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Research Article: New Research | Disorders of the Nervous System

Post-Stroke Intranasal (+)-Naloxone Delivery Reduces Microglial Activation and Improves Behavioral Recovery from Ischemic Injury

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Abstract

Ischemic stroke is the leading cause of disability, and effective therapeutic strategies are needed to promote incomplete recovery. Neuroinflammation plays a significant role in stroke pathophysiology, and there is limited understanding how it affects recovery. The aim of the study was to characterize the spatiotemporal expression profile of microglial activation and to study whether dampening microglial/macrophage activation post-stroke facilitates the recovery. For dampening microglial/macrophage activation we chose intranasal administration of naloxone, a drug that is already in clinical use for opioid overdose and is known to decrease microglia/macrophage activation. We characterized the temporal progression of microglia/macrophage activation following cortical ischemic injury in rat and found the peak activation in cortex 7 days post-stroke. Unexpectedly, there was a chronic expression of phagocytic cells in the thalamus associated with neuronal loss. (+)-Naloxone, an enantiomer that reduces microglial activation without antagonizing opioid receptors, was administered intranasally starting 1 day post-stroke and continuing for 7 days. (+)-Naloxone treatment decreased microglia/macrophage activation in the striatum and thalamus, promoted behavioral recovery during the 14-day monitoring period, and reduced neuronal death in the lesioned cortex and ipsilateral thalamus. Our results are the first to show that post-stroke intranasal (+)-naloxone administration promotes short-term functional recovery and reduces microglia/macrophage activation. Therefore, (+)-naloxone is a promising drug for the treatment of ischemic stroke and further studies should be conducted.

Significance statement

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Ischemic stroke is one of the leading causes of adult disability and new drug treatments are needed as there is no drug that would promote the recovery. Neuroinflammation is suggested to play a role in the recovery process. Naloxone is a drug used clinically to treat opioid overdose. Its opioid receptor inactive form, (+)-naloxone, is known to reduce the activation of microglia, the immune cells of the brain. We show for the first time that repeated dosing of intranasal (+)-naloxone starting one day after stroke promotes short-term recovery in rats, reduces microglial activation and neuronal loss in the stroke brain. Our finding could be important for future stroke treatment and encourages further testing of (+)-naloxone in stroke patients.

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Introduction

Approximately 10 million patients survive a stroke each year, however there are currently no pharmacological options to promote recovery. Neurological deficits including impaired use of contralateral limbs, sensory and cognitive deficits and problems in speaking are the primary cause of disability and remain without effective treatment. Despite increased knowledge of the cellular and molecular mechanisms that mediate damage and recovery after stroke, the development of new drugs for stroke has been unsuccessful (Dirnagl, 2012). Most studies have focused on neuroprotection, and therefore, there is a great need to find novel drug targets, and develop new treatments that would improve the recovery from ischemic brain injury by targeting the post-ischemic pathological mechanisms such as inflammation. Modulation of brain inflammatory cascades following stroke is recognized as a viable therapeutic strategy to promote functional recovery from ischemic brain injury (Endres et al., 2008). However, very few studies have taken this approach, and little is known about recovery from stroke in relation to neuroinflammation.

The drug naloxone, an opiate antagonist, has been clinically used for opioid overdose for nearly 50 years, has anti-inflammatory properties and can attenuate microglial activation. Naloxone has two stereoisomers, the (-) and (+) enantiomers (Fig. 1). (-)-Naloxone has a high affinity for antagonizing μ, δ, and κ opioid receptors, whereas (+)-naloxone, has a very low affinity for opioid receptors (Iijima et al., 1978). Clinical reports indicate that intravenously administered (-)-naloxone ameliorates neurological deficits in acute stroke (Baskin and Hosobuchi, 1981; Jabaily and Davis, 1984). Studies examining the neuroprotective effect of (-)-naloxone in rat focal cerebral ischemia also showed a significant reduction in neuronal loss and inflammation

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(Chen et al., 2001; Liao et al., 2003), but (+)-naloxone did not affect the infarction volume when administered before ischemia. These differences between (-) and (+) isoforms suggest a role for opioid receptor antagonism in the neuroprotective effect (Liao et al., 2003). However, both enantiomers have been shown to reduce the number of activated microglia in a rat model of neuropathic pain (Hutchinson et al., 2008), and to inhibit superoxide production from microglia, and subsequent neurodegeneration, by interacting with NADPH oxidase (Liu et al., 2002; Qin et al., 2005; Wang et al., 2012). Also, both naloxone enantiomers reduce lipopolysaccharide (LPS)-induced microglial activation and protect neurons from LPS-induced neurodegeneration (Liu et al., 2000a; Liu et al., 2000b; Liu et al., 2000c). LPS signaling in microglia/macrophages requires Toll-like receptor 4 (TLR4) that is expressed abundantly on microglia (Lehnardt et al., 2003). Both (-)- and (+)-naloxone enantiomers decrease LPS-induced TLR4 signaling (Hutchinson et al., 2008; Wang et al., 2016) and both have been shown to antagonize the TLR4 coreceptor MD2 (Hutchinson et al., 2012). TLR4 activation leads to activation of NF-kB, and production of pro-inflammatory cytokines, interferons, and reactive oxygen/nitrogen species. TLR4 deficiency is neuroprotective in ischemic stroke in mice (Kilic et al., 2008), and increased TLR4 expression is associated with more severe stroke in patients (Yang et al., 2008).

Collectively, these studies suggest that naloxone can modulate immune and glial responses and has therapeutic potential in stroke. However, its effects on post-stroke microglial activation and recovery have not been studied. Since (+) and (-) forms have similar efficacy in reducing microglial activation, the advantage of the (+)-form is that side effects from antagonizing opioid receptors can be avoided. Thus, we aimed to study whether prolonged post-stroke administration of (+)-naloxone in rats would promote recovery and whether this recovery is associated with

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altered levels of activated microglia/macrophages. Furthermore, we took an approach of intranasal administration since recent advances with intranasal naloxone formulations have provided excellent bioavailability and enabled easy administration in patients. We first characterized the neuroinflammatory response following focal cortical ischemia-reperfusion injury since it is not well studied. As microglia/macrophages showed activation already on day 2 post-stroke in the ischemic cortex and maximum activation was observed at day 7, we administered (+)-naloxone during this accumulation period. Here we show how post-stroke intranasal (+)-naloxone promoted behavioral recovery during the short 14-day testing period, and decreased microglia/macrophage activation and neuronal loss.

Materials and Methods

Animals. Adult male Sprague-Dawley rats (200-250 g, Charles River) were maintained under a 12h light-dark cycle. Food and water were freely available in the home cage. Experimental procedures were approved by the NIDA Animal Care and Use Committee or by the National Animal Experiment Board of Finland (protocol approval number ESAVI/5459/04.10.03/2011), and followed the guidelines of the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health publication, 1996), local laws and regulations.

Cortical stroke model in rats with distal middle cerebral artery occlusion (dMCAo). To model focal cortical ischemic stroke in rats, the three-vessel occlusion method was used. In this model, the stroke damage is restricted to the cortex, and the relative size of the stroke is close to an average human stroke (Delavaran et al., 2013). Ligation of the right middle cerebral artery (MCA) and common carotid arteries (CCAs) bilaterally was performed as described previously (Airavaara et al., 2010; Airavaara et al., 2009; Chen et al., 1986; Harvey et al., 2011). Briefly,

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rats were anesthetized with chloral hydrate 0.4 g/kg intraperitoneally (i.p.). The bilateral CCAs were identified and isolated through a ventral midline cervical incision. The rats were placed in a stereotaxic apparatus, and a craniotomy was performed to expose the right MCA. The MCA was ligated with a 10-0 suture, and bilateral CCAs were ligated with non-traumatic arterial clamps. After 60 or 90 minutes of ischemia, the suture around the MCA and arterial clips on CCAs were removed to begin reperfusion. After recovery from anesthesia, the rats were returned to their home cage. Body temperature during and after surgery was maintained at 37ºC.

Intranasal administration of (-)-naloxone and (+)-naloxone. (-)-Naloxone and (+)-naloxone were synthesized in the laboratory of Dr. Kenner Rice (NIDA IRP, NIH). Dosing was estimated from a previously published study (Hutchinson et al., 2008). Drugs were administered intranasally under isoflurane anesthesia (Anesthesia auto flow system, E-Z Anesthesia) starting from post-stroke day 1, and were continued at 12-hour intervals for 7 days, a total of 14 times. Briefly, animals were placed in the induction chamber for 1-1.5 minutes, and 5% isoflurane was delivered at 1000cc/min. Animals were transferred to the nose cone where they received 1.5% isoflurane delivered at 500cc/min for 30 seconds prior to intranasal naloxone delivery. To ensure maximal delivery, animals remained in supine position during naloxone administration. When the animals regained consciousness, they were returned to the home cage. Naloxone solution was prepared fresh daily in sterile ultrapure water and stored at room temperature between administrations. Using a Rainin LTS L-20 pipette and sterile pipette tips, 10 µl of drug or vehicle was administered into each nostril as described (Luo et al., 2013).

Implantation of mini-osmotic pumps for continuous (+)-naloxone delivery. Two days following the dMCAo surgery the rats were anaesthetized with chloral hydrate (0.4 mg/kg i.p.) for mini-osmotic pump implantation (Alzet, Palo Alto, CA, USA; model 2002). The pump was

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implanted under the skin and the catheter was inserted into the right ventricle. The pumps were filled with vehicle (sterile, ultrapure water) or (+)-naloxone 96 mg/ml. The pumping rate was 0.5 µl/h and the pumps were left in place for 12 days.

Behavioral analysis. The elevated body swing test for body asymmetry (Borlongan et al., 1998), modified Bederson's score (Bederson et al., 1986) and the measurement of locomotor activity were performed as previously described (Airavaara et al., 2010; Airavaara et al., 2009). Briefly, body asymmetry was analyzed from 20 consecutive trials by suspending the rats 20 cm above the testing table by lifting their tails and counting the frequency of initial turnings of the head or upper body contralateral to the ischemic side (the maximum impairment in stroke animals is 20 contralateral turns whereas naïve animals turn in each direction with equal frequency). For Bederson's score, neurological deficits were scored using the following criteria: 0 = rats extend both forelimbs straight when lifted by the tail, no observable deficit; 1 point = rats keep the one forelimb to the breast and extend the other forelimb straight when lifted by the tail; 2 points = rats show decreased resistance to lateral push in addition to behavior in score 1; 3 points = rats twist the upper half of their body when lifted by the tail in addition to behavior in other scores. Locomotor activity was measured for 24h by placing the rat in a 42x42x31 cm plexiglass box with an infrared activity monitor (Accuscan, Columbus, OH). All the behavioral assessments were performed in a blinded manner and the experimenter did not know group allocation. Body asymmetry and Bederson's score were assessed at the middle of the day, so that the animals had fully recovered from isoflurane anesthesia needed for the period of intranasal administration in the morning and in the evening.

