Title: ***Perilesional treatment with Chondroitinase ABC and motor training promote functional recovery after stroke in rats.***

Running title: Recovery from stroke with cortical Chondroitinase

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Abstract

Ischemic stroke insults may lead to chronic functional limitations that adversely affect patient movements. Partial motor recovery is thought to be sustained by neuronal plasticity, particularly in areas close to the lesion site. It is still unknown if treatments acting exclusively on cortical plasticity of perilesional areas could result in behavioural amelioration. We tested whether enhancing plasticity in the ipsilesional cortex using local injections of chondroitinase ABC (ChABC) could promote recovery of skilled motor function in a focal cortical ischemia of forelimb motor cortex in rats. Using the skilled reaching test, we found that acute and delayed ChABC treatment induced recovery of impaired motor skills in treated rats. vGLUT1, vGLUT2, and vGAT staining indicated that functional recovery after acute ChABC treatment was associated with local plastic modification of the excitatory cortical circuitry positive for VGLUT2. ChABC effects on vGLUT2 staining were present only in rats undergoing behavioural training. Thus, the combination of treatments targeting the CSPG component of the extracellular matrix in perilesional areas and rehabilitation could be sufficient to enhance functional recovery from a focal stroke.

Keywords: plasticity, perineuronal nets, penumbra, skilled reaching

Stroke takes an extremely heavy toll on affected individuals and their families. Survivors are often chronically impaired in their movements and left with long-term disability (Schwab 2010). Several interventions using antinflammatory treatments (Wang et al 2012), antioxidant and antiapoptotic agents (Ikeda-Matsuo et al 2011), and neuroprotective gene therapy (Al-Jamal et al 2011; Chu et al 2007) have been investigated, however physical rehabilitation therapy (Steinle & Corbaley 2011) remains the first line intervention strategy to attenuate chronic impairment of sensory-motor function (Arya et al 2011). Experimental and clinical evidence indicates that functional recovery by rehabilitation therapy is based on the ability to alter brain organization in adaptive ways after damage (Nudo 2011). Studies in both animals and patients have demonstrated that stroke-induced neuronal plasticity drives the formation of new local circuits, intracortical connections and descending projection remodelling, suggesting that promoting neuronal plasticity in the affected brain can enhance functional recovery. This observation provided a rationale to studies testing whether treatments promoting plasticity could facilitate functional recovery from stroke effects. For example, inosine, which acts through a direct intracellular mechanism to stimulate axon growth in several types of neurons (Smith et al 2007; Zai et al 2011), Nogo-A antibodies (Buchli & Schwab 2005), or agents that block signalling through the Nogo receptor (Kilic et al 2010; Papadopoulos et al 2006; Tsai et al 2011) promoted functional recovery in animal models. Interestingly, behavioural effects of these treatments were associated with enhanced plasticity of cortico-spinal axons in the spinal cord.

One alternative strategy to enhance plasticity in the adult brain is to recreate a tissue environment free from growth inhibiting molecules forming perineuronal nets such as chondroitin sulphate proteoglycans (CSPGs). By digesting CSPG side chains, ChABC modifies extracellular matrix and allows axonal sprouting (Bruckner et al 1998; Crespo et al 2007). Moreover, ChABC was found to facilitate plasticity in the adult visual cortex (Pizzorusso et al 2002; Pizzorusso et al 2006), and after spinal cord injury (Barritt et al 2006; Carter et al 2011; Garcia-Alias et al 2009; Houle et al 2006; Lee et al 2010; Tom et al 2009). Recently, ChABC has been tested in models of

brain trauma (Harris et al 2010), however the therapeutic potential of ChABC after stroke is only partially explored (Hill et al 2012; Nakamura et al 2009; Soleman et al 2012). Importantly, it is not known whether selective targeting of cortical perilesional areas is sufficient to promote behavioural amelioration and synaptic plasticity. To answer these questions, we investigated whether the treatment with ChABC restricted to the ipsilesional cortex could promote functional recovery from the effects of focal ischemia of the motor cortex containing the representation of the forelimb. We evaluated the effects of rehabilitation training and timing of ChABC delivery on behavioural recovery and cortical synaptic plasticity. We found that the synergic effect of ChABC treatment and specific rehabilitation training re-established motor function of the affected limb and caused synaptic plasticity, supporting the possibility that interventions enhancing plasticity in the perilesional cortex could promote functional recovery from stroke induced impairments.

Methods

Animals

All procedures were performed according to the guidelines of the Italian Ministry of Health for care and maintenance of laboratory animals (law 116/92) and in strict compliance with the European Communities Council Directive 86/609/EEC. Adult Long Evans rats (300-400 g) were housed with a 12 hr/12 hr light/dark cycle with food and water available *ad libitum*. Animals were reared in a standard environment (3 adult rats in 30 × 40 × 20 cm laboratory cages). For the duration of the protocol of the skilled reaching test (SRT), rats were fed approximately 20 g of Purina rat chow once a day after the daily tests (Whishaw & Pellis 1990) in addition to the food pellets they obtained while performing the SRT (Bio-Serv dustless precision pellets product F0021). The weight of the rats was maintained at about 90–100% of their expected body weight.

