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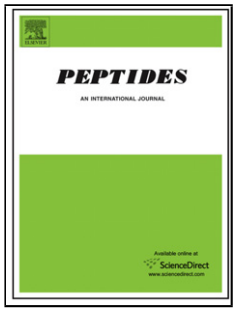

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Cyclic angiotensin-(1-7) contributes to rehabilitation of animal performance in a rat model of cerebral stroke

Anneke Kuipers[a], Gert N. Moll[a,b], Aharon Levy[c], Michael Krakovsky[c] and Rick Franklin[d]

[a]Lanthio Pharma, a MorphoSys AG company, 9727 DL Groningen, the Netherlands, kuipers@lanthiopharma.com, moll@lanthiopharma.com

[b]Department of Molecular Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, 9747 AG Groningen, the Netherlands, g.n.moll@rug.nl

[c]Pharmaseed Ltd, Hamazmera St 9, Ness-Ziona 74047 Israel, ronnie@pharmaseedltd.com, michael@pharmaseedltd.com

[d]Constant Therapeutics LLC, C/O Casner & Edwards, 303 Congress St. Boston, MA 02210 USA, rlf629@gmail.com

Correspondence to: G.N. Moll, Lanthio Pharma, 9727 DL Groningen, the Netherlands

Phone: +31 50 3050247, FAX: +31 50 3632348, e-mail: moll@lanthiopharma.com

Highlights Kuipers 2019b

- cAng-(1-7) induces an increase in cerebral capillary density

- cAng-(1-7) improves neurological severity score
- cAng-(1-7) improves performance in the stepping and forelimb placement tests
- cAng-(1-7) improves performance in the body swing test
- cAng-(1-7) is equally effective as bolus and via alzet pump

ABSTRACT

Peptidase-resistant, lanthionine-stabilized angiotensin-(1-7), termed cAng-(1-7), has shown therapeutic efficacy in animal models of cardiovascular, metabolic, kidney and pulmonary disease. Goal of the present study was testing the capacity of subcutaneously administered cAng-(1-7) to induce rehabilitation of animal performance in the transient middle cerebral artery occlusion rat model of cerebral stroke. 24 hours after ischemic stroke induction, cAng-(1-7) was administered for 28 days at a dose of 500 µg/kg/day, either daily via subcutaneous injection or continuously via an alzet pump. Both ways of administration of cAng-(1-7) were equally effective. Measurements were continued until day 50. Compared to vehicle, cAng-(1-7) clearly demonstrated significantly increased capillary density ($p<0.01$) in the affected hemisphere and improved motor and somatosensory functioning. The modified neurological severity score ($p<0.001$ at days 15 and 50), stepping test ($p<0.001$ at days 36 to 50), forelimb placement test ($p<0.001$ at day 50), body swing test ($p<0.001$ at days 43 and 50) all demonstrated that cAng-(1-7) caused significantly improved animal performance. Taken together the data convincingly indicate rehabilitating capacity of subcutaneously injected cAng-(1-7) in cerebral ischemic stroke.

Key words: lanthionine; angiotensin-(1-7); lanthipeptide; endothelium; arteries; brain

1. Introduction

Cerebral ischemic stroke is a prominent cause of serious, long-term disability and the second leading cause of death worldwide. Ischemic stroke, initiated by occlusion of brain vessels, and hemorrhagic stroke account for, respectively, 80% and 20% of the cerebrovascular accidents. The only FDA-approved treatment for acute ischemic stroke is intravenously administered tissue plasminogen activator. Unfortunately, its therapeutic window is limited to the first three hours of symptom onset, and it confers risk of bleeding. Hence, new treatments for stroke are needed.

Hypertension is a risk factor for the occurrence of stroke [3]. Arterial pressure and cardiovascular disease are largely controlled by the renin-angiotensin system (RAS). The heptapeptide Ang-(1-7) is a key factor of the protective arm of this system and exerts, next to cardiovascular roles, many other therapeutic effects. However, Ang-(1-7) is rapidly degraded by ACE and other peptidases. Upregulation of the angiotensin converting enzyme 2 (ACE2) / angiotensin-(1-7) (Ang-(1-7)) pathway has broad therapeutic potential in stroke [3,36,49].

In contrast to natural Ang-(1-7), lanthionine-stabilized cAng-(1-7), shows resistance to peptidases and is fully resistant to ACE. Its prolonged half-life in vivo allows pulmonary delivery. CAng-(1-7) demonstrated therapeutic efficacy in animal models of, amongst others, myocardial infarction, lung fibrosis, neonatal lung injury, pulmonary arterial hypertension, kidney disease and type 1 and 2 diabetes mellitus [7,21,23].

In this study, we tested cAng-(1-7) for the first time in the transient middle cerebral artery occlusion (tMCAO) rat stroke model. Therapeutic efficacy of cAng-(1-7) was assessed by the following analyses: modified neurological severity score, stepping test, forelimb placement test, body swing test and histology with respect to capillary density.

2. Materials and Methods

2.1. Peptide

4,7 D,L lanthionine-stabilized angiotensin-(1–7) peptide (cAng-(1–7)) was synthesized as previously [23]. In preliminary experiments (data not shown), daily subcutaneous administration by alzet pump of 50 µg/kg/d and 500 µg/kg/d of cAng-(1–7) was applied. The dose of 500 µg/kg/d, used in this study, gave overall a slightly better effect. As vehicle, DPBS buffer of Sigma has been used.

2.2. Animals

Male Sprague-Dawley (SD) rats with an average body weight of 314 g at study initiation (day 1) were obtained from Harlan Laboratories (Israel) and were housed in polyethylene cages (5/cage), measuring 35 × 30 × 15 cm. Rats were housed under standard laboratory conditions, with adequate fresh air supply, a photo cycle of 12 h of light and 12 h of dark and at ambient temperature of 20–24 ^{0}C. Animals were fed ad libitum on a commercial rodent diet (Teklad Certified Global 18% Protein Diet cat #: 106S8216) and had free access to autoclaved and acidified drinking water (pH 2.5 - 3.5). Male rats were used in this study with the purpose to reduce the variability within the groups due to gender, and avoid the female hormonal cycle.

The studies were performed at Pharmaseed Ltd. (Israel) and animal handling was performed according to guidelines of the National Institute of Health (NIH) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The study was performed in compliance with "The Israel Animal Welfare Act" and following approval of the

"The Israel Board for Animal Experiments".

2.3. tMCAO occlusion

On the day of surgery, anesthesia was induced by 4% isoflurane in a mixture of 70% N_2O and 30% O_2 and maintained with 1.5-2% isoflurane. Transient middle cerebral artery occlusion (tMCAO) was performed in which the mid cerebral artery of the right hemisphere was occluded. The tMCAO procedure day is defined as "day 1". A 4-0 monofilament nylon suture, coated with polylysine of 4 cm length was used for the occlusion. The surgical wound was closed and the animals were returned to their cages to recover from anesthesia. Two hours after occlusion rats were re-anesthetized, monofilament was withdrawn to allow reperfusion, the surgical wound was closed and rats were returned to their cages.

Animals were subjected to a modified Neurological Severity Scale (mNSS) test at 24 hours post reperfusion for exclusion criteria. Rats were stratified to the various groups according to the Neuro-Score results, in order to get even groups with similar stroke severity. Ten rats died following stroke induction and were not used. No animals were excluded due to neuroscore criterion. Only animals with an overall score of ≥ 10 were kept included in the study [8]. For all the behavioural tests, the individual who performed the tests was blinded as to the type (vehicle/drug/dose) of the treatment.

2.4. Administration of cAng-(1-7)

Animals in the vehicle-injected group and in the cAng-(1-7)-injected group received daily subcutaneous injections of, respectively, vehicle or 500 µg/kg/d cAng-(1-7) starting on day 2, 24 hours post-surgery, continued for 28 days. In animals of the cAng-(1-7) – alzet pump group

an osmotic alzet pump was implanted subcutaneously for continuous administration of 500 µg/kg/d cAng-(1-7) during 28 days. After the evaluation with the neuroscore for exclusion criteria, each group contained 14 rats.

