Secondary Pathology of the Thalamus after Focal Cortical Stroke in Rats is not Associated with Thermal or Mechanical Hypersensitivity and is Not Alleviated by Intra-Thalamic Post-Stroke Delivery of Recombinant CDNF or MANF

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Abstract

A stroke affecting the somatosensory pathway can trigger central post-stroke pain syndrome (CPSP). The symptoms often include hyperalgesia, which has also been described in rodents after the direct damage of the thalamus. Previous studies have shown that hemorrhagic stroke or ischemia caused by vasoconstriction in the thalamus induces increased pain sensitivity. We investigated whether inducing secondary damage in the thalamus by a cortical stroke causes similar pain hypersensitivity as has previously been reported with direct ischemic injury. We induced a focal cortical ischemia-reperfusion injury in male rats, quantified the amount of secondary neurodegeneration in the thalamus, and measured whether the thalamic neurodegeneration is associated with thermal or mechanical hypersensitivity. After one month, we observed extensive neuronal degeneration and found approximately 40% decrease in the number of NeuN+ cells in the ipsilateral thalamus. At the same time, there was a massive accumulation—a 30-fold increase—of phagocytic cells in the ipsilateral thalamus. However, despite the evident damage in the thalamus, we did not observe thermal or mechanical sensitization. Thus, thalamic neurodegeneration after cortical ischemia-reperfusion does not induce CPSP-like symptoms in rats, and these results suggest that direct ischemic damage is needed for CPSP induction. Despite not observing hyperalgesia, we investigated whether administration of cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF) into the ipsilateral thalamus would reduce the secondary damage. We gave a single injection (10 μg) of recombinant CDNF or MANF protein into the thalamus at 7 days post-stroke. Both CDNF and MANF treatment promoted the functional recovery but had no effect on the neuronal loss or the amount of phagocytic cells in the thalamus.

Introduction

Central post-stroke pain (CPSP) is a neuropathic pain syndrome developing typically months after stroke[1,2]. The prevalence of CPSP in patients is reported to vary between 1% and 14%[2–4]. The symptoms respond poorly to drug treatment and consist of spontaneous or evoked pain that may include hyperalgesia which is induced by nociceptive stimuli at a lower threshold than normally, and allodynia which is induced by non-nociceptive stimuli[3,4]. Abnormalities in thermal or mechanical pain sensation occur in more than 90% of patients with CPSP[3]. The pathophysiology behind CPSP is still unclear. Allodynia is thought to be triggered by central disinhibition leading to over-activation of the thalamus, whereas the mechanism for hyperalgesia has been proposed to be central sensitization resulting from

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deafferentation of neurons[4]. Patients suffering from thalamic infarcts at the ventral posterolateral thalamic nucleus (VPL)[4] or the ventral posterior nucleus-pulvinar (in rodents: posterior thalamic nuclear group; Po) border zone have a higher risk for developing CPSP[5]. However, non-thalamic infarcts anywhere in the somatosensory pathways, including the cortex, can also induce CPSP[3,4].

In rodents, the development of CPSP has been shown to be region specific after hemorrhagic lesion, and to be associated with a lesion of the VPL/ventral posteromedial thalamic nucleus (VPM)-Po system[6]. Several studies using hemorrhagic lesions of VPL/VPM have found mechanical and thermal hyperalgesia starting 7 days post-hemorrhage and lasting at least several weeks[7–9]. In some studies, only mechanical hyperalgesia was found after VPL/VPM hemorrhage[6,10]. Similarly to rodents, primates develop mechanical and thermal hyperalgesia after a hemorrhagic stroke of the VPL[11]. In addition, in rodent ischemic stroke model, sensitization to mechanical[12] and electrical[12,13] stimuli has been shown to occur 3 days after intraluminal middle cerebral artery occlusion and to persist at least 2 weeks. Furthermore, focal thalamic ischemia caused by endothelin-1 injection is known to induce local ischemia and neuronal loss in the thalamus and sensitization to thermal, but not mechanical, stimuli 4 weeks after the endothelin-1 injection[14]. In all of these studies, the lesion extended to the thalamus causing direct ischemic or hemorrhagic damage in the thalamus.

A cortical infarct induces delayed neuronal loss in the ipsilateral thalamus as a secondary effect due to connecting thalamocortical and corticothalamic pathways[15,16]. This phenomenon, called "exo-focal post-ischemic neuronal death," was originally described by Nagasawa and Kogure in 1990[16]. Secondary atrophy of the thalamus after a middle cerebral artery infarct has been observed in patients as well[17–20]. Moreover, microglia are activated in the thalamus after cortical stroke in rodents[15] and humans[21,22]. Microglial activation seems to play a role in the development of hyperalgesia and neuropathic pain, since treatment with microglial inhibitor minocycline has been shown to reduce hyperalgesia in a hemorrhagic CPSP model[23], and microglial activation in the spinal cord has been shown to play a role in the maintenance of chronic pain that develops after spinal cord injury[24–26]. Also, transient activation of VPL microglia with a local chemokine injection induces mechanical and thermal hypersensitivity[27]. In patients, spinal cord injury can lead to delayed loss of connected neurons in the thalamus and secondary somatosensory cortex, but the severity of neurodegeneration does not correlate with neuropathic pain, and sometimes more severe disruption of the somatosensory pathway can lead to numbness and loss of pain sensation rather than hypersensitivity[28]. Given the many uncertainties in the cause of neuropathic pain after injury in the somatosensory pathway, and the intriguing findings on the role of microglial activation in pain sensitization, we wanted to further extend the studies to cortical ischemia. Thus, our study aimed to investigate whether pure cortical ischemia caused by distal middle cerebral artery occlusion (dMCAo) and the following secondary thalamic neurodegeneration and microglial activation is associated with thermal or mechanical hypersensitivity. We are the first to study pain sensitization in the dMCAo ischemia-reperfusion model, and show here extensive thalamic neurodegeneration and inflammation which is not related to CPSP-like symptoms. These data indicate that neither neuronal loss nor microglial activation *per se* cause CPSP, and may thus bring more insight into the complex mechanism of CPSP development.

As an attempt to reduce the secondary damage, we targeted proteins with known *in vivo* neuroprotective effects to the thalamus where the delayed secondary damage occurs. Since the secondary damage occurs in a delayed manner, it would be an attractive target from a clinical point of view as the time window for acute neuroprotective treatment is extremely limited. Cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF) are endoplasmic reticulum (ER) resident proteins and form a family of proteins that differs structurally and functionally from classical neurotrophic factors[29–33]. The mechanism of action of CDNF and MANF is still unclear, but MANF has been shown to be important factor for ER homeostasis[34,35] and to protect from ER stress-induced cell death *in vitro*[36]. MANF and CDNF are neuroprotective *in vivo* in ischemic cerebral injury[37–40] and protect mouse primary cultures from oxygen glucose deprivation *in vitro*[39,41]. Both CDNF and MANF protect dopaminergic neurons and restore dopaminergic neurocircuitry in Parkinson's disease toxin model[42,43]. CDNF has been shown to decrease microglial activation in the substantia nigra of 6-hydroxydopamine treated rats[44]. Also, MANF has been shown to have anti-inflammatory effects by downregulating cytokine expression[45] and the NF-κB pathway[46,47], and to mediate tissue repair by increasing the alternative M2-like activation of innate immune cells after retinal damage in *Drosophila* and mouse[48]. We have shown before that when MANF is targeted into the peri-infarct area with an adeno-associated viral (AAV) vector 2 days after dMCAo, the number of phagocytic cells is transiently increased at 4 days post-stroke and the functional recovery of the rats is hastened[49]. Overall, there is rather strong evidence that MANF, and possibly CDNF, can modulate inflammation and reduce neuronal cell death. Thus, the aim was to test if post-stroke delivery of recombinant human CDNF (rhCDNF) or MANF (rhMANF) directly into the thalamus is able to alleviate the secondary pathology and promote behavioral recovery after dMCAo.