Focal ischemic lesion and treatment

After SRT completion, rats were anaesthetized with avertin prior to surgery. The head was fixed in a stereotaxic frame and the scalp retracted. Body temperature was monitored throughout the procedure using a rectal probe and maintained at 37^{o}C with a homeothermic blanket (Harvard Apparatus, Ltd, Edenbridge, Kent, UK). Unilateral lesions were performed in the hemisphere contralateral to the dominant forelimb. The two sites of injection were identified in correspondence to the forelimb motor cortex (A-P 0 mm, M-L 2,5 mm; A-P 2.5 mm, M-L 2,3 mm). A 1mm burr hole was drilled through the scalp at the two sites while continuously applying saline over the area to prevent damage to the brain. The underlying dura was pierced during microinjections by a glass micropipette (0.03 mm tip). Two 0.75 µl injections of ET-1 (Sigma; 40 pmol/µl in sterile saline) were delivered via a glass micropipette connected to a syringe, at a depth of 0.7 mm from the brain surface at each injection site. ET-1 was delivered at a rate of 0.5 µl/min with a 1-min interval before retract the micropipette from the tissue.

After induction of focal ischemia, the protocol for acute chondroitinase treatment was initiated. Animals received the first dose of ChABC (40 mU/µl; 1 µl for each site) or saline immediately after injury. An equivalent second dose of ChABC or saline was delivered in a subsequent surgery session one week later through the already existing apertures in the skull. At the end of any surgical session and prior to suturing, lidocaine gel was gently applied on animal skulls. Animals were allowed to recover from anaesthesia in a recovery box until fully conscious and paracetamol (100 mg/kg) was administered in the water.

The protocol for delayed ChABC treatment was initiated two weeks after stroke induction. At this time, animals underwent surgical preparation as described and were injected with a first dose of chABC. The second dose of ChABC treatment was delivered a week later.

Behavioural testing

Animals were handled every day for 1 week before the onset of the experiment while let familiarising with the arena box used for in the SRT (pre-training). All behavioural testing was

carried out by an experimenter blind to group membership of the animals. Animals were trained on a single pellet reaching task modified from Whishaw & Pellis, 1990. The boxes were made of clear Plexiglas with dimensions of 45 × 14 × 35 cm. In the centre of the front wall there was a vertical slit, 1 cm wide. On the outside of the wall, in front of the slit, a 2 cm wide shelf was mounted 3 cm above the floor. Two indentations on the surface of the shelf were located 1 cm from the inside of the wall and were aligned with the edges of the slit (1 cm apart) where rats could reach the pellet with either paw. A pellet was placed in the indentation contralateral to the limb which the rat preferred for reaching. The lateral placement of the food pellet prevented the rats from using the tongue to lap the pellet also preventing them from successfully using the non-preferred paw to grasp the food (Whishaw & Pellis 1990). A metal bar is located in front of the slit at circa 1 cm from the horizontal shelf preventing the animal to drag the food after reaching, avoiding performing a full correct movement of grasping.

Training. Training was performed as previously described (Gharbawie & Whishaw 2006). The first five days consisted in a shaping phase during which a number of food pellets were placed on the shelf to attract the animals. At the end of this period rats reliably retrieved the food from the shelf using the preferred limb on each trial. Then, a single pellet was placed in the food-indentation contralateral to the rat preferred paw and the training phase consisting in 25 trials repeated three times in each daily session for 7 days was performed prior to receiving surgery. If the rat obtained a pellet and subsequently consumed it without dropping it, a success was scored (Gharbawie & Whishaw 2006).

Histological procedure

After anaesthesia with an overdose of chloral hydrate, the thoracic cavity was open to expose the heart. A needle was inserted in the left ventricle, and the right atrium was cut open. By means of a peristaltic pump, vessels were washed with PBS, and then the animal was perfused in a 4% w/v solution of freshly prepared paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brain

was then quickly removed, postfixed by immersion in the same fixative solution for 24 hr, then cryoprotected overnight in 30% w/v sucrose in 0.1 M PB. Finally, brains were frozen by immersion in isopenthane and stored at –80 C; 12 hr before cutting, samples were put at –20 C. Free floating brain coronal sections of about 50 micron thickness cut with a cryostat (Leica Microsystems) were collected in PBS and distributed for further analysis.

Volume measurement

Brains were sectioned using a cryostat in 50-μm sections, and cresyl violet staining was performed. Images were acquired on a camera-mounted Zeiss Axioskop microscope, and lesion area was measured using the Metamorph software (Molecular Devices, Downingtown, Pennsylvania). The lesion area was identified and measured in all sections that contained ischemic lesion and a volume measurement computed by summation of areas multiplied by the interslice distance. The experimental group of any treatment consisted of at least 5 animals.

