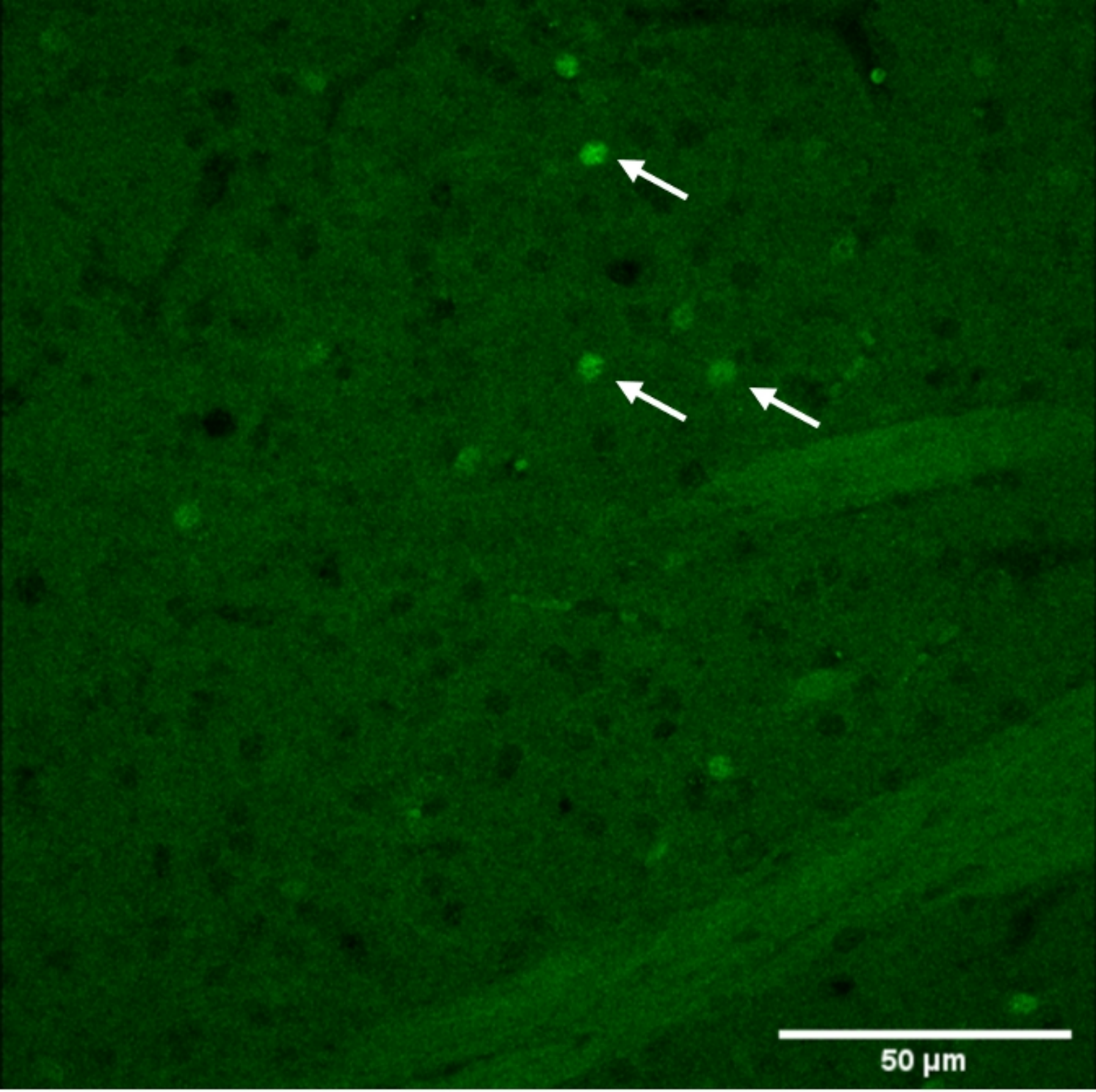

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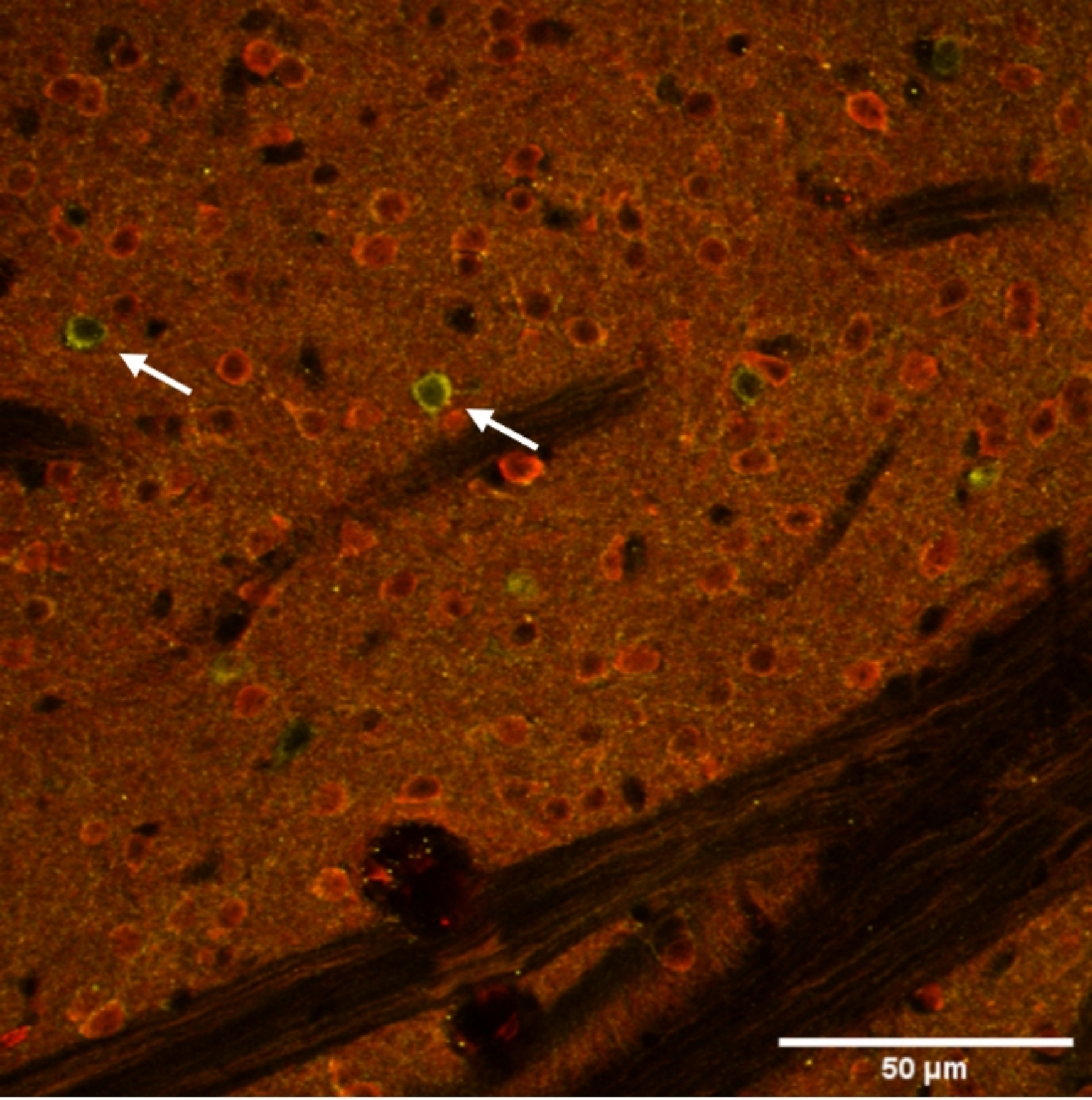