Histology. The rats were deeply anaesthetized with pentobarbital (90 mg/kg i.p.) and transcardially perfused with 200 ml saline followed by 500 ml of 4% paraformaldehyde. Brains

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were processed for either paraffin or free-floating sections and stained with anti-Iba1, anti-CD68, anti-NeuN, anti-GFAP, or anti-MBP antibodies.

Immunostaining of paraffin sections. Brains were post-fixed in 4% paraformaldehyde for 2 days, dehydrated in a series of ethanol and xylene, and embedded in paraffin. Brains were cut into 5 µm thick sagittal sections using a Leica HM355S microtome and mounted on Superfrost Plus slides (Thermo Scientific, Waltham, MA). Sections were deparaffinized and antigen retrieval was performed by heating in 0.05% citraconic anhydride (Sigma Aldrich, St. Louis, MO), pH 7.4. Endogenous peroxidase activity was blocked with 0.6% hydrogen peroxide (Sigma Aldrich), non-specific antibody binding was blocked with 1.5% normal goat or horse serum (Vector Laboratories, Burlingame, CA), followed by incubation with primary antibody (rabbit anti-Iba1 1:1000, cat#019-19741, RRID:AB_839504, Wako, Richmond, VA; mouse anti-CD68 1:500, cat#MCA341R, RRID:AB_2291300, AbD Serotec, Kidlington, UK; mouse anti-GFAP 1:1000, cat#MAB360, RRID:AB_2109815, Millipore, Billerica, MA; mouse anti-NeuN 1:200, cat#MAB377, RRID:AB_2298772, Millipore; rabbit anti-MBP 1:500, cat#ab40390, RRID:AB_1141521, Abcam, Cambridge, UK) at 4°C overnight. The next day, sections were incubated with secondary antibody (goat anti-rabbit, RRID:AB_2336820, or horse anti-mouse, RRID:AB_2336811, biotinylated secondary antibody 1:200, Vector Laboratories) followed by incubation with avidin-biotin complex (ABC kit, Vector Laboratories). Color was developed using peroxidase reaction with 3´,3´-diaminobenzidine (DAB; RRID:AB_2336382, Vector Laboratories). Cresyl violet staining was used as background staining with anti-CD68. For immunofluorescence staining, goat anti-rabbit Alexa488 (1:500, cat#A11034, RRID:AB_2576217, Life Technologies, Paisley, UK) and goat anti-mouse Alexa568 (1:500, cat#A11004, RRID:AB_2534072, Life Technologies) were used as secondary antibodies.

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Immunostaining of free-floating sections. Brains were dehydrated in 30% sucrose at 4°C and sectioned coronally into 40 µm thick slices using a Leica CM3050 Cryostat. Sections were taken from 2.1 to -1.0 mm (striatum) and -2.4 to -4.0 mm (thalamus) relative to bregma, then stored in 1x PBS for short-term storage, or cryopreservant for long-term storage (20% glycerol, 2% DMSO in 1x PBS). Sections were blocked with 0.3% hydrogen peroxidase and 4% bovine serum albumin (Sigma Aldrich) + 0.3% Triton-x-100 (Sigma Aldrich), then incubated with primary antibody (rabbit anti-Iba1 1:2000, RRID:AB_839504; mouse anti-NeuN 1:1000, RRID:AB_2298772) overnight at 4°C. The following day, sections were incubated with secondary antibody, followed by incubation with avidin-biotin complex and DAB as above.

Unbiased stereological counting of Iba1+ cells in the striatum. Iba1-positive (Iba1+) cells in the striatum were counted from 40 µm thick free-floating sections using unbiased stereology with a stereomicroscope (Olympus BX51) and StereoInvestigator 6 program (MBF Bioscience) as previously described (Mijatovic et al., 2007). The optical fractionator method, which involves a three-dimensional probe for counting the cell of interest by randomly and systematically sampling each section, was used. It is unbiased since it does not involve cell size and shape, and is unaffected by tissue processing. Cell population is computed from the volume fraction. The volume fraction consists of information on the thickness of tissue sampled, the number of sections sampled, and the area in each section sampled. Three 40 µm thick coronal slices were selected based on their relative location to bregma (+0.2, -0.26, and -0.4/-0.5 mm) to obtain results relatively free of bias in the distribution of the cells. Only Iba1+ cells with clear microglia morphology were counted. The contralateral and ipsilateral striata were first traced, and then the area was analyzed for each slice. Approximately 60 to 80 randomly selected sites per area/slice were analyzed to ensure accuracy and minimize error.

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Analysis of CD68+, Iba1+, and NeuN+ cells in the thalamus. The number of CD68+, Iba1+ and NeuN+ cells in the thalamus was analyzed using Image-Pro Analyzer 7.0 program. Slides were scanned with a 3DHISTECH Pannoramic 250 FLASH II digital slide scanner (Budapest, Hungary; scanning service provided by the Institute of Biotechnology, University of Helsinki; http://www.biocenter.helsinki.fi/bi/histoscanner/index.html) and 10x magnification images of the thalamus were taken with Pannoramic Viewer version 1.15.3. To estimate the number of immunopositive cells in the ipsilateral thalamus, the thalamus was traced and the Iba1 immunoreactive area was counted from 3-6 coronal sections (in 3 rats there were only 2 sections counted). The object count of NeuN+ cells in the thalamus was counted from 4-6 coronal sections per brain. Sections were taken every 300-400 µm between -2.4 and -4.0 mm relative to bregma, and an average for each brain was used for further analysis. The average number of NeuN+ cells and the Iba1 immunoreactive area are expressed as a percentage compared to the contralateral side. To estimate the number of CD68+ cells in the thalamus, the object count of CD68+ cells was analyzed from 3-4 sagittal sections per hemisphere, taken every 500 µm between 1.9 and 3.4 mm or -1.9 and -3.4 mm relative to bregma, and the average cell count per hemisphere was used for further analysis.

Quantitation of infarction size. At day 14 post-stroke, the average infarction size was quantified from 6 anti-NeuN stained coronal brain sections, taken every 800 µm between 1.80 and -3.00 mm relative to bregma. The area devoid of NeuN+ cells and the total area of the brain section were defined in Pannoramic Viewer version 1.15.3. Infarction size is expressed as a percentage of the total area of the section. At day 2 post-stroke, the infarction volume was quantified with 2,3,5-triphenyltetrazolium chloride (TTC) staining from 2 mm brain slices as described previously (Airavaara et al., 2009).

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Measurement of TNF-α secretion from microglia/macrophages isolated from the stroke cortex. The CD11b immunopositive cortical microglia/macrophages were isolated by magnetic activated cell sorting (MACS). The rats were anesthetized with pentobarbital (90 mg/kg i.p.) at 7 days post-stroke after 90 min dMCAo, and perfused with 200 ml of saline. The ischemic cortex and the corresponding contralateral cortex were dissected on ice in HBSS without Ca^{2+} and Mg^{2+}. The tissue was dissociated using Neural tissue dissociation kit (T) (Miltenyi Biotec, San Diego, CA, USA) and the gentleMACS Dissociator (Miltenyi Biotec). After dissociation, the cells were suspended in 0.5% BSA in PBS and incubated with Myelin Removal Beads II (1:10, Miltenyi Biotec) for 15 minutes at 4°C. The cells were washed and resuspended in 0.5% BSA in PBS and filtered through an LS column (Miltenyi Biotec) using a QuadroMACS Separator (Miltenyi Biotec). The total effluent was collected and resuspended in 0.5% BSA with 2 mM EDTA in PBS. The cells were incubated at 4°C for 10 minutes with mouse anti-CD11b:FITC antibody (1:10, cat# MCA275FA, RRID:AB_2129486, AbD Serotec). The cells were washed, resuspended in 0.5% BSA with 2 mM EDTA in PBS and incubated with anti-FITC MicroBeads (1:10, RRID:AB_244371, Miltenyi Biotec) for 15 minutes at 4°C. The cells were washed and resuspended in 0.5% BSA with 2 mM EDTA in PBS. The cell suspension was applied to an LS column placed on a QuadroMACS Separator, and the magnetically labeled cells were used for further experiments. The cells were plated on poly-ornithin-coated glass coverslips on a 48-well plate in the density of 30 000 cells/well in DMEM:F12 medium (Life Technologies) containing 10% FBS and 0.2% primocin (Invivogen, San Diego, CA). LPS or naloxone were added immediately after the plating. The culture medium was collected 20h later and analyzed using the Rat TNF alpha ELISA Ready-SET-Go!® kit (cat#88-7340-22, RRID:AB_2575088, eBioscience, San Diego, CA). The results are presented from three independent experiments. The

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CD11b+ cells were isolated from 2-3 rats in each experiment. The values for each well within an experiment were normalized to the mean value of the control wells within the corresponding experiment.

Experimental Design and Statistical Analysis. All the experiments and analyses were performed in a blinded manner. First, the time course of glial activation in the dMCAo model was characterized. To minimize the number of animals needed (n=24 in total; n=4 per group), 90 min occlusion time was used to create a robust lesion. Immunostaining of sagittal sections with anti-Iba1, anti-CD68 and anti-GFAP antibodies was used to visualize the temporal and spatial distribution of glial cells post-stroke. As the dMCAo model is unilateral, the contralateral hemisphere was used as a control after we confirmed that the expression of glial cells in naïve brain is similar to the contralateral hemisphere of ischemic brain. For quantitation of CD68+ cells, 3-4 sections per animal per hemisphere were used to result in an average.