Expression of synaptic markers

Glutamate vesicular transporter 1 and 2 (vGLUT1 and 2), and the GABA vesicular transporter (vGAT) were immunostained as described (Mainardi et al 2010). Coronal sections covering the primary motor cortex (50 micron) collected free-floating in PBS were blocked in 10% BSA, 0.3% Triton X-100 in PBS for 2 hr at room temperature, then incubated overnight at 4 C in 1% BSA, 0.1% Triton X-100, and 1:1,000 rabbit anti-vGLUT1 or -2 and vGAT (1:5000) primary antibody (Synaptic Systems) in PBS. The signal was detected by incubation in 1% BSA, 0.1% Triton X-100, and 1:400 anti-rabbit secondary antibody conjugated to Alexa Fluor-568 fluorophore (Molecular Probes, Eugene, OR) for 2.5 hr at room temperature, unless otherwise specified. Sections were mounted on glass slides using VectaShield mounting medium (Vector Laboratories, Burlingame, CA).

Images of vGLUT1-2 and vGAT immunoreactivity in the selected areas of the cortex were acquired with a confocal laser scanning microscope (Leica BM6000). Optimal acquisition parameters (photomultiplier gain, intensity offset, and laser excitation intensity) were adjusted at the beginning of each acquisition session and held constant. Acquired images were analysed off line using a dedicated software (Imaris, CH). A 63× oil objective (N.A. = 1.4) was used, with a 2.5 digital zoom. To achieve an optimal resolution on the z axis, 15 sequential focal planes, spaced 0.125 μm apart, were acquired. Each focal plane was then saved as a single TIFF file in an 8-bit gray-scale mode. For vGAT and vGLUT-2 immunoreactive puncta analysis, the image stacks were processed using the "spots" function of Imaris software (Bitplane, CH). A double threshold was applied to images to select puncta: the "spot quality threshold" parameter that threshold pixels above a certain value of luminance, and the "minimum spot diameter" parameter that select puncta above a certain dimension. In pilot analyses we manually adjusted both parameters to optimize puncta detection. As a compromise between automation and optimization, we choose to keep the "minimum spot diameter" constant for all cases with a value of 1 micron. To adjust for the different brightness of sections, the "spot quality threshold" was changed by the operator blind to the experimental case to optimize puncta detection.

The expression of these markers was detected in layers I-III selected regions of the brain cortex: for each slice section, areas of interest were defined as (a) lesion, where the damage was presents, (b) penumbra, the area bordering with the lesion, (c) ipsilateral, a cortical area distant from the lesion area in the same hemisphere (Par1), (d) contralateral, the homotopic area of the motor cortex in the opposite hemisphere. For each slide one image of each of these areas was collected representative of the fluorescence signal of the marker under investigation. For each animal, at least 6 slides were analysed and images collected.

Anterograde tracing of thalamocortical projection and colocalization analysis

To label thalamocortical pathway projecting into the primary motor cortex (M1), five adults Long Evans rats were anesthetized by intraperitoneal injection of avertin (1ml per 100gr body weight) and stereotaxically injected with 2 µl of AAV8-GFP viral vector (9,9 E+11 genomic copies/mL) solution into the ventrolateral-ventromedial (VL-VM) thalamic motor nuclei (-3,0 mm posterior to Bregma; 1,8 mm lateral to the midline, 6,00 mm depth) by pressure through a glass micropipette attached to Picospritzer (PDES-02 TX, npi electronic system, GmbH, Germany). The mixture containing the AAV8-GFP was delivered at a rate of 0.05 µl/min and the tip of the micropipette was held in place for 10 minutes after the injection and was then slowly withdrawn. During the same surgery, all the animals underwent the focal ischemia induction injury by ET-1 intracortical injections, and the ChABC or saline solution 0,9% perilesional treatment given both immediately after injury and one week later, as previously described. After SRT recall, rats were perfused and coronal sections covering the primary motor cortex (50 micron thickness) were prepared for immunostaining for vGLUT2 and vGLUT1 as described. A rabbit anti-GFP (1:400) primary antibody (Molecular Probes, Invitrogen) was added to the solution containing either vGLUT1 or vGLUT2 primary antibody. The signal was detected by incubation in 1% BSA, 0.1% Triton X-100, 1:400 anti-rabbit secondary antibody conjugated to Alexa Fluor-488 fluorophore (Molecular Probes, Eugene, OR) and 1:400 anti-guinea pig secondary antibody coniugated to Alexa Fluor-568 fluorophore (Molecular Probes, Eugene, OR) for 2.5 hr at room temperature. After rinsing twice in PBS, sections were mounted on glass slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Images of vGLUT1-2 immunoreactivity in the perilesional area of the cortex were acquired (the two channels acquired separately to minimize cross-talk) with a confocal laser scanning microscope (Leica BM6000). Synaptic marker positive puncta overlapping with GFP were counted offline using Imaris by an operator blind to the synaptic marker under analysis.

