Rho GTPase-dependent plasticity of dendritic spines in the adult brain

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INTRODUCTION

Brain activity is associated with structural changes in the neural connections. However, in vivo imaging of the outer cortical layers has shown that dendritic spines, on which most excitatory synapses insist, are predominantly stable in adulthood. Changes in dendritic spines are governed by small GTPases of the Rho family through modulation of the actin cytoskeleton. Yet, while there are abundant data about this functional effect of Rho GTPases in vitro, there is limited evidence that Rho GTPase signaling in the brain is associated with changes in neuronal morphology. In the present work, both chronic in vivo two-photon imaging and Golgi staining reveal that the activation of Rho GTPases in the adult mouse brain is associated with little change of dendritic spines in the apical dendrites of primary visual cortex pyramidal neurons. On the contrary, considerable increase in spine density is observed (i) in the basal dendrites of the same neurons (ii) in both basal and apical dendrites of the hippocampal CA1 pyramidal cells. While confirming that Rho GTPase-dependent increase in spine density can be substantial, the study indicates region and dendrite selectivity with relative stability of superficial cortical circuits.

Keywords: dendritic spines, two-photon microscopy, Golgi staining, Rho GTPases, cytotoxic necrotizing factor 1, brain plasticity, mice
changes observed was investigated by in vitro recording of activity-dependent plasticity phenomena, such as paired-pulse facilitation (PPF) and long-term potentiation (LTP). Altogether, our results indicate that Rho-dependent structural plasticity is substantial and widespread in hippocampal CA1 pyramidal neurons and weak in apical dendrites of V1 pyramidal neurons, thus suggesting a regional and dendritic selectivity.

MATERIALS AND METHODS

MORPHOMETRICAL ANALYSIS of Golgi-Cox STAINED SECTIONS

Animals

The experiments were carried out on eight male C57BL/6J mice (Harlan Italy, S. Pietro al Natisone, Udine, Italy) aged 3 months at the time of the treatment. The mice were housed at 21 ± 1°C at constant humidity (55%) and in a 12/12 h dark-light cycle, with light phase from 08:00 to 20:00. Food and water were provided ad libitum. The use and care of the animals was compliant with the Italian law (DL 116/92) and with the guidelines of the European Communities Council (1986).

Intracerebroventricular injections

Under general anesthesia (sodium pentobarbital, 30 mg kg\(^{-1}\) i.p.), a 27G needle mounted on a 25 μl Hamilton microsyringe was placed in the right lateral cerebral ventricle with a stereotaxic technique (coordinates from bregma and skull bone: A/P = 1.0 mm, L/M = ±1.0 mm, D/V = −3.0 mm; Paxinos and Franklin, 2004). The mice were injected 3.3 μl of 1.0 fmol kg\(^{-1}\) i.p. CNF1 (GenBank accession M) in a subset of cortical neurons (Feng et al., 2000) were used. A thy-1 promoter drives the expression of green fluorescent protein (GFP, line M) in a subset of cortical neurons (Feng et al., 2000) were used for the study. Surgical procedures were performed as described previously (Hobinna et al., 2009, Laperchias et al., 2013). Briefly, mice were deeply anesthetized with an i.p. injection of avertin (2 mg kg\(^{-1}\) i.p.). Before surgery, dexamethasone (2 mg kg\(^{-1}\) i.p.) and carprofen (0.3 ml of a 0.50 mg ml\(^{-1}\) solution i.p.) were injected to prevent cerebral edema and inflammation and to limit pain.

Primary visual cortex was identified on the basis of stereotaxic coordinates (Paxinos and Franklin, 2004). The skull overlying visual cortex was removed, taking care not to damage the dura. The dura was covered with a coverglass (5 mm diameter, 0.15 mm
FIGURE 1 | Rho GTPase activation increases spine density and dendrite branching in hippocampal CA1 pyramidal neurons. (A,D) Photomicrographs (scale bar 100 μm); (B,E) Neurolucida tracings; (C,F) detail of apical dendrite (scale bar 2 μm) of representative Golgi stained neurons in hippocampal CA1 of C57BL/6J mice treated 10 days before histology either with vehicle (control, A,C) or 1.0 fmol kg$^{-1}$ CNF1 i.c.v. (D,F). Spine density (G,J), number of intersections (H,K), and average dendrite length (I,L) in basal (G,I) and apical (J,L) dendrites are plotted by distance from cell soma (μm). Mean ± SEM; n = 16 in each group.

