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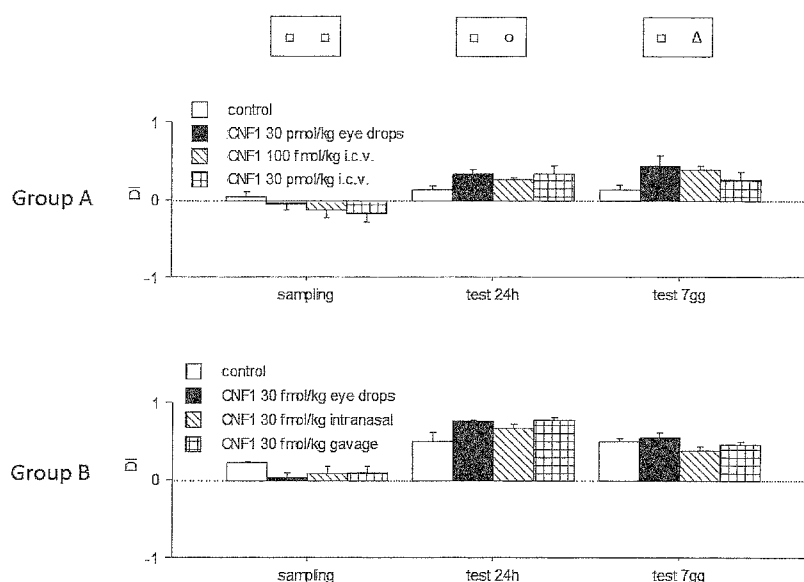


Fig. 1

(57) Abstract: The present invention concerns Rho GTPase activating bacterial toxins for use in treatment of cognitive impairment and neurodegenerative diseases such as Parkinson's disease by topical ocular, nasal or enteral administration, wherein said Rho GTPase activating protein toxins are chosen from the group consisting of Cytotoxic Necrotizing Factor 1 (CNF1) and Cytotoxic Necrotizing Factor 2 (CNF2).

Rho GTPase activating bacterial toxins for use in the treatment of disorders of the Central Nervous System by mucosal administration

The present invention concerns Rho GTPase activating bacterial toxins for use in the treatment of disorders of the Central Nervous System (CNS) by mucosal administration. Particularly, the present invention concerns Rho GTPase activating bacterial toxins for use in the treatment of disorders of the CNS chosen in the group consisting of cognitive impairment and neurodegenerative diseases such as Parkinson's disease, wherein said Rho GTPase activating bacterial toxin is administered by topical ocular, nasal or enteral (such as oral) administration. Preferably, Rho GTPase activating bacterial toxins are chosen from the group consisting of Cytotoxic Necrotizing Factor 1 (CNF1) and the similar protein Cytotoxic Necrotizing Factor 2 (CNF2).

Rho GTPases are a class of small GTP-binding proteins that encompasses Rho, Rac, and Cdc42 subfamilies. These regulatory proteins play a key role in the initiation, growth, guidance and branching of neural processes [1]. Beside cytoskeletal dynamics, Rho GTPases are key regulators of several mechanisms involved in neuronal physiology, including membrane trafficking, gene transcription, vesicle exocytosis and apoptosis [2, 3]. Not surprisingly, the derangement of Rho GTPase signaling has been associated with a number of CNS disorders, including familiar forms of intellectual disability [4], Alzheimer's disease [5, 6] and parkinsonisms [7, 8].

Cytotoxic Necrotizing Factor 1 (CNF1), a protein toxin produced by *E. coli*, causes a rearrangement of the cytoskeleton in intact cells through permanent activation of Rho GTPases. It was reported that this capability, previously observed in epithelial cells, extends to neurons [9, 10]. The effect is associated with enrichment in dendritic spines-like structures both in cultured neurons [9] and *in vivo* [11, 12]. More strikingly, the pharmacological modulation of Rho GTPase signaling by CNF1 enhances

learning, memory and motor activity in wild type mice. In addition, CNF1 has shown therapeutic potential in models of CNS disorders, including Rett syndrome [13] and Alzheimer's disease [14].

Cytotoxic Necrotizing Factor 1 is a protein of 1014 aminoacids and 113.7 kDa molecular weight. Therefore, its use for the therapy of CNS disorders requires local administration. In the above-mentioned studies, CNF1 was administered by intracerebroventricular (i.c.v.) injection [9] or by injection into a selected area of the CNS [11]. Notwithstanding the serious disorders for which the use of the molecule has been designated, and although i.c.v. administration was used in humans [15], this route is hardly exploitable in routine clinical use. As an alternative, it has been suggested that CNF1 might be used, for example, intrathecally by lumbar puncture, [16]. Yet, this route of administration is associated with a substantial degree of discomfort.

No study is available up to now concerning the administration of Rho GTPase activating bacterial toxins by an administration route different from i.c.v. injection or injection into selected areas of the CNS. Patent application WO2011/101881 mentions a possible administration of Rho GTPase activating bacterial toxins by ocular or nasal routes in the treatment of Rett syndrome. However, no experiments are described showing the effectiveness of the above mentioned administration routes. In addition, a possible efficacy in the treatment of Rett syndrome cannot suggest the same effect in other CNS disorders.

Based on the above, it is therefore apparent the need for new administration methods that could overcome the disadvantages of the known ones.

According to the present invention, it has now been found that the administration of CNF1 by topical ocular, intranasal or gavage routes enhances learning ability in wild type mice with even higher efficacy in behavioral tests than i.c.v. administration. Ocular and intranasal routes

also correct a Parkinson's disease model produced in the mouse by unilateral striatal injection of the neurotoxin 6-Hydroxydopamine (6-OHDA).

Surprisingly, it has been found that CNF1 shows efficacy after single administration by eye, intranasal drops or gavage. The effects are more ample than those following i.c.v. administration. The treatment substantially reduces anxiety levels, as well. Since the three routes of administrations are definitely less invasive and more feasible than i.c.v. administration, the present invention will greatly ease the medical use of bacterial toxins, such as CNF1.

In particular, it has been found that the administration of CNF1 by eye and nasal drops and by gavage administration:

- Improves the discrimination index in an object recognition (OR) task at both 24 h and 7 days post sampling;
- Is associated with increased exploration of objects the mice were exposed to;
- Improves the ability of locating a hidden platform in water maze tasks;
- Causes substantial reduction of anxiety as compared to i.c.v. route.

Moreover, ocular and nasal routes correct the behavioral asymmetries of a mouse model of Parkinson's disease produced by unilateral injection of 6-OHDA.

These findings suggest that the molecule could be transported through peripheral tissues and nerves and enter the CNS, where it exerts its proven beneficial effects.

All the three routes of administration, i.e. intranasal, ocular and gavage, can substantially ease the administration of CNF1 and make it possible to treat a number of CNS disorders by simply, as an example, applying small volumes of solutions containing CNF1 in the conjunctival

sac or in the naris or administering it orally (*per os*).

The effects of CNF1 are long lasting. For example, the mice used in the accompanying examples were tested between 10 and 30 days after the administration of low doses of CNF1 and clearly demonstrated the expected behavioral improvement. The prolonged efficacy of CNF1 seems to parallel the long lasting activation of cerebral Rac, which is still observed 4 weeks post-injection [9].

Given the sustained efficacy of CNF1 by topical ocular, intranasal route and gavage, such administrations need only be performed on no more than a weekly or monthly basis, and may be performed on a more infrequent basis, such as twice yearly, or preferably on a 2- or 3 -monthly basis or even more sparsely, such as once in the life. The molecules could be administered at any life time, including early postnatal to prevent the effects of molecular deficits underlying the pathogenesis of inherited disorders, such as some forms of intellectual disability.

The molecules of the present invention may be formulated in any suitable manner, such as in buffering and/or isotonic agents. Quantities to be administered may be any that are readily determined by the skilled physician, taking into account such factors as age, weight and sex, but will generally vary between about 0.001 fmol/kg and 100 μ mol/kg, preferably between about 0.01 fmol/kg and 1 μ mol/kg, and more preferably between 0.1 fmol/kg and 100 pmol/kg.