Wisteria Floribunda Agglutinin (WFA) and CSPG stubs staining

Three days after stroke four ChABC treated rats were perfused with cold 4% paraformaldehyde and post-fixed overnight. 50 micron sections were blocked in 3% goat or donkey serum 3% bovine serum albumin, in Tris buffered saline with 0.2% triton X-100. WFA histochemistry was performed by incubating sections in biotinylated WFA (20 mg/ml, Sigma, Haverhill, UK) overnight at 4 C (Hobohm et al 2005). Labelling was revealed using TRITC-labelled Streptavidin (Sigma) and images were acquired at the confocal microscope. An additional group of three animals were perfused with cold 4% paraformaldehyde and post-fixed overnight. 50 microns sections were blocked in 10% BSA, 0,3% Triton X-100 in PBS for 1 hr at room temperature, then incubated overnight at 4 °C in 1% BSA, 0,1% Triton X-100 and monoclonal 2B6 antibody (MD Bioproducts, CH) primary antibody (1:100) in PBS. The signal was detected by incubation in 1% BSA, 0.1% Triton X-100, 1:400 anti-mouse secondary antibody conjugated to Alexa Fluor-568 fluorophore (Molecular Probes, Eugene, OR) for 2 hr at room temperature. After rinsing twice in PBS, sections were mounted on glass slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Images of 2B6 immunoreactivity in injured hemisphere were acquired with a confocal laser scanning microscope (Leica BM6000).

Analysis of reactive astrogliosis

Two groups of animals (N=4 each) treated with ChABC or control were perfused three days after stroke with cold 4% paraformaldehyde and post-fixed overnight. 50 microns sections were blocked in 10% normal goat serum NGS, 0,3% Triton X-100 in PBS for 1 hr at room temperature, then incubated overnight at 4 C in 1% NGS, 0,1% Triton X-100 and monoclonal mouse anti-GFAP (1:500) primary antibody (Sigma Aldrich) in PBS. The signal was detected by incubation in 1% NGS, 0.1% Triton X-100, 1:400 anti-mouse secondary antibody conjugated to Alexa Fluor-568 fluorophore (Molecular Probes, Eugene, OR) for 2 hr at room temperature. After rinsing twice in

PBS, sections were mounted on glass slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Images of GFAP immunoreactivity in injured hemisphere were acquired with a confocal laser scanning microscope (Leica BM6000). Six fields (700x700 microns) were acquired in the penumbra area for each slice and four slices were analysed for each rat. For each image, we first choose a fluorescence intensity threshold to exclude background. Then, the area of the image containing pixels above threshold was multiplied by their average fluorescence to obtain the integrated fluorescence.

Results

Skilled reaching task (SRT) learning

SRT, which is a complex motor cortex-dependent behaviour, was analyzed in the single pellet retrieval task. This test is normally used to assess the ability of rats to perform forelimb grasping fine movements (Starkey et al 2011). Animals were trained to successfully retrieve a food pellet from a tray through a narrow window using their preferred forelimb. For each animal, the grasping ability was measured during the training period (day 1-6) when animals learned to perform the task, improving steadily their retrieving rate (Fig. 1A; n=57 One way ANOVA Repeated Measures, $P<0.001$). After completion of the SRT training rats were randomly assigned to one of the experimental groups.

Effects of ChABC cortical administration on CSPGs and astroglia

Before assessing ChABC effects on motor behaviour, we assessed the spatial coverage of CSPG glycosaminoglycan (GAG) digestion by ChABC using WFA staining. Intracortical injection of ChABC into the adult rat motor cortex resulted in a reduction of WFA staining in an area covering almost all the medio-lateral extent of the treated cortex and affecting all cortical layers. The area devoid of WFA staining did not spread into the contralateral hemisphere. Similar results were obtained using 2B6 antibody that labels the CSPG stubs left by ChABC digestion (Fig. 1B).

It has been suggested that ChABC could reduce glial scar formation assessed by GFAP immunostaining after severing nigro-striatal pathway (Li et al 2013). We analyzed GFAP staining three days after stroke in saline and ChABC treated rats. Our data show a trend of borderline statistical significance towards reduced GFAP staining in ChABC treated animals (Fig. 1C).

Rats treated with ChABC show motor impairment at the beginning of the recall phase

Fine movement impairment induced by the focal stroke was analyzed by comparing animal performance in the SRT before and after damage. The effects of intracortical ChABC treatment were studied applying two different protocols (Fig. 1D): an acute protocol, in which ChABC was administered immediately after lesion and seven days later, or a delayed protocol in which the two ChABC injections began two weeks after lesion. Motor function was assessed one week after the last ChABC administration. All animals continued to use the preferred limb even if the corresponding cortex was lesioned.

To induce a cortical focal lesion we injected ET-1, a potent vase constrictor at two sites targeting the forelimb motor cortex (Gilmour et al 2004). M1 focal ischemia caused a significant reduction of successful retrievals in the SRT on the first day of recall. This impairment was present both in ChABC and control rats regardless of the timing of application of the treatment. Indeed, on day 6 of the acute protocol, controls performed 37.8 ± 2.7 correct retrievals (n=16) while the mean value of the correct retrievals measured the first day of retraining (day 20) was significantly decreased (18.5 ± 2.7 correct retrievals, paired t-test, $P<0.005$). Similarly, the ChABC acute group managed 41.0 ± 2.5 positive trials (n=16) on day 6, but only 27.1 ± 2.8 positive trials on day 20 (paired t test, $P<0.005$).