Second, to test the efficacy of post-stroke (+)-naloxone (0.32 mg/kg; n=27), repeated dosing (twice a day for 7 days) was used due to the short half-life of naloxone. To make the setting clinically relevant, the treatment was started one day after stroke and was given intranasally, and a smaller infarct using 60 min dMCAo was induced. Behavioral recovery was monitored for 14 days with body asymmetry test, Bederson's score and measurement of spontaneous locomotor activity. Vehicle (n=25) and no treatment groups (n=13) were used as a control in the behavioral assays. As both naloxone enantiomers have anti-inflammatory effects on microglia, only the (-) form antagonizes opioid receptors. Thus, we included also (-)-naloxone (n=7) group in the behavioral assays to provide information whether (+)- and (-)-naloxone have a similar effect on recovery. Dose-response of (+)-naloxone was tested in a separate experiment (0.0008 mg/kg: n=8; 0.008 mg/kg: n=8; 0.08 mg/kg: n=7; 0.8 mg/kg: n=8; vehicle: n=11). The

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amount of microglial activation in the striatum and thalamus, and neuronal loss in the cortex and thalamus were analyzed with immunohistochemistry. For quantitation of Iba1+ and NeuN+ cells, 3-6 sections from corresponding coronal planes from each animal were used.

Statistical analyses were performed with IBM SPSS Statistics software version 24.0. Normal distribution of each dataset was analyzed by Levene's test for equality of variances and analyzed with either one-way ANOVA or Kruskal-Wallis nonparametric ANOVA, or with Student's t-test or nonparametric Mann-Whitney U test in the case of only two groups. One-way ANOVA was followed by Bonferroni's or Dunnett's post hoc test and Kruskal-Wallis test was followed by pairwise comparison with Mann-Whitney U test. Since the immunohistochemical data did not differ between the vehicle and no treatment groups or between (+)-naloxone doses 0.32 mg/kg and 0.8 mg/kg, the two groups were combined as one control group and one (+)-naloxone group. Statistical significance was considered at $p<0.05$. The results are presented as mean ± standard error of the mean.

Table 1: Statistical table

	Dataset	Data structure	Type of test	Power
a	Fig. 2P	Non-normal distribution (unequal variances)	Kruskal-Wallis test, Mann-Whitney U	H(11)=41.5, p=0.000 Ipsilateral hemisphere: d2 vs. d7: p=0.021 d2 vs. d14: p=0.021 d2 vs. d28: p=0.021 d2 vs. d56: p=0.021 d2 vs. d112: p=0.021 d7 vs. d14: p=0.021 d7 vs. d28: p=0.021 d7 vs. d56: p=0.083 d7 vs. 112: p=0.083 d14 vs. d28: p=1.00 d14 vs. d56: p=0.043 d14 vs. d112: p=0.021

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				d28 vs. d56: p=0.083 d28 vs. d112: p=0.043 d56 s. d112: p=0.773 Ipsilateral vs. contralateral: d2: p=0.773 d7: p=0.021 d14: p=0.021 d28: p=0.021 d56: p=0.021 d112: p=0.021
b	Fig. 5B	Non-normal distribution	Kruskal-Wallis test, Mann-Whitney U	d1: H(2)= 2.26, p=0.323 d3: H(2)=4.93, p=0.085 d7: H(2)=1.29, p=0.524 d10: H(2)=15.5, p=0.000 NT vs. (+)-Nal: p=0.006 Veh vs. (+)-Nal: p=0.000 NT vs. Veh: p=0.651 d14: H(2)=12.3, p=0.002 NT vs. (+)-Nal: p=0.012 Veh vs. (+)-Nal: p=0.001 NT vs. Veh: p=0.485
c	Fig. 5C	Non-normal distribution	Kruskal-Wallis test, Mann-Whitney U	d1: H(2)= 3.25, p=0.197 d3: H(2)=6.92, p=0.032 NT vs. (+)-Nal: p=0.017 Veh vs. (+)-Nal: p=0.059 NT vs. Veh: p=0.393 d7: H(2)=3.26, p=0.196 d10: H(2)=15.4, p=0.000 NT vs. (+)-Nal: p=0.002 Veh vs. (+)-Nal: p=0.002 NT vs. Veh: p=0.203 d14: H(2)=19.1, p=0.000 NT vs. (+)-Nal: p=0.000 Veh vs. (+)-Nal: p=0.000 NT vs. Veh: p=0.209
d	Fig. 5D	Non-normal distribution	Kruskal-Wallis test, Mann-Whitney U	H(4)=15.1, p=0.004 Veh vs. $8\cdot10^{-4}$: p=0.206 Veh vs. $8\cdot10^{-3}$: p=0.966 Veh vs. $8\cdot10^{-2}$: p=0.002 Veh vs. $8\cdot10^{-1}$: p=0.059 $8\cdot10^{-4}$ vs. $8\cdot10^{-3}$: p=0.552 $8\cdot10^{-4}$ vs. $8\cdot10^{-2}$: p=0.001 $8\cdot10^{-4}$ vs. $8\cdot10^{-1}$: p=0.019 $8\cdot10^{-3}$ vs. $8\cdot10^{-2}$: p=0.031 $8\cdot10^{-3}$ vs. $8\cdot10^{-1}$: p=0.142 $8\cdot10^{-2}$ vs. $8\cdot10^{-1}$: p=0.488

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e	Fig. 5E	Non-normal distribution	Kruskal-Wallis test, Mann-Whitney U	H(4)=6.38, p=0.041 Veh vs. $8{\cdot}10^{-4}$: p=0.690 Veh vs. $8{\cdot}10^{-3}$: p=0.487 Veh vs. $8{\cdot}10^{-2}$: p=0.016 Veh vs. $8{\cdot}10^{-1}$: p=0.038 $8{\cdot}10^{-4}$ vs. $8{\cdot}10^{-3}$: p=0.314 $8{\cdot}10^{-4}$ vs. $8{\cdot}10^{-2}$: p=0.006 $8{\cdot}10^{-4}$ vs. $8{\cdot}10^{-1}$: p=0.025 $8{\cdot}10^{-3}$ vs. $8{\cdot}10^{-2}$: p=0.144 $8{\cdot}10^{-3}$ vs. $8{\cdot}10^{-1}$: p=0.208 $8{\cdot}10^{-2}$ vs. $8{\cdot}10^{-1}$: p=0.730
f	Fig. 5F	Normal distribution	One-way ANOVA, Bonferroni	F(2,42)=0.054 NT vs. (+)-Nal: p=0.064 Veh vs. (+)-Nal: p=0.242 NT vs. Veh: p=1.00
g	Fig. 5G	Non-normal distribution (unequal variances)	Kruskal-Wallis test, Mann-Whitney U	H(2)=6.82, p=0.033 NT vs. (+)-Nal: p=0.004 Veh vs. (+)-Nal: p=0.243 NT vs. Veh: p=0.313
h	Fig. 5H	Non-normal distribution	Mann-Whitney U	d1: p=0.663 d3: p=0.663 d7: p=0.963 d10: p=0.001 d14: p=0.000
i	Fig. 5I	Non-normal distribution	Mann-Whitney U	d1: p=0.159 d3: p=0.401 d7: p=0.565 d10: p=0.002 d14: p=0.005
j	Fig. 5J	Normal distribution	t-test	d7: t(16)=0.47, p=0.647 d14: t(16)=0.06, p=0.953
k	Fig. 5K	Normal distribution	One-way ANOVA, Bonferroni	d7: F(2,59)=5.27, p=0.008 NT vs. (+)-Nal: p=0.574 Veh vs. (+)-Nal: p=0.113 NT vs. Veh: p=0.009 d14: F(2,62)=1.79, p=0.175
l	Fig. 6A	Normal distribution	t-test	t(26)=2.51, p=0.019
m	Fig. 6B	Non-normal distribution (unequal variances)	Kruskal-Wallis test, Mann-Whitney U	H(2)=11.4, p=0.003 Naïve vs. Ctrl: p=0.002 Naïve vs. (+)-Nal: p=0.157 Ctrl vs. (+)-Nal: p=0.036
n	Fig. 7A	Normal distribution	One-way ANOVA, Bonferroni	F(2,31)=8.63, p=0.001 Naïve vs. Ctrl: p=0.003 Naïve vs. (+)-Nal: p=1.00

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				Ctrl vs. (+)-Nal: p=0.013
o	Fig. 7B	Non-normal distribution (unequal variances)	Kruskal-Wallis test, Mann-Whitney U	H(2)=5.77, p=0.056
p	Fig. 7C	Non-normal distribution (unequal variances)	Kruskal-Wallis test, Mann-Whitney U	H(2)=17.6, p=0.000 Naïve vs. Ctrl: p=0.000 Naïve vs. (+)-Nal: p=0.001 Ctrl vs. (+)-Nal: p=0.027
q	Fig. 8B	Normal distribution	t-test	t(13)=0.89, p=0.389
r	Fig. 8D	Non-normal distribution	Mann-Whitney U	d2: p=0.861 d6: p=0.825 d10: p=0.279 d16: p=0.066
s	Fig. 8F	Non-normal distribution	Mann-Whitney U	d1: p=0.800 d3: p=0.861 d7: p=0.516 d10: p=0.391 d14: p=0.694
t	Fig. 8G	Normal distribution	Two-way RM ANOVA	F(4,52)=0.29, p=0.88
u	Fig. 9A	Non-normal distribution (unequal variances)	Mann-Whitney U	p=0.006
v	Fig. 9B	Normal distribution	One-way ANOVA, Dunnett	F(6,54)=2.74, p=0.022 20µM (-)-Nal: p=0.669 50µM (-)-Nal: p=0.020 100µM (-)-Nal: p=0.218 20µM (+)-Nal: p=0.492 50µM (+)-Nal: p=0.637 100µM (+)-Nal: p=0.006

d = post-stroke day; NT = no treatment; (+)-Nal = (+)-naloxone; Veh = vehicle; Ctrl = control; (-)-Nal = (-)-naloxone; RM = repeated measures

Results

Long-lasting microglial and astrocyte activation in the ipsilateral hemisphere after dMCAo

We first characterized the neuroinflammatory response in proximal (cortex) and distal (thalamus) regions following transient 90 min dMCAo, and second, tested the efficacy of post-stroke intranasal naloxone treatment in adult rats after 60 min dMCAo. At day 2 post-stroke, microglia/macrophage activation was observed mainly in the peri-infarct region, and few