2.5. Mortality

Death was recorded daily; the dead animal was excluded from the study.

2.6. Body weight

Body weight was measured on days 1, 2 and weekly thereafter, using a laboratory balance.

2.7. Neurological test score

The Modified Neurological Severity Scale (mNSS) analysis, including a set of clinical-neurological tests such as the composite of motor -, sensory -, reflex and balance tests, was applied. The neurological severity scale with a total score 18 was used [8].

2.8. Stepping test

Animals were tested with respect to forelimb akinesia using the stepping test. The animal was held with its hind limbs and one forelimb fixed with one hand and the unrestrained fore-paw was drawn along the table. The number of adjusting steps was counted while the animal was moved sideways along the table surface, 85 cm in approximately five seconds, in the forehand and backhand direction for both forelimbs [35].

2.9. Forelimb placing

For the forelimb-placing test, the examiner, blinded to treatment assignment, held the rat close to a tabletop and scored the rat's ability to place the forelimb on the tabletop in response to whisker, visual, tactile, or proprioceptive stimulation. Separate sub-scores were obtained for each mode of sensory input and added to give total scores (0 = normal, 12 = maximally impaired). Accordingly, the forelimb placing test score (0-12) was composed of the following scores: whisker placing (0-2); visual placing (forward (0-2), sideways (0-2)); tactile placing (dorsal (0-2), lateral (0-2)); proprioceptive placing (0-2). For each subtest, animals were scored as follows: 0.0 = immediate response; 0.5 = response within 2 seconds; 1.0 = response of 2-3 seconds; 1.5 = response of > 3 seconds; 2.0 = no response [17].

2.10. Body swing test

The body swing test was performed as described [5]. The rat was held approximately one inch from the base of its tail. It was then elevated to an inch above a surface of a table. The rat was held in the vertical axis, defined as no more than 10° to either the left or the right side. A swing was recorded whenever the rat moved its head out of the vertical axis to either side. Before attempting another swing, the rat was returned to the vertical position for the next swing to be counted. Twenty total swings were counted. A normal rat typically has an equal number of swings to either side. Following focal ischemia, the rat tends to swing to the contralateral side, which is the left side here. Body swing scores were expressed as delta left turn - right turn.

2.11. Sample Collection and Sacrifice

On day 51 or 52 after stroke induction, all rats from the s.c.-injected vehicle group and the s.c.-injected cAng-(1-7) group (500 μg/kg/d) were anesthetized by ketamine/xylazine, transcardially perfused by paraformaldehyde solution and three sections of brain samples were taken from the same areas of all animals. The sections were taken from the parietal lobe brain region at the infarct area, between -1.8 mm to -2.3 mm relative to Bregma (according to Paxinos Atlas). The sections were stained for immunohistochemical analysis by Smooth Muscle Actin (SMA) and Factor 8 antibodies (Thermo Fisher Scientific, Cheshire UK). For the SMA antibody, the kit's instructions were followed and the histology slides were incubated with the antibody for 20 minutes at room temperature. For the Factor 8 antibody, slides were incubated with the antibody in 2% normal horse serum in PBS, for 30 minutes at 37 °C. On the next day, after three washes with PBS, slides were incubated with biotinylated (AP)-conjugated secondary antibody for one hour at RT. Capillaries were identified under the microscope (Olympus BX43 with x10 magnification) in a total of three random fields from each section and photographed with high-resolution camera connected to a computer. Vascular density count was performed by using Image analysis software mainly used - Image Pro Plus Ver. 5.1 by Media Cybernetics. (+ optional software - Image Scope by Aperio, USA + Image-J by NIH). Density was expressed as the mean number of capillaries per field of view.

2.12. Statistics

Data are presented as the mean with standard error (SEM). Statistical analysis was performed by two-way ANOVA for repeated measures, followed by Bonferroni post hoc tests or, in Fig 6E, by an unpaired t-test. Calculations were performed using GraphPad Prism 8.1.1 software (GraphPad Software, San Diego California USA). Differences between groups, indicated by

asterisks in the figures and corresponding p values in the legends, were considered significant at $p < 0.05$.

3. Results

3.1. Mortality

In the s.c.-injected vehicle group of 14 rats, one rat died after the first dosing. No rats from either the s.c.-injected cAng-(1-7) or alzet pump-administered cAng-(1-7) died during the treatment.

3.2. Body Weight

Throughout the study, no statistically significant differences in body weight were observed among any of the different groups of animals (Fig. 1).

3.3. Neurological test score

As expected, in all groups of rats a sharp decline in neurological score was observed 24 hours after tMCAO followed by spontaneous improvement over time thereafter. However, a clear neurological improvement was observed at study day 15 and lasted up to at least day 50 in both cAng-(1-7)-treated groups compared to vehicle treated control (Fig. 2).

3.4. Stepping test score

Animals were also tested with respect to forelimb akinesia in a stepping test, commonly used for measurement of neuromuscular functions, as an index for motoric functioning of the animal. Spontaneous improvement in motor function over time was observed in all animals that were subjected to tMCAO. However, functional improvement in rats treated with cAng-(1-7) was more pronounced compared to vehicle-treated control. For alzet-pump-administered cAng-(1-7) and for s.c.-injected cAng-(1-7) this improvement reached statistical significance compared to vehicle on days 22 and 29, respectively, which lasted up to at least day 50 (Fig. 3).

3.5. Forelimb placement test score

A forelimb placement test was applied to measure somatosensory and sensory motor deficits. Similar to other tests spontaneous improvement in sensory motor deficit over time was observed in all animals that were subjected to tMCAO. There was additional functional sensory motor improvement in all rats that were cAng-(1-7)-treated compared to vehicle-treated control. This statistically significant improvement was more pronounced in treated animals from both groups on days 36-50 (Fig. 4).

3.6. Body swing test score

Animals were also tested for forelimb akinesia in a body swing test, commonly used for measurement of neuromuscular functions. CAng-(1-7) subcutaneously injected or administered by alzet pump clearly enhanced the recovery with respect to forelimb akinesia and reached statistical significance, compared to vehicle treated control, at day 29 which remained until day 50 (Fig. 5).

3.7. CAng-(1-7) caused an increase in the number of capillaries

Animals, except the alzet-administered cAng-(1-7) group, were examined in case of treatment with cAng-(1-7) (500 µg/kg/d, s.c., n=14) compared to treatment with vehicle (s.c., n=13). Treatment with cAng-(1-7) increased the number of capillaries evaluated by immunostaining and measured by image analysis (Fig. 6ABCDE).

4. Discussion

The RAS system may play a role in cerebral stroke and enhanced signaling via its AT_1 receptor (AT_1R) of centrally delivered angiotensin II can provoke ischemic stroke and neuronal damage [44]. Inhibition of this axis by the use of peripherally delivered ACE inhibitors (<500 Da) showed neuroprotection in rodents with focal cerebral ischemia [10]. ACE inhibitor captopril-induced elevation of Ang-(1-7) levels caused neuroprotection in rat cerebral ischemia [45].

Counterbalancing of the ACE / Ang II / AT_1R axis by the protective ACE2 / Ang-(1-7) pathway resulted in neuroprotection in stroke models [3]. Ischemic cerebral stroke itself caused in rats increased levels of ACE2 and Ang-(1-7) [32]. Overexpression of ACE2 led to brain protective effects [9,50,51]. Intracerebroventricular infusion of an ACE2 activator significantly attenuated the cerebral infarct size and neurological deficits [33]. In vitro, Mas receptor agonist AVE 0991 exerted direct protective effects on neuronal cells [27]. Intranasal delivery of AVE 0991 attenuated neuronal apoptosis via the MasR/PKA/CREB/UCP-2 pathway after hemorrhage in rat [34].