Performance after lesion was also affected in control and ChABC rats treated adopting the delayed protocol: in controls corrected retrievals at day 6, last day before lesion, were 39.4 ± 2.4 whereas at day 35, the first day of assessment after lesion, they were significantly reduced to 25.0 ± 2.3 (n=11, paired t-test, $P<0,005$). In ChABC rats, corrected retrievals at day 6 were 40.5 ± 1.2 whereas they were 28.1 ± 3.2 at day 35 (n=13, paired t-test, $P<0.005$). Thus, motor ability learned before lesion was lost after stroke and was not preserved by ChABC treatment.

Acute and delayed ChABC treatment promotes motor learning after cortical stroke

SRT performance of the acute ChABC and control groups dramatically diverged during recall (Fig. 2A,B). Indeed, at the end of the recall phase the number of successful pellet retrieval recovered to pre-lesion values (paired t-test, day 6 vs day 25, paired t-test P=0.38) in ChABC treated rats. Conversely, in control rats performance at the end of the recall period remained significantly lower than the pre-lesion level (paired t-test; day 6 vs day 25, paired t-test P<0,001). To better visualize post-lesion behavioural recovery in ChABC treated rats we also plotted the data normalized to the pre-lesion performance of each animal (Fig. 2C,D). This analysis nulls between-subject variability in the final level of pre-lesion learning. Using this processing of the data, it is even more evident how stroke rats treated with ChABC were able to relearn the motor task.

ChABC delayed treatment similarly improved the reaching skills of treated animals (Fig. 3). Successful retrieval at the end of the recall phase was significantly lower than pre-lesion values in control rats (at day 6 39.27 ± 2.36 retrieved pellets, at day 41 25.0 ± 2,30 retrieved pellets, paired t-test, P<0.001). On the contrary, ChABC delayed treatment induced a complete recovery of skilled reaching. Indeed, correct successful retrieval values at the end of the recall phase in the delayed ChABC group was not different from pre-injury levels (at day 6 40.5 ± 1.17 retrieved pellets, at day 41 36.21 ± 11.51 retrieved pellets, paired t-test p=0.44). This profile is clearly visualized when normalized results are examined (Fig. 3C,D). The occurrence of alternative retrieving strategies was not noted during performance measurement. Thus, local cortical treatment with ChABC promotes functional restoration of impaired forepaw use in stroke injured rats even if performed two weeks after injury.

ChABC treatment does not affect damage size in ischemic rats

We controlled whether ChABC might have influenced lesion size. The effect of the acute and delayed ChABC treatments was evaluated measuring the volume of the lesion in the brain of

animals sacrificed at the end of the behavioural protocol. The lesion volume comprised most of the primary forelimb motor cortex. For both the acute (control 0,99 ± 0,21mm^3 n=8; ChABC treated 0,75 ± 0,15 mm^3 n=10, t test, P=0,34) and the delayed protocol (control 2,32 ± 0,83 mm^3 n=5; ChABC treated 2,24 ± 0,83 mm^3 n=5, t test, P=0,1) the measurement of stroke volume revealed no significant differences between groups (Fig 4A). No correlation was observed between volume of the lesions and skilled reaching motor performance in any of the experimental groups (fig 4B, Pearson Product Moment Correlation, P > 0,050 for all groups).

ChABC and behavioural training altered expression of synaptic markers

As ChABC is known to promote cortical plasticity in the adult CNS after sensory deprivation (Carulli et al 2010; Nakamura et al 2009; Pizzorusso et al 2006) and in the spinal cord after damage (García-Alías et al., 2009), we investigated whether the cortical treatment with ChABC could promote plasticity of synaptic connections. Therefore at day 26 of the acute protocol, we performed an immunohistochemical analysis of synaptic markers in control and ChABC treated rats. At this time point, ChABC treated rats show complete functional recovery and are maximally different from control animals.

To assess synaptic connectivity, we measured punctate staining for markers of glutamatergic (vGLUT1 and vGLUT2) and GABAergic (vGAT) presynaptic terminals in different brain regions. Punctate marker staining was measured in the lesioned area (Les), in the penumbra (Pen), an area bordering with the lesion where plasticity has been reported to occur after damage, and in a cortical area located in parietal area Par1 ipsilateral to the lesion (Ipsi) and not damaged by stroke. We also measured marker expression in the homotopic area contralateral (Contra) to the lesion (Fig 5A).

For all proteins, expression was extremely scarce in the lesion area of both control and ChABC treated animals due to the loss of neuronal cells occurring after damage in either treated or control animals (Fig 5B,D; Table 1). vGLUT1 and vGAT puncta density was not significantly affected by ChABC treatment coupled with behavioural training in none of the other analyzed areas (Table 1).

On the contrary, ChABC treated rats showed a significant higher density of vGLUT2 positive puncta in the penumbra area (Fig 5B) with respect to the other areas of the same animals. This effect was specific for the ChABC treated penumbra, indeed vGLUT2 positive puncta density in trained ChABC rats was higher than both trained control rats or untrained ChABC treated rats (t-test, $p<0.05$). Therefore, training in the SRT coupled with ChABC is able to promote plasticity of vGLUT2 positive terminals in the penumbra area surrounding the stroke lesion.