Conditions that can be treated according to the present invention include all those in which cognitive enhancement could be of therapeutic value, such as: dementia, mild cognitive impairment, intellectual disability and any other condition associated with cognitive impairment, including attention deficit and hyperactivity disorder (ADHD), schizophrenia, metabolic diseases, cerebrovascular diseases, diabetic encephalopathy, psychic depression, intellectual disability of any type, Huntington's chorea, diffuse cerebral cortical atrophy, Lewy-body dementia, Pick's disease;

mesolimbocortical dementia and familial dementia with spastic paraparesis, brain tumors, including astrocytoma, oligodendroglioma and meningioma, epilepsy, coma and other disorders of consciousness. In addition, neurodegenerative, metabolic, toxic and lesional nervous system disorders will also directly benefit from the widespread effect of the treatment on the cytoskeleton and the consequent beneficial effect on the connectivity of nervous cells, as suggested by the results obtained in the 6-OHDA model of unilateral lesion. These conditions include progressive supranuclear palsy (PSP); multiple system atrophy (MSA); vascular parkinsonism; parkinsonism caused by Alzheimer's type pathology; familial syndromes associated with degeneration of substantia nigra; corticobasal atrophy; alpha synucleinopathies in general, which include striatonigral degeneration, olivopontocerebellar atrophy, Shy-Drager syndrome (SDS); dementia with Lewy bodies; Parkinson's disease.

The activators of the present invention may further be used to increase the performance of healthy subjects.

It is therefore specific object of the present invention a Rho GTPase activating bacterial toxin for use in the treatment of disorders of the CNS chosen from the group consisting of cognitive impairment and neurodegenerative diseases, wherein said Rho GTPase activating bacterial toxin is administered by topical ocular, nasal or enteral, such as oral, administration.

The Rho GTPase activating bacterial toxin that can be used according to the present invention are, for example, Cytotoxic Necrotizing Factor 1 (CNF1) and the Cytotoxic Necrotizing Factor 2 (CNF2), a molecule the sequence of which almost matches that of CNF1. The Rho GTPase activating bacterial toxin can be administered by a single administration, by a single weekly administration, by a single monthly administration or by a single administration every 2 or 3 months or even more sparsely, such as once in the life.

The cognitive impairment that can be treated according to the present invention are, for example, intellectual disabilities or dementias, such as Alzheimer's disease. The neurodegenerative diseases that can be treated according to the present invention are, for example, Parkinson's disease, progressive supranuclear palsy (PSP); multiple system atrophy (MSA); vascular parkinsonism; parkinsonism caused by Alzheimer's type pathology; familial syndromes associated with degeneration of substantia nigra, corticobasal atrophy; alpha synucleinopathies, including striatonigral degeneration, olivopontocerebellar atrophy, Shy-Drager syndrome (SDS); dementia with Lewy bodies; more in general, degenerative, genetic, inflammatory, metabolic, toxic, traumatic, neoplastic, convulsive, vascular disorders of the CNS.

The present invention concerns also a pharmaceutical composition comprising or consisting of at least one Rho GTPase activating bacterial toxin in association with one or more pharmaceutically acceptable excipients and/or adjuvants for use in treatment of disorders of the CNS chosen from the group consisting of cognitive impairment and neurodegenerative diseases, wherein said pharmaceutical composition is in a form suitable for topical ocular, nasal or enteral, such as oral, administration.

The Rho GTPase activating bacterial protein toxin can be chosen from the group consisting of Cytotoxic Necrotizing Factor 1 (CNF1) and Cytotoxic Necrotizing Factor 2 (CNF2).

The pharmaceutical composition of the invention can be administered by a single administration, by a single weekly administration, by a single monthly administration or by a single administration every 2 or 3 months or even more sparsely, such as once in the life.

The cognitive impairment that can be treated according to the present invention are, for example, intellectual disabilities or dementias, such as Alzheimer's disease. The neurodegenerative diseases that can be

treated according to the present invention are, for example, Parkinson's disease, progressive supranuclear palsy (PSP); multiple system atrophy (MSA); vascular parkinsonism; parkinsonism caused by Alzheimer's type pathology; familial syndromes associated with degeneration of substantia nigra, corticobasal atrophy; alpha synucleinopathies, which include striatonigral degeneration, olivopontocerebellar atrophy, Shy-Drager syndrome (SDS); dementia with Lewy bodies, and, more in general, degenerative, genetic, inflammatory, metabolic, toxic, traumatic, neoplastic, convulsive, vascular disorders of the CNS.

The pharmaceutical composition can further comprise at least one CNS drug different from Rho GTPase activating bacterial toxins, molecules able to facilitate penetration of drugs in the CNS and increase bioavailability or molecules that selectively modulate Rho GTPase signaling. For instance, the above mentioned CNS drug can be chosen from the group consisting of antidepressant drugs or cognitive enhancers. Molecules able to facilitate penetration of drugs in the CNS and increase bioavailability are for example adjuvant peptides; whereas molecules which selectively modulate Rho GTPase signaling can be chosen from the group consisting of selective inhibitors of Rho kinase, such as Fasudil and Y-27632.

The present invention concerns also a combination of at least one Rho GTPase activating bacterial toxin with at least one CNS drug different from Rho GTPase activating bacterial toxins, molecules able to facilitate penetration of drugs in the CNS or molecules which selectively modulate Rho GTPase signaling, for the separate or sequential use in the treatment of disorders of the CNS chosen from the group consisting of cognitive impairment and neurodegenerative diseases, wherein said Rho GTPase activating bacterial toxin is administered by topical ocular, nasal or enteral, such as oral, administration.

“Separate use” is understood as meaning the administration, at the same time, of the two compounds of the composition according to the invention in distinct pharmaceutical forms.

“Sequential use” is understood as meaning the successive
5 administration of the two compounds of the composition according to the invention, each in a distinct pharmaceutical form.

As mentioned above, said at least one CNS drug can be chosen from the group consisting of antidepressants, cognitive enhancers; molecules able to facilitate penetration of drugs in the CNS and increase
10 bioavailability are for example adjuvant peptides; whereas molecules which selectively modulate Rho GTPase signaling can be chosen from the group consisting of selective inhibitors of Rho kinase, such as Fasudil and Y-27632

According to the present invention, Rho GTPase activating
15 bacterial toxin can be chosen from the group consisting of Cytotoxic Necrotizing Factor 1 (CNF1) and Cytotoxic Necrotizing Factor 2 (CNF2).

Rho GTPase activating bacterial toxin can be administered by a single administration, by a single weekly administration, by a single monthly administration, by a single administration every 2 or 3 months or
20 more sparsely, such as once in the life.

As mentioned above, cognitive impairments that can be treated according to the present invention are, for example, intellectual disabilities or dementias, such as Alzheimer's disease. The neurodegenerative diseases that can be treated according to the present invention are, for
25 example, Parkinson's disease, progressive supranuclear palsy (PSP); multiple system atrophy (MSA); vascular parkinsonism; parkinsonism caused by Alzheimer's type pathology; familial syndromes associated with degeneration of substantia nigra, corticobasal atrophy; alpha synucleinopathies, which include striatonigral degeneration,
30 olivopontocerebellar atrophy, Shy-Drager syndrome (SDS); dementia with

Lewy bodies; more in general, degenerative, genetic, inflammatory, metabolic, toxic, traumatic, neoplastic, convulsive, vascular disorders of the CNS.

The present invention will be now described by illustrative but not
5 limitative way according to preferred embodiment thereof with particular reference to the enclosed drawings, wherein:

Figure 1 shows the effects of CNF1 on object recognition. The bar graphs represent the summary of discrimination indexes (DIs). Test solutions were administered 11 days before the task. Group A: two groups of mice were injected 100 fmol/kg or 30 pmol/kg CNF1 i.c.v. (3.3 µl final
10 volume, n = 4 in each group); two groups of mice were injected 3.3 µl vehicle i.c.v. followed by the administration of a) 15 pmol/kg CNF1 in 5 µl in each eye (n = 5) or 5 µl vehicle in each eye (control, n = 5). Group B: 4 groups of mice were administered a) 15 fmol/kg CNF1 in 5 µl in each eye
15 (n = 5) b) 15 fmol/kg CNF1 in 5 µl in each naris (n = 5) c) 5 µl vehicle in each eye (control, n = 4) d) 30 fmol/kg CNF1 in 1 ml by gavage (n = 4). Vehicle was 20 mM TRIS-HCl buffer, pH 7.5. Twenty-four hour after the exploration of two identical objects (*sampling, left column*), one familiar object (F) was replaced with a novel one (N, *test, center column*). The
20 novel object was substituted with a novel one (N2) and the test session was repeated 6 days later (*re-test, left column*). Top: schematic representation of object placement during the task. Data are mean ± SEM.