Natural Ang-(1-7) has been suggested to act in vivo via the Mas receptor [42]. The neuroprotective effect of natural Ang-(1-7) in stroke could be partly or fully inhibited by A779

[15,18,19,33,39]. Moreover, the anti-inflammatory effects caused by Ang-(1-7) were not reversed by PD123319, an angiotensin II type 2 receptor antagonist [18]. A recent in vitro study showed that Ang-(1-7) amplified MasR-induced negative modulation of cAMP levels and suggested that discrepancies with other in vitro studies [13,46,47] might be due to differences in experimental design [6]. Ang-(1-7) might also act as AT_1-R-biased agonist, selectively promoting β-arrestin activation while blocking the unfavorable Ang II/AT_1-R/Gq axis [14]. In addition, Mas/AT_1 receptor heterodimerization [22] and / or Mas/AT_2 receptor heterodimerization [28] urge to more than usual prudence in the interpretation of Mas receptor specificity and activation in both in vitro and in vivo studies.

Significant cerebroprotective actions of intracerebroventricularly administered Ang-(1–7) (899 Da) have been clearly demonstrated in many studies [1,18,19,33,39,40]. Cerebral stroke may initially cause BBB dysfunction (20). Therefore it can not be excluded that cAng-(1-7) passes the BBB early after the stroke induction. Intracerebrally administered Ang-(1-7) exerts protective effects on the BBB integrity, which may contribute to recovery after cerebral stroke, by regulating the tight junction protein expression [30,48]. Restoration of BBB integrity importantly contributes to reducing stroke-related neurological damage [2].

Interestingly, an orally delivered cyclodextrin-formulated Ang-(1-7) reduced in cerebral infarct volume and improved neurological functions. The authors speculated that Ang-(1-7) might pass the BBB that might be compromised during stroke [4]. In healthy rats, some intravenously injected natural Ang-(1-7) (899 Da) and more of the glucosylation-stabilized Ang-(1-7) (1278 Da) could be retrieved from cerebrospinal fluid [16], however, no mechanism of BBB passage was proposed and the used dosage of 10 mg/kg was high.

The BBB protects the central nervous system and limits the entry of, not only harmful molecules and endogenous molecules, but also of therapeutic peptides to the brain. Therefore,

nose-to-brain delivery of peptides is being investigated as an alternative route. CAng-(1-7) does not comprise a transport-specific ligand that would allow transport across the BBB. It is larger than the common upper limit of 500 Da for passive permeation across a membrane. CAng-(1-7) is very hydrophilic with high hydrogen bonding potential and –although cyclized- definitely lacks the lipophilicity that would facilitate crossing the BBB by diffusion [11,26]. Consistent with difficult delivery across the BBB, natural Ang-(1-7) has been administered by intracerebral infusion in many studies [1,18,19,30,33,39,40,48]. In essence, the efficient delivery of peptides across the intact BBB to the brain requires invasive techniques. It has been recognized that the major hurdle in exploiting the ACE2/Ang-(1–7) cerebroprotective axis is to find post-stroke treatments that can be administered non-invasively [38]. Peptides that may exert important cerebroprotective action after peripheral s.c. administration, like observed in the present study, are therefore clearly of special interest.

CAng-(1-7) activity was abolished or reduced by the Ang-(1-7) receptor antagonist D-Pro7/D-Ala7 [21]. CAng-(1-7) and Ang-(1-7) stimulate the proliferation of endothelial progenitor cells in the bone marrow [41,43]. After stroke bone-marrow-originating endothelial progenitor cells migrate to the injured area to contribute to vascular remodeling and angiogenesis. They can interact with endothelial cells, extravasate, and reach the injured site, where they incorporate into the vascular wall thus repairing endothelial damage occurring in stroke. They also produce trophic factors supporting neuroprotection. In stroke, angiogenesis and neurogenesis are coupled by signaling from exosomes [2,12]. CAng-(1-7) clearly caused significant increase in capillary density. A previous study on natural intracerebrally administered Ang-(1-7) demonstrated that administration prior to stroke induction promotes endothelial cell proliferation and increases brain capillary density [19]. Li et al demonstrated that cerebral capillary density supports health after ischemia [29]. Accordingly, patients with a greater cerebral capillary density have a better ischemic tolerance than patients with lower

capillary density [24]. Furthermore cAng-(1-7) might improve vasodilatory capacity [25,31], exert anti-inflammatory effects [18] and/or stimulate brain-derived neurotrophic factor [37].

The rat stroke tMCAO model is a traditionally accepted model for screening and evaluation of neuroprotective and rehabilitative treatments. In this study, cAng-(1-7) clearly and significantly improved motor functions evaluated by neuroscore, stepping test and body swing test. Sensory motor functions were also sensitive to the treatment with cAng-(1-7) witnessed by the forelimb placement test. The effect of cAng-(1-7) reached its highest significance in the period of day 29 to 50. In this rat model administered at a dose of 500 µg/kg/d, cAng-(1-7) exerted a comparable effect when administered continuously via the alzet pump or when injected. Taken together these data indicate that rehabilitation of performance found in the present study might result from enhanced Mas receptor stimulation.

CAng-(1-7) has strongly enhanced resistance to peptidase-mediated breakdown [21]. Taken together with the data in the current study, further studies on the therapeutic capacity of this cyclized angiotensin-(1-7) receptor agonist may be of interest. It will be important to assess whether or not the therapeutic potential of this receptor agonist differentiates from AT_1R blockers and / or ACE inhibitors.

5. Conclusion

It can be concluded that under the conditions of the present study, subcutaneously administered cAng-(1-7) treatment clearly improved the animal performance with respect to motor and somatosensory deficits in the rat stroke model. Furthermore, cAng-(1-7) significantly increased capillary density. Taken together, cAng-(1-7) is of interest as potential treatment for recovery after ischemic stroke.

Conflict of interest statement

Declarations of interest: none.

Author contributions

Pharmaseed Ltd is acknowledged for performing the experiments. Author contributions: A.K. performed statistical analyses and wrote the manuscript, G.N.M. contributed to writing the manuscript, R.L. and M.K. supervised the experiments and R.F. designed the experiments.

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LEGENDS TO THE FIGURES

Fig. 1. CAng-(1-7) did not alter body weight of rats after stroke.

cAng-(1–7) (500 µg/kg/d) subcutaneously injected (n=14) or administered by alzet pump (n=14) does not significantly alter body weight, when compared with vehicle (n=13). Data are presented as means ± SEM.

Fig. 2. CAng-(1-7) significantly improves the neuroscore after stroke.

After stroke induction, rats were treated with s.c.-injected vehicle or with subcutaneously injected cAng-(1-7) (500 µg/kg/d) or with alzet pump-administered cAng-(1-7) (500 µg/kg/d). Modified neurological severity scale data, up to a score of 18, which represents the most severe neurological situation, are presented as means + SEM. There were no significant differences between the cAng-(1-7) treated groups. Statistically significant differences of each cAng-(1-7) group compared to vehicle are indicated by asterisks in the figure, *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 3. CAng-(1-7) reduces forelimb akinesia after stroke.

Stepping test data are presented as means + SEM. No significant differences between the two cAng-(1-7)-treated groups were measured. Statistically significant differences were found between vehicle-treated rats (s.c, n=13) compared to the cAng-(1-7)-treated rats (500 µg/kg/d, s.c., n=14) and to the cAng-(1-7) treated rats (500 µg/kg/d alzet, n=14) on days 22-50. Significance is indicated by asterisks in the figure, *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 4. CAng-(1-7) improves somatosensory and sensory motor deficits after stroke.