VGLUT2 has been described to be expressed primarily by afferent thalamic fibers. To check whether the increase in vGLUT2 positive puncta was associated with thalamic axons also in ChABC treated and SRT trained rats, we traced thalamic fibers ascending from the VL/VM thalamic nuclei by microinjection of AAV carrying a GFP expression cassette into VL/VM nuclei. We then counted vGLUT2 positive puncta colocalizing with GFP positive fibers present in the penumbra area. vGLUT1 puncta were also counted to assess specificity of the colocalization for vGLUT2. The results showed a significantly higher colocalization for vGLUT2 than for vGLUT1 (Fig 5E) suggesting that VL/VM thalamic fibers predominantly express vGLUT2.

Lack of synaptic marker alteration in untrained rats treated with ChABC

To dissect out the role of ChABC administration from SRT training in promoting cortical synaptic plasticity, we measured the expression of vGAT, vGLUT1 and vGLUT2 throughout the Les, Pen, Ipsi, and Contra cortical areas of animals that underwent the acute treatment with ChABC or control, but did not receive any SRT training.

Consistently with our previous results, the expression of vGLUT1, vGLUT2 and vGAT was reduced in the lesion area regardless of the treatment (Fig. 5C and Table 2). Moreover, vGLUT1 and vGAT puncta density did not change throughout the different areas of the cortex in both ChABC and control rats. At difference with the results obtained in trained rats, vGLUT2 was also unaffected in the penumbra of ChABC treated, untrained rats. In these animals, vGLUT2 positive

puncta density remained similar to the levels measured in Ipsi and Contra areas of the same animals and in the penumbra area of control animals.

The lack of increase in penumbral vGLUT2 punctate staining after ChABC treatment suggests that ChABC *per se* is not sufficient to sustain presynaptic plasticity in the motor cortex after focal ischemic damage.

Discussion

We showed that specific motor training combined with intracortical ChABC treatment promotes functional recovery from the effects of a focal cortical ischemia induced by ET-1 injection in the forelimb M1 area. Skilled forelimb motor behaviour was tested using SRT, one of the most sensitive indicators of forelimb motor deficits during the chronic post-injury period (Adkins & Jones 2005). Both acute and delayed ChABC treatments were effective, albeit the final level of motor learning reached by the acutely treated rats was higher than that reached with the delayed ChABC treatment. This could be due to an additional effect of ChABC on counteracting inflammatory and swelling reaction in the short time after stroke (Elkin et al 2011) or to a facilitation of intrinsic plasticity mechanisms occurring during the first weeks after lesion and fading with time.

Mechanisms of action of ChABC: ChABC digests the GAG chains of CSPGs into soluble disaccharides, and leaves behind the core protein. In several lesion models this treatment has been shown to promote functional recovery (Garcia-Alias et al 2009; Garcia-Alias & Fawcett 2012; Hyatt et al 2010; Jefferson et al 2011; Tester et al 2007), suggesting that most of the inhibitory activity of CSPGs is mediated by their GAG moieties. Several molecular mechanisms have been proposed for the inhibitory activity of GAGs on functional recovery after lesion (Garcia-Alias et al 2009). Receptor protein tyrosine phosphatase sigma (RPTPσ) has been identified as a CSPG

receptor, through which CSPGs can trigger growth cone collapse when the GAG chains bind to the surface of the first immunoglobulin-like domain of the receptor (Shen et al 2009). In addition, other mechanisms could also participate in the inhibitory nature of the CSPGs. For instance, the high negative charges of the sulphate groups can induce repulsive allosteric interaction with the axonal growth cone and with charged molecules and ions (Suttkus et al 2012), impeding their growth on CSPG enriched areas (Gilbert et al 2005), or CSPGs could mask some growth-promoting substrates, such as laminin (McKeon et al 1995). Proteoglycans often act by attracting active molecules to their GAG chains, then forming a ternary complex with their receptor. During development, several axon guidance molecules such as slits rely on this mechanism for their localization and action and, in the adult CNS, semaphorin3 family molecules bind to CSPGs and may rely on this for their inhibitory actions (Vo et al 2013). Overall, all these mechanisms should impinge on the plasticity of the neuronal connections involved in functional recovery. Indeed, ChABC treatment was effective also in models that specifically involve plasticity mechanisms to achieve functional recovery such as recovery from amblyopia in the adult visual cortex (Pizzorusso et al 2006). Furthermore, recent work indicates that ChABC can modulate plasticity mechanisms underlying learning and memory (Gogolla et al 2009; Romberg et al 2013). Thus, ChABC-facilitated plasticity could enhance functional recovery, either by enhanced sprouting of existing axons or by the formation of new connections at the injury site (Bradbury et al 2002). Sprouting of axons in the perilesional cortex has been documented during early post-cortical contusion (Carmichael et al 2005; Harris et al 2010) and post-ischemic (Carmichael et al 2005) periods. We examined presynaptic markers of inhibitory and excitatory terminals and we found that ChABC enhanced density of vGLUT2 positive puncta only when its delivery was combined with motor training. Thus, sprouting of excitatory fibres in the cortical area close to the lesion could contribute to the skilled reaching learning observed in lesion animals treated with ChABC. On a functional level, the increase of glutamatergic presynaptic terminals might tip the excitatory/inhibitory balance in the penumbra areas towards excitation. This mechanism would counteract the decrease in excitability of the neuronal circuits in peri-infarct

cortex due to the high levels of tonic GABA (Carmichael 2012), a factor shown to be important to hinder remapping of motor and sensory function after stroke.