Figure 2 shows the effects of CNF1 on working memory for object recognition: the bar graphs represent the summary of discrimination
25 indexes (DI). Test solutions were administered 17 days before the task. Group A: two groups of mice were injected 100 fmol/kg or 30 pmol/kg CNF1 i.c.v. (3.3 µl final volume, n = 4 in each group); two groups of mice were injected 3.3 µl vehicle i.c.v. followed by the administration of a) 15 pmol/kg CNF1 in 5 µl in each eye (n = 5) or 5 µl vehicle in each eye
30 (control, n = 5). Group B: 4 groups of mice were administered a) 15 fmol/kg

CNF1 in 5 μ l in each eye (n =5) b) 15 fmol/kg CNF1 in 5 μ l in each naris (n = 5) c) 5 μ l vehicle in each eye (control, n = 4) d) 30 fmol/kg CNF1 in 1 ml vehicle by gavage (n = 4). Vehicle was 20 mM TRIS–HCl buffer, pH 7.5. Sixty minutes after the exploration of two identical objects (*sampling, left column*), one familiar object (F) was replaced with a novel one (N, *test, right column*). Top: schematic representation of object placement during the task. Data are mean $\pm$ SEM.

Figure 3 shows the effects of CNF1 on place (left) and reversal (right) learning in a water maze task. Test solutions were administered 24 days before the task. Group A: two groups of mice were injected 100 fmol/kg (n = 8) or 30 pmol/kg (n = 9) CNF1 i.c.v. (3.3 μ l final volume); three groups of mice were injected 3.3 μ l vehicle i.c.v. followed by the administration of a) 15 pmol/kg CNF1 in 5 μ l in each eye (n =5) b) 15 pmol/kg CNF1 in 5 μ l in each naris (n = 5) or c) 5 μ l vehicle in each eye (control, n = 6). Group B: 4 groups of mice were administered a) 15 fmol/kg CNF1 in 5 μ l in each eye (n =5) b) 15 fmol/kg CNF1 in 5 μ l in each naris (n = 5) c) 5 μ l vehicle in each eye (control, n = 5) d) 30 fmol/kg CNF1 in 1 ml vehicle by gavage (n = 5). Vehicle was 20 mM TRIS–HCl buffer, pH 7.5. Place learning consisted of one daily session of 3 consecutive trials in 5 consecutive days with the platform in a fixed position. On day 8 from the beginning of the test, after moving the platform to a different position, reversal learning was studied in one session of 3 consecutive trials. Data are mean $\pm$ SEM.

Figure 4 shows the effects of CNF1 in a 6-OHDA mouse model of Parkinson's disease. Animals lesioned with 6-OHDA (2 mg/ml of saline containing 0.2% ascorbic acid into the left striatum; 3 μ l) were treated with 30 fmol/kg CNF1 by either nasal or ocular drops or vehicle by nasal drops (control) 14 weeks after 6-OHDA lesion. Mean $\pm$ s.e.m.; n = 8 in each group.

Figure 5 shows that CNF1 causes hypertrophy of the mouse

substantia nigra. Animals lesioned with 6-OHDA (2 mg/ml of saline containing 0.2% ascorbic acid into the left striatum; 3 μ l) were treated with 30 fmol/kg CNF1 or vehicle (control) by nasal drops 14 weeks after 6-OHDA lesion. Photomicrographs (40x) of TH-ir fibers in the substantia nigra *pars reticulata* of the 6-OHDA-lesioned or intact hemisphere in control and CNF1-treated mice .

Example 1: *Study on new methods of administration of Cytotoxic Necrotizing Factor 1 and other Rho GTPase activating toxins for medical use*

1. Materials and Methods

1.1. Molecules

Expression of recombinant CNF1 was carried out in *E. coli*. The constructs had T7 promoter, His-SUMO tag, SUMO protease. C-terminally His-tagged proteins were purified under native conditions by Ni²⁺ nitrilotriacetic acid affinity chromatography. The His-tag was removed by SUMO protease digestion and tag-free proteins were separated by Ni²⁺ nitrilotriacetic acid chromatography.

The biological activity of the molecules was tested in cultured HeLa cells after 48 h incubation by detection of multinucleation and cell enlargement.

1.2. Animals

The experiments were carried out on CD-1 and C57Bl6j mice (Charles River, Italy) aged 9 weeks at the time of CNF1 administration. The use and care of the animals followed the European Communities Council Directive of 24 November 1986 (86/609/EEC). The mice were housed at 21 $\pm$ 1 °C at constant humidity (55%) and in a 12/12 h dark–light cycle, with light phase from 08:00 to 20:00. They were divided into three groups. In a first group of CD1 mice (group A), the effects of intranasal and ocular administration of CNF1 were compared with those following i.c.v. administration. Since the results of group A showed effects of the protein

by all three routes of administration, the second group of CD1 mice (group B) was treated by intraocular, intranasal and gavage administration at doses of three orders of magnitude lower. The third group (group C, C57Bl6j mice) underwent ocular and intranasal CNF1 administration 14 weeks after unilateral 6-OHDA lesion of the left striatum.

1.3. *Molecule administration*

1.3.1. *CNF1 administration*

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

Mice that underwent i.c.v. administration were randomly assigned to the following 5 groups of treatment: CNF1 100 pmol/Kg or 30 pmol/Kg (n = 9 each); vehicle (20 mM TRIS-HCl buffer, pH 7.5; three groups). The first vehicle-treated group (n = 7) served as control. The second vehicle-injected group (n = 5) was treated during the surgical session using an Eppendorf automatic pipette with 15 pmol/Kg of CNF1 in 5 μ L in each eye. The third vehicle-injected group (n = 5) was treated during the surgical session using an Eppendorf automatic pipette with 15 pmol/Kg of CNF1 in 5 μ L in each naris and used for water maze experiments only.

Group B, which was not i.c.v. injected, was treated with 30 fmol/kg CNF1 intraocular, intranasal or by gavage (n = 5 in each group of treatment; for gavage administration CNF1 was dissolved in 1 ml Tris-HCl buffer, pH 7.5).

Group C was treated with 30 fmol/kg CNF1 intraocular or intranasal or vehicle intranasal (control; n = 8 in each group of treatment) 14 weeks after unilateral 6-OHDA lesion of the left striatum.

1.3.2 6-OHDA injection

Under general anaesthesia (sodium pentobarbital, 50 mg/kg intraperitoneally, ip), the mice were injected with a 27G needle mounted on a 25 µL Hamilton microsyringe using a stereotaxic apparatus (Kopf, Stoelting) and following coordinates from bregma and skull bone [17]. Five min post-injection, the needle was removed and the surgical wound sutured.

Six-OHDA (3 µl of 2 mg/ml solution in saline containing 0.2% ascorbic acid) was injected into the left striatum (A/P = +0.4, L/M = +1.8, D/V = -3.5 mm) at the rate of 1 µl/min.

1.4. Behavior

1.4.1. Object Recognition

1.4.1.1. Experimental apparatus

The arena for Object Recognition was a plastic box (42 x 26 x 16 cm, l x w x h), positioned in a silent room at constant, dim light. 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.

1.4.1.2. Experimental procedure

All experiments were performed in the light phase of the day (h 10.00 to 15.00). One day before object recognition (OR; day 10 post treatment), the mice were placed in the arena in a predefined starting point and were allowed to explore the apparatus for a 10 min habituation session.

For OR, 2 objects were placed at the center of 2 arena sectors (Fig. 1 and 2). At the beginning of each session, the mouse was placed at the center of the arena and allowed to explore for 10 min. Each event during which the animal 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. At the end of each session, the objects were carefully cleaned with 70% ethanol to remove olfactory traces.

The first experiment consisted of 3 sessions: during *sampling* (day 11 post treatment), the animals were exposed to 2 identical objects ($\square$; Fig. 1). On the subsequent day, one of the 2 familiar objects was substituted with a novel one ($\circ$, *test*, Fig. 1). In the *re-test*, performed 19 days post injection, the novel object was substituted by a novel one (Δ , Fig. 1) and the mice were allowed to explore again the set of objects.

The second experiment (working memory test) was carried out 21 days post treatment. A 60 min interval elapsed between *sampling* and *test* session and no subsequent testing was performed. The objects used in the working memory test ($\diamond$ and $\triangle$, Fig. 2) were novel to the mice, i.e. they had not been used during the previous OR.