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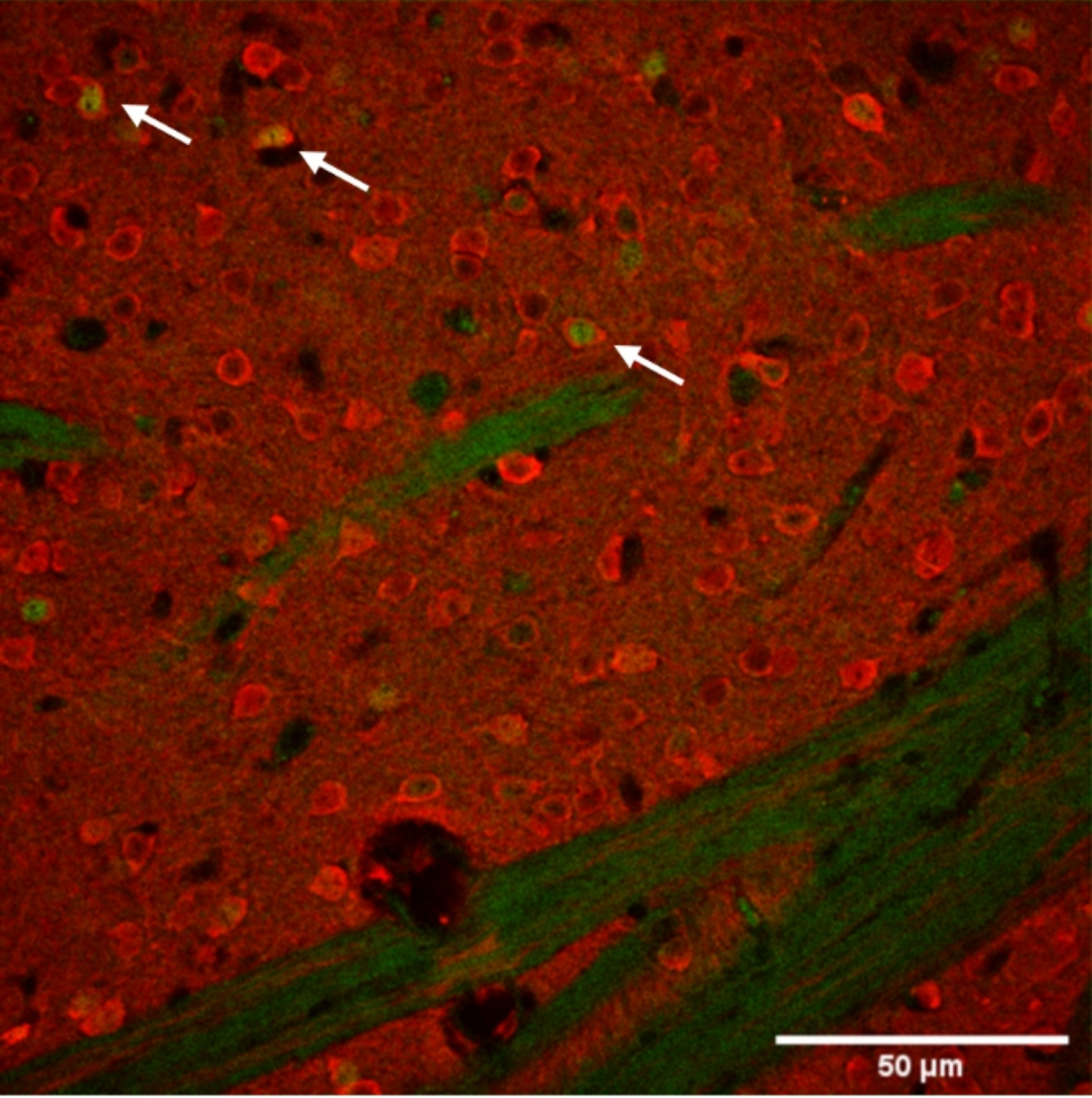

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