Materials and Methods

Animals

A total of 59 male Sprague Dawley rats (age 8–9 weeks, weight 250–300 g, Envigo, Netherlands) were used for the experiments. Rats were housed in groups of 4 animals in

individually ventilated cages with *ad libitum* access to food and water under a 12h/12h dark–light cycle. The wellbeing of the animals was monitored daily. All behavioral experiments were performed during the light phase and the animals in different experimental groups were assessed in a random order by a blinded investigator. All animal experiments were conducted according to the 3 R principles of EU directive 2010/63/EU on the care and use of experimental animals, local laws and regulations, and were approved by the national Animal Experiment Board of Finland (protocol approval number ESAVI/7812/04.10.07/2015). The sample size was calculated based on a pilot experiment on Hargreaves' test, with 0.05 significance level, 0.95 power, and the calculated Cohen's *d* (2.21) as the effect size. All behavioral experiments and analyses were performed in a blinded manner, and the results are reported according to the ARRIVE guidelines. One animal from the stroke group was excluded because it did not have observable lesion. One animal died due to anesthesia during dMCAo surgery and one animal from the sham group died from an unknown reason 3 days after the sham operation. There was no further mortality.

Distal Middle Cerebral Artery Occlusion Model

To model cortical ischemia-reperfusion injury, a dMCAo was performed as described before[37,38,50]. Briefly, the rats were anesthetized with an intraperitoneal injection of 4% chloral hydrate (0.4 g/kg; Sigma Aldrich, St. Louis, MO, USA). Both common carotid arteries (CCAs) were isolated through a cervical incicion, and a craniotomy was made on the right hemisphere to expose the middle cerebral artery (MCA). The distal branch of the right MCA was ligated with 10-0 suture and the CCAs were occluded with non-traumatic arterial clips. After 90 min the suture and clips were removed to allow reperfusion. Sham-operated rats went through all the same procedures as stroke rats but the arteries were not occluded. Lidocaine (10 mg/ml, Orion Pharma, Espoo, Finland) was used locally on the skin during surgery and a single dose of carprofen (5 mg/kg s.c.; Rimadyl, Zoetis, Louvain-la-Neuve, Belgium) was given after the surgery for postoperative pain. Saline (4–5 ml s.c.) was given after the surgery to prevent dehydration. Body temperature of the rats was maintained at 37°C until the animals had recovered from anesthesia and were returned to their home cages.

Although chloral hydrate anesthesia is still used in stroke research[51–56], the use has been criticized for ethical and safety reasons[57]. Therefore, we would like to point out that chloral hydrate is not an optimal anesthetic for surgical procedures in rodents and the anesthesia protocol should be further refined.

Neurological Tests

Body asymmetry test[58] and modified Bederson's score[59] were used to monitor the level of neurological deficits caused by ischemia[37,38]. The body asymmetry was analyzed from 20 consecutive trials by lifting the rats above the testing table by the 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 resulting in 10 contralateral turns). In modified Bederson's score the neurological deficits were scored according to the following criteria: 0 = no observable deficit; 1 point = rats show decreased resistance to lateral push; 2 points = rats keep the contralateral forelimb to the breast and extend the other forelimb straight when lifted by the tail in addition to behavior in score 1; 3 points = rats twist the upper half of their body toward the contralateral side when lifted by the tail in addition to behavior in other scores.

Hargreaves' Test

Hargreaves' test was used to measure thermal hyperalgesia from all the paws. The rats were placed into Plexiglas boxes on Plexiglas floor, and were allowed to habituate for 10–15 min until they ceased exploratory behavior. An infrared beam (intensity 70 mW/cm^2) was positioned beneath the plantar surface of each paw using an automated device with a switch-off function as a response to moving the paw (Plantar test 37370, Ugo Basile, Gemonio, Italy). The cut-off time was set to 30 s. Each measurement was repeated three times with several minutes in between trials. A rapid withdrawal of the paw followed by shaking and/or licking the paw was interpreted as a reaction to pain. An average of three trials was used for the data analysis. We included an additional group of naïve rats in the experiments to control for the effect of repeated testing, and the animals were assigned to different groups (naïve, sham, stroke) based on the basal latency to withdraw paw in Hargreaves' test to form equivalent groups.

Mechanical Stimulation with Dynamic Plantar Aesthesiometer

Dynamic Plantar Aesthesiometer was used to measure sensitivity toward light mechanical touch from the left (contralateral) hindpaw. The rats were placed into Plexiglas boxes on a metal mesh surface and were allowed to habituate for 10–15 min until they ceased exploratory behavior. A stainless steel probe (diameter 0.5 mm) attached to a touch stimulator (Dynamic Plantar Aesthesiometer 37450, Ugo Basile) was lifted toward the plantar surface of the left hindpaw from below. The touch stimulator had an automated switch-off function as a response to movement of the paw. A rapid withdrawal of the paw was interpreted as a reaction to the stimulus. Maximum force exerted was set to 50 g in 20 s. As in Hargreaves' test, each measurement was repeated three times with several minutes in between trials.

Intracranial Administration of rhCDNF and rhMANF

The rats were balanced into groups based on the body swing and Bederson's score on day 4 post-stroke. We chose to give the protein injection on day 7 post-stroke since there are not yet many phagocytic cells in the thalamus at that time point[60]. The stereotaxic surgery was performed under isoflurane anesthesia (4.5% during induction, 2.5% during maintenance). After placing the animal in a stereotaxic frame (Stoelting, Wood Dale, IL, USA), the skull was exposed and a small hole was made with a dental drill. Using coordinates according to The Rat Brain in Stereotaxic Coordinates[61], 4 μl of vehicle (phosphate buffered saline; PBS), rhCDNF (2.5 μg/μl) or rhMANF (2.5 μg/μl) was injected into the right thalamus (A/P –3.0; M/L –3.0; D/V –6.0) at speed 0.5 μl/min with 33G blunt needle (Nanofil; World Precision Instruments, Sarasota, FL, USA). The needle was kept in place for 4 min after the injection to prevent backflow. RhCDNF and rhMANF (Icosagen, Tartu, Estonia) were produced in a Chinese hamster ovarian (CHO)-based cell line.

The distribution of rhCDNF in the brain after intrathalamic injection was tested in naïve rats by injecting rhCDNF as described above, and sacrificing the animals 2 h after the stereotaxic injection for immunohistochemistry with anti-hCDNF antibody.