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 experiment was done in order to select objects the mice showed similar degrees of preference for, i.e. objects that minimized the variance of exploration times.

Results of OR experiments are reported in Figure 1 and 2 as discrimination indexes (DIs), calculated as ("time exploring right object" – "time exploring left object")/("time exploring right object" + "time exploring left object").

1.4.2. Water Maze

1.4.2.1. Experimental apparatus

To explore the effects of CNF1 on spatial learning, we trained the

mice to find a hidden platform in a water maze paradigm. The maze was a circular pool (PVC, 80 cm in diameter, 31 cm high walls) positioned in a silent room at constant, dim light. The pool was surrounded by several objects that served as external cues, including an anti-vibration table (84 cm × 90 cm × 75 cm), a bookshelf (74 cm × 90 cm × 44 cm) containing colored books, a lab cabinet (240 cm × 120 cm × 50 cm), a painting (33 cm × 45 cm). It was arbitrarily divided in four equal quadrants and filled with water at 22 ± 2 °C up to the height of 21 cm. Water was made opaque by addition of milk. In one quadrant, at the center of the line from the pool wall to the pool center, a water-filled cylinder (Plexiglas, 8 cm in diameter, 20 cm high) was placed. The cylinder's upper surface, which had been made rough to facilitate climbing, was 1 cm under water and provided a platform on which the mice could climb to escape from water during the experiments. In these conditions, the platform was invisible to the animals.

1.4.2.2. *Experimental procedure*

The platform was held in a fixed position during the entire place learning. The mice were trained to learn the position in 3 consecutive daily trials. Altogether, the mice underwent 15 learning trials over 5 consecutive days. At the beginning of each trial, the animal was placed in the water with the head facing the pool wall. The starting point varied across trials and days according to a standard pseudorandom sequence. The mice were left in the water until they reached the invisible platform and climbed on it; then they were left on the platform for a 10 s reinforcement time. If the mice had not found the platform within 90 s (cut-off time), the experimenter placed them on it.

On days 9, the mice were trained to learn a novel platform position in a different quadrant (reversal learning) in 3 consecutive trials, which were run as described for place learning. Twenty-four hours later (day 10), the mice were trained to find a platform that had been made visible with a flag placed on it. The training consisted of four trials (intertrial time = 30

min). During this cued learning, the platform position and the starting point were changed every trial according to a pseudorandom sequence. This experiment was performed to test the animals' sensory-motor ability and motivation to reach the platform.

1.4.3. Behavioral analysis of 6-OHDA lesioned mice

The experiments were carried out in a silent, dimly lit testing room at 23 ± 6 °C. Six mice were tested in each session. Each animal was placed in a glass cylinder (27 cm height, 16 cm diameter). The animals were made invisible each other by placing sheets of black paper among the cylinders. The experiments were acquired by a video camera connected to a personal computer. Animals' movements were tracked and analyzed with dedicated software (ANY-maze™, Stoelting).

Four weeks post-lesion and 4 weeks post-treatment, the animals were injected amphetamine sulfate (AM, 2.5 mg/kg ip in saline) and immediately placed in the cylinders. The animals' turning behavior was analyzed for 60 min. Rotations were considered for the analysis only if the animal had completed a 360° turn. Only mice that had exhibited an average > 2.0 rotations/min ipsiversive to the lesion during the first AM test underwent treatment. Before treatment, the mice were ranked for ipsiversive rotations and stratified to obtain homogeneous groups (CNF1-treated groups: 3.910 ± 0.291 , n = 8; control group 3.612 ± 0.238 , n = 8; rotations/min, mean $\pm$ s.e.m.).

1.5. Brain sections and histology

Perfusion, tissue processing [18] and immunohistochemistry [19] were performed as previously described in mice selected from Group C, in particular in those treated with intranasal drops. Sections were immunostained with primary rabbit polyclonal antibody to TH - neuronal marker (Abcam, 1:1000 in 0.2% Triton X-100 and 5% horse serum in PBS). Images were acquired using a BX60 Olympus microscope equipped with a Spot Insight Color microscope camera (Diagnostic Instruments).

1.6. Statistical analysis

Data were analyzed by analysis of variance (ANOVA) and analysis of covariance (ANCOVA). Bonferroni's correction was used for post hoc individual comparisons. Mice treated with intranasal drops were not included in the analysis in consideration of their low number. Object exploration times were the averages of measurements carried out by two independent observers. Inter-rater reliability in time measurements was evaluated by linear regression analysis. All calculations were performed with Statistica 5.0 for Windows.

2. Results

2.1. CNF1 improves object recognition

The mice did not show any behavioral or physical abnormality until the end of the testing and no death occurred. Weight increase, food and water consumption were not significantly different among the groups. Before the placement in the experimental arena, the groups treated with CNF1 showed reduced anxiety. The effect was less conspicuous in the group treated i.c.v.

Object exploration times in the sampling session were analyzed by ANOVA for repeated measurements, in which "treatment" (levels: "vehicle", "CNF1 100 fmol/kg i.c.v.", "CNF1 30 pmol/kg i.c.v." and "CNF1 30 pmol/kg eye drops" in group A; "vehicle", "CNF1 30 pmol/kg eye drops", "CNF1 30 pmol/kg nasal drops" and "CNF1 30 pmol/kg gavage" in group B) was "between-subjects" factor and objects placed in the arena were "within-subjects" factors (levels: "left object" and "right object"). The mice showed similar levels of exploration for the two objects during the training session, thus indicating a lack in preference for object position. However, some treated groups showed an overall increase in object exploration ($F_{3,14} = 17.668$, $p < 0.0001$; $p < 0.005$ vs vehicle for ocular administration in group A; $F_{3,15} = 6.723$, $p = 0.0043$; $p < 0.05$ vs vehicle for gavage administration in group B; Fig. 1), suggesting a reduced level of anxiety.

In order to evaluate the effect of the treatment on the preference of the novel object relative to the familiar one during the two sessions of the test, exploration times of the novel object were analyzed by ANCOVA. Exploration times of the familiar object were used as covariates. In the 24 h retention test, CNF1 increased relative exploration time ($F_{3,13} = 4.922$, $p = 0.0169$ in group A, in which individual comparisons did not reach the significance after correction; $F_{3,15} = 12.385$, $p = 0.0002$ in group B, $p < 0.0001$, $p = 0.0074$ and $p < 0.0001$ vs control for gavage, nasal and ocular administration, respectively; Fig. 1). A substantial increase in the overall object exploration was observed in the treated groups. Seven days post sampling, the difference in novel object preference among the groups was still noticeable, but it did not reach the statistical significance in group A ($F_{3,13} = 2.323$, $p = 0.1228$; $F_{3,15} = 6.532$, $p = 0.0048$ in group B, $p < 0.001$, $p < 0.05$ and $p < 0.05$ vs control for gavage, nasal and ocular administration, respectively; Fig. 1). The lack of significance in Group A is possibly explained by reduced exploration of the familiar object in the vehicle-treated group.

On the whole, the data of OR task indicate an improvement in long-term object memory in mice treated with CNF1. The effect tended to decline in the 7-days *re-test*.

The effects of the treatments in the working memory test were then analyzed. In the sampling, the four groups did not show significant differences in the exploration of the two objects. Again, the overall time exploring was significantly different in the treatment groups, with a tendency to increase in the "eye drop" group ($F_{3,14} = 3.581$, $p = 0.0414$ in group A; $F_{3,16} = 1.746$, $p = 0.1980$ in group B; Fig. 2). After one hour, the mice were tested again after replacing one of the two familiar objects with a novel one. Times of exploration of the familiar object were used as covariates. The analysis revealed an effect of treatment that just approaches the significance in Group A ($F_{3,13} = 2.768$, $p = 0.0839$; $F_{3,15} =$

1.987, $p = 0.1592$ in group B; Fig. 2). The results show that CNF1 improves long-term memory. More experiments are needed to unfold the effects of the treatments on working memory.

The correlation coefficient (r) of the measurements performed by the two observers during the OR sessions was 0.92 (slope = 1.01), indicating an acceptable inter-rater reliability.