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phagocytic CD68+ (a marker for activated, phagocytic microglia/macrophages) cells were present (Fig. 2C-D). Microglia/macrophage activation peaked on day 7 post-stroke in the ischemic cortex when the core area was filled with Iba1+ (a marker for all microglia/macrophages) and CD68+ cells having a phagocytic/macrophage-type morphology (Fig. 2Ea, Fa). Microglia/macrophage activation was seen in the ipsilateral striatum starting from post-stroke day 7 (Fig. 2Ec). Interestingly, activated microglia/macrophages were aligned along fiber bundles, with a "stream-like" formation along the myelin embedded axons (Fig. 3). Iba1+ cells with activated morphology were evident in the ipsilateral thalamus at day 7 post-stroke, but only a few cells were CD68+ (Fig. 2Ed, Fd, P). CD68 immunoreactivity increased in the ipsilateral thalamus after post-stroke day 7, peaked at days 14-28, and remained elevated until day 112 ($H(11)=41.5$, $p<0.001$, Kruskal-Wallis, Table 1a; Fig. 2P). These results suggest that inflammation in the thalamus is long lasting and phagocytic cells are present in far distal areas at least 4 months after stroke (Fig. 2Nd, P). Neuronal loss was observed in the ipsilateral thalamus at post-stroke day 14 (Fig. 6B). At 112 days post-stroke, atrophy of the thalamus due to shrinkage of the ipsilateral thalamus was observed (Fig. 2Me, Ne, Fig. 4Ge). Analysis of spatiotemporal activation of GFAP+ cells (a marker for astrocytes) revealed a similar pattern as microglia/macrophage activation (Fig. 4). Similarly to microglia, astrocytes in the ischemic core died during the first couple of days. Astrogliosis was found in the peri-infarct area starting from day 2 post-stroke (Fig. 4Bb). There was clear astrocytic scar formation in the peri-infarct region starting from post-stroke day 7 (Fig. 4Cb, Ce). From day 7 onwards, astrogliosis was evident in the striatum and even in the thalamus (Fig. 4C-G). Astrogliosis in the ipsilateral thalamus persisted for up to 112 days post-stroke (Fig. 4Gd, Ge), suggesting glial scar formation without local ischemic damage.

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Post-stroke treatment with intranasal (+)-naloxone started on day 1 post-stroke promotes short-term behavioral recovery, reduces neuronal loss and decreases microglial activation

The efficacy of post-stroke (+)-naloxone treatment was tested in rats (n=65), and treatment was initiated one day after 60 min dMCAo, before extensive activation of microglia/macrophages, and it was continued for 7 days i.e. the period when microglia/macrophage activation progresses in the ischemic region. One day post-stroke, the rats were balanced into groups based on their neurological deficits as measured by body asymmetry and Bederson's score, and (+)-naloxone (0.32 mg/kg) or vehicle were administered intranasally every 12h for 7 days (i.e. 14 doses per animal, Fig. 5A). To identify the confounding effect of isoflurane, two control groups were included: (i) stroke rats receiving intranasal vehicle (including repeated isoflurane) and (ii) stroke rats with no intranasal treatment and no isoflurane. There were no significant differences in body asymmetry amongst the groups on days 1, 3, or 7 after dMCAo (Fig. 5B, Table 1b). However, on days 10 and 14 post-stroke, body asymmetry ($H(2)=15.5$, $p<0.001$ and $H(2)=12.3$, $p=0.002$, respectively, Kruskal-Wallis, Table 1b) and neurological deficits ($H(2)=15.4$, $p<0.001$ and $H(2)=19.1$, $p<0.001$, respectively, Table 1c) were significantly reduced in the (+)-naloxone treated rats (Fig. 5B-C). The reduction in body asymmetry ($H(4)=15.1$, $p=0.004$, Kruskal-Wallis, Table 1d) and neurological deficits ($H(4)=6.38$, $p=0.041$, Table 1e) was dose-dependent (Fig. 5D-E). As another indicator of hastened recovery, (+)-naloxone improved locomotor activity at day 14 post-stroke ($H(2)=6.82$, $p=0.033$, Kruskal-Wallis, Table 1g; Fig. 5G). Similarly, (-)-naloxone (0.32 mg/kg, intranasally) induced behavioral recovery on days 10 and 14 post-stroke (n=18) ($p=0.001$ and $p<0.001$, respectively, for body asymmetry test, Table 1h; and $p=0.002$ and $p=0.005$, respectively, for Bederson's score, Mann-Whitney U-test, Table 1i; Fig. 5H-I). It has

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been reported that continuous administration of the opioid receptor antagonist (-)-naltrexone decreases body weight and appetite (Atkinson, 1984). We found no effect on the body weight with naloxone treatment (Table 1j-k, Fig. 5J-K).

Post-stroke (+)-naloxone (0.32-0.8 mg/kg) reduced infarction size on day 14 after dMCAo ($t(26)=2.51$, $p=0.019$, unpaired t-test, Table 1l; Fig. 6A) and prevented delayed neuronal death in the ipsilateral thalamus ($H(2)=11.4$, $p=0.003$, Kruskal-Wallis, Table 1m; Fig. 6B). We observed a negative correlation between the number of neurons (NeuN+ cells) in the ipsilateral thalamus and infarction size (Pearson correlation $R = -0.691$, $p < 0.0001$), showing that the larger the lesion in the cortex, the more extensive neuronal loss in the thalamus. (+)-Naloxone significantly decreased the Iba1+ cell number in the ipsilateral striatum at post-stroke day 14 ($F(2,31)=8.63$, $p=0.001$, one-way ANOVA, Table 1n; Fig. 7A). There was no statistical difference in the contralateral striatum (Table 1o, Fig. 7B). Similarly, a significant reduction of Iba1 immunoreactivity by (+)-naloxone was found in the ipsilateral thalamus ($H(2)=17.6$, $p<0.001$, Kruskal-Wallis, Table 1p; Fig. 7C).

Pre-treatment or delayed post-stroke treatment with (+)-naloxone is not beneficial in dMCAo model

We tested the neuroprotective effect of (+)-naloxone (n=15) by giving (+)-naloxone (0.32 mg/kg) or vehicle intranasally three times: 12h and 1h before 60 min dMCAo and immediately after reperfusion (Fig. 8A). The infarction volume was determined by TTC staining 2 days after stroke. We found no significant differences in the infarction volume between the groups ($p=0.389$, unpaired t-test, Table 1q; Fig. 8B). To answer the question whether more delayed (+)-

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naloxone treatment would give similar effect on recovery as the treatment started on post-stroke day 1, we administered (+)-naloxone (0.8 mg/kg) or vehicle intranasally twice daily starting from day 3 after 60 min dMCAo and continuing for 7 days (n=13; Fig. 8C). There was an evident recovery effect over time in the body asymmetry test, but there were no statistically significant differences between the vehicle and (+)-naloxone groups (Table 1r; Fig. 8D). On post-stroke day 16, there was a tendency (p=0.066, Mann-Whitney U test, Table 1r) in the (+)-naloxone group for milder neurological deficits in the body asymmetry test, but it did not reach statistical significance. To study whether longer, continuous administration of (+)-naloxone would further enhance recovery after 60 min dMCAo, we delivered (+)-naloxone (1.15 mg/24 h) or vehicle into the ventricle using mini-osmotic pumps from post-stroke day 2 until post-stroke day 14 (n=15; Fig. 8E). There was again evident recovery effect in the body asymmetry test, but there were no differences between the vehicle and (+)-naloxone groups (Table 1s; Fig. 8F). Nor were there any differences in the body weight between the groups (Table 1t; Fig. 8G).

Naloxone decreases TNF-α secretion from microglia/macrophages

To test whether naloxone enantiomers affect cytokine secretion, we isolated CD11b+ microglia/macrophages from the infarct area at day 7 post-stroke and measured the secretion of TNF-α, a cytokine with well-characterized pro-inflammatory effect downstream of TLR4 signaling. First, we tested whether the isolated cells respond to treatment with LPS by increasing the secretion of TNF-α. Overnight treatment with LPS increased the amount of secreted TNF-α approximately 3.5-fold (p=0.006, Mann-Whitney U, Table 1u; Fig. 9A), confirming that the isolated cell population had properties characteristic of microglia and macrophages. Overnight treatment with both naloxone enantiomers decreased the amount of TNF-α in the culture medium

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statistically significantly ($F(6,54)=2.74$, $p=0.022$, one-way ANOVA, Table 1v; Fig. 9B). (-)-Naloxone 50 μM and (+)-naloxone 100 μM decreased the unstimulated secretion of TNF-α by approximately 15% compared to the control.

Discussion

We show for the first time that intranasal post-stroke administration of naloxone enantiomers reduces inflammation and hastens recovery during short-term behavioral monitoring. Our data support a therapeutic window for initiation of twice daily (+)-naloxone treatment between 16-36h post-stroke and continuing the treatment for 7 days. Our study also indicates that modulation of microglia/macrophage activation in the ischemic cerebral cortex and remote regions in the striatum and thalamus is a potential therapeutic drug strategy. Since microglia/macrophages can phagocytose viable neurons after ischemia (Neher et al., 2013), neuroinflammation may lead to secondary neuronal loss. An ischemic brain injury leads to activation of microglia, the release of pro-inflammatory factors, and further potentiates neuronal damage days to weeks after dMCAo (Dirnagl et al., 1999). It has been suggested, that during an ischemic event the brain remains in a continual state of neurotoxicity and microglia become over-activated, releasing pro-inflammatory factors that can contribute to further damage (Glass et al., 2010). It is known that over-reactive microglia can cause increased levels of cytokines, specifically TNF-α and interleukin-1β and -6 (Block et al., 2007; Lee et al., 1993). Therefore anti-inflammatory drugs, such as minocycline, have been used to alleviate this neuroinflammation and improve stroke outcome (Yrjanheikki et al., 1999). Liebigt *et al.* have shown that post-stroke application of minocycline and indomethacin in rats, combined with rehabilitative training, produces improved functional recovery compared to training alone (Liebigt et al., 2012).