Immunohistochemistry

The rats were anesthetized with a lethal dose of pentobarbital (90 mg/kg i.p.; Mebunat, Orion Pharma) and transcardially perfused with 200 ml of PBS followed by 500 ml of 4% paraformaldehyde (Sigma Aldrich) in PBS. The brains were post-fixed in 4% paraformaldehyde for at least 2 days before they were dehydrated in a series of ethanol and xylene, and embedded in paraffin. Brains were cut into 5 μm thick coronal sections using a Leica HM355 S microtome and mounted on Labsolute microscope slides (Th. Geyer, Renningen, Germany). Sections were deparaffinized in xylene, rehydrated in a series of ethanol, and heated in 0.05% citraconic anhydride (Sigma Aldrich), pH 7.4, for antigen retrieval. Endogenous peroxidase activity was blocked with 0.6% hydrogen peroxide (Sigma Aldrich). Non-specific antibody binding was blocked with 1.5% normal horse or goat serum (Vector Laboratories, Burlingame, CA, USA), followed by incubation with primary antibody (mouse anti-CD68 1:500, cat#MCA341 R, AbD Serotec, Kidlington, UK; mouse anti-NeuN 1:200, cat#MAB377, Millipore, Billerica, MA, USA; rabbit anti-MBP 1:500, cat#40390, Abcam, Cambridge, UK; rabbit anti-hCDNF 1:500, DDV1, a gift from Dr. Johan Peränen, University of Helsinki, Finland) at 4°C overnight. The next day, sections were incubated with horse anti-mouse 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 (Vector Laboratories). Anti-CD68 immunostained sections were additionally stained with cresyl violet to visualize the whole section for cell counting. For immunofluorescence staining, goat anti-rabbit Alexa Fluor 488 (1:500, cat#A11034, Invitrogen, Carlsbad, CA, USA) and goat anti-mouse Alexa Fluor 568 (1:500, cat#A11004, Invitrogen) were used as secondary antibodies. Immunofluorescence was imaged with a Leica TCS SP5 MP confocal microscope.

Image Analysis

The stained 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; https://www.helsinki.fi/en/infrastructures/histotechnology-and-laboratory-animal-pathology/bi-histoscanner) and images of stained sections were taken with Pannoramic Viewer version 1.15.3 using 10× magnification. The thalamus was outlined according to The Rat Brain in Stereotaxic Coordinates[61] and the number of NeuN+ and CD68+ cells in the thalamus was quantified with Image-Pro Analyzer version 7.0. The cells were counted from a minimum of three coronal sections per brain between –2.9 and –3.9 mm relative to bregma. The average was counted for each animal and was used for further analysis. The number of NeuN+ cells in the ipsilateral thalamus was expressed as a ratio of the number of NeuN+ cells in the contralateral thalamus of each section to decrease variation due to differences in the background staining. The neuronal loss in the thalamus, outlined according to The Rat Brain in Stereotaxic Coordinates[61], on day 7 post-stroke was quantified from three sagittal sections per hemisphere (every 0.5 mm between 2.4 and 3.4 mm, and –2.4 and –3.4 mm relative to bregma) stained with cresyl violet by manually counting the Nissl+ neurons. The neuronal counts were expressed as a ratio of the contralateral thalamus. The average infarction size was quantified from a minimum of four anti-NeuN stained sections of the caudal brain, between –2.3 and –4.4 relative to bregma. The area devoid of NeuN+ cells was delineated in Pannoramic Viewer version 1.15.3 and the infarction size was expressed as a percentage of the whole section. In the CDNF/MANF experiment, the infarct size was similarly quantified from four sections stained with cresyl violet between 1.2 and –3.6 relative to bregma.

Statistical Analysis

All the statistical analysis was done with IBM SPSS Statistics version 24 and all values are reported as mean ± standard deviation (SD). Hargreaves' test, Dynamic Plantar Aesthesiometer and body weight were analyzed with two-way repeated measures ANOVA followed by Bonferroni's post hoc test. Body asymmetry and Bederson's score were analyzed with Kruskall–Wallis test and pairwise Mann–Whitney U test using the exact p-value. Immunohistological data were analyzed by one-way ANOVA followed by Bonferroni's post hoc test. The unit of analysis was a single
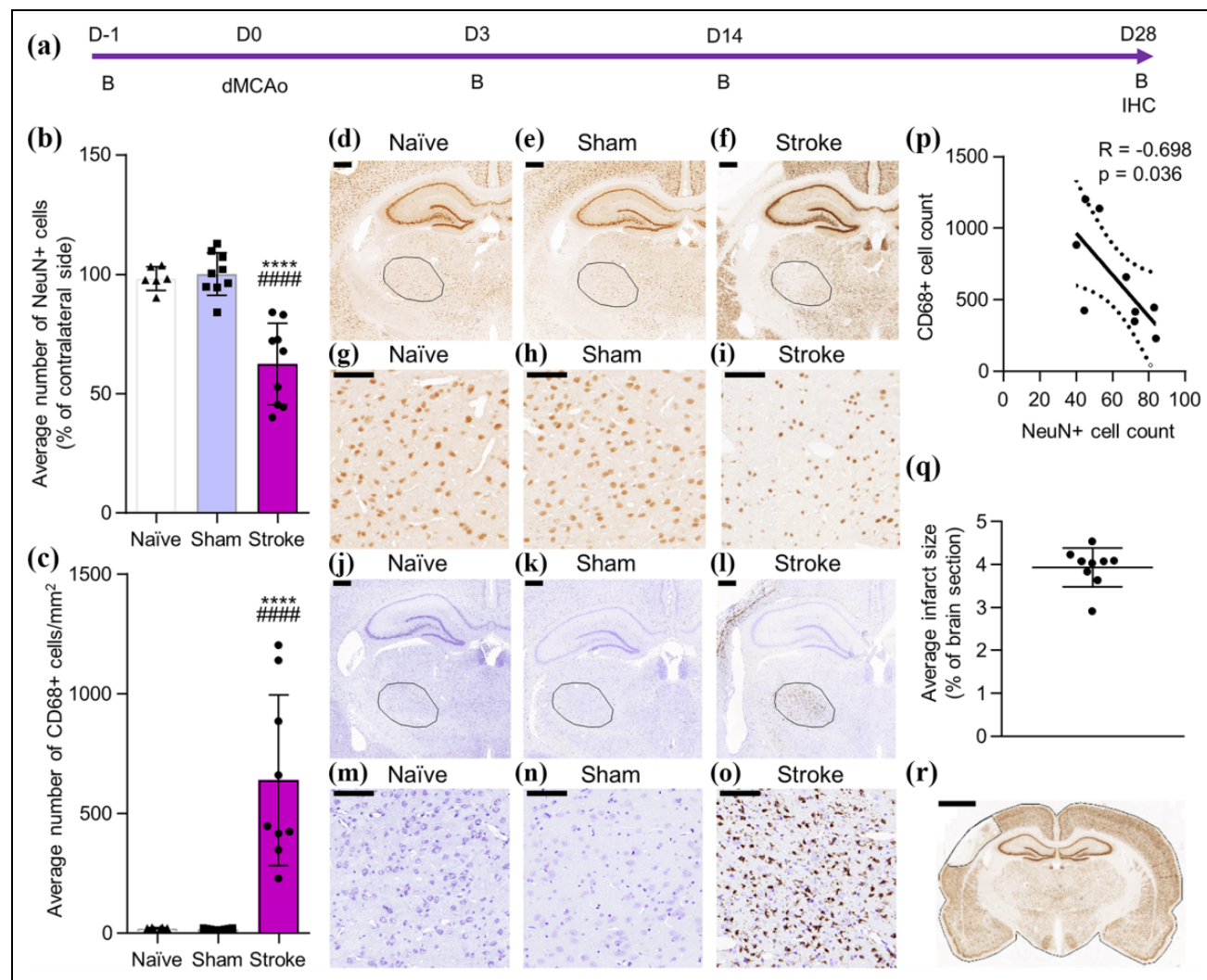
Fig. 1. Delayed neuronal loss and phagocytosis occurs in the ipsilateral thalamus after cortical ischemia-reperfusion injury. (a) Experimental timeline. D = indicated post-stroke day; B = behavioral experiment; dMCAo = distal middle cerebral artery occlusion; IHC = immunohistochemistry. (b) The ratio of NeuN+ cells in the ipsilateral thalamus compared with the contralateral thalamus in naïve rats and 28 days after cortical stroke or sham operation. (c) The number of phagocytic CD68+ cells in the ipsilateral thalamus in naïve rats and 28 days after cortical stroke or sham operation. Representative images of anti-NeuN (d–i) and anti-CD68 (j–o) immunostained brain sections from naïve (d, g, j, m), sham (e, h, k, n), and stroke (f, i, l, o) groups. The delineated area in d–f; j–l indicates the area analyzed. Scale bar is 500 μm in low magnification images and 100 μm in high magnification images. Naïve $n = 6$, sham $n = 9$, stroke $n = 9$. ****($p < 0.0001$) indicates comparison with the sham group, #### ($p < 0.0001$) indicates comparison with the naïve group. (p) Pearson correlation with 95% confidence intervals of neuronal loss (b) and the number of phagocytic cells (c) in the thalamus 28 days post-stroke. (q) The average infarct size in the caudal brain (between −2.3 and −4.4 relative to bregma) at 28 days post-stroke expressed as a percentage of the whole section. (r) Representative image of anti-NeuN stained section showing the NeuN negative infarct area on the cortex. Scale bar is 2000 μm. All values are reported as mean ± SD.

