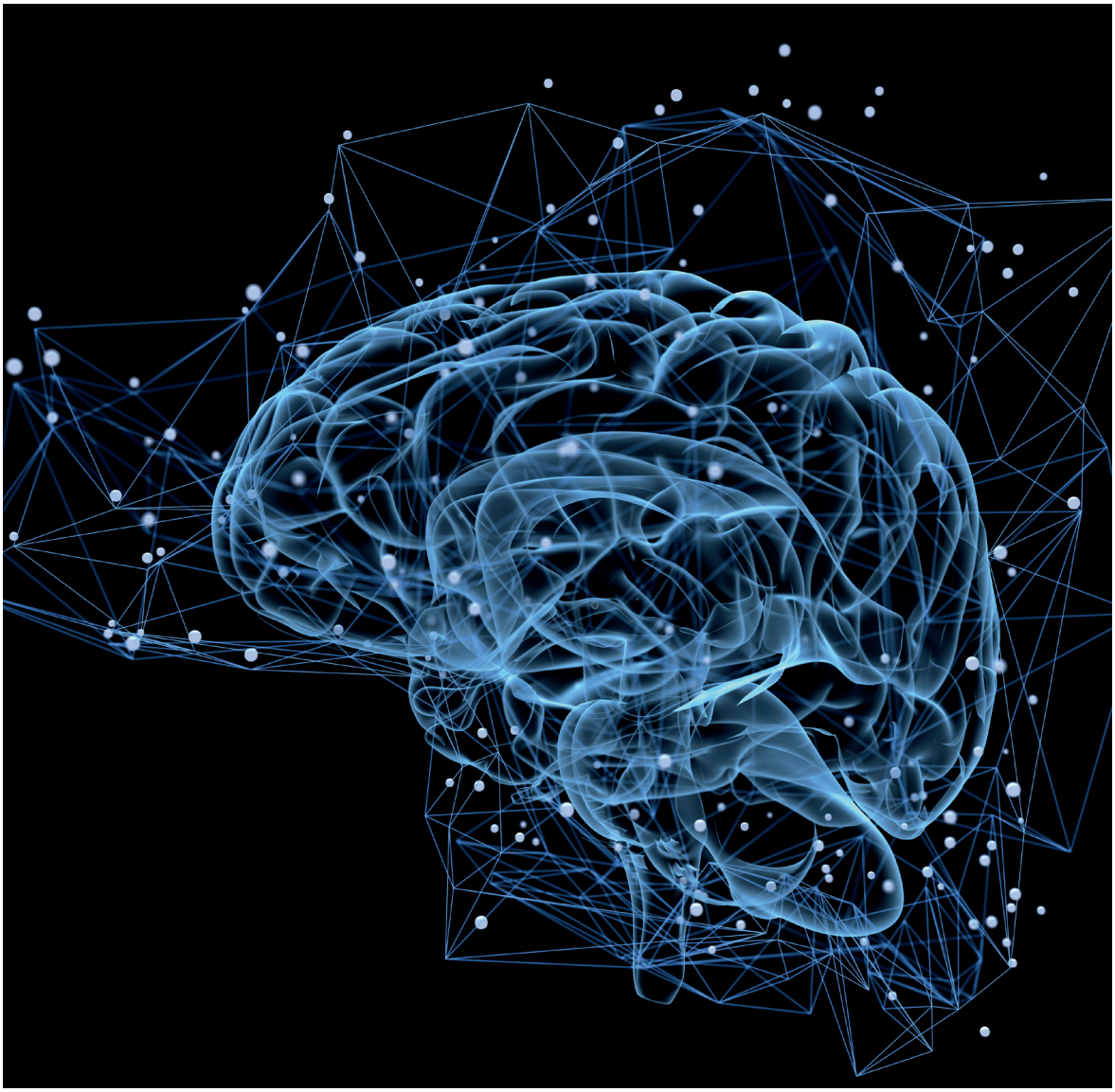
GUNA-**BDNF**

Research studies and clinical experiences on low-dose Brain-derived Neurotrophic Factor



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L. Milani

BDNF
BRAIN DERIVED NEUROTROPHIC FACTOR
– AN EXTRAORDINARY AND POWERFUL MASTER REGULATOR OF THE BRAIN

Time it was, and what a time it was,
it was a time of innocence, a time of confidences.
Long ago, it must be.
I have a photograph.
Preserve your memories, they're all that's left you.