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Our data indicate that twice a day post-stroke treatment with (+)-naloxone for a week improves short-term behavioral recovery and reduces neuronal damage and infarction size. The behavioral effect of (+)-naloxone correlated with suppression of activated microglia in the striatum and thalamus and was not observed until day 10 post-stroke, which suggests that (+)-naloxone targets delayed post-stroke pathophysiological mechanisms such as inflammation. The administration of (+)-naloxone was targeted to the period when the number of activated microglia/macrophages is peaking in the cerebral cortex and before microglial activation is evident in the striatum or thalamus. We, therefore, propose that dampening microglia/macrophage activation with post-stroke (+)-naloxone restricts inflammation to limit associated lesion-expansion that occurs up to 24-48h after transient MCAo (Li et al., 2000; Liu et al., 2009). This is further supported by the fact that (+)-naloxone treatment starting on days 2-3 post-stroke did not have a significant effect on recovery. Thus, the therapeutic window for (+)-naloxone seems to be between 16-36h post-stroke when there is not yet substantial activation of microglia. However, when using mini-osmotic pumps the drug kinetics and concentrations at the target tissues over the administration period are only estimations until quantitative assessments of delivery are performed. When the intranasal (+)-naloxone treatment was started at day 3 post-stroke and continued for 7 days, there was a trend for milder neurological deficits on day 16 post-stroke ($p=0.066$). Due to the small number of animals, the data is inconclusive and further studies are needed to fully optimize the dosing and timing of administration. Yet, pre-stroke treatment with (+)-naloxone was not neuroprotective as has been shown before by Liao *et al.* (Liao et al., 2003), and supports our hypothesis that (+)-naloxone should be administered during the inflammation period.

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We also show that naloxone enantiomers decrease unstimulated TNF-α secretion from microglia/macrophages isolated from the stroke cortex at day 7 post-stroke. Although the characteristics and phenotypes of microglia are altered in *in vitro* conditions (Gosselin et al., 2017), this data strengthen the view that (+)-naloxone can regulate the pro-inflammatory function of microglia/macrophages. Moreover, microglia phenotype is highly dependent on the environment where it is and therefore the data should be interpreted carefully. It is noteworthy though that this study is the first to show that (-)- or (+)-naloxone inhibits basal TNF-α secretion from stroke-activated microglia/macrophages without extra stimulus.

The time course of microglial and astrocyte activation in the thalamus following cortical stroke is less studied. We observed activated microglia/macrophages and increased GFAP immunoreactivity in the thalamus still at 4 months post-stroke, and indeed, also in the intraluminal MCAo model microglial activation in the thalamus has been reported to be long-lasting, for up to 6 months post-stroke (Justicia et al., 2008). However, in the intraluminal MCAo model the ischemic lesion is somewhat close to the thalamus and in some cases extends into it. Microglia and astrocytes have been shown to be activated already after day 3 post-stroke in the ipsilateral thalamus in the transient intraluminal MCAo model (Loos et al., 2003). In our model, microglial and astrocyte activation was evident in the ipsilateral thalamus at 7 days post-stroke, but interestingly, microglia were not phagocytic until at day 14. Previously, it has been reported that neuronal degeneration in the ipsilateral thalamus is evident at 14 days post-stroke in the transient intraluminal MCAo model (Loos at al 2003), similar to our findings. The ischemia-induced neuronal loss and microglia/macrophage activation in the thalamus probably reflects retrograde/anterograde degeneration caused by cortical damage. However, inflammation has

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been implicated in secondary neurodegeneration (Block et al., 2005; Schroeter et al., 2006) and (+)-naloxone reduces inflammation and neurodegeneration at both primary (cortex) and secondary (thalamus) sites of injury. Although it remains unclear whether the beneficial effects of (+)-naloxone occur directly in cells in the thalamus or indirectly by reducing inflammation in the cortex, or both, intranasal (+)-naloxone is apparently beneficial to recovery from stroke. The thalamus regulates multiple sensory and motor functions that are also controlled by other brain regions. Furthermore, the thalamus is a relay-station connecting the right and left hemispheres, and a unilateral lesion of the thalamus usually has little behavioral consequences (Carrera and Bogousslavsky, 2006). Overall, the role of thalamic neurodegeneration and inflammation following cortical infarction remains unclear, and it is not known whether the secondary pathology affects the behavioral recovery. Our study supports the need for future research into the role of thalamic injury in cortical stroke.

Single (+)-naloxone doses that had effect on recovery in our study were 0.08 mg/kg, 0.32 mg/kg and 0.8 mg/kg. As a comparison, the FDA-approved Narcan® nasal spray to treat opioid overdose contains 4 mg of (-)-naloxone hydrochloride being equivalent to 0.08 mg/kg dose for a person weighing 50 kg. Based on pharmacokinetic data on naloxone (Dowling et al., 2008; Krieter et al., 2016), we estimated that (+)-naloxone levels in rat brain reached nanomolar concentrations. The pharmacokinetic profile of naloxone is favorable for stroke treatment since it is efficiently transported to the brain. However, its short elimination half-life [1.57±0.784h (Lewis et al., 2012)] requires repeated dosing. Short-term (up to 2 days post-stroke) intravenous (-)-naloxone (dose from 0.4 mg to 4 mg) has been tested in patients having an acute ischemic stroke, and it has rapidly, within minutes, improved neurological deficits (Baskin and Hosobuchi,

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1981; Jabaily and Davis, 1984). However, later clinical studies on the efficacy of naloxone have been inconclusive. A phase II open trial studying the safety of a high loading dose followed by 24h infusion of (-)-naloxone did not find benefit at 3 months post-stroke when the treatment was started within 48h of stroke onset (Olinger et al., 1990). A subsequent double-blind, randomized pilot trial using the 24h infusion found no significant differences between the naloxone and placebo groups when the treatment was initiated within 12h of the onset of symptoms (Federico et al., 1991). At the time when these studies were performed, the anti-inflammatory effects of naloxone were not known. The lack of effect in these clinical studies may have resulted from the short treatment regimen. Treatment for only 24h may not be enough to dampen the post-stroke microglial response. Moreover, the clinical studies have been based on the assumption that opioid antagonism is at least partly behind the beneficial effect of naloxone in acute stroke that was reported in the early case studies in the 1980's. In our experiment, both (+)- and (-)-naloxone had a similar effect on recovery starting from post-stroke day 10 onwards, implying that opioid receptor antagonism is not necessary for the recovery promoting effect in the chronic phase of stroke. Thus, we propose additional clinical studies that would employ different dosing paradigms to optimize the post-stroke (+)-naloxone dosing regimen.

The Stroke Therapy Academic Industry Roundtable (STAIR) recommendations to improve the quality of preclinical stroke studies emphasize the importance of dose-response and therapeutic window studies together with histological and functional outcome monitoring. According to STAIR recommendations, the therapies should be tested in several animal species using both sexes as well as aged, and comorbid animals. Therefore, we warrant further studies using (+)-naloxone for stroke treatment in female, aged and comorbid animals to better reflect the clinical

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554 situation within the heterogeneous patient population. Also, behavioral testing should be carried
555 out for more than 14 days to confirm the beneficial effect of (+)-naloxone on long-term
556 behavioral recovery. Regarding the safety of naloxone therapy, naloxone has been already shown
557 to be safe and well tolerated in patients with the corresponding dose range we have been using in
558 rats.
559
560 In conclusion, characterization of the neuroinflammatory response in rat cortical stroke revealed
561 long-lasting microglia/macrophage and astrocyte activation as well as neuronal death in the
562 ipsilateral thalamus. Phagocytic cells were present in the thalamus for up to 4 months post-
563 stroke. This delayed neuronal loss and phagocytosis in the thalamus could serve as a new target
564 for drug treatment after stroke with a larger therapeutic window than exists for current post-
565 stroke treatment (i.e. tissue plasminogen activator). Most importantly, we found that intermittent
566 post-stroke intranasal (+)-naloxone treatment starting on day 1 post-stroke promoted short-term
567 behavioral recovery, reduced microglial activation in the striatum and thalamus, and decreased
568 neuronal loss in the cortex and thalamus. It is likely that (+)-naloxone mediates its positive
569 effects on stroke via mechanisms where TLR signaling and reduction of oxidative stress are
570 involved. (+)-Naloxone is thus a promising drug for the treatment of ischemic stroke.
571

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705 **Legends**

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Figure 1. Chemical structure of (-)-naloxone and (+)-naloxone enantiomers. Ki of (-)-naloxone for antagonizing opioid receptors is in the 1 nM range whereas (+)-naloxone has a very low affinity for opioid receptors with a Ki of 10 000 nM (Iijima et al., 1978).

Figure 2. Time course of microglia/macrophage activation after cortical stroke. Representative images of immunostaining for all microglia/macrophages (Iba1) and phagocytic microglia/macrophages (CD68) from a: ischemic core, b: peri-infarct area, c: striatum, d: thalamus and e: whole brain sagittal section at 2 (**C, D**), 7 (**E, F**), 14 (**G, H**), 28 (**I, J**), 56 (**K, L**) and 112 (**M, N**) days after 90 min dMCAO in rat. Control images (**A, B**) are from the contralateral hemisphere of the stroke brain. Scale bar is 50 µm (high magnification) and 2000 µm (low magnification). **O**: An example image of anti-NeuN immunostaining at post-stroke day 2 showing in more detail the regions a-d. **P:** Quantitation of CD68+ cells in the thalamus (d) at different time points showing the accumulation and clearance of phagocytic microglia/macrophages in the ipsilateral thalamus. * $p<0.05$ indicates statistical difference between the ipsilateral thalamus at different time points; # $p<0.05$ indicates statistical difference between the ipsilateral and contralateral thalamus in each time point, Mann-Whitney U test after Kruskal-Wallis test; n=4 in each group. The data represent mean ± SEM.