animal in all analyses. Statistical significance was considered at $p < 0.05$.

Results

Neurodegeneration and Microglial Activation in the Ipsilateral Thalamus at Day 28 Post-Stroke

We characterized the secondary thalamic neurodegeneration by immunostaining with anti-NeuN (a marker for neurons)

and anti-CD68 (a marker for activated, phagocytic microglia/macrophages) antibodies (Fig. 1a). At 28 days post-stroke, 38% of the neurons in the ipsilateral thalamus were lost (Fig. 1b; d–i). The amount of NeuN+ cells in the ipsilateral thalamus was 98%, 100%, and 62% of the amount in the contralateral thalamus in the naïve, sham, and stroke groups, respectively. The stroke rats had significantly fewer neurons in the ipsilateral thalamus ($F_{2,21} = 26.11$, $p < 0.0001$, one-way ANOVA) than the rats in naïve ($p <$
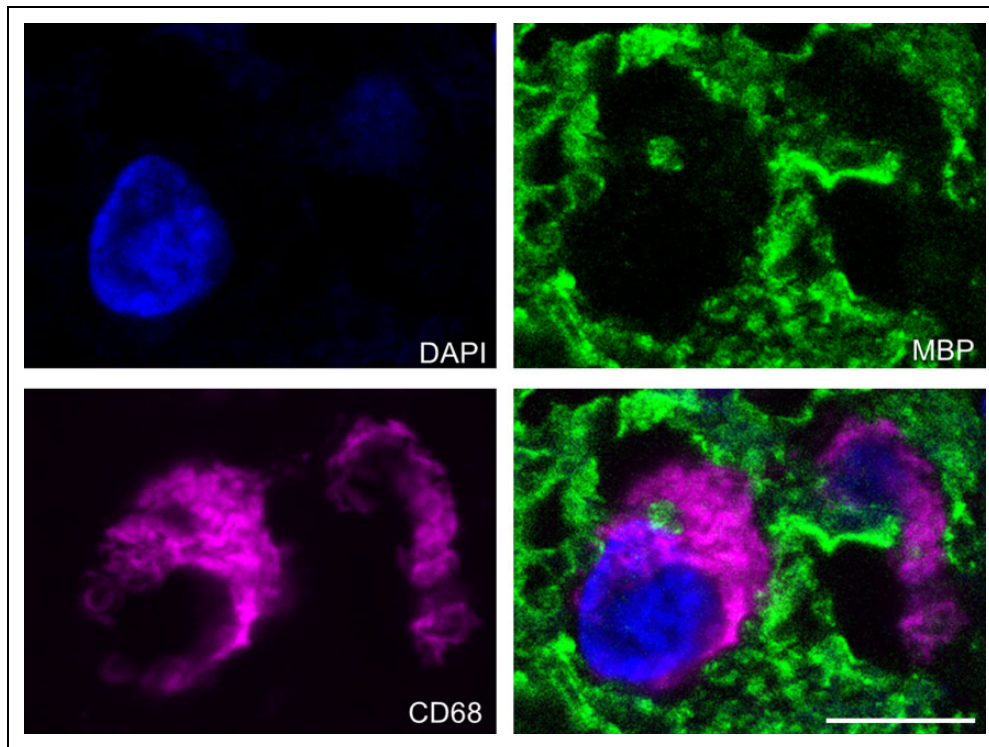
Fig. 2. CD68+ cells are phagocytosing myelin in the ipsilateral thalamus at 28 days post-stroke. Myelin basic protein (MBP)-CD68 double immunofluorescence staining of the thalamus showing a phagocytosing cell (magenta) with MBP (green) inclusion. Cellular nuclei is shown in blue color with DAPI staining. Scale bar is 10 μm.

0.0001) and sham ($p < 0.0001$) groups. There was no difference between the naïve and sham groups, and sham operation did not cause any detectable damage to the brain. Also, the stroke rats had significantly more CD68+ cells in the ipsilateral thalamus (640 cells/mm^2; $F_{2,21} = 22.57$, $p < 0.0001$, one-way ANOVA) when compared with the naïve (20 cells/mm^2; $p < 0.0001$) and sham (16 cells/mm^2; $p < 0.0001$) groups (Fig. 1c; j–o). There was a negative correlation between the number of CD68+ cells and NeuN+ cells in the ipsilateral thalamus at 28 days post-stroke (Pearson correlation $R = -0.698$, $p = 0.036$; Fig. 1p). The average infarct size in the caudal brain was 3.9% of the brain section and the infarct was restricted to the cortex in all animals (Fig. 1q–r).

Next, we clarified the location of phagocytic cells in relation to myelin debris in the thalamus. Some of the CD68+ cells colocalized with myelin in the ipsilateral thalamus (Fig. 2). However, we did not observe significant differences in the amount of myelin between the ipsilateral and contralateral thalamus on post-stroke day 28.

Cortical Stroke Induced Long-Term Neurological Deficits but no Thermal or Mechanical Sensitization

The stroke rats had significantly more severe neurological deficits in the body asymmetry test than the sham-operated rats at all time points (day 3: $p < 0.0001$; day 14: $p < 0.0001$;

day 28: $p = 0.014$, Mann–Whitney U test; Fig. 3a). Also, in Bederson's score test, the neurological deficits were more severe in the stroke group at days 3 ($p < 0.0001$) and 14 ($p < 0.0001$) post-stroke, but there was a spontaneous recovery at post-stroke day 28 as the stroke rats had significantly milder deficits compared with day 3 ($p = 0.014$) and there was no difference between the sham and stroke groups ($p = 0.050$; Fig. 3b). The stroke rats gained body weight slower than the naïve rats (time effect $F_{2,50} = 806.2$, $p < 0.0001$; group effect: $F_{2,25} = 4.167$, $p = 0.027$, two-way repeated measures ANOVA), and the body weight was reduced in stroke rats on days 3, 14, and 28 ($p < 0.01$; $p < 0.05$; $p < 0.05$, respectively) when compared with naïve rats, but there was no difference between the stroke and sham groups ($p = 0.469$; Fig. 3c).