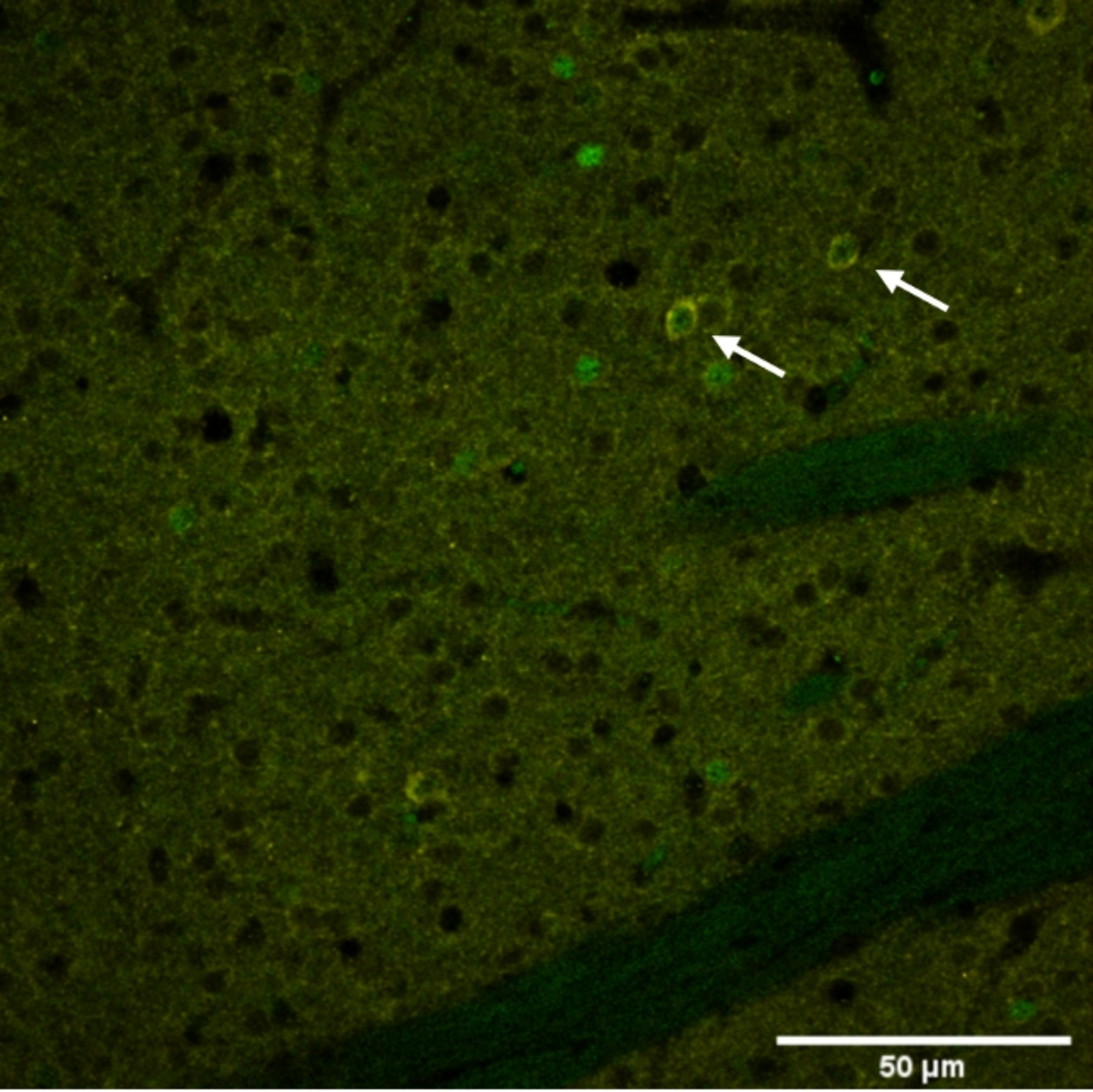

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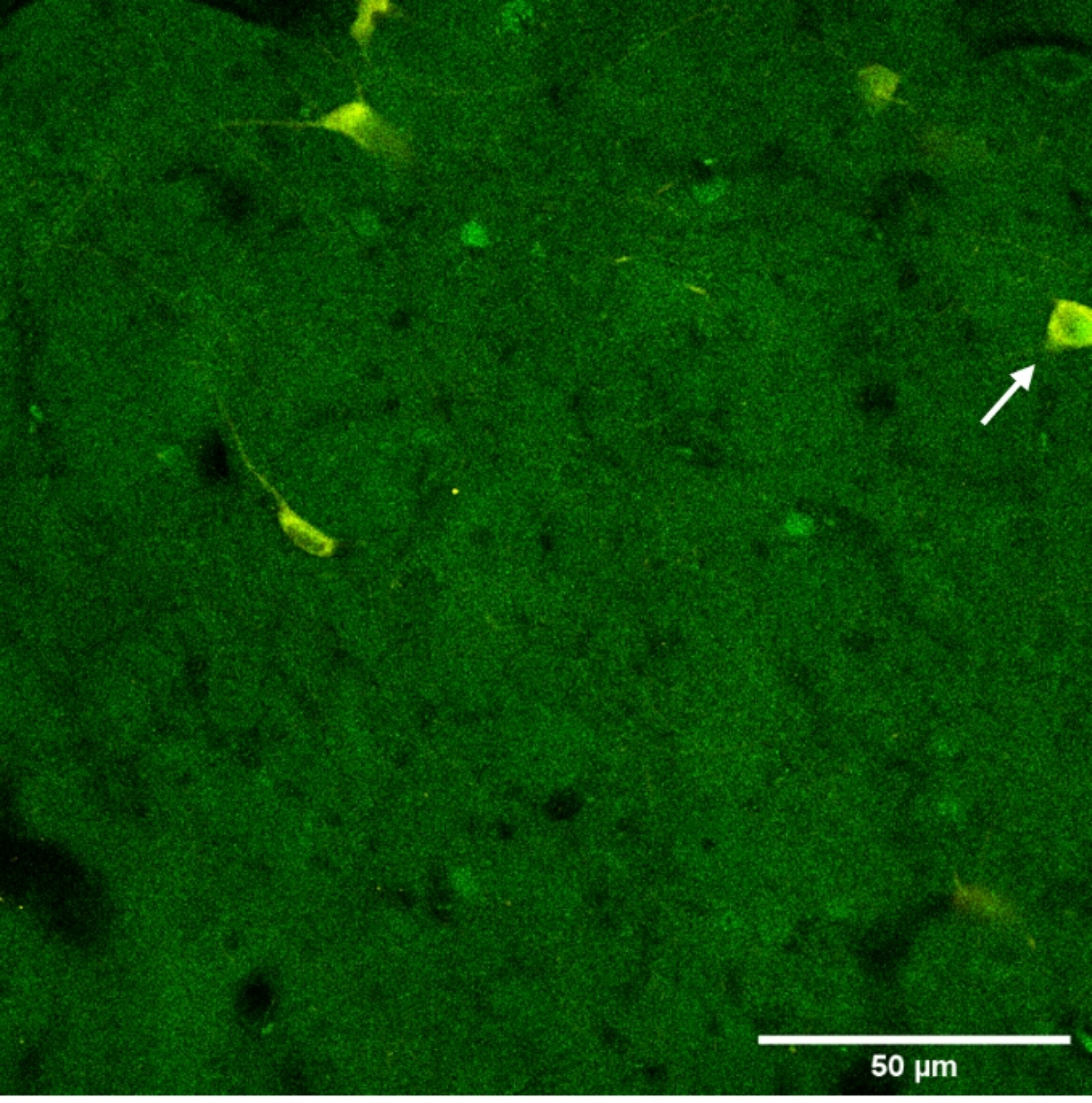