In each animal, the apical dendritic tufts of layer V pyramidal neurons were imaged for 16 days. In each session, the dendritic tufts were localized using as a reference the vascular pattern of the cortical region, and then using low-magnification 2PLSM imaging to identify the cell of interest by the unique branching pattern of its apical dendrites. For high-magnification spine imaging, 7–10 fields were selected for each cell. Image stacks consisted of sections (512 × 512 pixels; 90 nm/pixel, pixel dwell time 2.5 μs) collected in 1 μm z-step size. Care was taken to achieve almost identical fluorescence levels across imaged regions and imaging sessions. All images in the figures are maximum intensity projections (MIPs) of z-stacks.

Spine turnover analysis
A total of 2830 spines were tracked in time-lapse images on day 1 before treatment administration and subsequently on day 5, 10, and 15 post-injection. All clear protrusions emanating laterally from the dendritic shaft were measured. Evaluation of spine appearance/disappearance was based on the following criteria: spines were considered as lost if they disappeared into the haze of the dendrite, whereas spines were considered as gained if they showed clear protrusion from the dendrite.

Animals were anesthetized with isoflurane. In vivo images of GFP-expressing neurons were acquired by a two-photon laser scanning microscope (2PLSM, TCS-SP5, Leica Microsystems, Germany) equipped with a Ti:Sapphire tunable laser, (680–1080 nm; Chameleon Ultra, Coherent Inc, CA, USA). The objective was water immersion HCX APO L 20x/1.00 NA (Leica Microsystems, Germany). The GFP was excited at 920 nm and the signal was collected by non-descanned detectors in the range 500–530 nm.
Turnover ratios (TORs), i.e., the fraction of spines appearing and disappearing from an imaging session to the following one were calculated as \( \text{TOR} = \frac{N(t) - N(0)}{2 \times N(0)} \), where \( N(0) \) is the number of spines at \( t = 0 \), and \( N(t) \) is the number of spines of the original set surviving after time \( t \). By definition, \( \text{TOR} \) is a monotonically decreasing function of time, and \( \text{TOR}(0) = 1 \) (Holtmaat et al., 2005).

**Hippocampal and Cortical Slice Preparation and Electrophysiology**

Two groups of C57BL/6J mice, treated with either 1.0 fmol kg\(^{-1}\) CNF1 or vehicle were used for in vitro electrophysiology experiments. Intracerebroventricular administration of the test solution was carried out as described above. Ten–eighteen days post-treatment, the mice were deeply anesthetized with urethane (1.5 g kg\(^{-1}\) i.p.) and decapitated. The brains were removed and the hippocampus and primary visual cortex were isolated. Transverse hippocampal or cortical slices, 400 \( \mu \)m thick, were cut with a tissue chopper (The Mickle Laboratory Engineering Co. Ltd., Gomshall, Surrey, UK), transferred to an incubation glass chamber containing artificial cerebrospinal fluid (ACSF) saturated with a gas mixture of 95% O\(_2\) and 5% CO\(_2\) and maintained at room temperature for at least 2 h. ACSF is a water solution (pH 7.4) containing (mM): 126 NaCl, 3.5 KCl, 1.2 NaH\(_2\)PO\(_4\), 25 NaHCO\(_3\), 2 CaCl\(_2\), 1.3 MgCl\(_2\), 11 glucose. For electrophysiological experiments, slices were transferred to a submerged-type recording chamber, placed about 100 \( \mu \)m below the surface and perfused with oxygenated ACSF (24 ± 1°C) with a peristaltic pump (Gilson Minipuls3, WI, USA) at a constant flow rate (2.5–3 ml min\(^{-1}\)). An electrode (stainless steel, 250 \( \mu \)m diameter, tapered tip size 8\(^{\circ}\), 5 M\(_{Omega}\); A-M Systems Inc., WA, USA) was placed into the stratum radiatum within the CA1 area to stimulate the Schaffer’s collateral commissural fibers or in the layer IV of the V1 area of the visual cortex. Glass micropipettes (OD 1.0 mm, ID 0.7 mm, 1.5–2 M\(_{Omega}\)) filled with ACSF were placed in the hippocampal dendritic layer of the CA1 area or in the layer III of V1 for extracellular recording of field excitatory post-synaptic potentials (fEPSPs). The depth of the electrodes was adjusted in order to maximize the height of the fEPSPs, which were evoked by regular stimulation (0.033 Hz; squared waves, 100 \( \mu \)s; constant current). The responses were amplified 1000 times and filtered at 10 kHz (L-C low pass filter, 40 dB/decade). The signals were then sampled at 40 kHz, digitized and stored on disk for subsequent off-line analysis.