We tested the animals for thermal and mechanical sensitivity before and after stroke or sham surgery. There were no differences between the groups in mechanical sensitivity (Fig. 3d). The absolute basal latencies were 10.32 s and 25.77 g for the naïve, 8.97 s and 22.41 g for the sham, and 8.81 s and 22.02 g for the stroke group. There was a significant time effect ($F_{2,50} = 7,292$, $p = 0.0017$) in two-way repeated measures ANOVA but no time × group interaction ($p = 0.9793$). In Hargreaves' test for thermal sensitivity there was no difference between the sham and stroke groups at any time points, nor was there an asymmetry between the ipsilateral and contralateral paws (Fig. 3e–h). The absolute basal latencies were for the left forepaw 5.75 s, 5.62 s, and
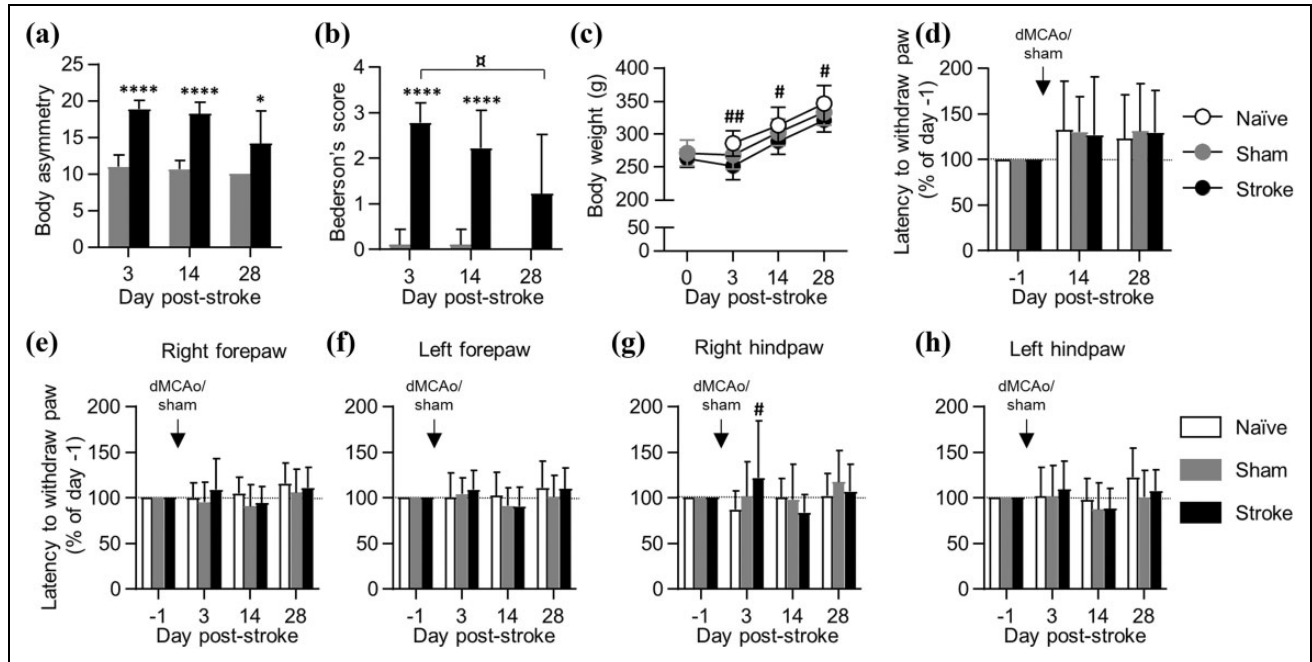
Fig. 3. Cortical ischemia-reperfusion injury does not induce thermal or mechanical hypersensitivity. (a) Body asymmetry test and (b) Bederson's score test were performed for stroke and sham-operated rats. (c) Body weight. (d) Mechanical sensitivity was measured from left hindpaw with Dynamic Plantar Aesthesiometer. (e–h) Hargreaves' test was performed for all the paws. In (d–h), the latency to withdraw paw (s) is expressed in relation to the result on day −1 before stroke/sham operation. Naïve $n = 10$, sham $n = 9$, stroke $n = 9$. * indicates comparison between sham and stroke groups; # indicates comparison between naïve and stroke groups; ¤ indicates comparison inside the stroke group at different time points. **/#/¤*$p < 0.05$; *##*$p < 0.01$; ******$p < 0.0001$. All values are reported as mean $\pm$ SD.

5.49 s; for the right forepaw 5.65 s, 5.86 s, and 5.46 s; for the left hindpaw 5.74 s, 6.25 s, and 5.78 s; and for the right hindpaw 6.28 s, 6.24 s, and 5.81 s, for the naïve, sham, and stroke groups, respectively. There was a time × group interaction in two-way repeated measures ANOVA ($F_{6,75} = 2.388$, $p = 0.036$) for the right hindpaw, and at 3 days post-stroke, the stroke rats exhibited a reduced sensitivity for thermal stimulus when compared with naïve animals ($p < 0.05$; Fig. 3g). For the right forepaw, left forepaw, and left hindpaw, there was a significant time effect ($F_{3,75} = 4.863$, $p = 0.004$; $F_{3,75} = 3.229$, $p = 0.027$; $F_{3,75} = 4.215$, $p = 0.008$; respectively) but no time × group interaction ($p = 0.4035$; $p = 0.4936$; $p = 0.5110$; respectively).

Post-Stroke Intra-Thalamic CDNF and MANF Promote Recovery but Have no Effect on the Neuronal Loss or Microglial Activation in the Thalamus

Even though the cortical infarct did not induce hyperalgesia, we wanted to study if an injection of CDNF and MANF could alleviate the neuronal loss in the ipsilateral thalamus. We chose to give the treatment on post-stroke day 7 as we have previously shown that there are not yet many phagocytic cells in the thalamus[60]. Also, we quantified the number of neurons in the ipsilateral thalamus at day 7 post-stroke and found no decrease in the neuronal count, although there was

a clear loss in 1 out of 4 animals (Fig. 4a). Furthermore, the size of the neuronal nuclei appeared smaller especially in the ventral part of the VPM in the ipsilateral thalamus (Fig. 4c) compared with the contralateral side (Fig. 4b), indicating that some neuronal damage had already occurred. These results further indicated that the time chosen for the rhCDNF and rhMANF infusion was justified in terms of observing neuroprotective effect.