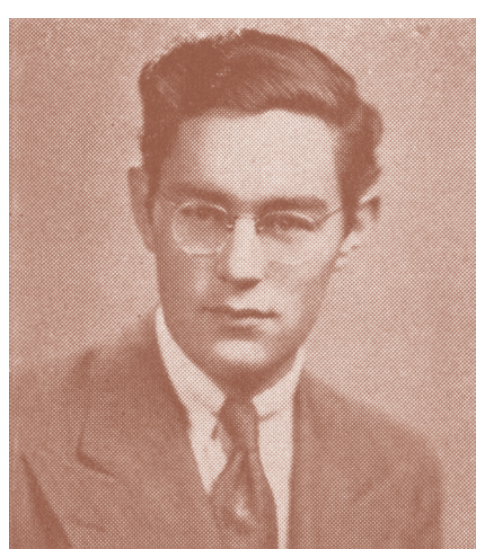
– Paul Simon & Art Garfunkel. Bookends Theme, Columbia Records, 1968.

THE INCREDIBLE CASE OF HENRY MOLAISON – THE AMNESIAC WE WILL NOT FORGET

In 1953, Henry G. Molaison, 27, from Manchester, Connecticut (USA) underwent a major bilateral anteromedial temporal lobectomy, with extensive resection of the anterior 2/3 of the hippocampi, parahippocampal cortices, entorhinal cortices, piriform cortices, and amygdalae (FIG. 1), in an attempt to stop his severe, worsening, drug-resistant, tonic-clonic seizures (15-20 per day), which had afflicted him since childhood and had probably been caused by a road accident at the age of 9, which had resulted in a fractured skull and brain concussion.

– I quote Dr William B. Scoville, the expert neurosurgeon who had operated on him:
"(…) *we performed a bilateral resection of the medial temporal lobes, advancing posteriorly for a distance of 8 cm from the central point of the edge of the temporal lobe, so that the temporal horn formed the lateral margin of the resection*".

The neurosurgeon had already performed several partial ablations of other areas of the brain in cases of severe psychosis (at that time they were defined as "psychosurgery procedures") with no significant side effects (Scoville, 1951).
– The procedure carried out on Henry Molaison had had a favourable impact on his epileptic fits, which were considerably fewer and of shorter duration, but the patient had become incapable of creating new memories: he only had a blurred memory of a few events concerning his family and



– Henry G. Molaison (1926-2008). Photograph taken 2 years before his bilateral anteromedial temporal lobectomy. – One of the most widely studied patients in the History of Medicine.

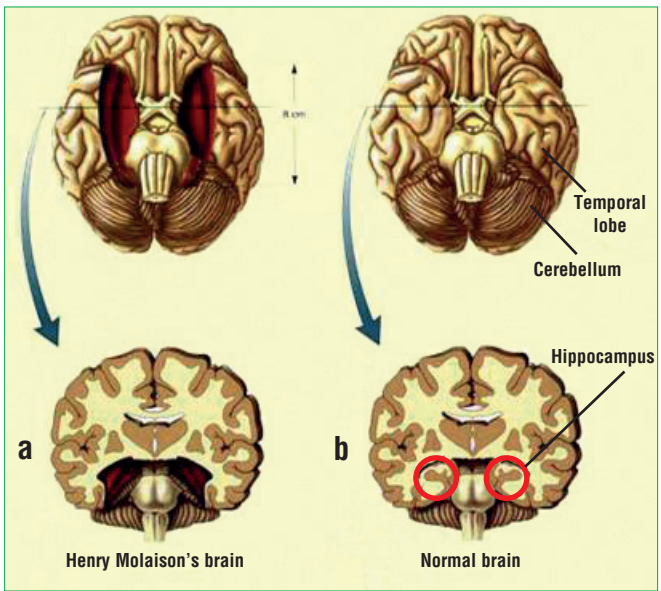


FIG. 1

a) Extensive ablation of the anterior $^2/_3$ of the hippocampi, parahippocampal cortices, entorhinal cortices, piriform cortices, and amygdalae; b) Normal brain.
Top: Inferior view of normal encephalon.
Bottom: Cross section at the level of the temporal lobe of Henry Molaison's brain (a) and normal brain (b).

childhood; nevertheless, he could play the piano, ride a bicycle and perform even complex actions that the brain transmits to the somatic musculature due to conditioned motor patterns, "motor memories", which are currently defined as "intuitive", but which the patient did not remember having learned.

The patient's motor cortex, basal ganglia, and cerebellum were intact, therefore his coordinated automatic motor skills were correctly preserved.
His ability to formulate procedural memory remained intact.

– Despite the severe memory problems, the patient performed well in intelligence and articulated language skill tests, a clear indication of how some memory functions – short-term storage (max. 1-2 minutes), vocabulary, phonemes, etc. – had not been compromised by the surgery (Corkin, 2002; Smith & Kosslyn, 2007).

Before the Molaison case, neuroscientists had considered memory to be "monolithic": the entire brain was involved in the memorisation processes. It is currently recognised that short-term memory is created by trains of electrical impulses conveyed from the cortex to the hippocampus, leaving no trace in the DNA of the hippocampal cells.