2.2. CNF1 enhances spatial memory

The escape latencies to reach the platform were analyzed by repeated measurement ANOVA. In the analysis, two ways were assigned to “within-subject” factors (session, levels “1” to “5” and trial, levels “1” to “3”). The escape latency decreased across training, indicating that the mice learned the platform position (effect of session, Group A: $F_{4,112} = 27.387$, $p < 0.0001$; Group B: $F_{4,64} = 25.003$, $p < 0.0001$; Figure 3 left). The mice learned the platform position within each learning session (effect of trial, Group A: $F_{2,56} = 10.191$, $p = 0.0002$; Group B: $F_{2,32} = 17.255$, $p < 0.0001$). The effect of treatment was significant only in group B ($F_{3,16} = 3.311$, $p = 0.0470$, CNF1 gavage significantly different from vehicle, $p < 0.05$ by individual ANOVA with Bonferroni’s correction, other individual comparisons only approached the significativity after correction), whereas its interaction with both session and trial were not significant. The interaction trial x session and trial x session x treatment were not significant, as well. It can be concluded that CNF1 by gavage improves place learning, whereas the effects of the molecule by other routes of administration just approach the significativity.

The results of the reversal learning were analyzed as for place learning, except that the “session” factor had only one level. The analysis of the escape latencies suggests that CNF1 enhances the ability to find the platform (Group A: effect of treatment: $F_{4,28} = 2.844$, $p = 0.0426$; interaction treatment x trial: $F_{8,56} = 2.851$, $p = 0.0099$; CNF1 eye drop and CNF1 30 pmol/kg i.c.v. significantly different from vehicle, $p < 0.05$, CNF1

100 fmol/kg i.c.v. significantly different from control, $p < 0.01$ by individual ANOVA with Bonferroni's correction; Group B: $F_{3,16} = 1.451$, $p = 0.2653$; interaction treatment x trial: $F_{6,32} = 3.882$, $p = 0.0050$; all individual treatment x trial significantly different from vehicle, $p < 0.05$ by individual ANOVA with Bonferroni's correction; Fig. 3 right). No other effects resulted from the ANOVA on reversal data. The results of the reversal learning suggest an effect of CNF1 either on spatial learning and/or on working memory (the 3 trials were strictly consecutive). No differences among groups were observed in the cued learning experiment. The follow up of treated animals revealed that they were in good conditions for months after the administration. However, CNF1-treated animals looked in better conditions, and this effect was more marked in the "eye drop" group.

2.3 CNF1 corrects asymmetries and restore the trophism of substantia nigra DA neurons in the 6-OHDA model of parkinsonism

The mice were treated with vehicle (control) or CNF1 (either nasal or eye drops) 14 weeks after 6-OHDA injection. They were tested for AM-induced turning 4 and 18 weeks after the lesion. The data were analyzed by ANOVA for repeated measurements, where "treatment" ("eye drops" "nasal drops" and "control") was "between-subjects" factor and "time of AM challenge" (levels: "basal", "18 weeks") was "within-subject" factor. The number of rotations was reduced in CNF1-treated groups ($F_{2,21} = 5.120$, $p = 0.0155$; treatment with AM: $F_{1,21} = 94.804$, $p < 0.0001$; treatment x time of AM: $F_{2,21} = 16.905$, $p < 0.0001$; Figure 4). We concluded that both intraocular and intranasal CNF1 effectively corrected the motor asymmetry caused by nigrostriatal lesion.

Hypertrophic changes of DA neurons were observed in the substantia nigra of representative 6-OHDA lesioned mice in both hemispheres. The TH-ir fibers showed increased varicosity, length and thickness (Figure 5). The area occupied by TH-ir tissue was substantially increased by the treatment. The thickness of TH-ir fibers was increased by

CNF1, as well. Overall, CNF1 induced hypertrophy of DA cells in both hemispheres, and corrected the effects of 6-OHDA injection on the TH-ir tissue.

3. Discussion

5 The activation of cerebral Rho GTPases by CNF1 is followed by enrichment in the dendritic tree spines/arborization both “in vitro” [9] and “in vivo” [12]. Behavioral studies in wild type [9, 20] and genetically modified mice modeling brain disorders [13, 14] showed the cognition enhancing effect of the molecule.

10 The present study confirms that CNF1 improves OR and spatial learning. The two dose levels used in i.c.v. administration do not display substantial differences in efficacy. This is consistent with the mechanism of action of the molecule: being an enzyme, its effects are already complete at very low doses. This lack of dose-response effect was already
15 reported in [9].

 Yet, and surprisingly, eye and nasal drop administration and gavage administration exhibit efficacy in both learning tasks. In addition, eye and nasal drop, as well as gavage administration of CNF1 shows better efficacy than i.c.v. administration. This increased efficacy can be
20 explained by several mechanisms. First, these routes of administration (i.e. ocular, intranasal, and gavage) might follow pathways that allow for a more widespread and more efficacious delivery of the molecule to the nervous tissue. In fact, molecules administered by i.c.v. injection have to overcome the barrier represented by the ependymal cells that upholster
25 the cerebral ventricles. Second, bilateral administration (as in the case of ocular and intranasal) might better distribute the protein to the two hemispheres as compared to unilateral i.c.v. administration. Since according to the present study, unilateral eye drop administration was not performed, this latter hypothesis cannot be confirmed or excluded. Against
30 it, it has been reported that the morphological effects of unilateral i.c.v.

administration are evenly distributed in the two hemispheres [12], a finding that seems to challenge this assumption. Third, i.c.v. injections are less reliable than local administration, such as those obtained by eye or nasal drops and by gavage. Indeed, in spite of the careful execution of the stereotaxic surgery, the molecule can not always be correctly injected into the lateral ventricle.

The performance in OR and water maze is sensitive to aging [21]. Object recognition is impaired in animal models of Alzheimer's disease [22] and ID, such as Down syndrome [23, 24] and Fragile X syndrome [25]. On the other hand, water maze performance is altered in mice affected by Alzheimer-type pathology [14] and intellectual disability [26]. In these models, either the derangement of Rho signaling [5, 6] or the alterations of the dendritic tree/spines [27-29] has been reported. Therefore, the results of the present study support the administration of CNF1 by eye drops, nasal spray/drops and enteral in the therapy of a number of neurological disorders, such as the above-mentioned.

While it has been suggested that CNF1 might be administered intrathecally or in the perispinal space by sporadic if not single injection, intraocular, intranasal and enteral administrations represent a more practical route. A number of neurological disorders with few therapeutic options at present time might be treated by simple local administration of CNF1. Based on the available evidence, it is conceivable a therapeutic use of eye drops, nasal drops or enteral CNF1 in conditions associated with the decline of cognitive functions, e.g. in dementia, Fragile X syndrome, Down syndrome and other forms of intellectual disability, neurodegenerative, cerebrovascular, metabolic, toxic, traumatic, inflammatory disorders. These routes of administration of the bacterial protein toxin might thus represent an entirely new tool for the treatment of these conditions.

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Tanghe A, et al. Reduced spine density in specific regions of CA1 pyramidal neurons in two transgenic mouse models of Alzheimer's disease. J Neurosci. 2011;31:3926-34.

CLAIMS

1) Rho GTPase activating bacterial toxin for use in treatment of disorders of the Central Nervous System chosen from the group consisting of cognitive impairment and neurodegenerative diseases, wherein said
5 Rho GTPase activating bacterial toxin is administered by topical ocular, nasal or enteral, such as oral, administration.

2) Rho GTPase activating bacterial toxin for use according to claim 1, wherein said Rho GTPase activating bacterial toxin is chosen from the group consisting of Cytotoxic Necrotizing Factor 1 (CNF1) and Cytotoxic
10 Necrotizing Factor 2 (CNF2).

3) Rho GTPase activating bacterial toxin for use according to anyone of the claims 1-2, wherein said Rho GTPase activating bacterial toxin is administered by a single administration, by a single weekly administration, by a single monthly administration, by a single administration every 2 or 3
15 months or more sparsely, such as once in the life.

4) Rho GTPase activating bacterial toxin for use according to anyone of the claims 1-3, wherein said cognitive impairment is chosen from the group consisting of intellectual disabilities or dementias, such as Alzheimer's disease.

5) Rho GTPase activating bacterial toxin for use according to anyone of the claims 1-3, wherein said neurodegenerative diseases are chosen from the group consisting of Parkinson's disease, progressive supranuclear palsy (PSP), multiple system atrophy (MSA), vascular parkinsonism, parkinsonism caused by Alzheimer's type pathology; familial
20 syndromes associated with degeneration of substantia nigra; corticobasal atrophy; alpha synucleinopathies, which include striatonigral degeneration, olivopontocerebellar atrophy, Shy-Drager syndrome; dementia with Lewy bodies.
25

6) Pharmaceutical composition comprising or consisting of at least one

Rho GTPase activating bacterial toxin in association with one or more pharmaceutically acceptable excipients and/or coadjuvants for use in treatment of disorders of the Central Nervous System chosen from the group consisting of cognitive impairment and neurodegenerative diseases, wherein said pharmaceutical composition is in a form suitable for topical ocular, nasal or enteral, such as oral, administration.