Site of action of ChABC: There is abundant literature on the use of ChABC in the spinal cord or in the cerebrospinal fluid in presence of spinal damage; however it was not clear whether a cortical ChABC treatment could be beneficial in stroke models. Recently, it was shown that spinal delivery of ChABC promotes functional recovery in aged rats (Soleman et al 2012). Furthermore, intracerebral infusion of ChABC in medial cerebral artery occlusion rat model, resulting in a large lesion involving cortical and subcortical structures, also promoted functional amelioration (Hill et al 2012). Our study demonstrates that ChABC treatment limited to cortical perilesional areas is sufficient for functional recovery and creates a permissive environment for training induced synaptic reorganization.

An obvious target of our ChABC treatment is cortical connectivity. This possibility is strengthened by the plastic rewiring observed using presynaptic puncta staining in the perilesional areas of trained, ChABC treated rats. Intriguingly, an enhancement of puncta density was present in vGLUT2 positive and not in vGLUT1 or vGAT positive terminals. vGLUT2 is thought be present prominently on thalamic terminals present in cortical tissue (Fattorini et al 2009; Kaneko & Fujiyama 2002), the mechanisms involving this population in the effect of ChABC are still to be elucidated. Furthermore, lack of alteration of puncta density should be interpreted cautiously because vGLUT1 and vGAT positive terminals could still participate to the plasticity process by changing their strength, localization, and postsynaptic partner.

Plasticity of cortical connectivity and enhanced glutamatergic input could preserve firing of descending neurons promoting plastic changes occurring also subcortically. Indeed, spared cortical descending fibres may strengthen their synaptic efficacy on those spinal neurons deprived of innervation. Lesioned spinal axons can sprout into denervated spinal domains; moreover enhancement of this sprouting is associated with better behavioural recovery from stroke effects

(Lee et al 2004). Recently, lentivirus mediated gene expression of bacterial ChABC in the motor cortex was found to enhance sprouting of lesioned corticospinal neurons (Zhao et al 2011). This effect could be mediated by the transport of ChABC into the spinal cord along axons of transduced corticospinal neurons as shown by the presence of digested CSPG stubs (Zhao et al 2011).

It has been reported that, in presence of wide damaged areas including corpus callosum white matter and sub cortical regions, structural and functional reorganization is not limited to spared areas of the injured hemisphere, but may occur in homotopic regions of the intact hemisphere (Jones & Schallert 1992). We investigated the presynaptic vesicular transporters expression in the homotopic contralateral region without finding any significant change in both glutamatergic and GABAergic transporters, thus making unlikely that plastic rearrangements of contralateral presynaptic terminals could be induced by ChABC in our model of focal ischemia. As following a cortical injury animals adjust forelimb movements to compensate for deficits in the affected limb (Whishaw et al 2004), true recovery may be masked (Whishaw 2000; Whishaw et al 1991), or even hindered (Alaverdashvili et al 2008; Alaverdashvili et al 2007), by the use of alternative movement strategies, often resulting in postural compensation (Krasovsky & Levin 2010). Lack of plasticity in the contralateral motor cortex may preserve perilesional circuitry from minimal maladaptive interference, often invoked to justify inappropriate stimulation evoked movements.

The amelioration of skilled movements observed with cortical treatment with ChABC together with the lack of maladaptive forms of plasticity underscores the importance of cortical plasticity in functional recovery from stroke. Furthermore, treatments targeting CSPGs could be effective in restoring motor function not only after traumatic lesions of the spinal cord, but also in cortical stroke patients.

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Table 1. SRT training

vGLUT1			vGAT	
	ChABC	Control	ChABC	Control
Les	216,3 ± 24,1	236,2 ± 35,1	366,6 ± 54,2	344,1 ± 64,2
Pen	1973,7 ± 65,6	1925,6 ± 96,5	1006,7 ± 93,9	1025,4 ± 31,7
Ipsi	2053,9 ± 84,5	2213, 0 ± 94,5	1158,5 ± 146,3	1079,1 ± 112,4
Contra	1908,7 ± 107,4	2123,4 ± 79,6	1225,4 ± 84,3	982,8 ± 100,0

Table 1. vGLUT1 and vGAT puncta density is altered exclusively in the Les area with respect to all other areas (n=6 for chABC and n=5 for controls for both stainings, one-way RM ANOVA $p<0.01$, post-hoc Holm-Sidak test $p<0.05$)

Table 2. No SRT training				
vGLUT1			vGAT	
	ChABC	Control	ChABC	Control
Les	165.0 ± 36,1	145,9 ±30,9	189,7±13,2	206,7 ± 14,4
Pen	989,4 ± 67,5	1117,9 ±167,0	1424,2±72,2	1439,2 ± 33,7
Ipsi	1069,8 ± 92,8	1127,4 ±118,8	1436,7±55,2	1449,2 ± 69,3
Contra	1059,7 ±128,3	1198,4 ±155,8	1404,8±52,8	1454,2 ± 45,3

Table 2. vGLUT1 and vGAT puncta density is altered exclusively in the Les area with respect to all other areas (n=3 for both treatments and stainings; one-way RM ANOVA $p<0.01$, post-hoc Holm-Sidak test $p<0.05$).

