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Long-lasting efficacy of the cognitive enhancer Cytotoxic Necrotizing Factor 1

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ABSTRACT

Rho GTPases are key regulators of the activity-dependent changes of neural circuits. Besides being involved in nervous system development and repair, this neural structural plasticity is believed to constitute the cellular basis of learning and memory. Here we report that concurrent modulation of cerebral Rho GTPases, including Rac, Rho and Cdc42 subfamilies, by Cytotoxic Necrotizing Factor 1 (CNF1, 10 fmol/kg intracerebroventricularly) improves object recognition in both C57BL/6J and CD1 mice. The improvement is long lasting, as it is still observed 90 days post treatment. At this time, the treatment is associated with enhancement of neurotransmission and long-term potentiation. The effects depend on changes in Rho GTPase status, since the recombinant molecule CNF1 C866S, in which the enzymatic activity was abolished through substitution of serine to cysteine at position 866, is ineffective. The study confirms the role of Rho GTPases in learning and suggests that a single administration of CNF1 is effective for a long time after administration. In general, the long-lasting cognition enhancing effect of CNF1 might be beneficial for the treatment of CNS disorders.

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1. Introduction

Learning is thought to be associated with changes in synaptic connections. Long-Term Potentiation (LTP), a laboratory phenomenon involving activity-dependent, long-lasting changes in synaptic efficacy, has been widely used to model synaptic plasticity (Bliss and Collingridge, 1993). Synaptic plasticity occurs by different steps, which involve membrane receptors, calcium flows, second messengers, proteins synthesis and changes in the shape of the dendritic tree. In order to treat Central Nervous System (CNS) disorders associated with the impairment of cognitive functions, the pharmacological modulation of these mechanisms has been attempted. Examples are agents that regulate or bind to NMDA or AMPA-receptors, which are involved in the induction of LTP. Although this approach may not have reached its full potential, these experimental therapeutic attempts are endowed with little efficacy, so far (Berry-Kravis et al., 2006). In fact, most of the aforementioned mechanisms have little relevance to the pathophysiology of cognitive abilities.

It has been shown that disorders associated with cognitive deficits are associated with dysregulation of actin cytoskeleton (Newey et al., 2005; Ramakers, 2002). Typically, in some forms of intellectual disability (ID) changes in the organization of the neuronal tree, in particular at the level of dendritic spines, are consistently observed (Huttenlocher, 1970; Purpura, 1974). These structural aspects are under the control of Rho GTPases, small regulatory proteins involved in a number of cellular functions, including the regulation of actin assembly/disassembly (Etienne-Manneville and Hall, 2002; Hall and Lalli, 2010). In the brain, this process is essential for determining the shape of the neuronal dendritic tree and its extreme terminations, namely dendritic spines (Hayashi et al., 2007). Therefore, Rho GTPases represent key effectors of a series of biological events leading to changes in synaptic arrangement and neural connectivity. Rho GTPases are involved in LTP (O'Kane et al., 2004; Rex et al., 2009). In particular, Rho GTPase signaling is involved in activity-dependent plasticity of single spines (Murakoshi et al., 2011). In addition, a number of studies have shown that mutations in genes code for proteins related to Rho GTPase signaling are consistently associated with genetic forms of ID. Therefore, the modulation of Rho GTPase signaling may impact on a mechanism that is truly involved in disorders affecting cognition. This therapeutic avenue might be of value in conditions such as ID and Alzheimer's disease (AD).

The pharmacological modulation of Rho GTPase signaling leads to changes in learning abilities. The manipulation of this pathway is associated with deficits in spatial memory (Dash et al., 2004), fear memory (Lamprecht et al., 2002, 2006) and social recognition (Zhao et al., 2006). Cytotoxic Necrotizing Factor 1 (CNF1), a protein toxin produced by *Escherichia coli*, determines a rearrangement of the cytoskeleton in intact cells through permanent activation of

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Rho GTPases, including Rho, Rac1 and Cdc42 (Boquet, 2001). It was reported that this capability, previously observed in epithelial cells, extends to neurons (Diana et al., 2007; Pavone et al., 2009). The effect is associated with enrichment in dendritic-spines-like structures both in cultured neurons (Diana et al., 2007) and *in vivo* (Cerri et al., 2011). More strikingly, mice treated with CNF1 display a substantial improvement both in a water maze and in a fear conditioning task (Diana et al., 2007).