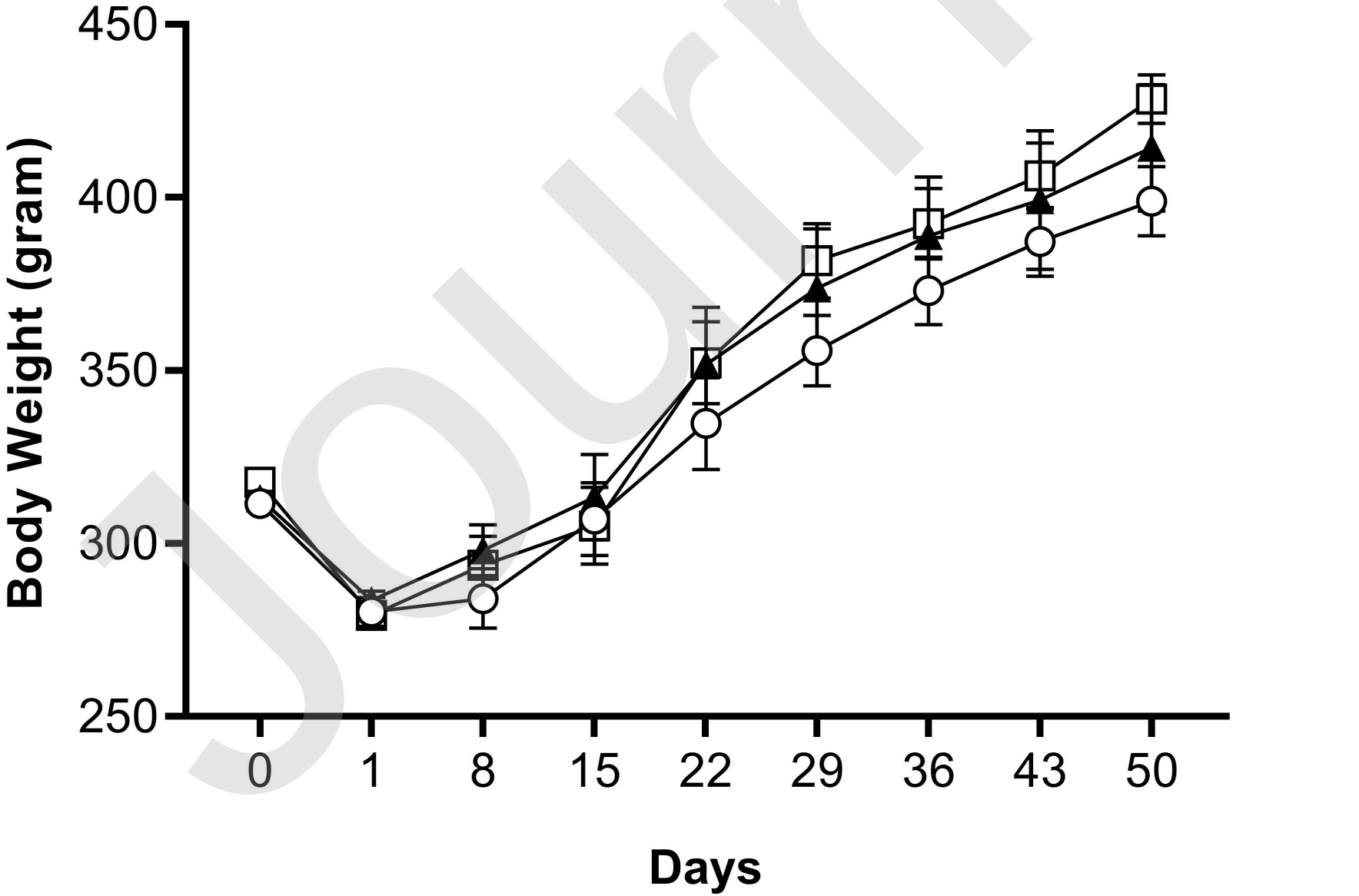
Data from a forelimb placement test between (0) and maximally impaired (12) are presented as means + SEM. No significant differences were measured between both cAng-(1-7)-treated groups. Statistically significant differences were found between vehicle, (s.c., n=13) compared with cAng-(1-7) (500 µg/kg/d, s.c., n=14) or cAng-(1-7) (500 µg/kg/d alzet, n=14) as indicated by asterisks in the figure, *p < 0.05; ** p < 0.01; *** p < 0.001.

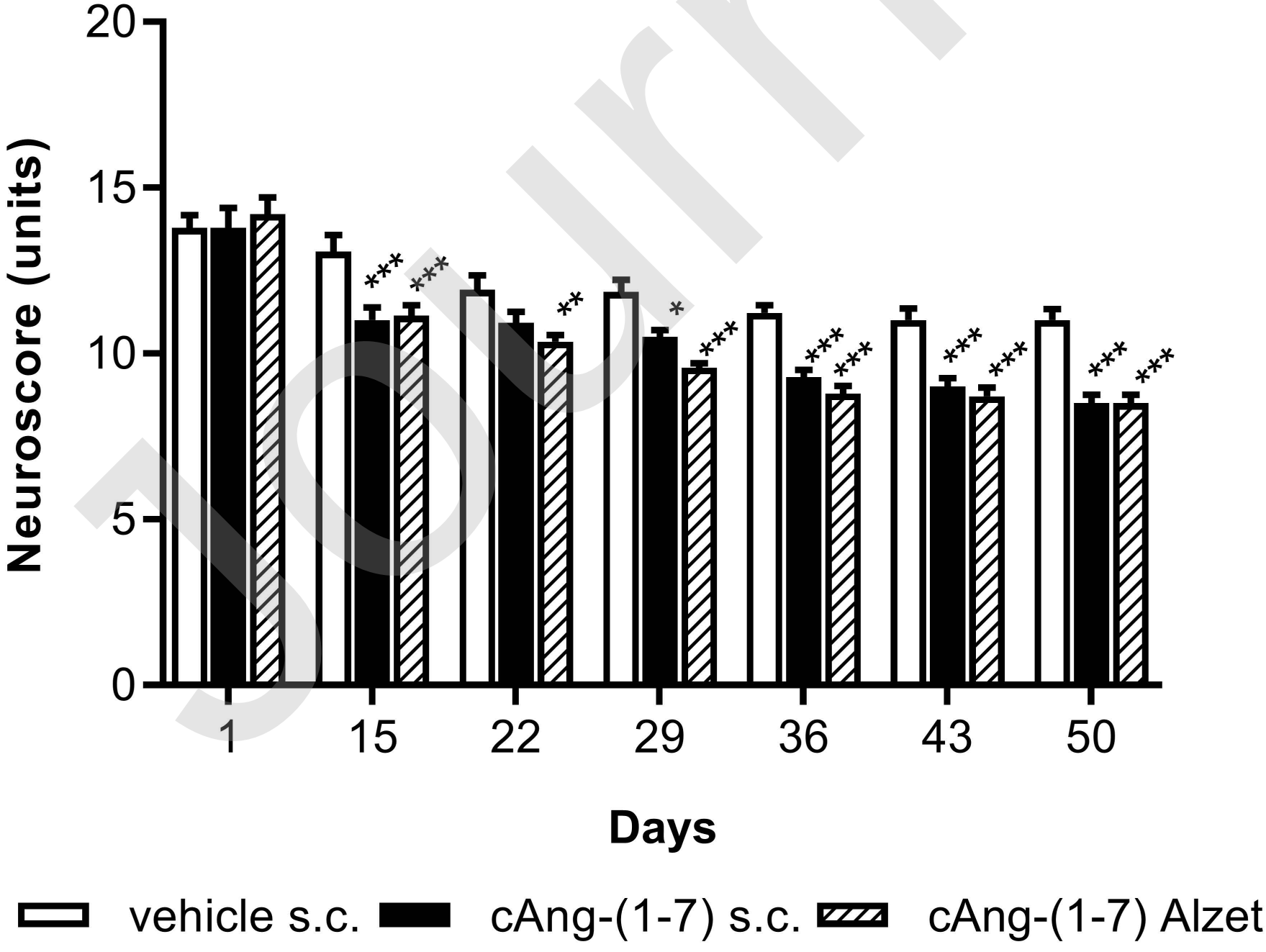
Fig. 5. CAng-(1-7) improves rat's functioning in the body swing test delta after stroke.

Data are represented as means + SEM. No significant differences between the cAng-(1-7)-treated groups were observed. Statistically significant differences were measured between vehicle (s.c., n=13) compared with cAng-(1-7) (500 µg/kg/d s.c., n=14) or with cAng-(1-7) (500 µg/kg/d, alzet, n=14) as indicated by the asterisks in the figure, *p < 0.05; ** p < 0.01; *** p < 0.001.

Fig. 6ABCDE. CAng-(1-7) increases capillary density after stroke.