– If the experience is emotionally meaningful or repeated *(repetita iuvant)*, the information stabilises, **it is imprinted in the DNA of the hippocampal cells**, which send engrams to the cortex that are now recorded and persist over time.

This is how long-term memory is formed.

In the incredible case of Henry Molaison, the hippocampus and neighbouring cortices (FIGS. 1, 2) had been partially removed, which meant that "his" time had stopped on 1 September 1953, the date of his surgery; after that date ... nothing.

– From 1955, the patient was constantly monitored for a few hours a day by neuropsychologist Brenda Milner; every day – without exception – Henry could not remember ever meeting her before. Every day throughout the many years of observation, Henry would hold out his hand to her and introduce himself: *"Hi… I am Henry, nice to meet you, Ma'am"*

Henry lived to the age of 82, in a temporary present moment, in the here and now... always and only for a few seconds, in a state of "perfect" mindfulness.

• The recent past did not exist, the future would never be remembered; there was only the fragmented remote past, which was in any event prior to the date of his surgery.

– In 2014, Molaison's brain became the focus of very detailed, sophisticated studies (Annese *et* Al., 2014); it is now preserved at the San Diego School of Medicine Institute for Brain and Society Brain Observatory, San Diego, California (USA) (2401 very thin fronto-occipital "slices"; inter: 70 μm).

From these studies it emerged that the amount of brain tissue removed was less than what was reported at the time, but... there was no post-surgery NMRI in 1953.

– Even after his death, neuroscientists continue and will continue to learn from the "amnesiac we will not forget", which is the title of an article by Benedict Carey published in the New York Times on 4 December, 2008.

Paradoxically, the man without a memory is and will continue to be a lasting memory for all neuroscientists, and others.

BIZZOZERO'S ERROR AND THE NEUROTROPHINS

According to the classification formulated in 1894 by Giulio Bizzozero (1846-1901) (Vigliani, 2002), the cells of an organism can be divided into 3 groups:

1) labile, which continuously reduplicate, for example the mucous membranes, epidermis and endometrium (lining epithelial cells);
2) stable, which do not normally reduplicate continuously, but can undergo mitosis in response to homoeostatic requirements, for example in glandular tissues or in the liver after a hepatectomy;

3) perennial, highly specialised, incapable of cell division, for example **nerve cells**.

– This classification, which was used until the final decades of the last century (Chiarugi & Bucciante, 1968), was later radically deconstructed following the discovery of Nerve Growth Factor (NGF) (Levi-Montalcini & Hamburger, 1951).

– **NGF** is a peptide-based neurotrophic factor primarily involved in the proliferation, growth, maintenance and survival of some target neurons.

• Bizzozero's perennial cells… do not exist.

The following belong to a group of Neurotrophic Factors **not** related to NGF: **1)** CNTF (Ciliary NeuroTrophic Factor); **2)** GDNF (Glial-cell-line Derived Neurotrophic Factor); **3)** TGFα/β (Trophic Growth Factor α/β); **4)** FGFs (Fibroblast Growth Factors); **5)** EGF (Epidermal Growth Factor) and **6)** PDGF (Platelet-Derived Growth Factor).

– Since NGF, **3 further neurotrophins (NTs)** have been discovered in humans: **BDNF** (Barde *et* Al., 1982), **NT3** (Maisonpierre *et* Al.,1990) and **NT4** (NT4/5) (Hallbook *et* Al., 1991), each with a distinct profile of trophic effects on specific central and peripheral neuronal subpopulations.

• Of particular significance is BDNF (Brain Derived Neurotrophic Factor), which was first isolated by Yves-Alain Barde, Dave Edgard & Hans Thoenen in the laboratories of the Neurochemistry Section of the Max-Planck Institut für Psychiatrie in Munich - Germany.
The research group was coordinated by the Swiss Prof. Thoenen.
– There follows a quote from part of his original description of the discovery of BDNF: "(…) *we are reporting on the purification of a factor, taken from the brain of pigs, which promotes the survival and growth of the sensory neurons in cultured chicken embryos (…); approximately 1 microgramme of this factor was isolated from 1.5 kg of pig brain (5 brains, Ed.); this factor is the first purified neurotrophic factor since NGF (Nerve Growth Factor), from which it can be clearly distinguishable due to its different antigenic and functional properties*".

Thoenen studied Neurotrophic Factors throughout his life (Thoenen, 1995, 2000; Thoenen & Sendtener, 2002), "a modest man whose discoveries have had profound impact on modern neuroscience" (Iversen, 2013).

A human brain should contain, on average **0.85 ≈ microgrammes** (one microgramme = a millionth of a gramme).