Both water maze (Morris et al., 1982) and context conditioning (Phillips and LeDoux, 1992) depend on hippocampal functioning. In the present work, we aimed at better characterizing the effects of CNF1 on learning by testing the effects of the protein in an object recognition (OR) task. Object recognition involves specific brain circuits (Parron and Save, 2004; Winters and Bussey, 2005; Xavier et al., 1990), therefore providing complementary information to tasks based on hippocampal functioning. This ethologically relevant test is based on the rodents' innate curiosity to explore novel objects, requires no reinforcers or aversive stimuli and implies very little stress for the animals.

Object recognition is a simple test, thus suitable for quick assessment of memory performances. Due to its mechanism of action, it has been claimed that the increase in learning ability induced by CNF1 might be long-lasting (Diana et al., 2007). We aimed at studying the effects of the bacterial protein on OR up to 3 months after single administration. Given the proven predictivity of OR (Ennaceur et al., 1989; Scali et al., 1994), the study might add evidence to the therapeutic potential of CNF1 in dementia, mild cognitive impairment, ID and any other condition associated with cognitive impairment.

Since the effects of CNF1 are associated with enhanced glutamatergic transmission and synaptic plasticity in the hippocampal CA1, we also sought to determine whether LTP is changed 3 months post CNF1 administration in the same area.

2. Materials and methods

2.1. Molecules

Expression of recombinant CNF1 and C866 CNF1 proteins was carried out in *E. coli*. N-terminally His-tagged proteins were purified under native conditions by Ni^{2+} nitrilotriacetic acid affinity chromatography. The His-tag was removed and tag-free proteins were separated by Ni^{2+} nitrilotriacetic acid chromatography.

2.2. Animals

The experiments were carried out on male C57BL/6J or CD1 mice (Harlan Italy, S. Pietro al Natisone, UD, Italy) aged 2 months at the time of toxin injection. The use and care of the animals followed the guidelines of the European Communities Council (1986). 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.

2.3. Surgery and drug administration

Under general anesthesia (sodium pentobarbital, 50 mg/kg ip), a 27G needle, mounted on a 25 μL Hamilton microsyringe was placed in the right cerebral lateral ventricle with a stereotaxic technique. The mice were injected 3.3 μL of the test solution Five min post injection, the needle was removed and the surgical wound sutured. From this time on, the mice were housed in individual cages and monitored for general conditions for the following 7 days.

A first group of C57BL/6J and CD1 mice was used for OR studies 10 days post treatment. Mice from the CD1 strain were randomly assigned to the following 2 groups of treatment: 10 fmol/kg CNF1 ($n = 16$) or vehicle (20 mM TRIS–HCl buffer, pH 7.5; $n = 18$). Mice from the C57BL/6J strain were randomly assigned to the following 3 groups of treatment ($n = 12$ in each group): 10 fmol/kg CNF1, 10 fmol/kg CNF1 C866S (a recombinant CNF1 in which the enzymatic activity was abolished through substitution of serine to cysteine at position 866) or vehicle. A second group of C57BL/6J mice, treated with either 10 fmol/kg CNF1 or vehicle ($n = 12$ in each group) was used for OR experiments 90 days post injection. A third group of C57BL/6J mice, treated with either 10 fmol/kg CNF1 ($n = 11$) or vehicle ($n = 12$) was used for *in vitro* electrophysiology experiments 92–110 days post treatment.

The dose of CNF1 and CNF1 C866S used in this work (10 fmol/kg) was significantly higher than those used in previous studies (0.6–1 fmol/kg) (De Filippis et al., 2011; De Viti et al., 2010; Diana et al., 2007; Pavone et al., 2009). This was done because in a preliminary set of *in vitro* electrophysiology experiments, mice treated with CNF1 1.0 fmo/kg displayed inconsistent changes in basal hippocampal CA1 responses.