Figure A, B, C and D are representative images from the parietal lobe of 13 (A,C) and 14 (B,D) animals. The scale bar in Figures A, B, C, D represents a distance of 50 µm. A, SMA staining of vehicle-treated animal. B, SMA staining of cAng-(1-7)-treated animal. C, Factor 8 staining of vehicle-treated animal. D, Factor 8 staining of cAng-(1-7)-treated animal. E, capillary density ratio (ipsilateral/contralateral hemisphere) in treated rats compared to vehicle control on day 50. Data are presented as percentage relative to the left undamaged hemisphere + SEM. Statistically significant difference were found between the cAng-(1-7)-treated group compared to the vehicle-treated control group as indicated by asterisks in the figure, ** p < 0.01.


450
400
350
300
250
Body Weight (gram)
0
1
8
15
22
29
36
43
50
Days
vehicle s.c.
cAng-(1-7) s.c.
cAng-(1-7) Alzet


20
15
10
5
0
Neuroscore (units)
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*
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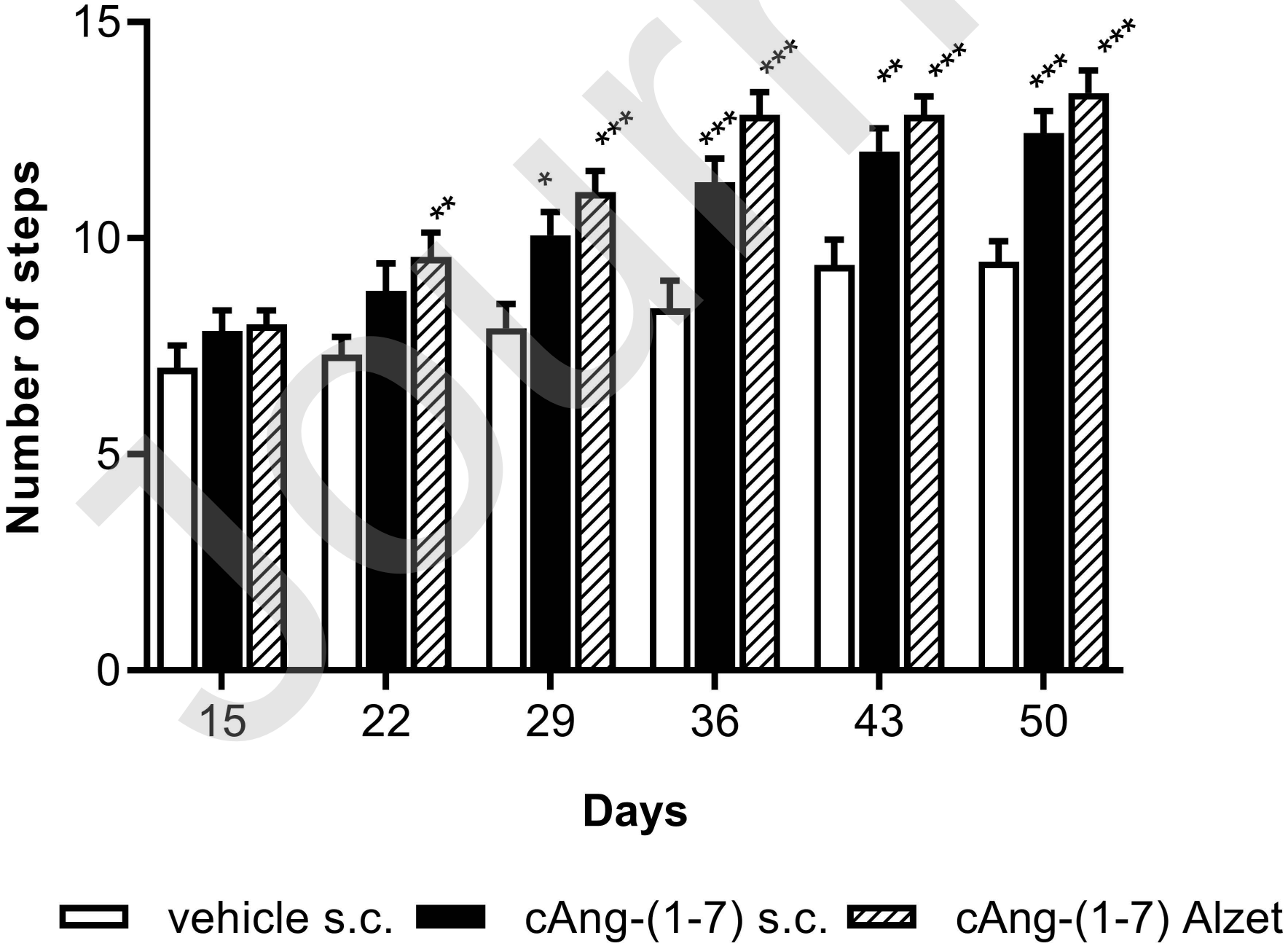
1
15
22
29
36
43
50
Days
vehicle s.c.
cAng-(1-7) s.c.
cAng-(1-7) Alzet


15
10
5
0
Number of steps
15
22
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36
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Days
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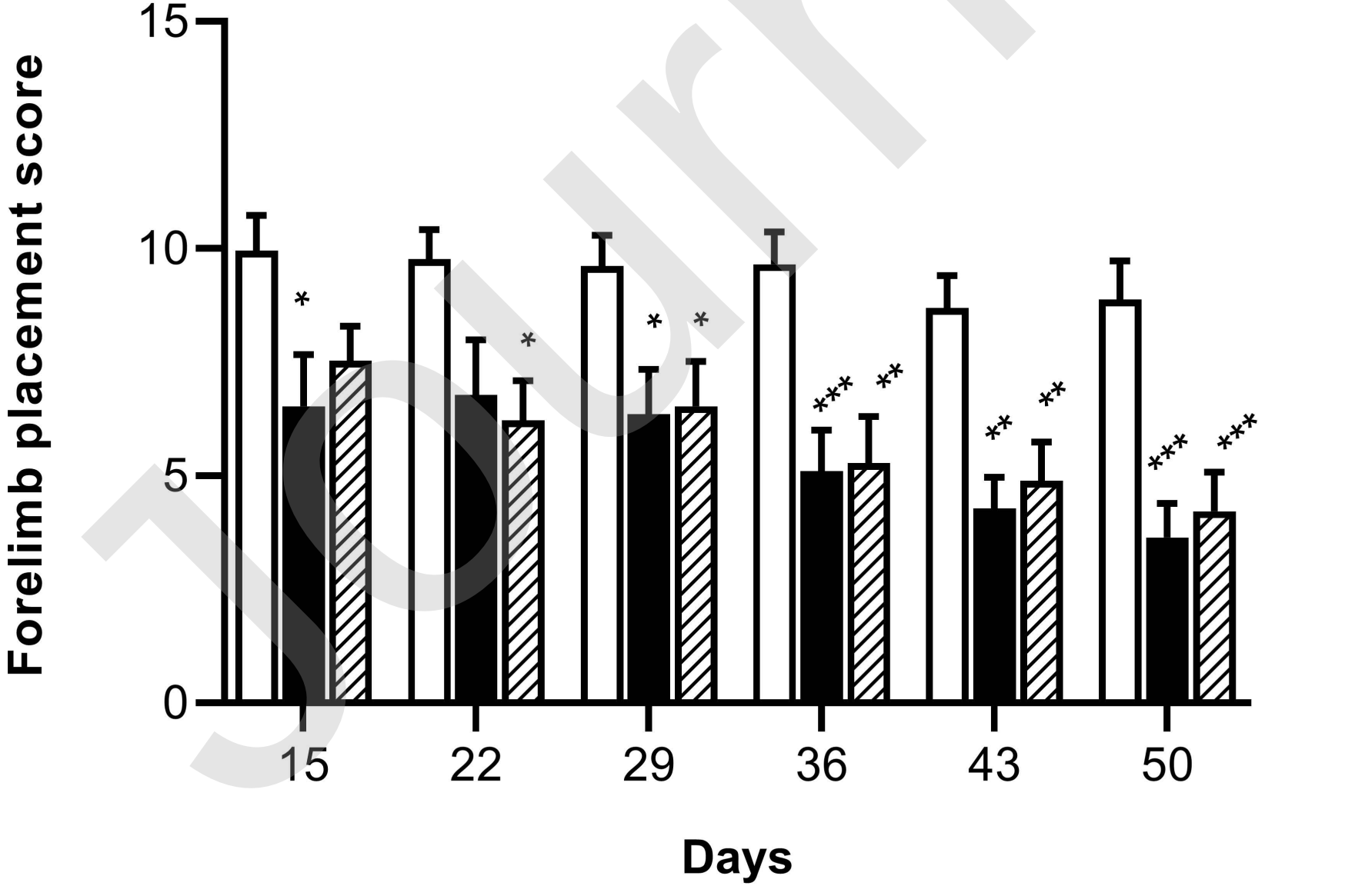
vehicle s.c.
cAng-(1-7) s.c.
cAng-(1-7) Alzet