• Mature BDNF is a protein composed of 119 amino acids, with a molar mass of 14 KDa ≈ (13.2-15.19 KDa), an iso-

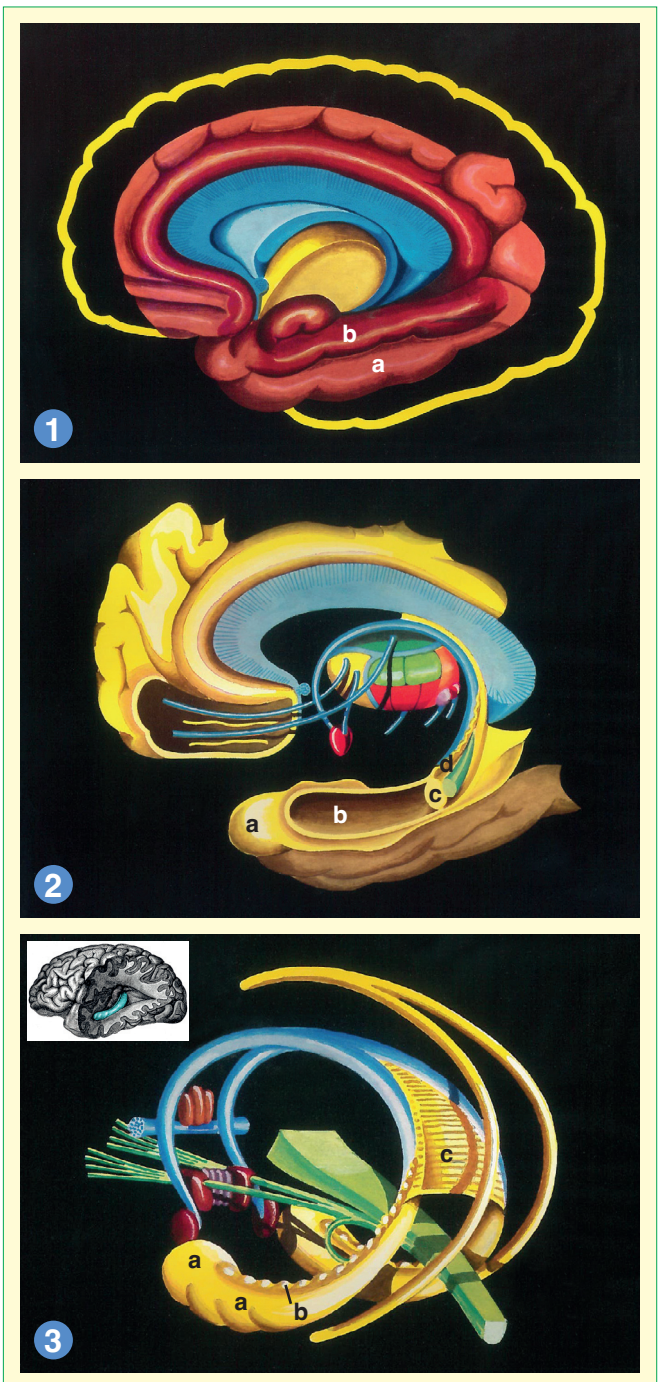


FIG. 2

The hippocampus is very difficult to successfully identify in anatomical dissection. This is because it is located deep in the temporal lobe, and because there are some differences in the terminology used by the various schools of anatomy. Moreover, its course does not follow the major axis of the temporal lobe, but is inclined from bottom to top and from the outside to the inside.
– Its correct location is shown on the 3 images.
1 left hemi-encephalon: a) temporal gyrus; b) hippocampal gyrus;
2 left semi-encephalon: a) temporal lobe, anterior portion; b) empty space that accommodates head and body of hippocampus; c) hippocampus sectioned $^{2}/_{3}$ anterior - $^{1}/_{3}$ posterior (tail); d) fimbria of hippocampus;
3 a) head and body of hippocampus; b) fimbria of hippocampus.
– Note that the hippocampi of the 2 hemispheres approach the midline and, in the region of their tails, they are paired; (c) tentorium of hippocampus.

Once attached to its main TrKB receptor, a dimer, BDNF initiates 3 distinct PATHWAYS.
From top, left: in the 1st PATHWAY, the sequential concatenation of 5 protein complexes (Shc, Grb2, Sos, GAB1, PI3K) stimulates PIP2, a phospholipid component of the cell membranes, which – in turn – stimulates another phospholipid, PIP3.
Through the intermediation of PDK1 (Phosphoinositide-dependent kinase) this produces AkT, also known as Protein-Kinase B. This facilitates neuronal survival, neuronal growth, neuroplasticity, and short-term memory (cytoplasmic activity).
AkT can enter the nucleus and modify the DNA: in this case the information materialises in the long-term memory.
–In the 2nd PATHWAY, intramembrane PIP2, stimulated by PLCγ, a protein attached to the 2nd arm of the TrKB dimer, stimulates IP3, which releases Ca^{++} and produces CAMK (or MAMK), a Ca^{++}/modulin-dependent protein-kinase that enters the nucleus (see above for AkT).
– The 3rd PATHWAY involves the sequential linking of the first 4 protein complexes to a series of promoters, as far as ERK (signal-regulated extra cellular kinase; it also enters the nucleus to modify the DNA).
• NOTE: the table shows 2 TrKB receptors for display and graphic purposes only.
In reality, the 3 PATHWAYS occur simultaneously following the interaction of BDNF with the TrKBs found on the neurons that expose them.