**FIGURE 2** Rho GTPase activation increases spine density in basal but not apical dendrites of V1 visual cortex. (A,D) Photomicrographs (scale bar 100 \( \mu \)m); (B,E) Neurolucida tracings; (C,F) detail of apical dendrite (scale bar 2 \( \mu \)m) of representative Golgi stained neurons in V1 visual cortex of C57BL/6J mice treated 10 days before histology either with vehicle (control, A,C) or 1.0 fmol kg\(^{-1}\) CNF1 i.c.v. (D,F). Spine density (G,J) number of intersections (H,K), and average dendrite length (I,L) in basal (G,I) and apical (J,L) dendrites are plotted by distance from cell soma (\( \mu \)m). Mean ± SEM; \( n = 16 \) in each group.
Ten minutes before the induction of LTP and 1 h after LTP; neurotransmission was studied by recording input–output curves, i.e., the response produced by 11 consecutive stimuli of increasing intensity (0–200 μA in steps of 20 μA). Stimulus intensity used throughout LTP experiments was selected so that fEPSP initial slopes ranged from 40 to 60% of the maximum obtained in the first input–output curve. For analysis, only slices that reached a steady response in 30 min were used. LTP was induced by three consecutive theta-burst stimulations (TBS, inter-stimulation interval = 30 s; 10 trains of 4 stimuli at 100 Hz; baseline intensity; inter-train interval = 200 ms) and recorded for at least 1 h. In non-potentiated slices, PPF was elicited at six interpulse intervals (25, 30, 100, 200, 300, and 400 ms, stimulation intensity selected as 0.626 ± (25, 50, 100, 200, 300, and 400 ms, stimulation intensity selected as for LTP experiments). Data were entered into analysis as a single subject, and therefore reflect individual mice.

**RESULTS**

**Rho GTPase ACTIVATION INCREASES SPINE DENSITY IN HIPPOCAMPAL CA1**

Activation of Rho GTPases produces a substantial increase in spine density on both basal and apical dendrites of hippocampal CA1 pyramidal neurons. In basal dendrites, spine density on segments that usually show little or no increased considerably on segments that usually show little or no (0.626 ± (25, 50, 100, 200, 300, and 400 ms, stimulation intensity selected as for LTP experiments). Data were entered into analysis as a single subject, and therefore reflect individual mice.

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Although CNF1 does not affect the density of spines and branch-

distance from cell soma: F_{3,28} = 2.342, P = 0.149; effect of
dependence from cell soma: F_{3,28} = 2.122, P < 0.0011; Figure 2H).
The average length of basal dendrites by distance from cell soma was
70.474 ± 3.921 in CNF1-treated and 76.472 ± 4.801 in
cell group (μm; F_{1,37} = 0.570, P = 0.5484; interaction dendrit-
length × radius: F_{3,26} = 1.552, P = 0.1306. Figure 2J). For apical
dendrites, the average length was 40.054 ± 1.488 and
38.562 ± 1.796 in CNF1-treated and control group, respectively
(F_{1,34} = 0.138, P = 0.7130; differences in the effects of treatment
by radius: F_{3,26} = 0.701, P = 0.8196; Figure 2L).