Figure 3. Activated microglia in the striatum are lined up along axonal bundles after cortical stroke. Immunostaining of rat striatum at post-stroke day 14 for **A**: phagocytic microglia/macrophages (CD68) and **B**: all microglia/macrophages (Iba1). **C**: Double immunofluorescence staining of rat striatum at post-stroke day 14 for phagocytic

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729 microglia/macrophages (CD68; red; **D**) and myelin (MBP; green; **E**) with DAPI (blue; **F**). A-B:
730 Scale bar is 200 µm. C-F: Scale bar is 50 µm.
731
732 **Figure 4. Time course of astrocyte activation after cortical stroke.** Representative images of
733 immunostaining for astrocytes (GFAP) from the ischemic core (a), peri-infarct area (b), striatum
734 (c) and thalamus (d) at 2 (**B**), 7 (**C**), 14 (**D**), 28 (**E**), 56 (**F**) and 112 (**G**) days after 90 min
735 dMCAo in sagittal rat brain paraffin sections. Control images (**A**) are from the contralateral
736 hemisphere of the stroke brain. Scale bar is 50 µm in high magnification images and 2000 µm in
737 low magnification images. The regions analyzed (a-d) are shown in more detail in Fig. 2O.
738
739 **Figure 5. Post-stroke intranasal administration of naloxone enantiomers promotes**
740 **functional recovery.** **A**: Experimental timeline. Intranasal naloxone (or vehicle) was
741 administered twice daily for 7 days post-stroke. D1-D14: post-stroke days 1-14, B = behavioral
742 assay. **B-C**: Effects of (+)-naloxone (0.32 mg/kg; n=27), vehicle (n=25) and stroke with no
743 treatment (n=13) on body asymmetry (B) and Bederson's neurological score test (C). ** $p<0.01$,
744 *** $p<0.001$ indicate post hoc comparison between (+)-naloxone and vehicle groups, and #
745 $p<0.05$, ## $p<0.01$, ### $p<0.001$ indicate post hoc analysis between (+)-naloxone and no
746 treatment groups with Mann-Whitney U test after Kruskal-Wallis test. **D-E**: Effects of different
747 doses of (+)-naloxone 0.0008 mg/kg (n=8), 0.008 mg/kg (n=8), 0.08 mg/kg (n=7) and 0.8 mg/kg
748 (n=8) compared to vehicle (n=11) on day 14 post-stroke on body asymmetry (D) and Bederson's
749 neurological score test (E). * $p<0.05$ and ** $p<0.01$ indicate pairwise comparison with vehicle, #
750 $p<0.05$ and ## $p<0.01$ indicate pairwise comparison with other (+)-naloxone doses with Mann-
751 Whitney U test after Kruskal-Wallis test. **F-G**: Effects of (+)-naloxone (0.32 mg/kg; n=16),

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vehicle (n=16) and stroke with no treatment (n=13) on vertical (F) and horizontal (G) activity measured for 24h on day 14. ## p<0.01, Mann-Whitney U test after Kruskal-Wallis test. **H-J**: Effects of (-)-naloxone (0.32 mg/kg; n=7) and vehicle (n=11) on body asymmetry test (H), Bederson's neurological score test (I) and body weight (J). ** p<0.01 and *** p<0.001 indicate comparison with vehicle group with Mann-Whitney U test. **K**: Effects of (+)-naloxone (0.32 mg/kg, n=27), vehicle (n=25) and no treatment (n=13) on body weight on days 7 and 14 post-stroke. ** p<0.01, one-way ANOVA, Bonferroni's post hoc test. The data represent mean ± SEM.

Figure 6. Post-stroke intranasal (+)-naloxone decreases infarction area and neuronal loss in the thalamus. A: Average infarction size calculated from NeuN negative area at day 14 post-stroke. * p<0.05, Student's *t*-test. **B**: The average number of neurons (NeuN+ cells) in the ipsilateral thalamus at day 14 post-stroke expressed as a percentage of the contralateral thalamus. * p<0.05 and ** p<0.01 indicate pairwise comparison with the control group with Mann-Whitney U test following Kruskal-Wallis test. **C**: Representative photomicrographs of anti-NeuN immunostained brain sections with infarction area delineated. **D-F**: Representative photomicrographs of anti-NeuN immunostaining of ipsilateral thalamus in naïve (D), control (E) and (+)-naloxone (F) treated rats. Scale bar is 150 μm. naïve = no stroked rats (n=6), control = stroke rats with vehicle or no treatment (n=18), (+)-naloxone (0.32-0.8 mg/kg; n=10). The data represent mean ± SEM.

Figure 7. Post-stroke intranasal (+)-naloxone decreases microglia/macrophage activation in the striatum and thalamus. A-B: Microglia/macrophages (Iba1+ cells) were counted with

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unbiased stereology in the ipsilateral (A) and contralateral (B) striatum. * $p<0.05$, ** $p<0.01$ indicate pairwise comparison with the control group with Bonferroni's post hoc test following one-way ANOVA. **C:** The area of Iba1+ cells in the ipsilateral thalamus expressed as a percentage of the contralateral thalamus. * $p<0.05$, *** $p<0.001$ indicate pairwise comparison with the control group; ## $p<0.01$ indicates comparison with the naïve group with Mann-Whitney U test following Kruskal-Wallis test. **D-K**: Representative photomicrographs of anti-Iba1 immunostaining of ipsilateral striatum (D-E) and thalamus (F-G) in control (D, F) and (+)-naloxone (E, G) treated rats; H-K show high magnification. Black arrow shows a typical Iba1+ cell. Scale bar is 1000 µm (D-G) and 50 µm (H-K). naïve = no stroked rats (n=6), control = stroke rats with vehicle or no treatment (n=18), (+)-naloxone (0.32-0.8 mg/kg; n=10). The data represent mean ± SEM.

Figure 8. The effect of different pre- and post-stroke treatment with (+)-naloxone on infarct volume and functional recovery. A: Experimental timeline. Intranasal (+)-naloxone or vehicle was administered three times: 12h and 1h before dMCAo and immediately after reperfusion. Infarction volume was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining 2 days after stroke. **B:** Average infarction volume (mm^3) on day 2 post-stroke in vehicle (n=7) and (+)-naloxone (0.32 mg/kg; n=8) pre-treated rats. Pre-stroke intranasal administration of (+)-naloxone was not neuroprotective in 60 min dMCAo. **C:** Experimental timeline. (+)-Naloxone was delivered intranasally twice daily for 7 days post-stroke starting from post-stroke day 3. **D:** The effect of (+)-naloxone (0.8 mg/kg; n=6) and vehicle (n=7) treatment from post-stroke day 3 to post-stroke day 10 on body asymmetry test. **E:** Experimental timeline. (+)-Naloxone was delivered into the ventricle via mini-osmotic pumps for 12 days post-stroke

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starting from post-stroke day 2. **F:** The effects of 12 day continuous delivery of (+)-naloxone (1.15 mg/24h; n=7) and vehicle (n=8) on body asymmetry test and **G:** body weight. In A, C and E: D = the indicated post-stroke day, B = behavioral assay. The data represent mean ± SEM.

Figure 9. Naloxone reduced TNF-α secretion from CD11b+ cells. A: LPS induced TNF-α secretion from CD11b-expressing cells isolated from the infarct area of rat brain 7 days after dMCAo. ** $p<0.01$, Mann-Whitney U test; control n=6, LPS n=5 in 2 independent experiments. **B:** CD11b+ cells were isolated from the infarct area and treated with different concentrations of naloxone as indicated for 20h. *$p<0.05$; **$p<0.01$ indicate pairwise comparison with the control group by Dunnett's post hoc test following one-way ANOVA; control n=9, naloxone n=8-9 in 3 independent experiments. The culture medium was analyzed using TNF-α ELISA. The data represent mean ± SEM.

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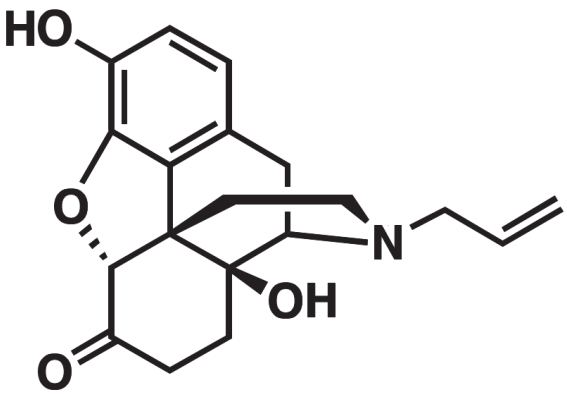

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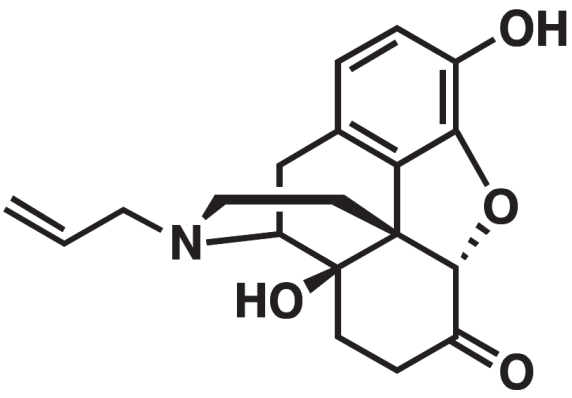

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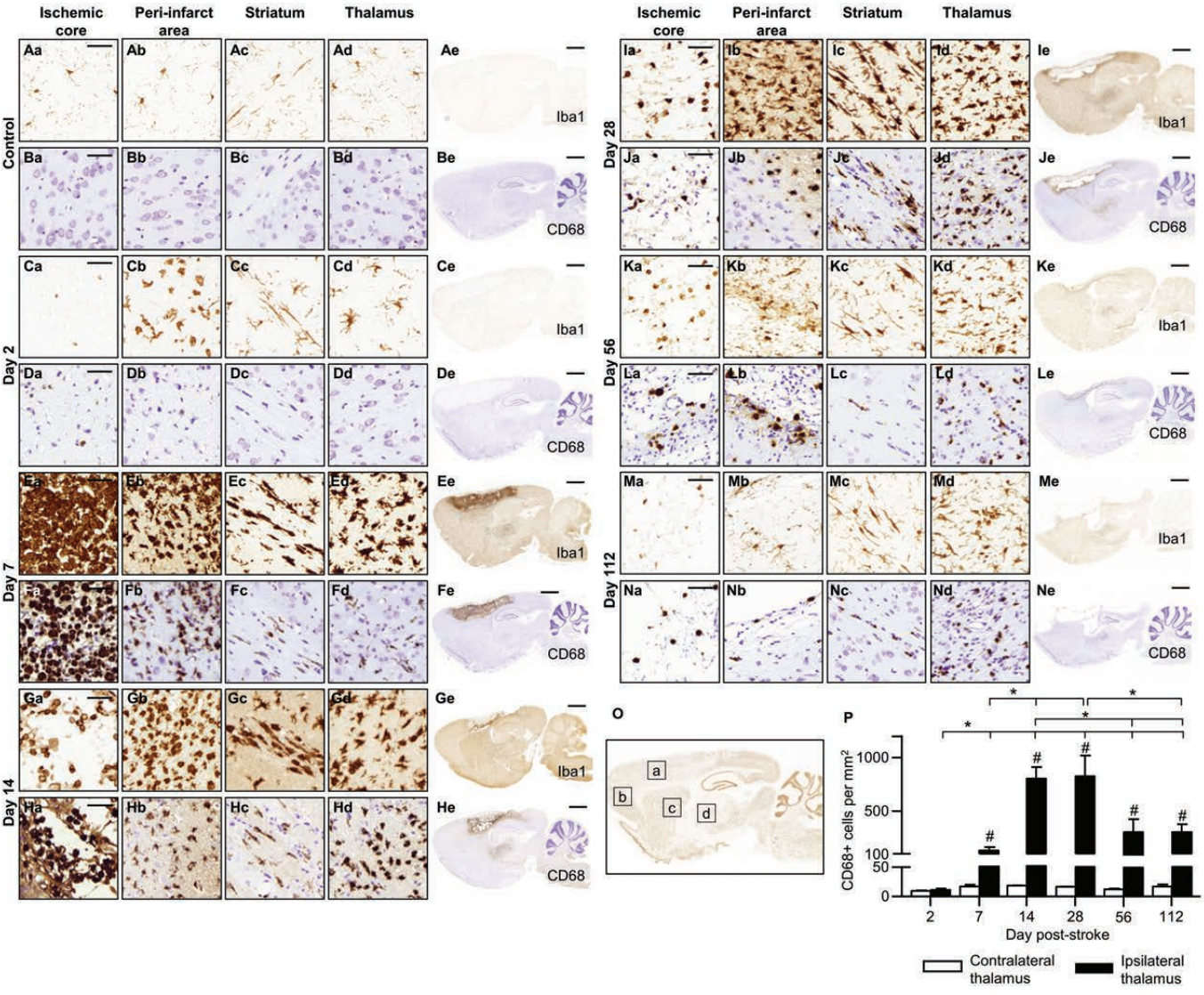