2.4. Behavior

2.4.1. Experimental apparatus

A circular arena (80 cm in diameter, 30 cm high wall), was positioned in a silent room at constant, dim light. The arena was surrounded by several objects that served as external cues, including an anti-vibration table (84 × 90 × 75 cm), a bookshelf (74 × 90 × 44 cm) containing colored books, a lab cabinet (240 × 120 × 50 cm), a painting (33 × 45 cm). A video-camera viewing the experimental area was positioned on the vertical from the center of the arena and connected to a monitor located in an adjacent room. Mice's behavior was analyzed by two independent observers.

2.4.2. Experimental procedure

All experiments were done in the light phase of the day (h 10.00–15.00). In the 3 days before OR, the mice were placed in the arena from a predefined starting point and were allowed to explore the apparatus for a daily 10 min habituation session. For OR, 2 objects were placed at the center of 2 arena sectors (Figs. 1 and 2). The experiment consisted of 2 or 3 sessions: during *sampling*, the animals were exposed to 2 identical objects. On the subsequent day, one of the 2 familiar objects was substituted with a novel one (*test*). In the test performed 10 days post injection, seven days after sampling, the mice were also allowed to re-explore the same set of objects (*re-test*).

At the beginning of each session, mice were placed at the center of the arena and allowed to explore for 5 min. Each event during which the mouse was touching or sniffing the object, being at a distance <2 cm from it, was considered as exploration. Sometimes, mice climbed on the top of the objects and remained on them for some seconds: those times were not considered as exploration; consequently, they were subtracted from the total exploration time. In order to avoid selection biases, no animals were excluded from the analysis. Times of contact were measured by two independent observers.

For the choice of objects to be used in OR, a preliminary experiment was carried out in a different set of control mice (results not shown). This was done in order to select objects mice showed similar degrees of preference for, i.e. objects that minimized the variance of exploration times. Two different sets of objects were used in the day 10 and day 90 post treatment tests. The 4 objects were a glass parallelepiped (10.7 × 6.5 × 6.5 cm) with 3 evenly distributed horizontal 1.3 cm wide black stripes (□, Fig. 1), a truncated cone (10 cm high, 6 cm in diameter at the base, 2 cm at the top) painted in brown (○, Fig. 1), a green pyramid (12 cm high, 5 cm in diameter at the base, ●, Fig. 2), a white cylinder (14 cm high, 7 cm in diameter) patterned with 6 × 4 cm black lozenges (Δ, Fig. 2). At the end of each session, the objects were carefully cleaned with 70% ethanol to remove olfactory traces.

2.5. Hippocampal slices preparation and electrophysiology

Mice were deeply anaesthetized with urethane (1.5 g/kg ip) and decapitated. The brains were removed and the hippocampus was isolated. Transverse hippocampal slices, 400 μm thick, were cut with a tissue chopper (The Mickle Laboratory Engineering Co. Ltd., Gomshall, Surrey, England), 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 (mmol): 126 NaCl, 3.5 KCl, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2 $CaCl_2$, 1.3 $MgCl_2$, 11 glucose. For electrophysiological experiments, slices were transferred in submerged-type recording chamber, placed about 100 μm below the surface and perfused with oxygenated ACSF (24 ± 1 °C) with a peristaltic pump (Gilson Minipuls3) at a constant flow rate (2.5–3 ml/min). An electrode (stainless steel, 250 μm diameter, tapered tip size 8°, 5 MΩ; A-M Systems Inc., Carlsborg, WA, USA) was placed into the stratum radiatum within the CA1 area to stimulate the Schaffer collateral-commissural fibers. Glass micropipettes (OD 1.0 mm, ID 0.7 mm, 1.5–2 MΩ) filled with ACSF were placed in the hippocampal dendritic layer of the CA1 area for extracellular recording of field excitatory postsynaptic 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 μs; 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.

Ten min before the induction of LTP and 1 h after LTP, basal neurotransmission was studied by recording input–output curves, i.e. the responses produced by 11 consecutive stimuli of linearly increasing intensity (0–200 μA in steps of 20 μA). Stimulus intensity used throughout the LTP experiments was selected so that fEPSP initial slopes ranged from 40% to 60% of the maximum obtained in the first input–output curves. For analysis, only slices that reached a steady response in 30 min were used. LTP was induced by three trains of tetanic stimuli (100 pulses, 100 Hz, 30 s inter-train interval, basal intensity) and recorded for at least 1 h. In non-potentiated slices, paired-pulse facilitation (PPF) was elicited at six interpulse