Rho GTPase activation does not affect spine dynamics in the superficial V1 visual cortex

Although CNF1 does not affect the density of spines and branch-
ing apical dendrites of layer V pyramidal neurons in V1 visual cortex,
changes in the turnover of spines or dendrite morphology may have occurred that are not reflected in the morphometry of
neurons of Golgi stained cells. For this reason, the stability of apical dendritic
trajectories under the effects of CNF1 was studied by repeated, time-lapse
in vivo two-photon imaging (Figures 3A-C). A group of control
mice was treated with CNF1 C866S, a recombinant CNF1 in which
the enzymatic activity was abolished by substitution of serine with
cysteine at position 866 (Schmidt et al., 1998; Figures 3D,F). A group of mice that received only vehicle injection and a group of
untreated, age-matched mice were used as additional controls
(Figures 3G and LN, respectively).

We monitored the overall effects of Rho GTPase activation during
the first two weeks after treatment. We did not see any change
in the morphology of the superficial processes of layer V pyrami-
dal neurons of V1 visual cortex. We then evaluated dendritic spine
turnover, stability, and density.

Individual spines observed in the first imaging session, a total 860,773,847 and 350 spines in CNF1-, CNF1 C866S-treated, vehicle
injected, and untreated group, respectively, were followed up.
The fraction of spines surviving until the last imaging session (sur-
vival fraction, SF) was calculated. No significant differences were
observed among the four groups in SF (CNF1: 79.0 ± 2.3%, n = 4;
CNF1 C866S: 82.4 ± 2.1%, n = 4; vehicle: 78.4 ± 6.0%, n = 4;
untreated mice: 83.0 ± 3.0%, n = 3, mean ± SEM; F_{3,26} = 0.654,
P = 0.606; Figure 3J).

A subpopulation of spines appeared and disappeared across imaging sessions (Figures 3L,JN). This phenomenon was
observed in all groups. The rate of spine turnover (TOR) was
slightly higher in CNF1 treated mice than in the controls, even
though not significantly (CNF1: 9.8 ± 2.5%, n = 4; CNF1 C866S:
8.5 ± 1.6%, n = 4; vehicle: 9.2 ± 1.8%, n = 4; untreated mice:
8.0 ± 1.5%, n = 3, mean ± SEM; F_{3,26} = 1.764, P = 0.153;
Figure 3P).

Spine density (average of all time points) was comparable
among the four treatment groups (CNF1: 0.154 ± 0.045, n = 4;
CNF1 C866S: 0.130 ± 0.033, n = 4; vehicle: 0.170 ± 0.050%,
n = 4; untreated mice: 0.152 ± 0.056, n = 3, spines/μm²;
mean ± SEM; F_{3,26} = 1.291, P = 0.279; Figure 3Q). In

In conclusion, the activation of Rho GTPases did not induce
changes in the superficial dendritic tree of V1 pyramidal neurons.

Rho GTPase activation enhances glutamatergic neurotransmission and long-term potentiation in hippocampal CA1 but not in V1 visual cortex

Input-output curves were analyzed by a one-way ANOVA for
repeated measurements, in which treatment (“control,” “CNF1”) was
“between-subjects” factor and both the effects of LTP “pre-
LTP” and “post-LTP)” and the trend of responses at increasing
stimulation intensity (11 levels, “0” to “200”) were “within-
subjects” factors.

The ANOVA on the initial slopes of the EPSP in the hippocampus showed that the responses recorded during the generation of input-output curves were significantly different in control and in CNF1-treated mice (F_{1,12} = 3.003, P = 0.045; Figure 4A). Field EPSP slopes were significantly affected by stimulation intensity (F_{9,120} = 23.032, P < 0.0001) and its interaction with treatment (F_{9,120} = 2.531, P = 0.0084) and LTP (F_{4,5} = 12.174, P = 0.0045; interaction LTP × stimulation intensity:
F_{9,120} = 4.970, P < 0.0001). Overall, the results indicate that
Rho GTPase activation increases excitatory neurotransmission in the hippocampal CA1.

The analysis of EPSP maximal slopes recorded in the layer
III of V1 visual cortex (Figure 4B) shows no significant effects of treatment (F_{1,12} = 0.027, P = 0.8711; interaction treat-
mant stimulation intensity: F_{9,120} = 0.261, P = 0.9982). The
effects of LTP (F_{1,12} = 5.291, P = 0.0482; interaction LTP × stim-
ulation intensity: F_{9,120} = 3.501, P = 0.004) were significant, as
they were the effects of stimulation intensity (F_{9,120} = 19.116, P = 0.0001). Apparently, in the layer III of V1 visual cortex,
the activation of Rho GTPases does not affect basal glutamatergic neurotransmission and LTP.