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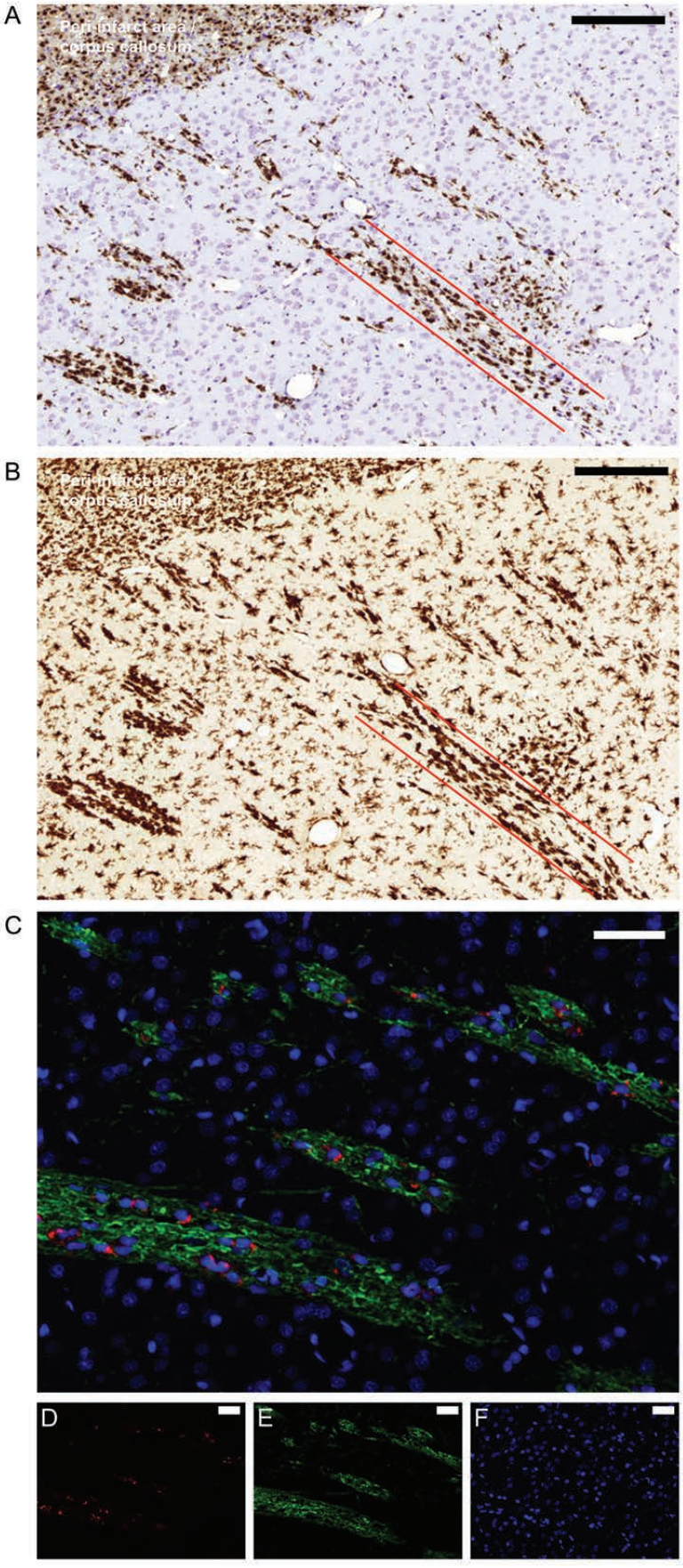

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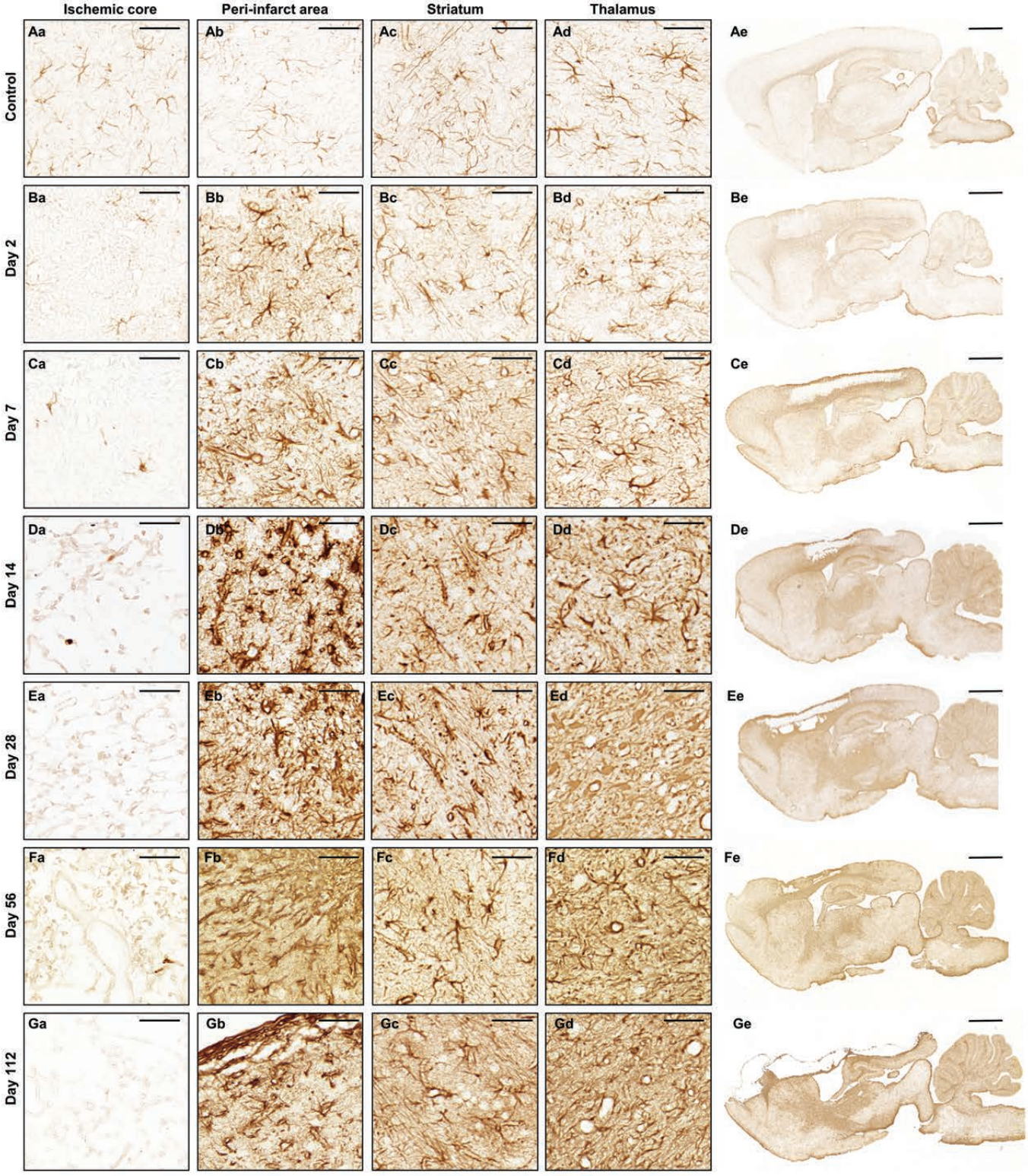

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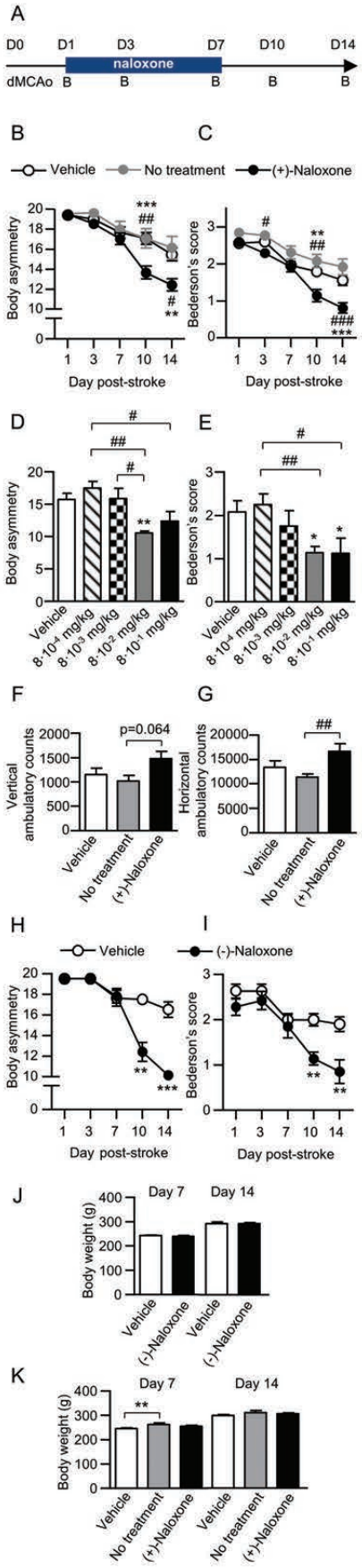