The distribution of rhCDNF in the brain after the intra-thalamic injection was tested, and rhCDNF spread widely from the injection site into the entire thalamus as well as into the caudal striatum (Fig. 5). This is in line with our previous finding[62], and indicates that CDNF diffuses well in the brain parenchyma. The close homolog MANF is presumed to have similar distribution pattern. Hence, rhCDNF and rhMANF as a single dose of 10 μg were injected into the ipsilateral thalamus at 7 days post-stroke (Fig. 6a). At day 14 post-stroke (day 7 post-injection) there was a statistically significant difference between the groups in body asymmetry test (Kruskal–Wallis test $K = 11.52$, $p = 0.0032$) and Bederson's score (Kruskal–Wallis test $K = 10.13$, $p = 0.0063$). Pairwise comparisons with Mann–Whitney U test revealed that both CDNF and MANF-treated rats performed better in body asymmetry test ($p = 0.0059$ and $p = 0.0007$, respectively; Fig. 6b) and Bederson's score ($p = 0.0259$ and $p = 0.0047$, respectively; Fig. 6c) than vehicle-treated rats. There was no difference in the horizontal or vertical locomotor activity
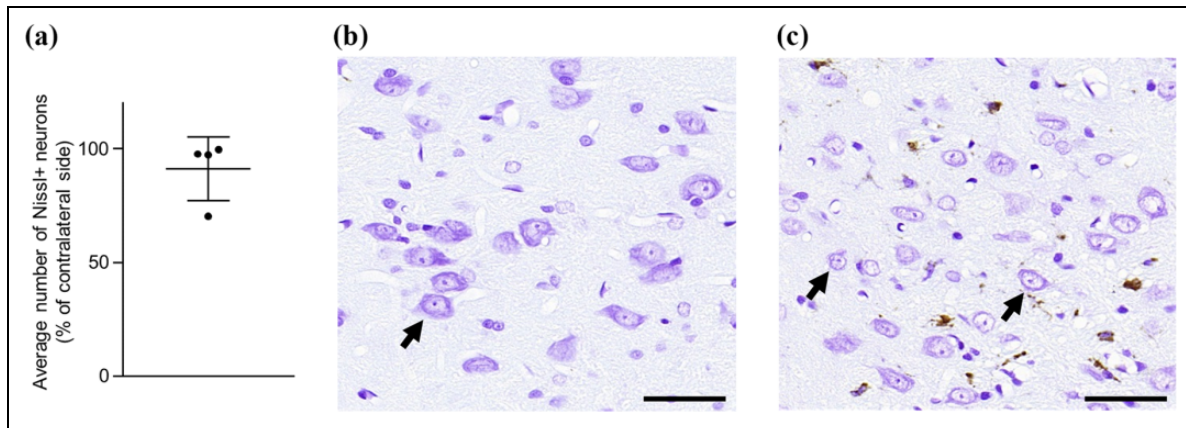
Fig. 4. Quantitation of neuronal damage in the thalamus at post-stroke day 7. (a) The ratio of Nissl+ neurons (mean ± SD) in the ipsilateral thalamus compared with the contralateral thalamus at day 7 post-stroke. (b) A representative image of the contralateral and (c) ipsilateral ventral posteromedial thalamic nucleus stained with cresyl violet and anti-CD68 antibody at post-stroke day 7. The black arrows indicate examples of Nissl stained neuronal nuclei. Scale bar is 50 μm.
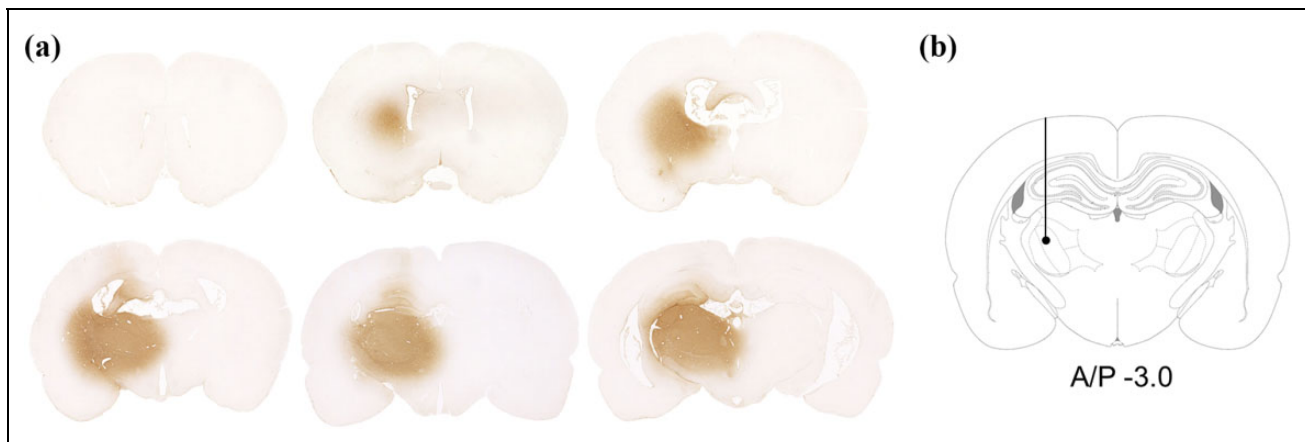
Fig. 5. The distribution of recombinant human CDNF in the rat brain 2 h after a single intra-thalamic injection. (a) RhCDNF (10 μg) was injected into the right thalamus (A/P −3.0; M/L −3.0; D/V −6.0 mm relative to bregma) and the brain sections were immunostained with anti-hCDNF antibody. (b) Illustration of the thalamic injection site (modified from Paxinos and Watson, 2005[61]).

[two-way repeated measures ANOVA; time × treatment interaction $F_{2,23} = 1.691$, $p = 0.21$ for horizontal activity; time × treatment interaction $F_{2,23} = 1.158$, $p = 0.33$ for vertical activity (data not shown)] or body weight [two-way repeated measures ANOVA; time × treatment interaction $F_{4,46} = 1.311$, $p = 0.28$ (data not shown)] between the groups. Despite the positive effect on behavior, CDNF and MANF did not alter the number of activated microglia/macrophages or the amount of neuronal loss in the ipsilateral thalamus. The number of phagocytic CD68+ cells in the thalamus was quantified and did not reveal any differences between the vehicle and CDNF or MANF groups (one-way ANOVA $F_{2,12} = 0.0439$, $p = 0.96$; Fig. 6d). Similarly, there was no difference in the amount of NeuN+ cells in the ipsilateral thalamus between the groups (one-way ANOVA $F_{2,12} = 0.5902$, $p = 0.57$; Fig. 6e). The infarct size was

quantified to verify that the groups had equally severe lesions. There was no difference in the average infarct size between the groups (one-way ANOVA $F_{2,12} = 0.0882$, $p = 0.92$; Fig. 6f).