All slices reached the criterion for LTP. Responses obtained at
baseline intensity were steady both pre and post-TBS. The trend
of EPSP maximal slopes after TBS in hippocampal CA1 and V1
visual cortex are illustrated in Figures 4C,D, respectively. Sixty
minutes post-TBS, the LTP in hippocampal CA1 area from CNF1-
treated mice was significantly higher than that observed in slices
from control mice (P < 0.05 by ANOVA, using average EPSP slope of 10 pre-TBS responses as covariate). On the contrary, the
difference was not significant in V1 visual cortex. These results
might reflect a plasticizing effect that is selective for hippocampal
CA1. However, the findings may also be explained by the different
trend of the input/output curves observed in CA1 and therefore
do not necessarily reflect a real increase in synaptic plasticity.

The analysis of EPSP maximal slopes during PPF by ANOVA
for repeated measurements using the slope of the first response as
covariate did not show any significant effect of the treatment or its
interaction with inter-stimulus interval, neither in hippocampal
CA1 (Figure 4E) nor in V1 visual cortex (Figure 4F).

The effects of CNF1 do not show hemispheric differences

The reported effects may be partly explained by a prominence effect,
i.e., the tendency of the treatment to selectively affect cell pro-
cesses that are closer to the injection side. To test this hypothesis,
we compared the effects of the treatment in the morphometry of
Golgi stained CA1 hippocampal neurons of the two brain
hemispheres. If a local effect exists, it should be observed in
the injected side. In general, the effect hemisphere was far from
FIGURE 3 | Rho GTPase activation does not increase spine density and turnover in apical dendrites of primary visual cortex. GFP-m mice were injected with CNF1 (1.0 fmol kg\(^{-1}\) i.c.v., \(n = 4\)), CNF1 C866S (1.0 fmol kg\(^{-1}\) i.c.v., \(n = 4\)), vehicle (20 mM TRIS-HCl buffer, pH 7.5, \(n = 4\)), or did not received any treatment (\(n = 3\)) and transcranial two-photon imaging in time-lapse of dendritic spines was performed. Examples of images collected from mice treated with CNF1 (A,D), CNF1 C866S (D,F), vehicle (G,I), and without treatment (L,N) (A,D,G,L) bright field views of the vasculature below cranial window. The arrows indicate the region where two-photon images were acquired (B,E,H,M) low-magnification images of a layer V dendritic arbor in visual cortex (C,F,I,N) time-lapse images of dendritic branches acquired 1 day before treatment (T0) and 5 (T5), 10 (T10), and 15 (T15) days after injection (from boxed regions in B,E,H,M, respectively). Figures show an example of persistent (yellow arrows) and transient spines (red arrows). Mean survival fraction (O), mean turnover ratios (P) and mean spine density (Q) are plotted as a function of time. Scale bars = 200 \(\mu\)m (A,D,G,L), 100 \(\mu\)m (B,E,H,M), 5 \(\mu\)m (C,F,I,N).
FIGURE 4 | Enhancement of glutamatergic neurotransmission and long-term potentiation in hippocampal CA1 but not in V1 visual cortex after Rho GTPase activation. Input–output curves of fEPSPs recorded 10 min before and 1 h after induction of long-term potentiation obtained by three consecutive theta-burst stimulation (TBS, inter-stimulation interval = 30 s; 10 trains of 4 stimuli at 100 Hz, baseline intensity; inter-train interval = 200 ms) in CNF1-treated (1.0 fmol kg⁻¹ i.c.v.), and control (vehicle-treated) C57BL/6J mice (control, n = 6; CNF1, n = 8). (A) hippocampal CA1 and (B) V1 visual cortex. Time course of fEPSP slopes pre- and post-TBS and representative traces (left, control; right, CNF1; horizontal scale bar: 1.5 ms; vertical scale bar: 1.5 mV) in (C) hippocampal CA1 (control, n = 12; CNF1, n = 13) and (D) V1 visual cortex (control, n = 12; CNF1, n = 10). Paired-pulse facilitation at different interstimulus intervals (control: n = 6, CNF1: n = 6) in (E) hippocampal CA1 and (F) V1 visual cortex. All recordings were performed 10–18 days post i.c.v. injections. Data are mean ± SEM.
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FIGURE 5 | Effects of Rho GTPase activation on spine density by cerebral hemisphere. The analysis of spine density by distance from cell soma (μm) in hippocampal CA1 pyramidal neurons of the left (L, n = 16) and right (R, n = 16) hemisphere. C57BL/6J mice were injected into the right lateral ventricle 10 days before histology. Spine density in basal (C,D) and apical (A,B) dendrites. Data are mean ± SEM.