Forelimb placement score
15
10
5
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Days
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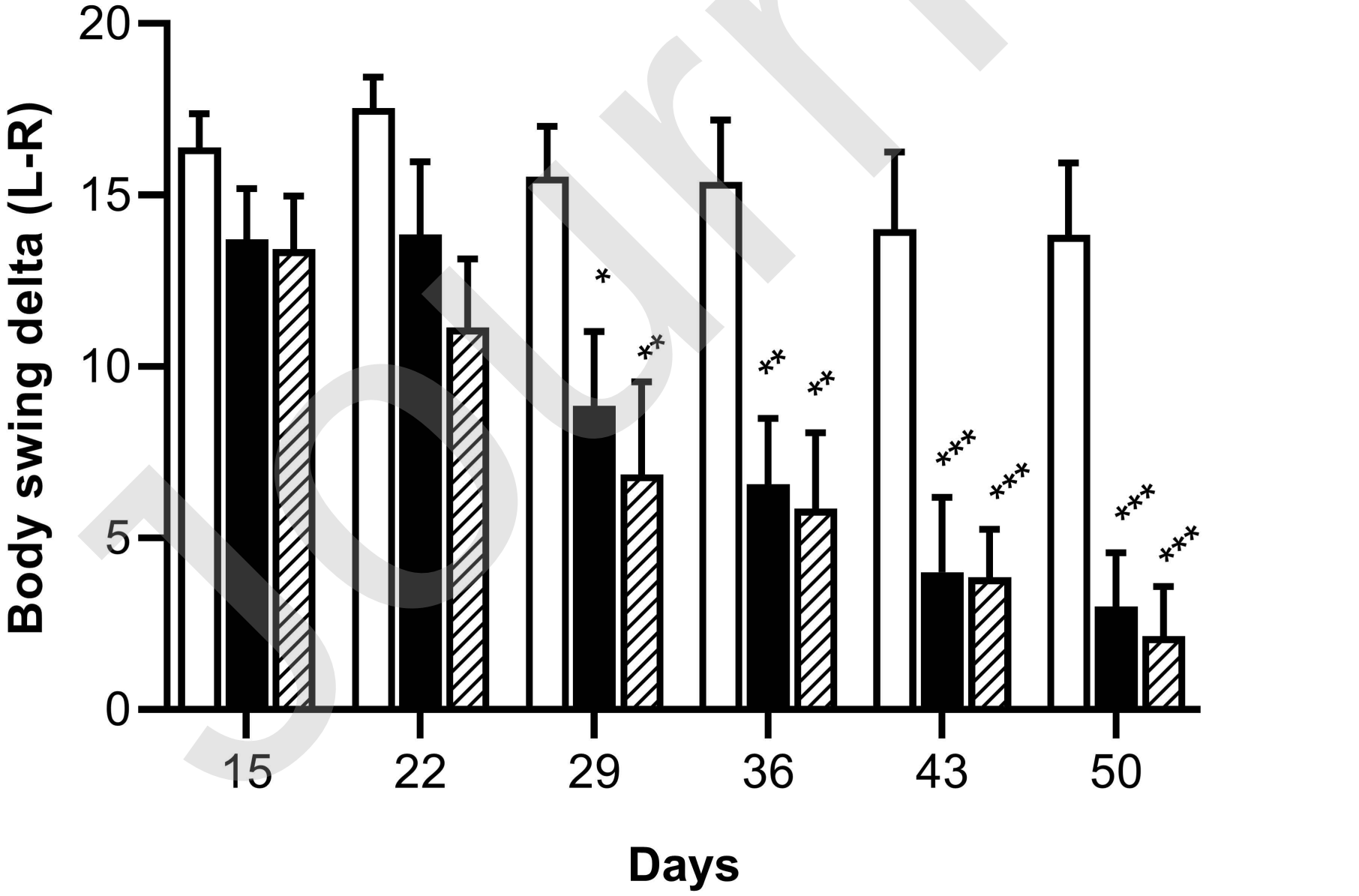
vehicle s.c.
cAng-(1-7) s.c.
cAng-(1-7) Alzet