Discussion

We found extensive neuronal degeneration and microglial activation in the ipsilateral thalamus one month after cortical stroke. We have also found prominent thalamic astrocyte activation at the same time point[60]. The secondary damage of the thalamus was first characterized by Nagasawa and Kogure in 1990 by inducing transient ischemic stroke by introducing an embolus into the internal carotid artery, leading to an infarct that extended to the cortex as well as to the striatum[16]. They observed ^{45}Ca accumulation in the
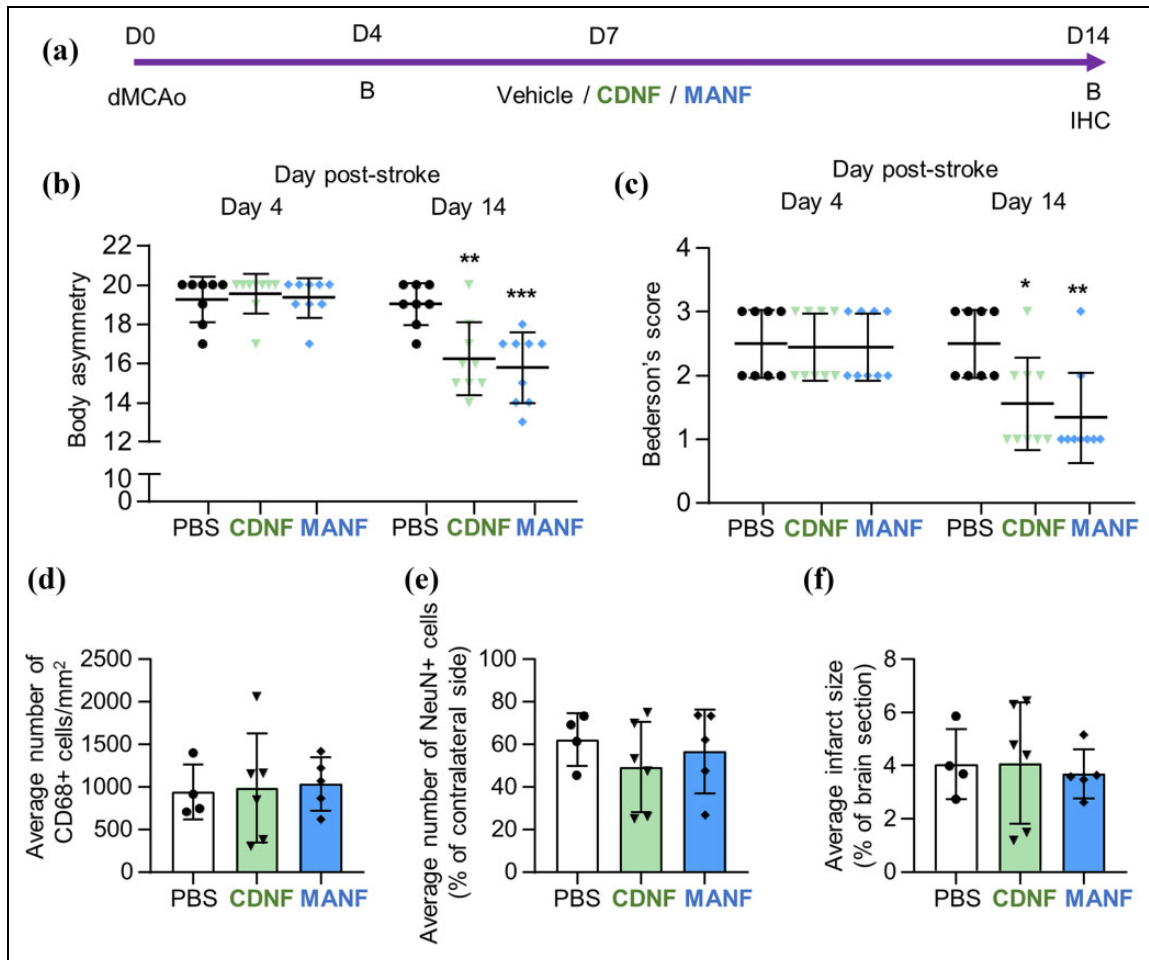
Fig. 6. Post-stroke intra-thalamic CDNF and MANF injection promotes functional recovery but does not reduce the amount of neuronal loss or CD68+ cells in the thalamus. (a) Experimental timeline. D = indicated post-stroke day; B = behavioral experiment; dMCAo = distal middle cerebral artery occlusion; IHC = immunohistochemistry. (b) Body asymmetry test and (c) Bederson's score test were performed on days 4 and 14 post-stroke, $n = 8–9$. (d) The number of phagocytic CD68+ cells in the ipsilateral thalamus at day 14 post-stroke. (e) The ratio of NeuN+ cells in the ipsilateral thalamus compared with the contralateral thalamus at day 14 post-stroke. (f) Average infarct size expressed as percentage of the whole section. *indicates comparison with PBS group; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. All values are reported as mean $\pm$ SD.

ipsilateral thalamus at 3 days post-stroke but no histological changes were observed until the next observation point, at 2 weeks post-stroke, when neuronal damage and gliosis was detected in the thalamus[16]. This original report is in line with our observations, and we found histological changes in the thalamic neurons already at 7 days post-stroke, the time point missing from the original study.

Despite the neuronal degeneration in the thalamus, we found no sensitization to thermal or mechanical stimuli at any time point. We observed a slight tendency for decreased paw withdrawal latency in Hargreaves' test in both sham and stroke rats 14 days post-operation when compared with naïve rats, but it did not reach statistical significance. This was repeated in two individual experiments (data not shown). The tendency for increased sensitivity for thermal stimulus may be due to general inflammation caused by the surgery and not by the neurodegeneration and inflammation

in the thalamus *per se*, since the same tendency was also observed in the sham-operated rats which experienced no neurodegeneration nor inflammation in the thalamus. In line with Blasi et al.[14], we did not observe any sensitization to mechanical stimuli.

The prevalence of CPSP in patients is relatively low (1–14%), and it is still unclear why some patients develop CPSP. The prevalence of CPSP in rodents may be on a similar level as in humans, making it more difficult to detect the sensitization for pain in rodent models of ischemic stroke. It has been shown that the development of CPSP in rodents is region specific in hemorrhagic stroke models, and requires damage of the VPL/VPM region[6]. VPL and VPM are important for the sensory functions and have been shown to be associated with thermal sensitization also after ischemic damage[14]. However, in all of the previously published preclinical studies, the infarction extended to the

thalamus causing direct ischemic or hemorrhagic damage, unlike in our dMCAo model. Indeed, we found no difference between the sham and stroke groups in sensitivity for thermal or mechanical stimuli, even though the secondary thalamic neurodegeneration after dMCAo mainly affected the VPL/VPM/Po region, implying that cortical infarcts do not induce CPSP in rats. Secondary neurodegeneration after cortical ischemia is likely affecting only neurons that project to the cortex, whereas direct thalamic ischemia damages also other neurons, and may explain why hyperalgesia occurs only after direct thalamic lesions. Furthermore, the mechanism of neuronal death in the secondary degenerating regions is most likely different from the one in the ischemic area, which may contribute to the fact that direct ischemic damage in the thalamus causes hyperalgesia and secondary damage does not. However, CPSP has been described in patients with cortical stroke[63], but the pathophysiology underlying thalamic and cortical stroke-induced CPSP may be different. Also, the prevalence of CPSP in cortical stroke patients is lower than in patients with thalamic stroke[4]. It is possible that the level of thalamic damage or the amount of microglial activation in our model is not enough to induce CPSP. Thus, it is unclear whether the secondary neurodegeneration in the thalamus following cortical infarction leads to any functional or sensory deficits and whether the secondary neurodegeneration has a role in the recovery from stroke. As a limitation of our study, we did not assess functions related to the thalamus other than hyperalgesia. In addition to regulating motor control and different sensory functions, the thalamus has a role in the regulation of wakefulness, consciousness, motivation, attention, emotional experiences, learning, and memory[64]. Moreover, it seems that the damage in the thalamus would need to be rather large before it manifests on a behavioral level. Also, our study was limited to assess only mechanical and thermal hyperalgesia and not all the features linked to CPSP such as cold hyperalgesia and allodynia. Furthermore, a longer observation period may have been required to detect possible late-onset hyperalgesia occurring only after a prolonged interval from the neuronal damage. Phantom pain shares similarities with CPSP and is thought to be caused by reorganization of the sensorimotor cortical networks, a phenomenon also occurring after stroke, and can be triggered after a long period from the initial deafferentation[65].