electric point of 9-10 (Chao & Bothwell, 2002). It shares **50% of its amino acid sequence with 3 other NT** (Narhi *et* Al., 1993; Marco Salazar, 2014) (FIG. 3).

– All 4 human NTs share a common ancestral gene (Dos Santos *et* Al., 2011; Covaceuszach *et* Al., 2021).

BDNF is encoded by the BDNF gene which, in humans, is located on chromosome 11, band p 15.
The BDNF gene is made up of 11 exons (exon = portion of the gene that is transcribed by RNA polymerases during transcription) associated with 8 functional promoters + 1 exon that codes for BDNF (Metsis *et* Al., 1993; Timmusk *et* Al., 1993).
– The significance of this complex transcriptional organisation is not well understood; the most accredited hypothesis is that it induces **multiple stages of regulation** by means of alternative promoters, different RNA stability or different subcellular localisations of both RNA and proteins.

– BDNF is synthesised in the neuronal endoplasmic reticulum as a **PRE-PRO-BDNF** complex which subsequently migrates into the Golgi apparatus and later into the trans Golgi system, a maturation system that faces towards the internal portion of the cell membrane.

The PRE portion (18 amino acids) undergoes proteolytic cutting in the endoplasmic reticulum of the nerve cell.
Mature BDNF subsequently splits from the PRO portion (112 amino acids) due to the action of enzymes found in both the intracellular and extracellular compartments.

If this latter splitting does not occur, mature BDNF is not formed. Instead the PRO-BDNF precursor is formed, and biological activity of this differs from that of mature NT (Lee *et* Al., 2001), which induces a neurotoxic effect, as occurs in Alzheimer-Perusini disease (Chen *et* Al., 2017; Fleitas *et* Al., 2018).

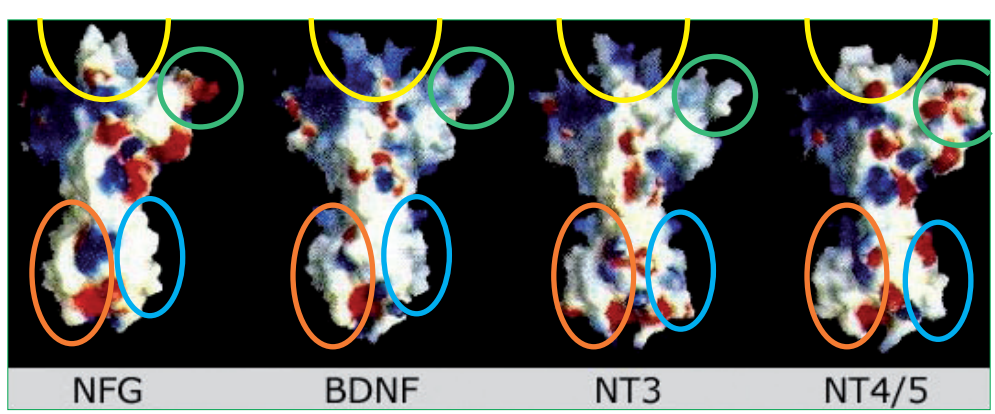
Each NT binds to one or more tropomyosin-kinase receptors, member(s) of the **tyrosine-kinase family (TrKs)** (Patapoutian & Reichhardt, 2001), which activate a wide range of intracellular signals, including – crucially – cyclic AMP phosphorylation (Ji *et* Al., 2005).
• In the case of BDNF, it binds and activates **2** different transmembrane receptors, both at the pre-synaptic and post-synaptic level: one with **high affinity** [**TrK** (pronunciation: Trak) **B**], mentioned above, the other belonging to the TNF superfamily (Tumour Necrosis Factor), **p 75**, with **low affinity**.

• These 2 receptors show no homology, or cytoplasmic domain, and they activate different signalling pathways. They probably appeared at different times in the course of evolution (Tettamanti *et* Al., 2010).