7) Pharmaceutical composition according to claim 6, for use according to claim 6, wherein said Rho GTPase activating bacterial toxin is chosen from the group consisting of Cytotoxic necrotizing Factor 1 (CNF1) and Cytotoxic Necrotizing Factor 2 (CNF2).

8) Pharmaceutical composition according to anyone of the claims 6-7, for use according to anyone of the claims 6-7, wherein said pharmaceutical composition is administered by a single administration, by a single weekly administration, by a single monthly administration, by a single administration every 2 or 3 months or more sparsely, such as once in the life.

9) Pharmaceutical composition according to anyone of the claims 6-8, for use according to anyone of the claims 6-8, wherein said cognitive impairment is chosen from the group consisting of intellectual disabilities or dementias, such as Alzheimer's disease.

10) Pharmaceutical composition according to anyone of the claims 6-8, for use according to anyone of the claims 6-8, wherein said neurodegenerative diseases are chosen from the group consisting of Parkinson's disease, progressive supranuclear palsy (PSP), multiple system atrophy (MSA), vascular parkinsonism, parkinsonism caused by Alzheimer's type pathology; familial syndromes associated with degeneration of substantia nigra; corticobasal atrophy; alpha synucleinopathies, which include striatonigral degeneration, olivopontocerebellar atrophy, Shy-Drager syndrome (SDS); dementia with

Lewy bodies.

11) Pharmaceutical composition according to anyone of the claims 6-10, for use according to anyone of the claims 6-10, wherein said pharmaceutical composition further comprises at least one CNS drug different from Rho GTPase activating bacterial toxins, molecules able to facilitate penetration of drugs in the CNS or molecules which selectively modulate Rho GTPase signaling.

12) Combination of at least one Rho GTPase activating bacterial toxin with at least one CNS drug different from Rho GTPase activating bacterial toxins, molecules able to facilitate penetration of drugs in the CNS or molecules which selectively modulate Rho GTPase signaling, for the separate or sequential use in treatment of disorders of the Central Nervous System chosen from the group consisting of cognitive impairment and neurodegenerative diseases, wherein said Rho GTPase activating bacterial toxin is administered by topical ocular, nasal or enteral, such as oral, administration.

13) Combination according to claim 12, for use according to claim 12, wherein said at least one CNS drug is chosen from the group consisting of antidepressants, cognitive enhancers.

14) Combination according to claim 12, for use according to claim 12, wherein said molecules able to facilitate penetration of drugs in the CNS are adjuvant peptides.

15) Combination according to claim 12, for use according to claim 12, wherein said molecules which selectively modulate Rho GTPase signaling are chosen from the group consisting of selective inhibitors of Rho kinase, such as Fasudil and Y-27632.

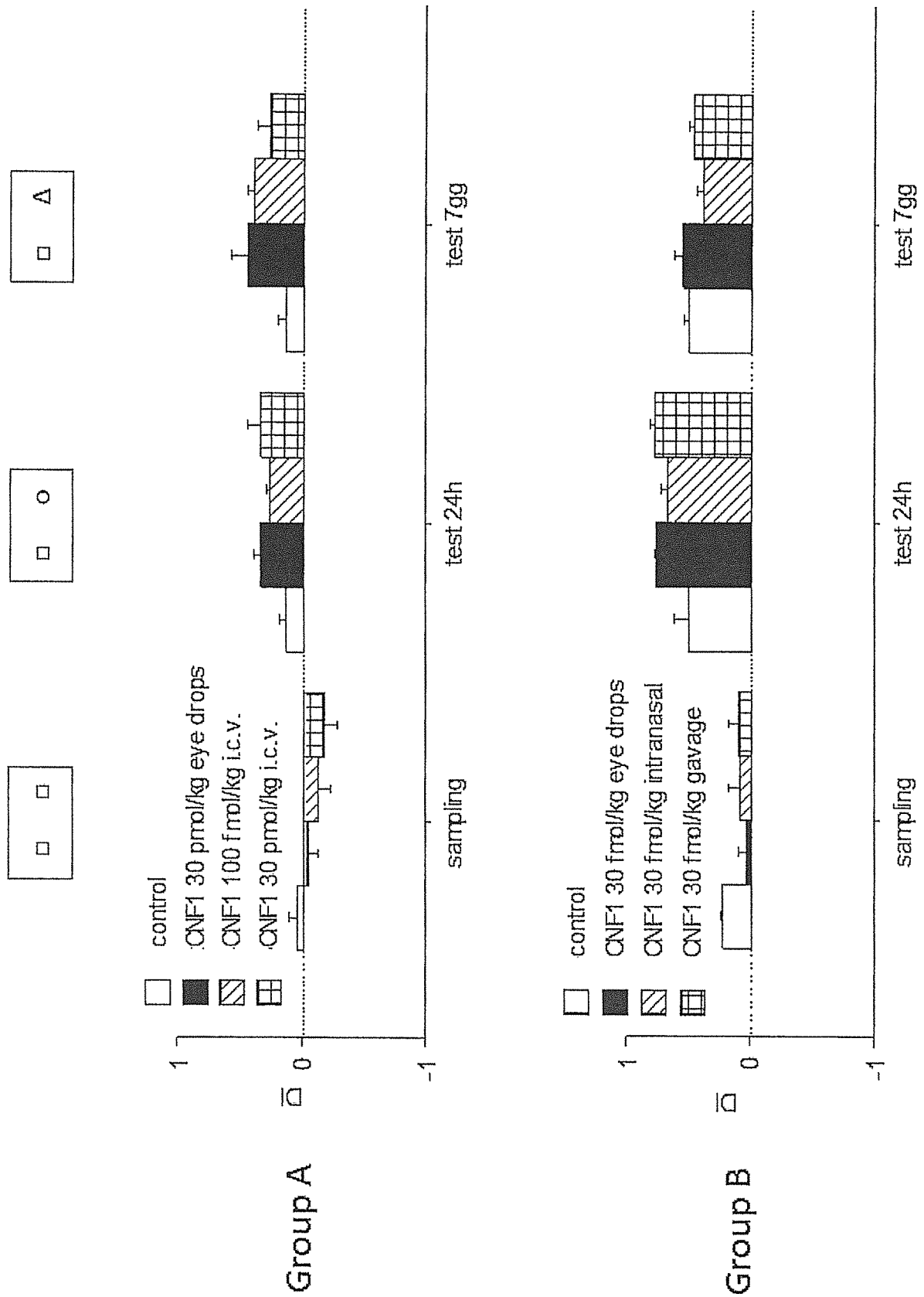
16) Combination according to anyone of the claims 12-15, for use according to anyone of the claims 12-15, wherein Rho GTPase activating bacterial toxin is chosen from the group consisting of Cytotoxic Necrotizing

Factor 1 (CNF1) and Cytotoxic Necrotizing Factor 2 (CNF2).

17) Combination according to anyone of the claims 12-16, for use according to anyone of the claims 12-16, wherein said Rho GTPase activating bacterial toxin is administered by a single administration, by a single weekly administration, by a single monthly administration, by a single administration every 2 or 3 months or more sparsely, such as once in the life.

18) Combination according to anyone of the claims 12-17, for use according to anyone of the claims 12-17, wherein said cognitive impairment is chosen from the group consisting of intellectual disabilities or dementias, such as Alzheimer's disease.

19) Combination according to anyone of the claims 12-17, for use according to anyone of the claims 12-17, wherein said neurodegenerative diseases are chosen from the group consisting of Parkinson's disease, progressive supranuclear palsy (PSP), multiple system atrophy (MSA), vascular parkinsonism, parkinsonism caused by Alzheimer's type pathology; familial syndromes associated with degeneration of substantia nigra; corticobasal atrophy; alpha synucleinopathies, which include striatonigral degeneration, olivopontocerebellar atrophy, Shy-Drager syndrome (SDS); dementia with Lewy bodies.