Body swing delta (L-R)
20
15
10
5
0
15
22
29
36
43
50
Days
*
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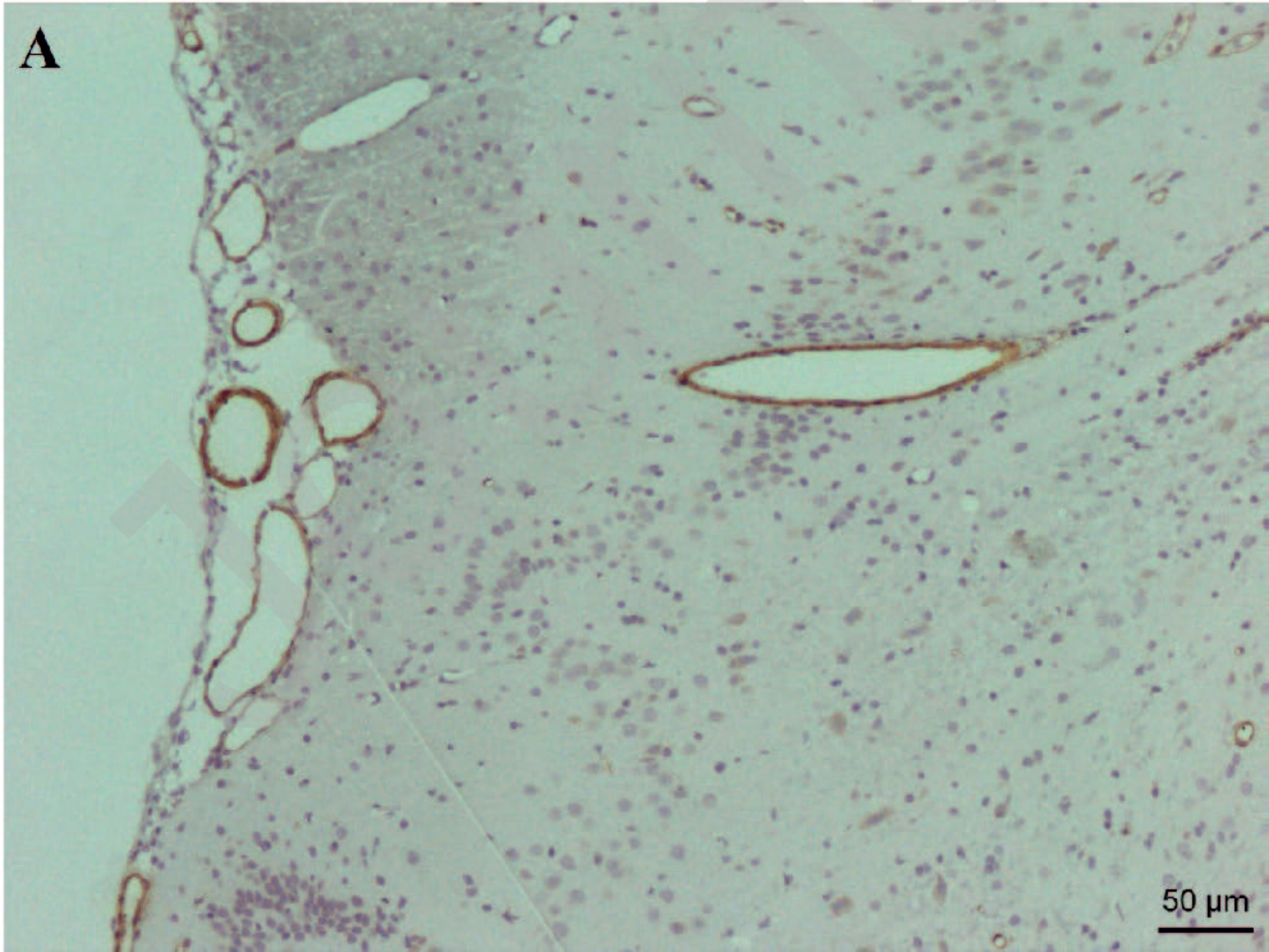
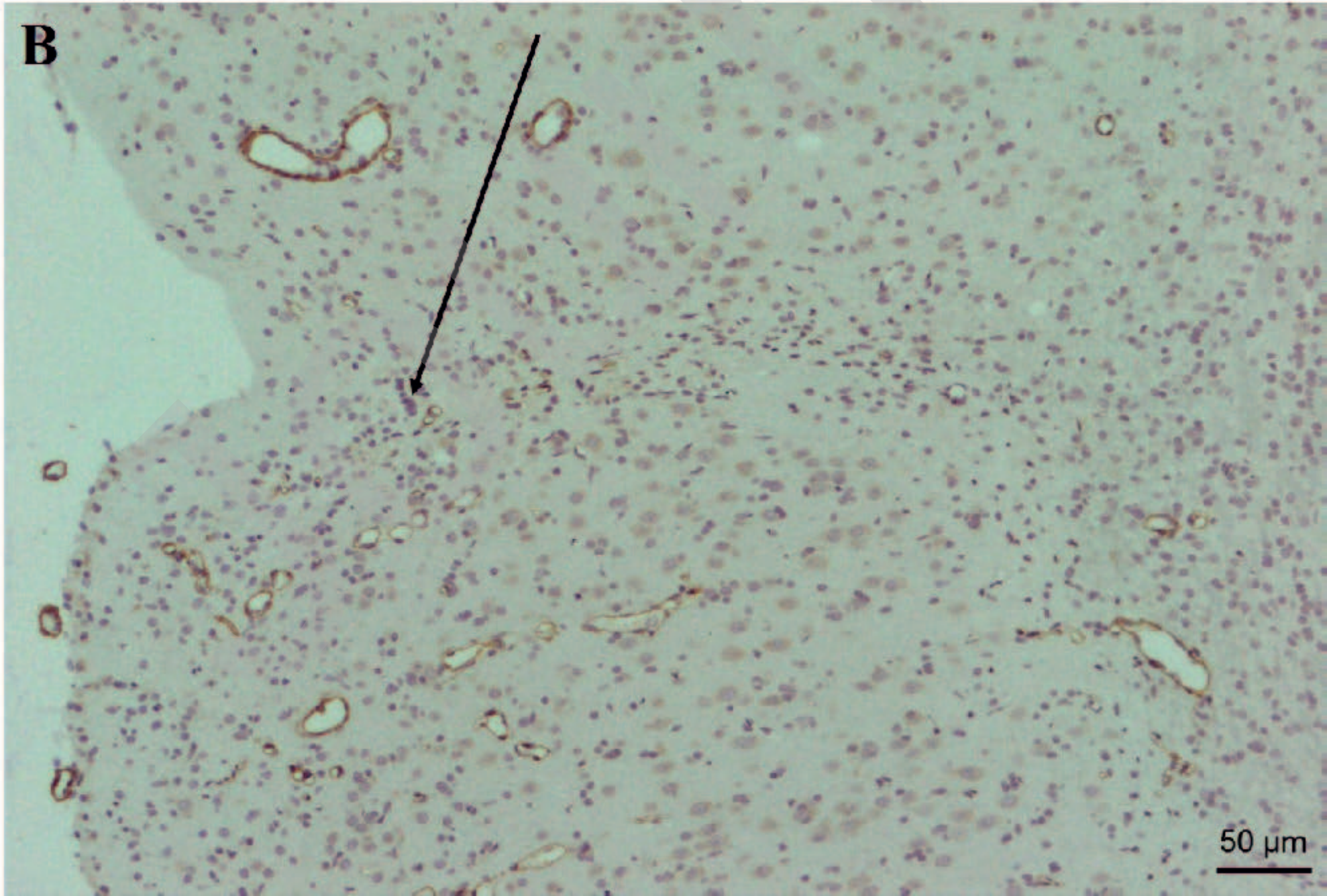
vehicle s.c.
cAng-(1-7) s.c.
cAng-(1-7) Alzet