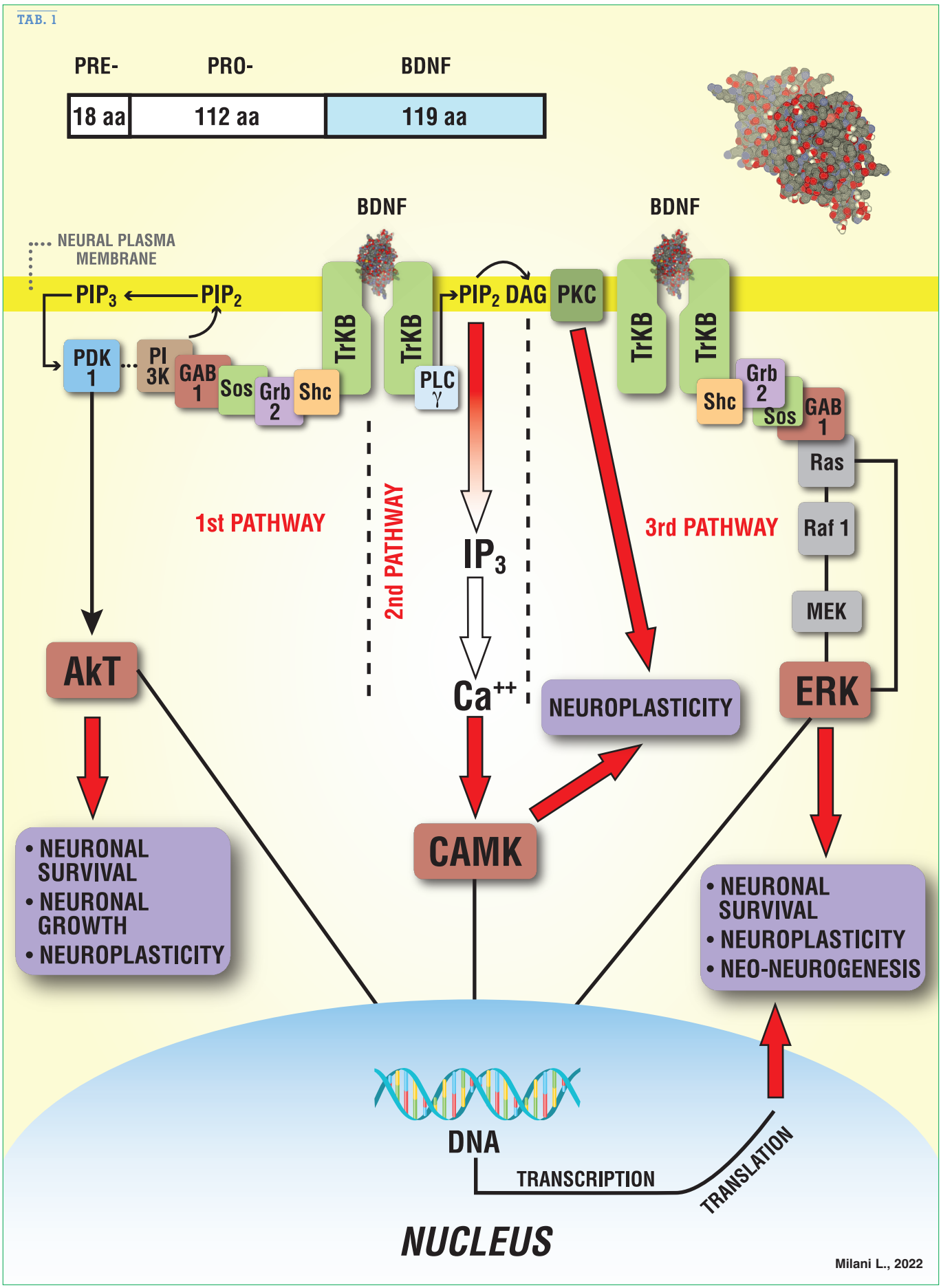
In the presynaptic neuron, BDNF is "packed" into dense, optically dark vesicles; it reaches the terminal plate of the neuron (nerve plate) through rapid axonal flow from the neuronal nucleus (Conner *et* Al., 1997).

FIG. 3

Despite having a similar protein molecular structure, neurotrophins NGF, BDNF, NT3 and NT4/5 show significant morphological differences.
– Author's highlights.