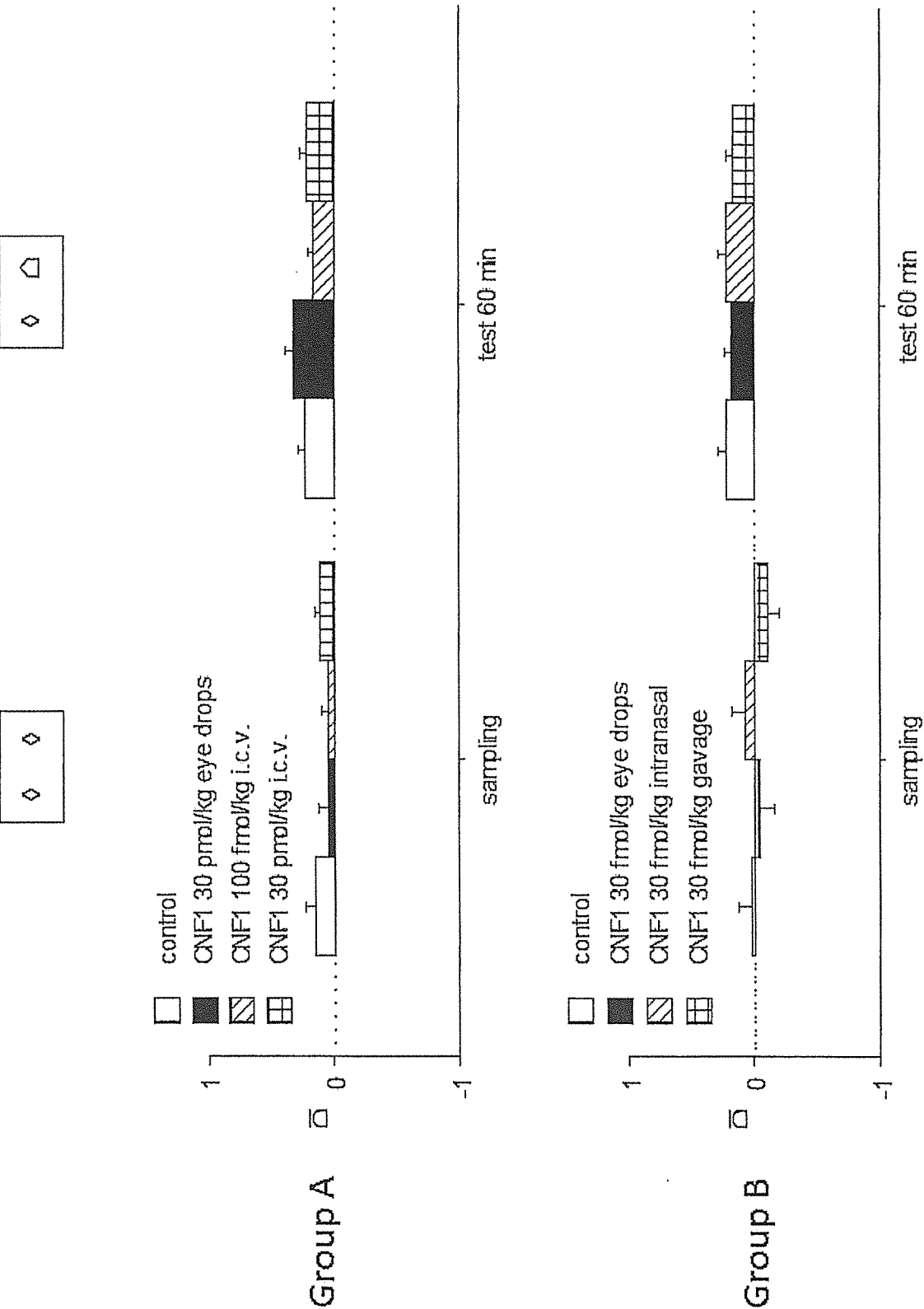


Fig. 2

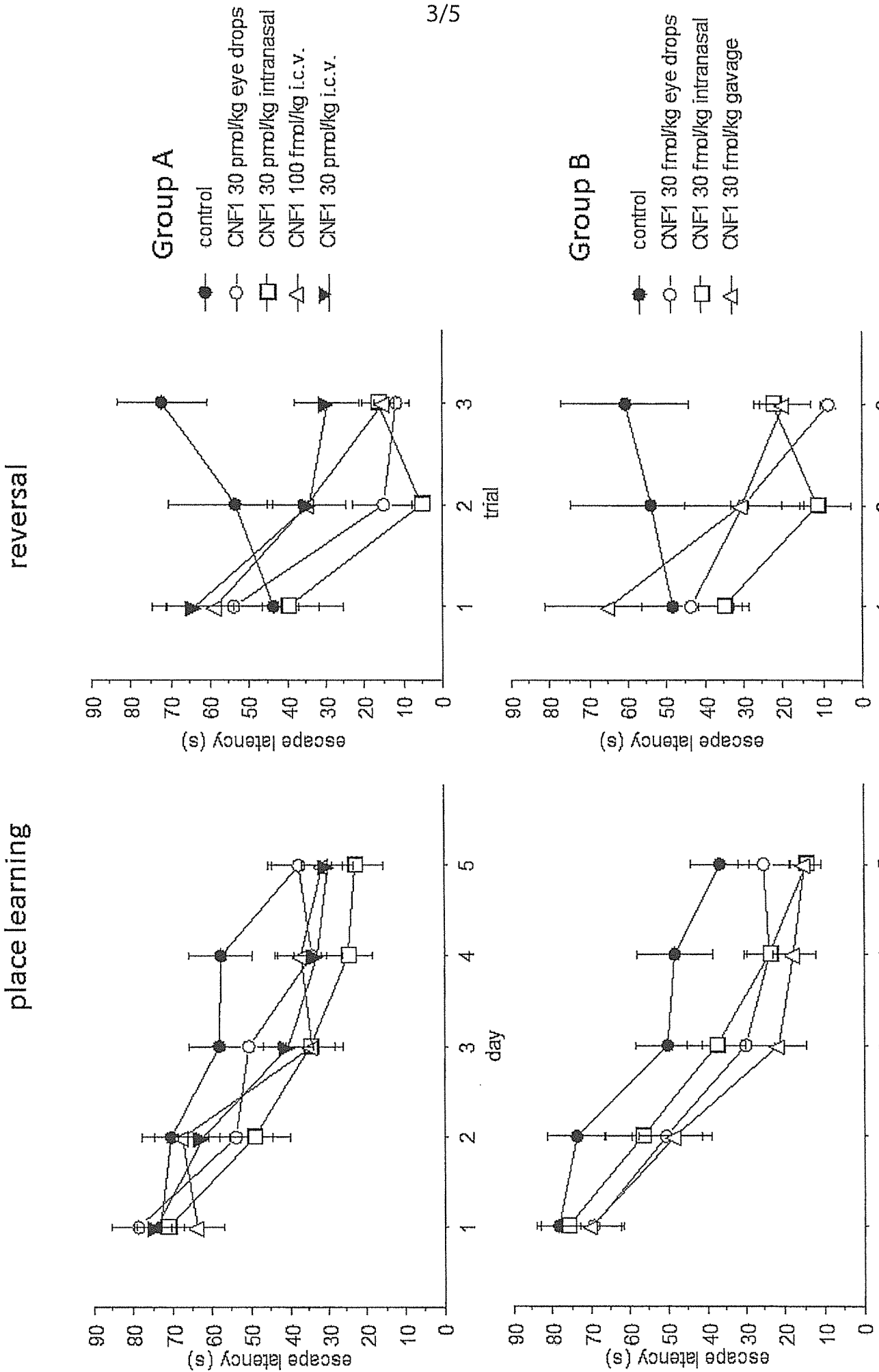


Fig. 3

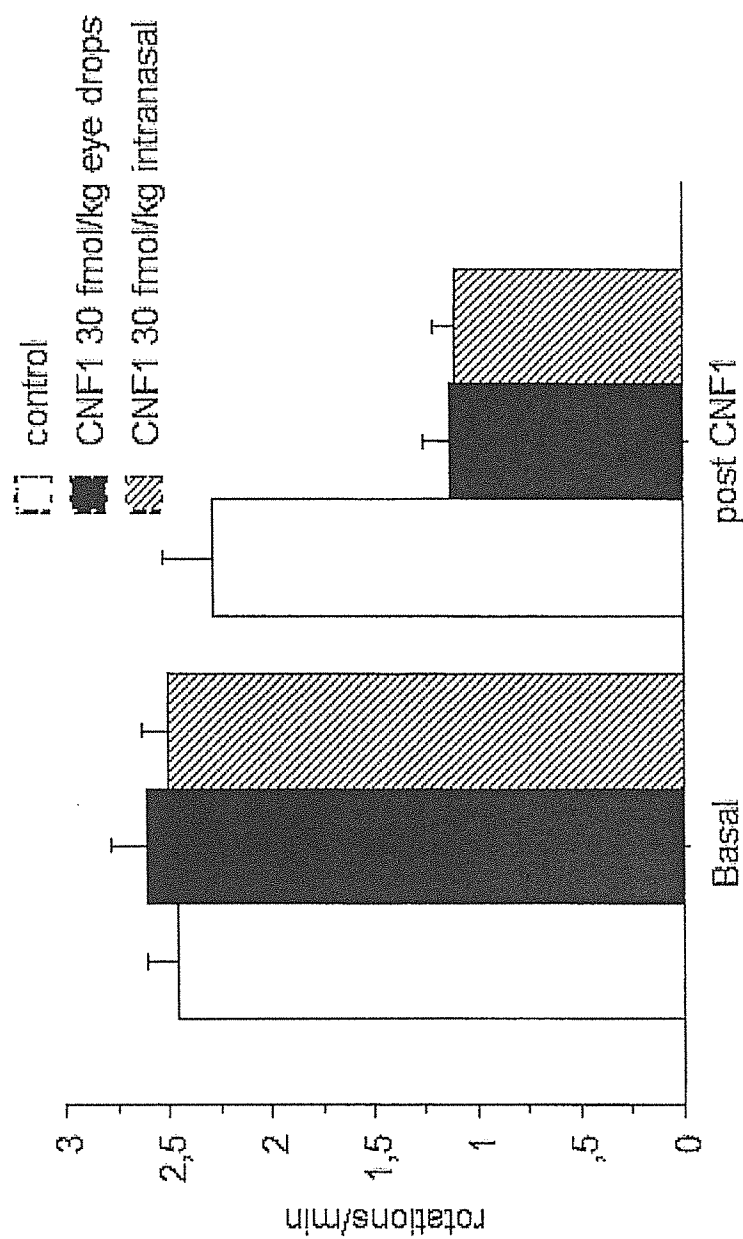


Fig. 4

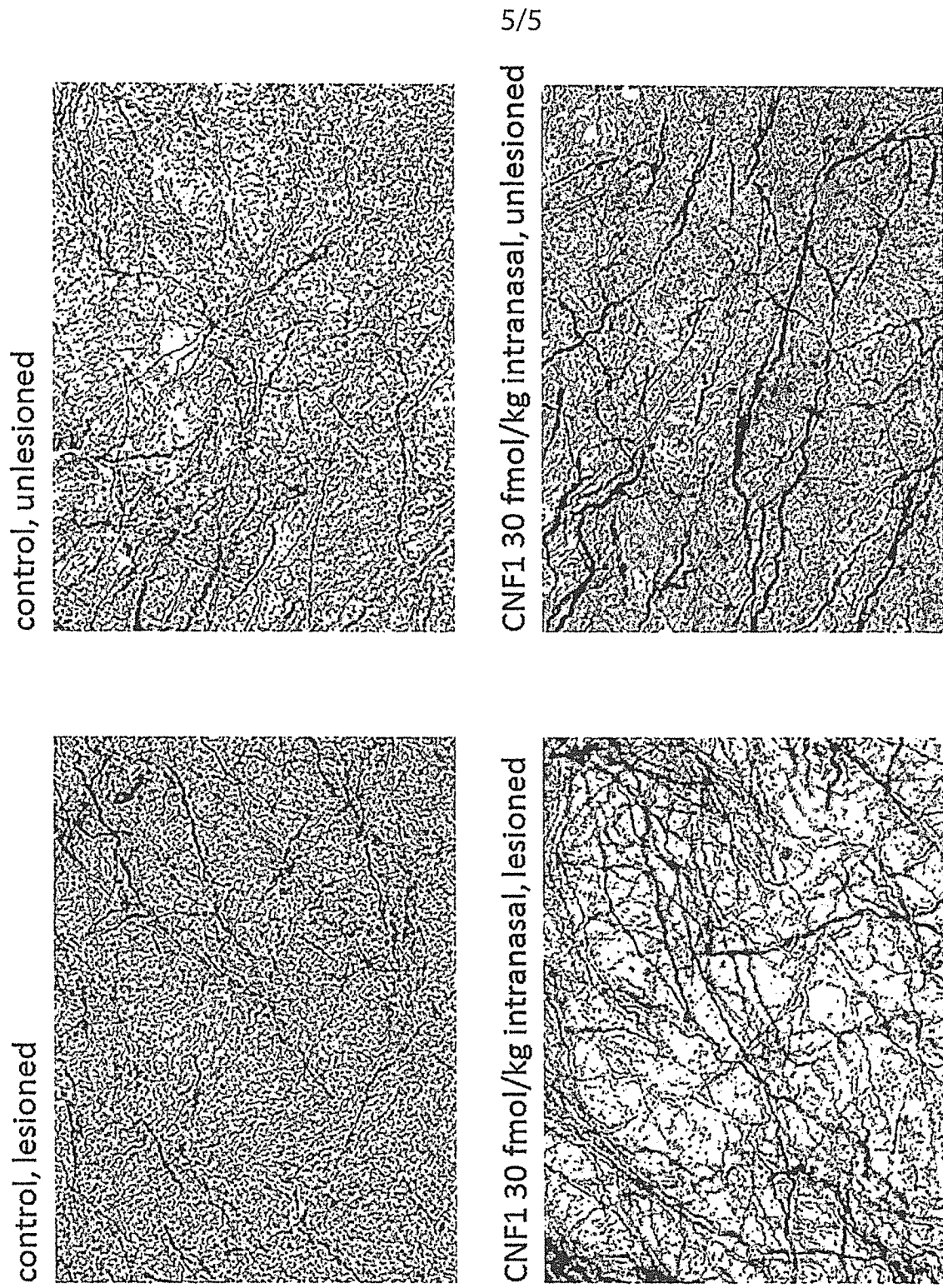


Fig. 5

INTERNATIONAL SEARCH REPORT

International application No

PCT/IT2016/000120

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K38/16 A61P25/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, INSPEC, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/105998 A2 (IST SUPERIORE SANITA [IT]; DIANA GIOVANNI [IT]; FIORENTINI CARLA [IT]) 12 October 2006 (2006-10-12)	1-10
Y	the whole document pages 8,9; claims	1-19
X	WO 2011/101881 A2 (IST SUPERIORE SANITA [IT]; FIORENTINI CARLA [IT]; LAVIOLA GIOVANNI [IT]) 25 August 2011 (2011-08-25)	1-4,6-9
Y	the whole document pages 3, 5; claims; examples	1-19
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Further documents are listed in the continuation of Box C.



See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

10 January 2017

Date of mailing of the international search report

31/01/2017

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INTERNATIONAL SEARCH REPORT

International application No

PCT/IT2016/000120