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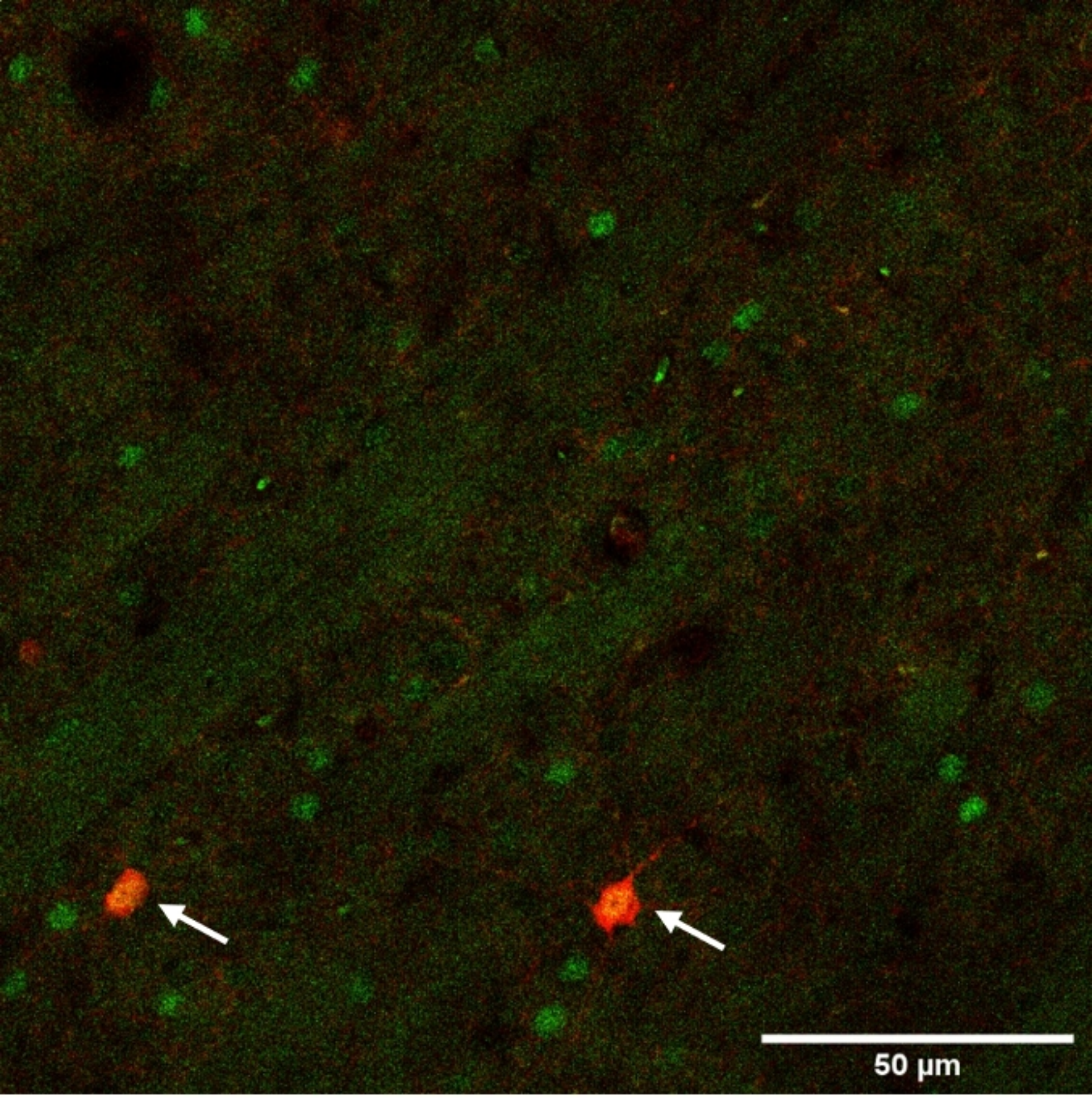

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