TAB. 1


PRE-
PRO-
BDNF
18 aa
112 aa
119 aa
BDNF
BDNF
NEURAL PLASMA MEMBRANE
PIP3
PIP2
PIP2 DAG
PKC
PDK 1
PI 3K
GAB 1
Sos
Grb 2
Shc
TrKB
TrKB
PLC γ
TrKB
TrKB
Shc
Grb 2
Sos
GAB 1
Ras
Raf 1
MEK
ERK
1st PATHWAY
2nd PATHWAY
3rd PATHWAY
IP3
Ca++
AkT
NEUROPLASTICITY
CAMK
• NEURONAL SURVIVAL
• NEURONAL GROWTH
• NEUROPLASTICITY
• NEURONAL SURVIVAL
• NEUROPLASTICITY
• NEO-NEUROGENESIS
DNA
TRANSCRIPTION
TRANSLATION
NUCLEUS
Milani L., 2022

– Upon arrival of the electrical impulse *(spike)*, BDNF binds to the 2 post-synaptic element receptors mentioned above, and there it triggers – on TrKB – **3 pathways**, with distinct cascade mechanisms.
TrKB is a transmembrane receptor with a short outer part and a long inner part, and is also a dimer (Shen & Maruyama, 2012) (TAB. 1).

– 1st PATHWAY: in an 8-step process [2 of which occur inside the neuronal plasma membrane (PIP2→PIP3)] we arrive at the formation of **Akt**, also known as Protein-Kinase B: this induces survival, neuronal growth and neuroplasticity whether it remains in the cytoplasm or enters the nucleus, and stimulates transcription → translation with the help of DNA.

– 2nd PATHWAY: **CAMK** (Ca^{2+}/modulin-dependent kinase) is synthesised in a 4-step process.
This enters the nucleus directly and – from this moment on – works in the same way as Akt in the 1st Pathway, in the intra-nuclear passage.

– 3rd PATHWAY: a 7-step process [the first 4 of which are identical to those of the 1st Pathway (Shc, Grb_2, Sos and GAB1)] results in the synthesis of **ERK** (Extracellular Signal-Regulated Kinase).
The terminal linking of 3 proteins (Ras, Raf 1 and MEK) to form a chain, identical to that which causes insulin to release glucose to the cells, thanks to DNA, also leads to the same effects as the 1st Pathway, thus highlighting the classic phenomenon of redundancy, as occurs in the cytokines (Milani, 2007).

• **Synthesis**:
BDNF = neuronal survival, neuronal growth, neuroplasticity, neo-neurogenesis and prevention of neuronal apoptosis.

SPECIFIC NATURE OF BDNF

- Its activity on the post-synaptic receptor depends only on the depolarisation of the pre-synaptic neuron (Mowla *et* Al., 1999; Goggi *et* Al., 2003).
- It can be transferred per se from a donor neuron to a recipient neuron (role of neurotransmitter too?) (Kohara *et* Al., 2001; Selvam, 2018).
- It excites the glutaminergic synapses and depresses the GABAergic synapses (hence the excitation) (Tanaka *et* Al., 1997).

– In Henry Molaison's hippocampi and neighbouring cortices, BDNF was overexpressed following his road accident, which explains his continuous, severe epileptic seizures and justifies the subsequent anteromedial temporal lobectomy with excision of part of both hippocampi.

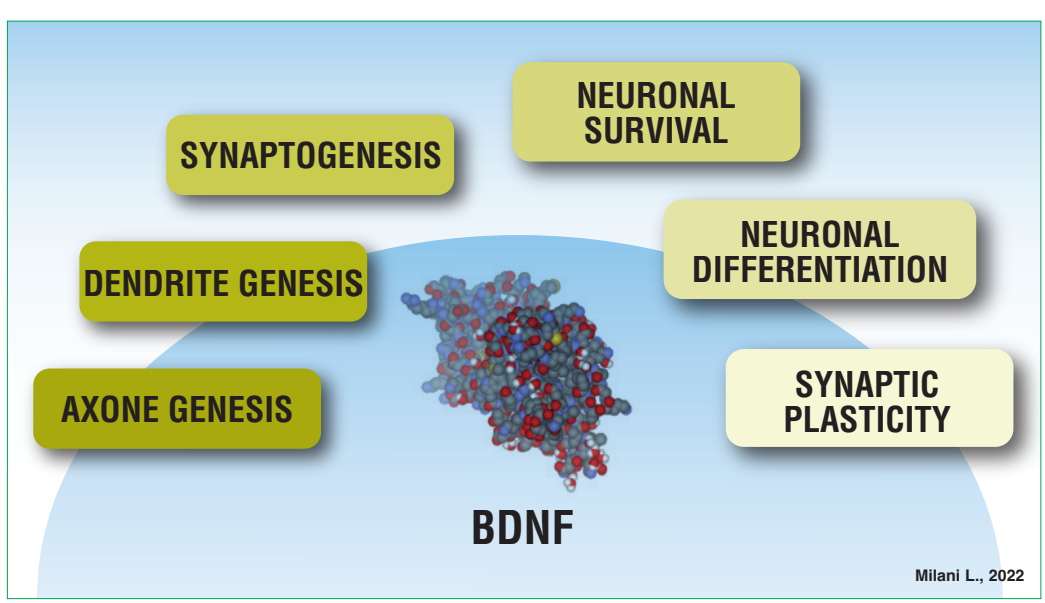
The activities performed by BDNF are the most widely, best characterised of the 4 known NTs.