Figure legends

Figure 1

A: Learning curve of the SRT test. Positive retrieves were measured during the training period (day 1-6, n=57 One way ANOVA Repeated Measures, $P<0.001$).

B. Visualization of the area of digestion by ChABC. Left: WFA staining in an ET-1 injected brain perfused three days after lesion shows two perilesional areas of digestion (marked by asterisks). Right: immuno staining for CSPG stubs using 2B6 antibody in a different rat. Calibration bar = 1 mm in left picture, 0.6 mm in right picture.

C: GFAP staining at the border of the lesion site (dark area at the top of pictures). Quantitation of the integrated GFAP staining fluorescence did not show a statistical difference between chABC treated (N=4) and control (N=4) rats three days after lesion (t-test, $p=0.06$). Calibration bar = 40 microns.

D. Treatment protocols. SRT was administered during Training an Re-call sessions.

Figure 2

Acute ChABC treatment promotes SRT re-learning after stroke. Learning curves of the control and ChABC groups did not stastically differ [two-way ANOVA, N=16 for both treatment groups, no significant effect of the treatment group ($p=0.41$) or time X treatment group interaction was observed ($p= 0.21$). The factor time was significant (factor time $p<0.001$)].Stroke caused a significant fine movement impairment in both control (A) and ChABC treated animals (B) measured on the first day of re training (day 20). After re-training, control animals failed to reach full recovery while ChABC treated animals fully recovered reaching motor skills. Data normalized to the pre-lesion level of learning (average of the last three days of pre-lesion training) show a similar profile for control (C) and ChABC treated (D) rats. * $P<0.05$ with respect to pre-lesion level (paired t-test).

Figure 3.

Delayed ChABC treatment promotes SRT re-learning after stroke. Fine forelimb movement are impaired in both control (A) and ChABC treated animals (B) measured on the first day of re-training (day 34). After re-training, control animals failed to reach full recovery while ChABC treated animals fully recovered reaching motor skills. Data normalized to the pre-lesion level of learning (average of the last three days of pre-lesion training) show a similar profile for control (C) and ChABC treated (D) rats. * $P<0.05$ with respect to pre-lesion level (paired t-test).

Figure 4.

Lack of effect of acute and delayed chABC treatment on lesion volume. No significant difference in lesion volume was measured between control and acute (A) or delayed (C) ChABC treated animals. No correlation was present between the volume and the retrieval performance in controls and chABC treated rats treated with the acute or the delayed protocol (B, D). Retrieval performance index was calculated as the mean performance during the last three days of the recall phase on normalized graphs shown in Fig 3 and 4.

Figure 5.

Punctate vGLUT2 staining is selectively increased in the Pen area of acutely ChABC treated rats receiving SRT training.

A: Cresyl violet staining of the lesion showing the location of lesion (Les), penumbra (Pen), ipsilateral unlesioned (Ipsi), and homotopic contralateral areas (Contra) are also shown.

B. Quantitation of vGLUT2 puncta density in control and ChABC treated rats trained in the SRT. In control treated rats the Les area significantly differed from the other areas (one way RM ANOVA $p<0.01$; Holm-Sidak post-hoc test $p<0.05$). Within ChABC treated rats both the Les and the Pen areas significantly differed from all the other areas (one way RM ANOVA $p<0.01$; Holm-Sidak post-hoc test $p<0.05$). The Pen area of controls and ChABC rats were significantly different (t-test,

$p<0.01$). * indicates $p<0.05$ with respect to the other groups within the same treatment. & indicates $p<0.05$ between Pen areas of ChABC and control treated rats.

C. Quantitation of vGLUT2 puncta density in control and ChABC treated rats without training. Both in control and ChABC treated rats only the Les area significantly differed from the other areas (one way RM ANOVA $p<0.01$; Holm-Sidak post-hoc test $p<0.05$). The Pen area of controls and ChABC rats were not significantly different (t-test, $p=0.64$). * indicates $p<0.05$ with respect to the other groups within the same treatment.

D. Representative maximum projection images of vGLUT2 staining in control and ChABC treated rats with and without SRT training. Calibration bar 15 microns.

E. vGLUT2 puncta density in GFP labeled thalamic fibers (N=37 axons) is significantly higher than vGLUT1 puncta density (N=35; t-test, $p<0.001$). Left: picture of a GFP positive fiber carrying vGLUT2 positive puncta. Calibration bar 6 microns.