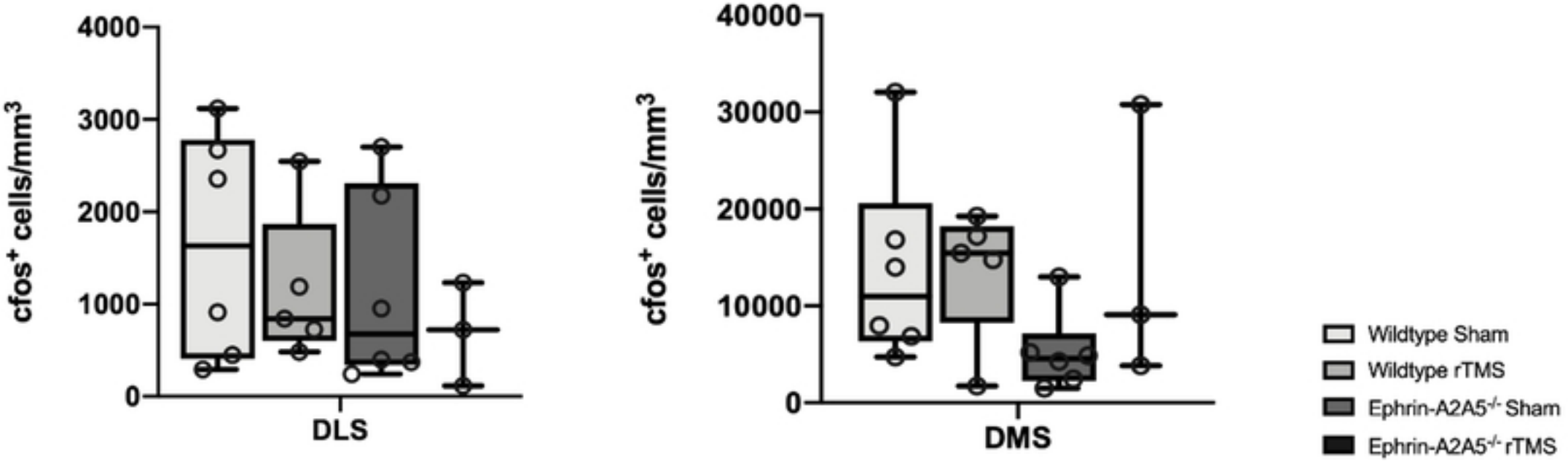
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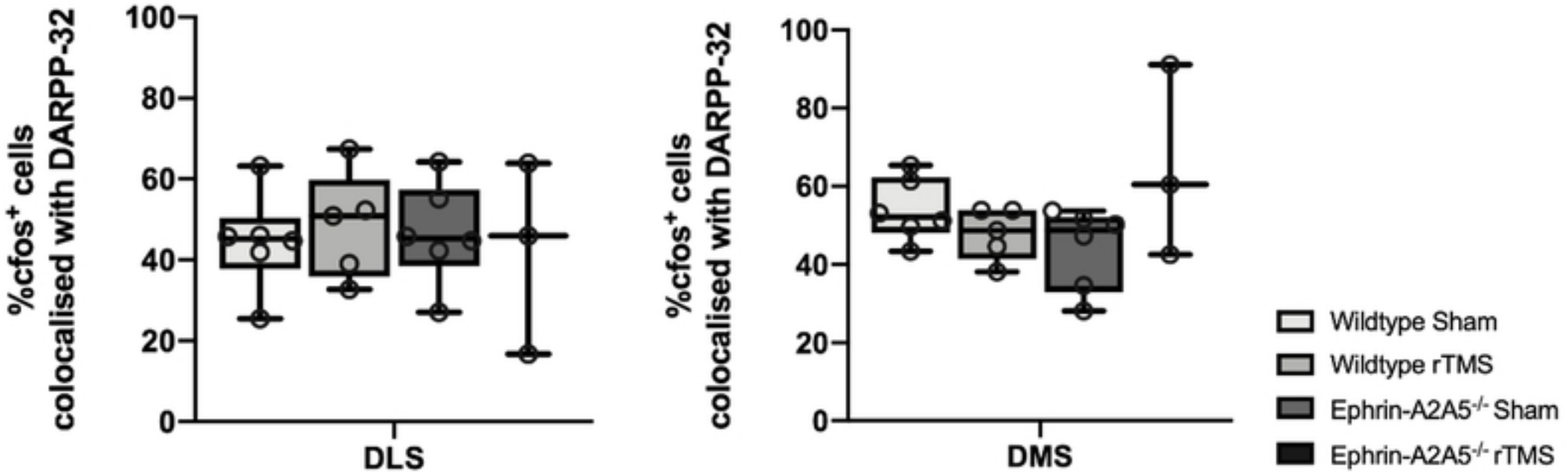
Figure