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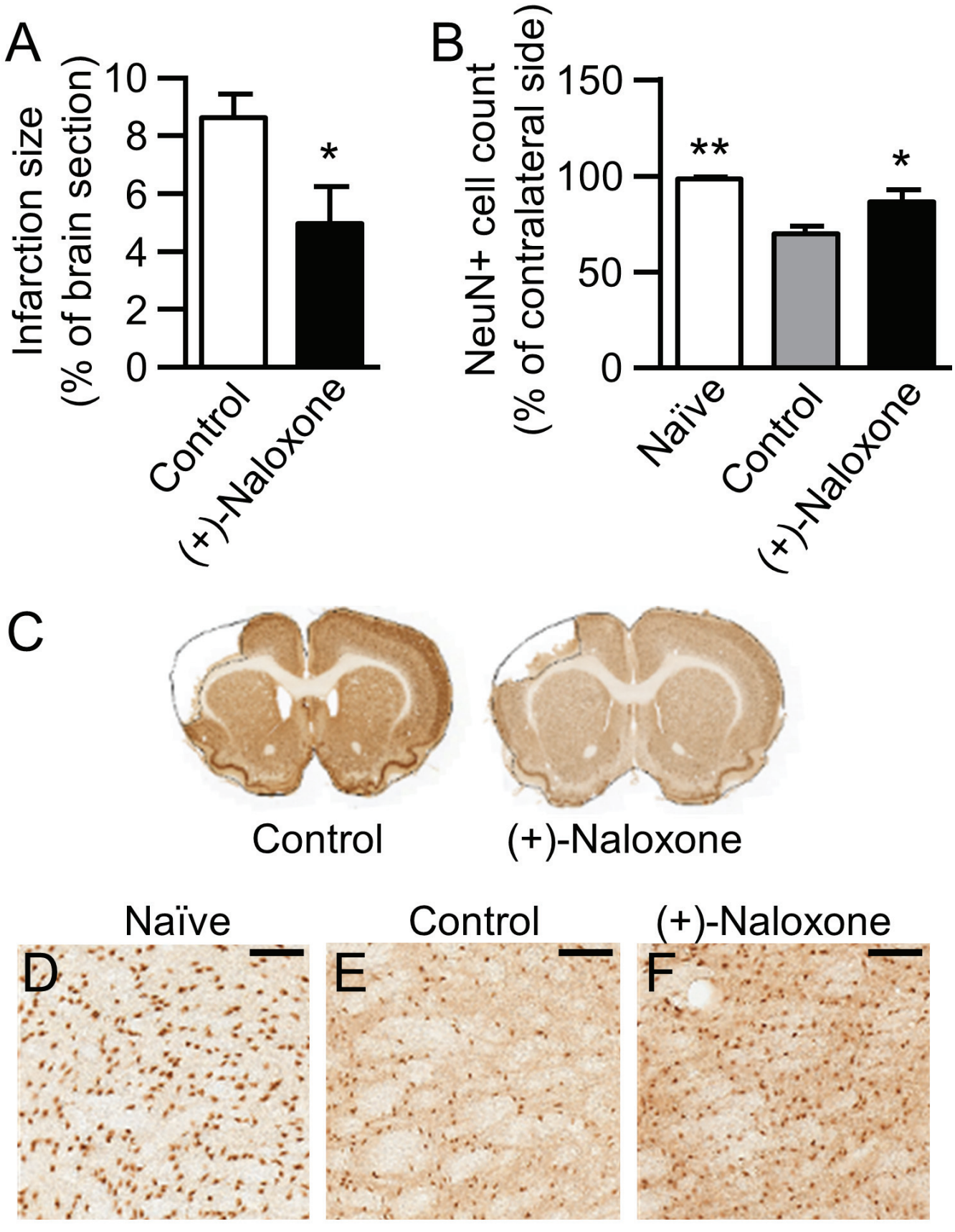

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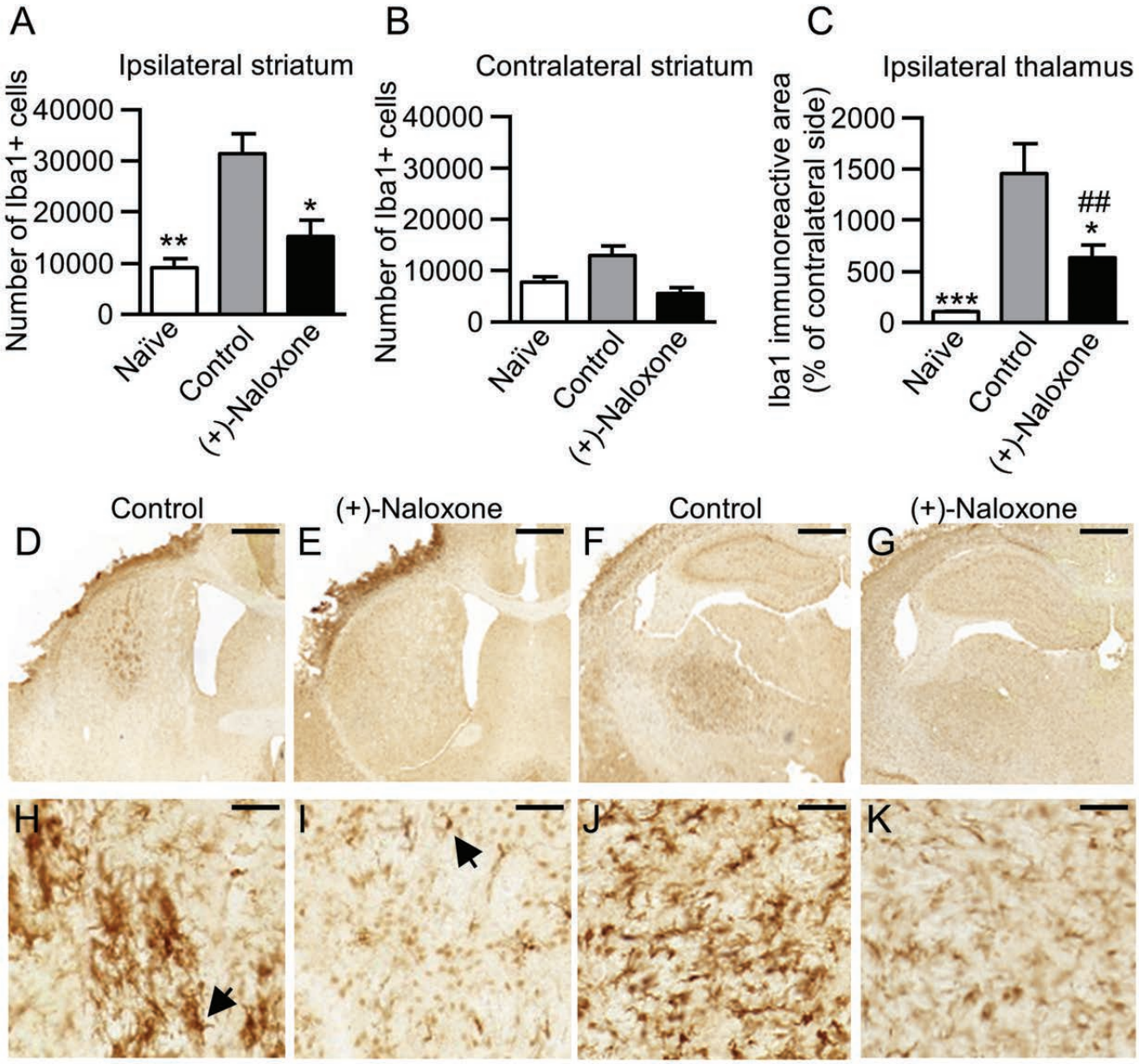

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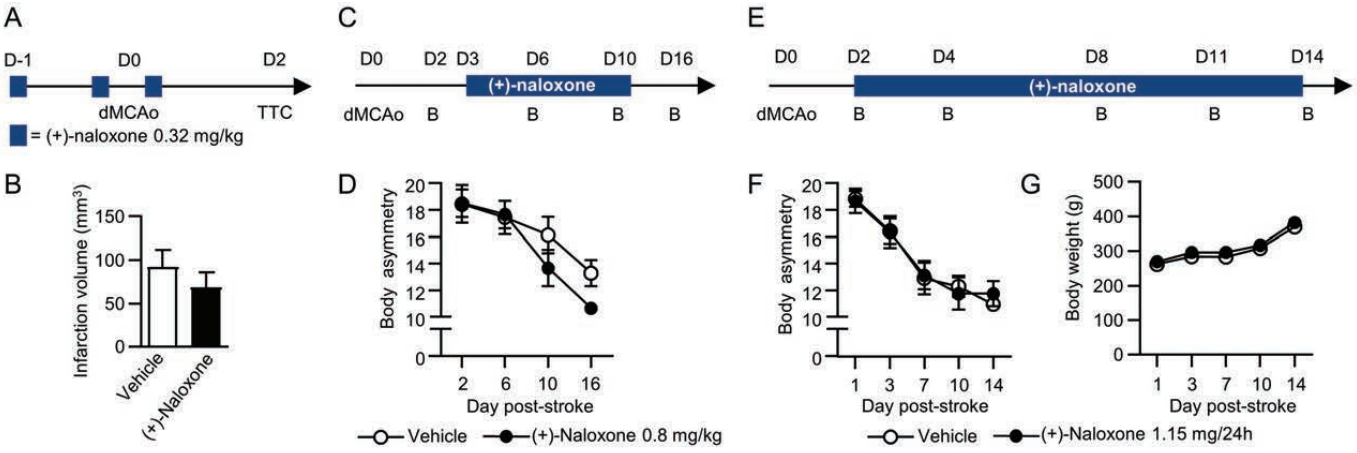

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