A
50 µm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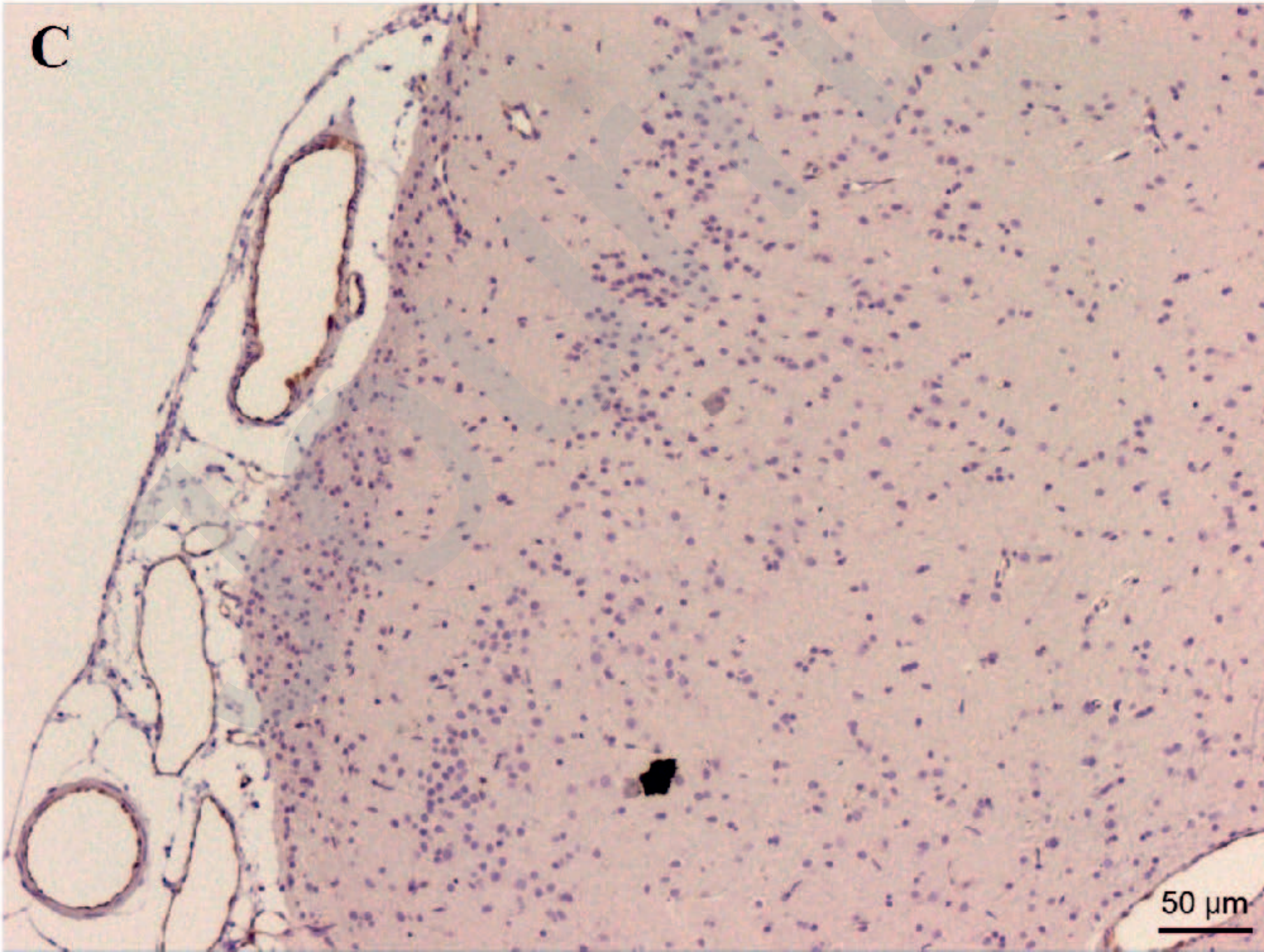
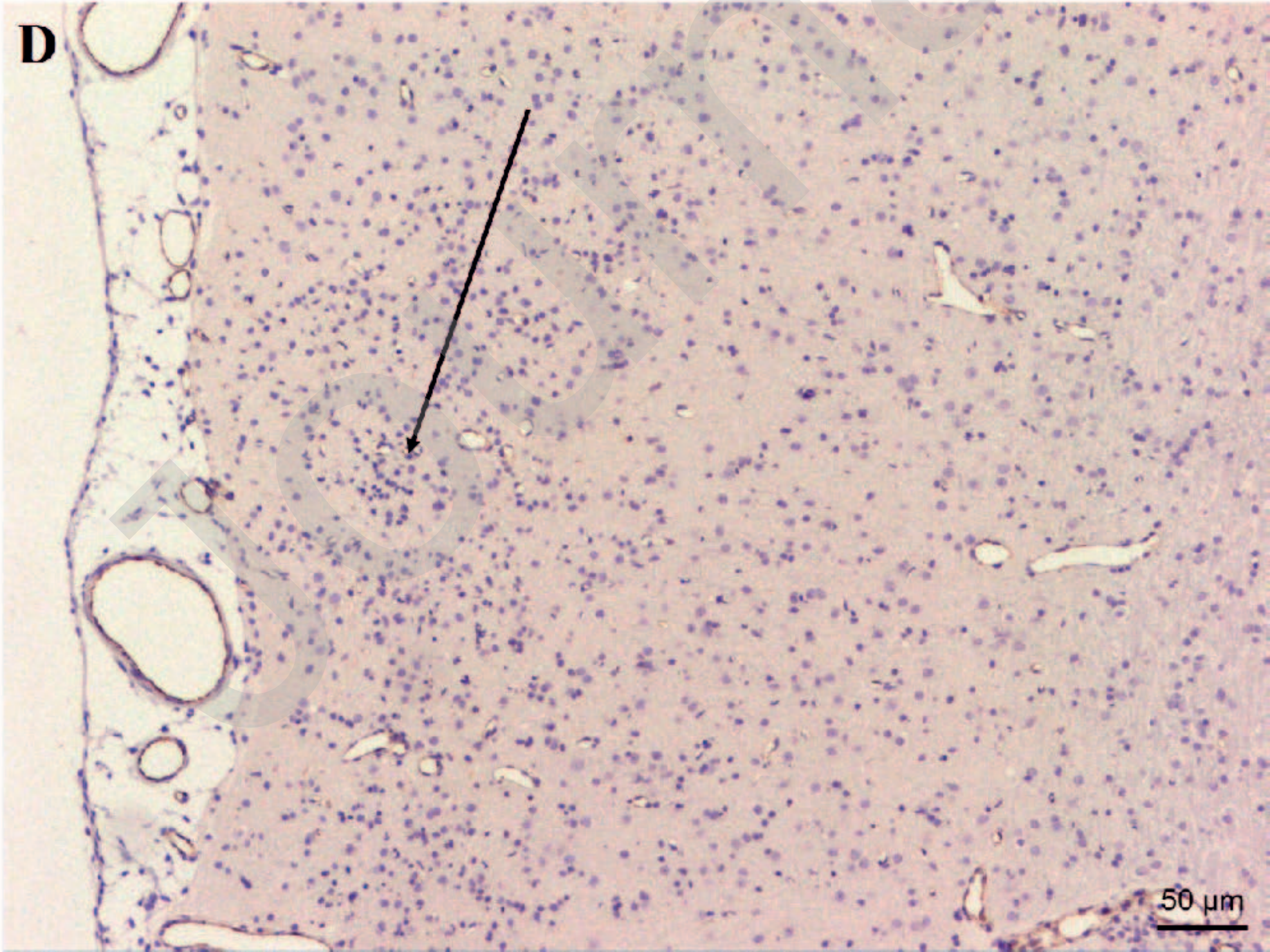
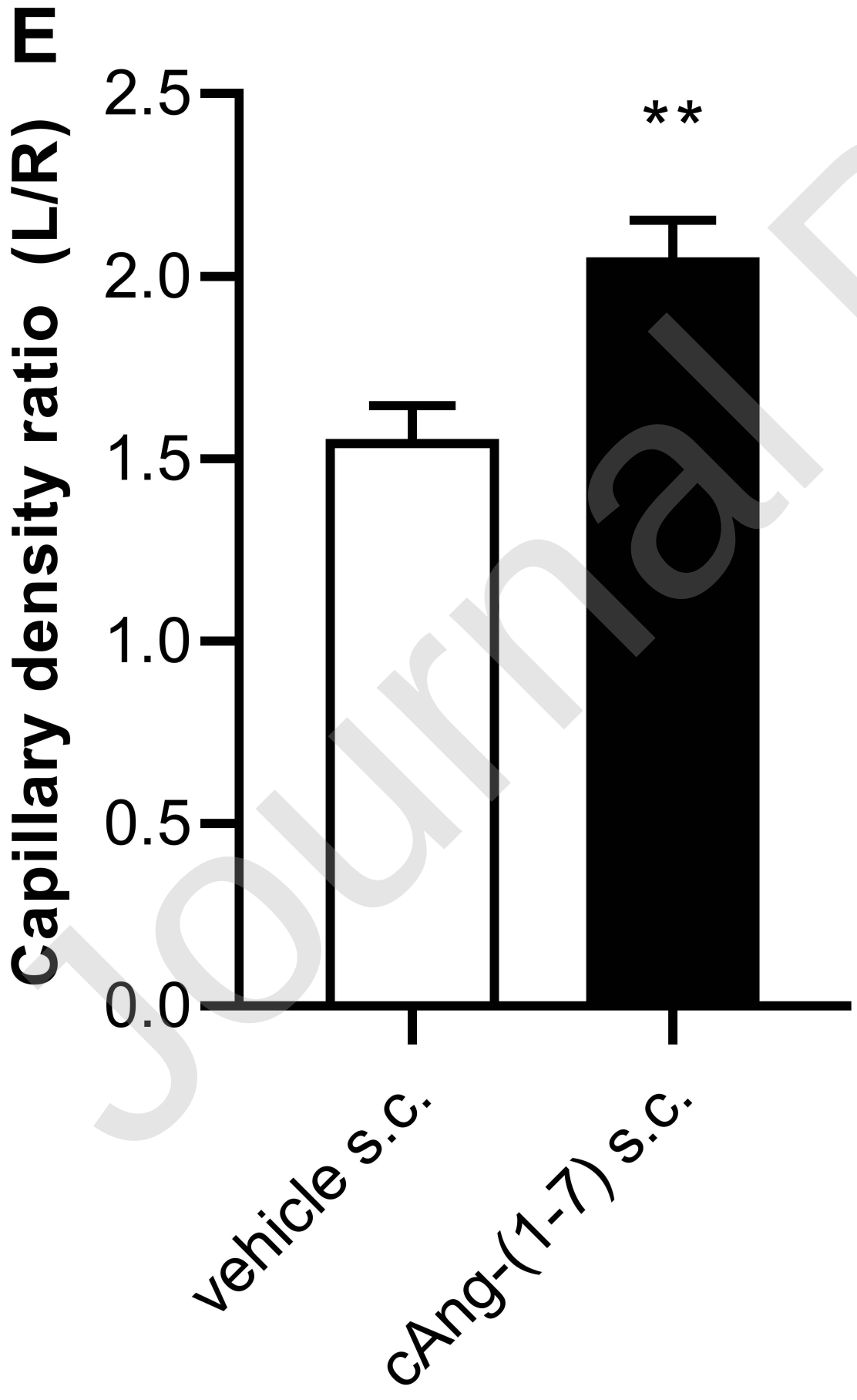
50 µm


C
50 µm


D
50 µm


Capillary density ratio (L/R)
2.5
2.0
1.5
1.0
0.5
0.0
**
vehicle s.c.
cAng-(1-7) s.c.