the physiological relevance of dendritic spine plasticity in adulthood. Different techniques permitting the in vivo study of deeper brain areas will be needed, as well.

Although we cannot rule out the possibility that other mechanisms modify dendritic spines on apical cortical dendrites, regional differences in Rho-GTPase signaling might represent the cause of the stability of cortical circuits. However, since synaptic plasticity follows coincident stimulation at single synaptic buttons, it is also possible that this event is not common in outer cortical layers. As a third explanation, functional synaptic plasticity, which triggers structural plasticity, might be reduced in the apical dendrites of cortical pyramidal neurons. This possibility is suggested by our electrophysiology findings and it was also previously hinted (Frankland et al., 2001).

Whether differences in Rho GTPase-dependent plasticity reflect different distribution of neural activity leading to structural plasticity or functionality of Rho signaling cascade, regional analysis of Rho GTPase-dependent plasticity may provide information about the importance of this process in different areas of the brain. Specifically, our findings might reflect differences in the physiological role of apical dendrites of cortical pyramidal neurons. Functional split of apical and basal dendrites of layer V cortical pyramidal neurons of the barrel cortex was reported (Petreanu et al., 2009). Differences in the degree of structural plasticity (absent in apical, present in basal dendrites) have also been observed in V1 visual cortex pyramidal neurons (Michalet et al., 2012). Although a full characterization of V1 inputs by subcellular areas of the neuron is still to be obtained, it seems that layer II and III and the upper layer IV, which contain apical dendrites of layer V pyramidal neurons, receive long-range connections from the contralateral hemisphere (Rochefort et al., 2009). Thus, the selective structural stability of outer cortical processes might be crucial for complex functions requiring the integration of multimodal inputs.

We also report an increased branching of the hippocampal dendritic tree. The increase was observed after modulation of a physiological pathway in healthy individuals. Thus, such changes might occur in the physiological functioning of the adult brain. This finding suggests that adult activity-dependent neural morphogenesis may not be limited to spines, but it might involve the entire neural tree. The presence of these changes in deep brain structure such as hippocampus, but not in visual cortex, might explain why this phenomenon has never been observed with in vivo multiphoton microscopy of adult mice.

Cytotoxic necrotizing factor 1 substantially increases neural connectivity. To our knowledge, no molecule, including synthetic drugs and nervous growth factors, parallels the size of the increase in the number of dendritic spines produced by CNF1 in vivo. The increase is equally distributed in the two hemispheres of the brain and was specially observed on the principal dendrite shaft, on oblique dendrites and in the terminal part of the hippocampal neuron. It is worth observing that the effect was seen in adult individuals of a mouse strain displaying excellent performance in hippocampal-dependent learning tasks (Rossi-Arnaud and Ammassari-Teule, 1998). In several disorders, such as those
associated with intellectual disability, the impoverishment of the dendritic tree is a consistent stigma (Ramakers, 2002). Reversing this feature by modulation of Rho GTPases might be a therapeut- ic value (Bongmba et al., 2013). This effect, together with those previously described on learning ability (Diana et al., 2007; De Viti et al., 2010; De Filippis et al., 2012; Borrelli et al., 2013), can be seen as regulators of morphological neu- roplasticity. Hence, the Rho GTPase-Ser/Thr protein kinase cascade might represent a novel potential therapeutic approach for the treatment of several disorders of the central nervous system (Mussil et al., 2013).

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