Microglia/macrophages are known to remove myelin debris by phagocytosis after stroke[49,66]. We have previously reported that in the striatum the phagocytic CD68+ cells colocalize with the myelin bundles at 2 weeks post-stroke and the CD68+ cells are present in the ipsilateral thalamus for up to 4 months after cortical stroke[60]. Whether the long-term presence of phagocytic cells in the thalamus is beneficial or detrimental is not known. The phagocytic cells are needed in the thalamus to remove the cell and myelin debris resulting from the retrograde and anterograde degeneration caused by the cortical infarction. On the other hand, microglia have been implicated in the development of CPSP[23]. It

has also been shown that in some cases viable cells can be phagocytosed, and by inhibiting this after ischemia, neuronal loss can be reduced[67].

We hypothesized that with CDNF and MANF we could modulate inflammation and simultaneously support the survival of thalamic neurons. In a model of retinal degeneration, rhMANF reduced apoptosis and degeneration of the retina by increasing the alternative activation of immune cells and shifting the environment toward favoring tissue repair.[48] We have previously shown that post-stroke peri-infarct targeting of AAV-hMANF transiently increases the number of phagocytic, CD68+ cells and causes upregulation of *complement component 3* (*C3*) and *Emr1* transcripts in the peri-infarct region at 4 days post-stroke. However, with the single injection of hCDNF and hMANF protein we did not observe any differences in the number of CD68+ cells in the thalamus nor in the neuronal loss. Likely a single dose of CDNF or MANF protein is not sufficient to modulate the post-stroke neuroinflammation, or the effect is too transient to prevent the delayed degeneration. In animal models of Parkinson's disease, CDNF and MANF are able to restore dopamine phenotype of the nigrostriatal neurons[68] and thus protect the dopaminergic neurons from death, whereas in the neuroprotection experiments in stroke models, CDNF and MANF have been shown to decrease apoptosis and acute damage[38–40,69,70]. However, the secondary neurodegeneration of the thalamus is slow and the mechanism of cell death is different than in acute ischemia, which may also explain why we did not observe neuroprotective effects on thalamic neurons. Furthermore, day 7 post-stroke may be already too late to rescue the degenerating thalamic neurons. It is possible that the thalamic neurons can no longer be rescued if they have lost their inputs to the cortex. We chose to give the treatment on post-stroke day 7 since we have previously shown that there are only few CD68+ cells in the thalamus at that time point[60]. However, the Iba1+ cells show already activated morphology, implying that the microglia are activated but not yet phagocytic[60]. We also found evidence of neuronal damage in the ipsilateral thalamus already at day 7 post-stroke even though the number of neurons was decreased only in 1 out of 4 animals.

As mentioned, there is extensive evidence that rhCDNF and rhMANF are neuroprotective *in vivo*[37–40,42,43], and that at least MANF is able to regulate ER homeostasis and protect cells from ER stress-induced cell death[34–36]. We have previously shown that rhCDNF is internalized by cortical and striatal neurons after intrastriatal injection[62] which likely explains how rhCDNF, and its homolog MANF, are able to exert their effects on neurons. The exogenous rhCDNF can also be retrogradely transported from the striatum to the dopaminergic neurons of substantia nigra[62]. In the striatal and cortical cells rhCDNF was localized inside endosomes and multivesicular bodies, indicating that it is endocytosed from the extracellular space by neurons, and also other cell types, widely and unspecifically[62]. RhCDNF was detected also in the cytoplasm of neurons, which could indicate that it

was localized to the ER as well[62]. However, the detection threshold of immunoelectron microscopy was not sufficient to detect rhCDNF in the ER lumen directly[62]. Thus, it seems likely that unspecific endocytosis of rhCDNF, and presumably also of rhMANF, is the primary mechanism that enables the intracellular effects of extracellularly injected proteins. Recent study indicated that MANF binding to sulfatides promotes cellular uptake, and thus, it may be that the neuroprotective effect is dependent on lipids[71]. CDNF and MANF differ from the traditional neurotrophic factors also in this regard as the classical trophic factors, such as glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF), mediate their effects via a cell surface receptor. So far, the only evidence indicating that CDNF and MANF may have a cell surface receptor is the KDEL-like canonical ER retention signal sequence that has a main function to retain them from the Golgi into the ER, but also has been indicated to be needed for MANF association to the plasma membrane[72].

Delayed intra-thalamic post-stroke treatment with rhCDNF and rhMANF promoted the behavioral recovery of the rats. The behavior-promoting effect is most likely mediated by a mechanism other than modulation of the thalamic secondary pathology. Recombinant CDNF and MANF proteins are known to diffuse well in the brain after an intracranial injection[43,62] and rhCDNF spread from the thalamus all the way to the peri-infarct region. Thus, CDNF and MANF may have some acute neuronal effects, such as modulation of neurotransmission, which could explain why the neuronal deficits of animals were alleviated. We have previously shown that post-stroke delivery of rhMANF on day 3 post-stroke and AAV-MANF on day 2 post-stroke to the peri-infarct area promotes functional recovery without affecting the infarct volume[49]. We suggested that this could be partly caused by faster clearance of debris due to the increased phagocytosis and could support the repair processes[49]. Glia have lately emerged as a putative major player in the repair processes occurring after ischemic stroke. Activated microglia secrete several different pro-inflammatory and anti-inflammatory cytokines and other factors, such as neurotrophic factor BDNF, which have an important role in recovery[73]. The M2 type microglia/macrophages can promote neurogenesis, axonal sprouting and remyelination, and regulate synaptogenesis, whereas the pro-inflammatory M1 type has been shown to inhibit these repair mechanisms[74]. However, it is known that behavioral improvement can be a result of enhanced local glia function that then facilitates neurotransmission. Studies with transplantation of human astrocytes into the mouse brain have shown that glia can be involved in learning[75], and that human glia transplantation can increase the life-span of dysmyelinated mice[76]. Therefore, further studies are needed to reveal the mechanism behind the recovery-promoting effect of post-stroke CDNF and MANF and the involvement of non-neuronal cells. However, it is significant that CDNF and MANF were able to ameliorate the neurological deficits in our study even though the treatment was given one week after the infarct, thus suggesting rather long time window for hastening recovery.

In conclusion, unilateral cortical infarction and the following secondary loss of connecting neurons, and microglial activation in the thalamus do not induce thermal or mechanical hypersensitivity. The reasons behind CPSP are likely complex, and thalamic neurodegeneration alone is not enough to trigger hyperalgesia after experimental stroke, and may require direct ischemic or hemorrhagic injury of the thalamus. Delayed intra-thalamic post-stroke treatment with CDNF and MANF reduced the neurological deficits but did not affect the secondary pathology. The current finding strengthens the potential of CDNF/MANF-based therapies as a prospective treatment to promote the post-stroke functional recovery.

Author Contributions

JEA and MA: conception and design; JEA, SP and MA: data collection, analysis and interpretation; JEA and SP: manuscript writing; MA: manuscript revision.

Ethical Approval

Ethical approval was obtained from the national Animal Experiment Board of Finland (protocol approval number ESAVI/7812/04.10.07/2015).

Statement of Human and Animal Rights

All of the experimental procedures involving animals were conducted in accordance with the the 3 R principles of EU directive 2010/63/EU on the care and use of experimental animals, local laws and regulations, and were approved by the national Animal Experiment Board of Finland.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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