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nicholette Zeliadt: "Brain stimulation could ease memory problems in Rett syndrome", Spectrum News, 2 November 2015 (2015-11-02), XP55333205, Retrieved from the Internet: URL:https://spectrumnews.org/news/brain-stimulation-could-ease-memory-problems-in-re-tt-syndrome/ [retrieved on 2017-01-09] the whole document	1-19
Y	----- MUSILLI MARCO ET AL: "Behavioral effects of Rho GTPase modulation in a model of Alzheimer's disease", BEHAVIOURAL BRAIN RESEARCH, vol. 237, 28 September 2012 (2012-09-28), pages 223-229, XP028958323, ISSN: 0166-4328, DOI: 10.1016/J.BBR.2012.09.043 the whole document	1-19
Y	----- SARA TRAVAGLIONE ET AL: "The E. coli CNF1 as a Pioneering Therapy for the Central Nervous System Diseases", TOXINS, vol. 6, no. 1, 7 January 2014 (2014-01-07) , pages 270-282, XP055198804, DOI: 10.3390/toxins6010270 the whole document	1-13

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IT2016/000120

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2006105998 A2	12-10-2006	EP 1865979 A2 US 2011212895 A1 WO 2006105998 A2	19-12-2007 01-09-2011 12-10-2006
WO 2011101881 A2	25-08-2011	EP 2536424 A2 IT 1398191 B1 WO 2011101881 A2	26-12-2012 14-02-2013 25-08-2011