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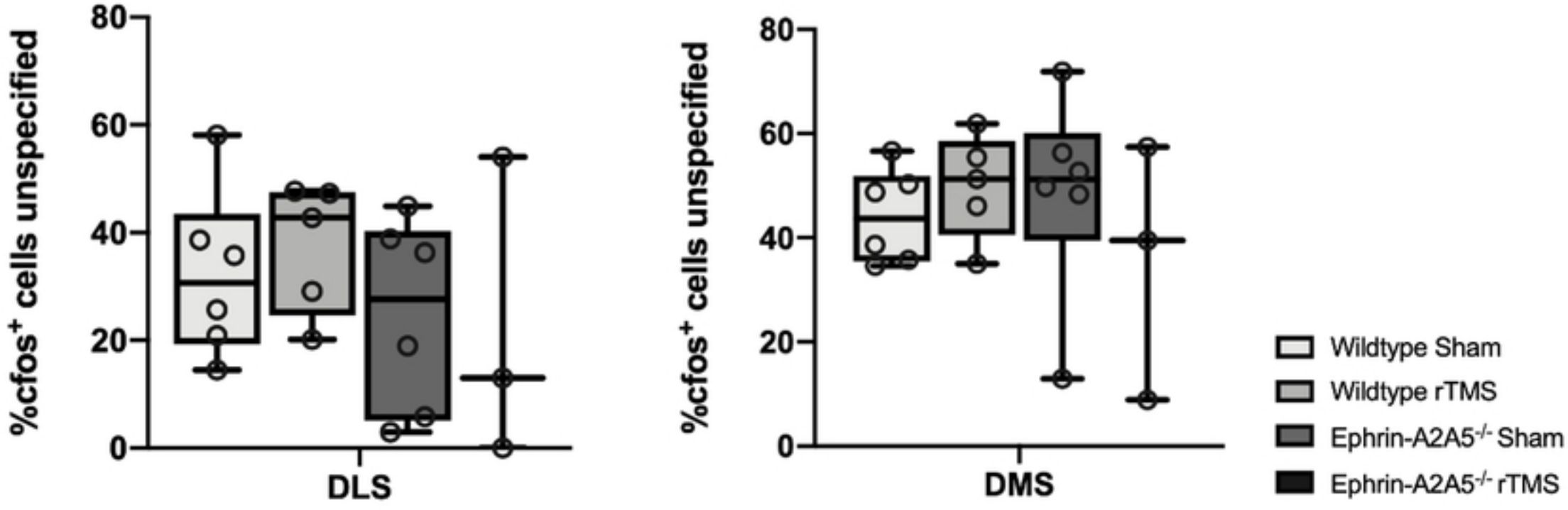
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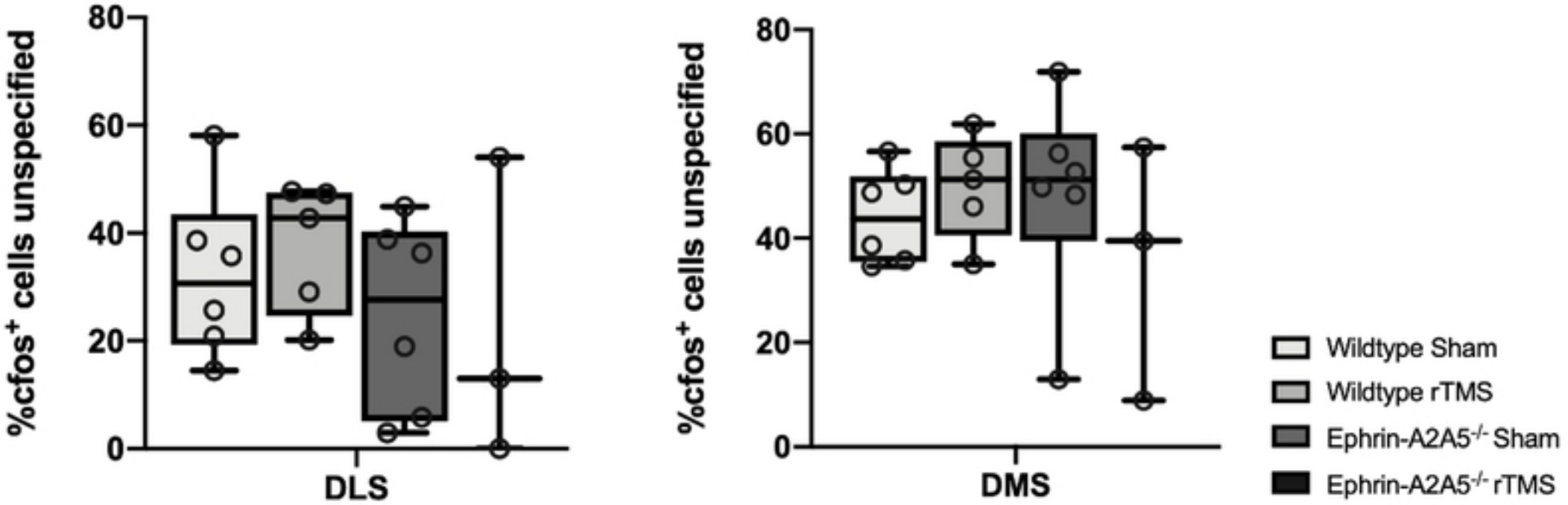
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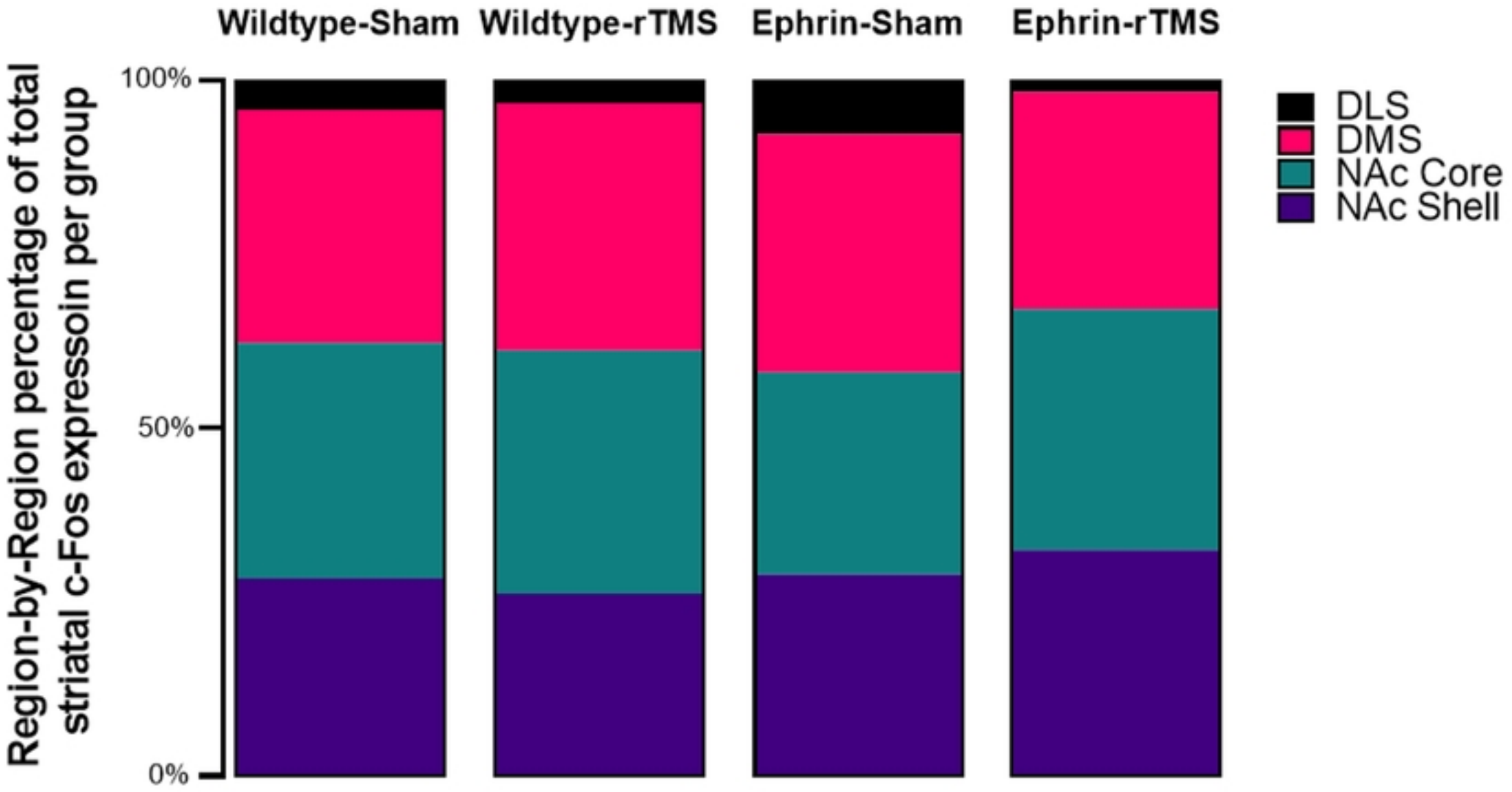
Wildtype-Sham
Wildtype-rTMS
Ephrin-Sham
Ephrin-rTMS
Region-by-Region percentage of total striatal c-Fos expressoin per group
100%
50%
0%
DLS
DMS
NAc Core
NAc Shell

Figure