▶ From the extensive medical-scientific literature consulted, the biological activities **related to BDNF** are: neuronal survival, neuronal differentiation, synaptogenesis, synaptic plasticity, increase in the arborisation of dendrites and new axons: a truly... extraordinary, powerful master regulator of the brain, both in prenatal life and throughout life (TAB. 2).

In the brain, a high concentration of BDNF can be detected in the **cerebral cortex**, especially the **pre-frontal**cortex, which is well-developed only in humans (Milani, 2018), in

TAB. 2

BDNF
– Main biological activities.



the cerebellum, the basal ganglia and – above all – the **hippocampus**, structure belonging to the limbic brain; outside the brain, in the retina, kidneys, heart, lungs, pancreas, prostate, skeletal muscle, and saliva (Pruunsild *et* Al., 2007).

In the prefrontal cortex ... for high level thinking, in the cerebellum ... for posture, in the basal ganglia ... for the control of extrapyramidal involuntary movements and in the hippocampus ... for the consolidation of long-term memory and the control of emotions.
– Binomial Prefrontal Cortex-Hippocampus… for learning.

• Not bad for a protein made up of only 119 amino acids.

BDNF has been synthesised in all **vertebrates** since the lower Carboniferous period, 360-318 million years ago, and is almost **identical in all mammals**. It has shown extraordinary success in the course of evolution (molecular stability and highly performing activities concerned with integration in the environment) (Götz *et* Al., 1992; Tettamanti *et* Al., 2010).

– However, there are two factors that compromise BDNF function; a single substitution of methionine instead of valine (BDNF val66met) along its amino acid sequence has been linked with the susceptibility, incidence and clinical picture of certain psychiatric disorders (anxiety, depression) and neurodegenerative disorders such as glaucoma, multiple sclerosis, Alzheimer-Perusini disease, amyotrophic lateral sclerosis, Parkinson's Disease and Rett Syndrome* (Egan *et* Al., 2003; Shen *et* Al., 2018).

BDNF val66met (variant) polymorphism occurs in **30%** ≈ of the population (source: University of Milan - La Statale Insalutenews 19 October 2016) and is not associated with serum BDNF (Terraciano *et* Al., 2013).

The other genetic error is BDNF L21, associated with congenital central alveolar hypoventilation syndrome (Weese-Mayer *et* Al., 2002) ("Ondine's curse"), a rare disease characterised by a major defect in the central control of respiration.

– These genetic errors have only been demonstrated in humans.

* **Rett syndrome**: Rare developmental disease that affects the CNS.
Almost all sufferers of the disease are female (the "girl children with beautiful eyes"), with an estimated prevalence of 1/9000 adolescents.
– The syndrome is characterised by apparently normal development in the first 5-20 months, after which there is a progressive loss of acquired motor function, stereotyped hand movements, an inability to interact and socialise, and loss of speech. At the age of 20-30 years, many patients develop scoliosis, sleep and breathing disorders and epileptic seizures.

• Some changes in BDNF levels lead to psychiatric disorders, depression, bipolar disorders, psychosis, eating disorders, post-traumatic stress disorder, anxiety disorders, self-harm, and suicidal behaviour.

Of all the NTs, it has emerged that BDNF is the major regulator of many neuronal types such as sensory neurons, retinal ganglion cells, spinal motor neurons, cholinergic neurons, and some clusters of dopaminergic neurons.

BDNF is widely distributed in the CNS; its expression is decreased in many degenerative diseases, as demonstrated in post-mortem studies on Alzheimer-Perusini disease (Phillips *et* Al., 1991; Hock *et* Al., 2000), Parkinson's disease (Mogi *et* Al., 1999; Phillips *et* Al., 1999), Huntington's disease (Ferrer *et* Al., 2000), and severe depression (Lee & Kim, 2010), in which BDNF increases as a result of antidepressant therapy (Shimizu *et* Al., 2003).

• Many neurons are eliminated during the period of intrauterine development in mammals: a large percentage of neurons already formed (**20%** to **70%**) undergoes degeneration; this takes place in order to regulate the optimal number of neurons in adult life. However, this number changes significantly throughout life: at the age of 50 years ≈ **most** of the neurons in the hippocampus are replaced *ex novo* and new neurons are formed, along with the associated dendritic arborisations and synapses. This is to prevent the loss of memories that the apoptotic neurons had preserved as engrams: the master regulator of these complex functions is – above all – BDNF.
– In fact, in the animal model of learning and memory, electrical stimulation of the hippocampus increases the expression of BDNF and NGF (Patterson *et* Al., 1992; Castrén *et* Al., 1993; Bramham *et* Al., 1996).

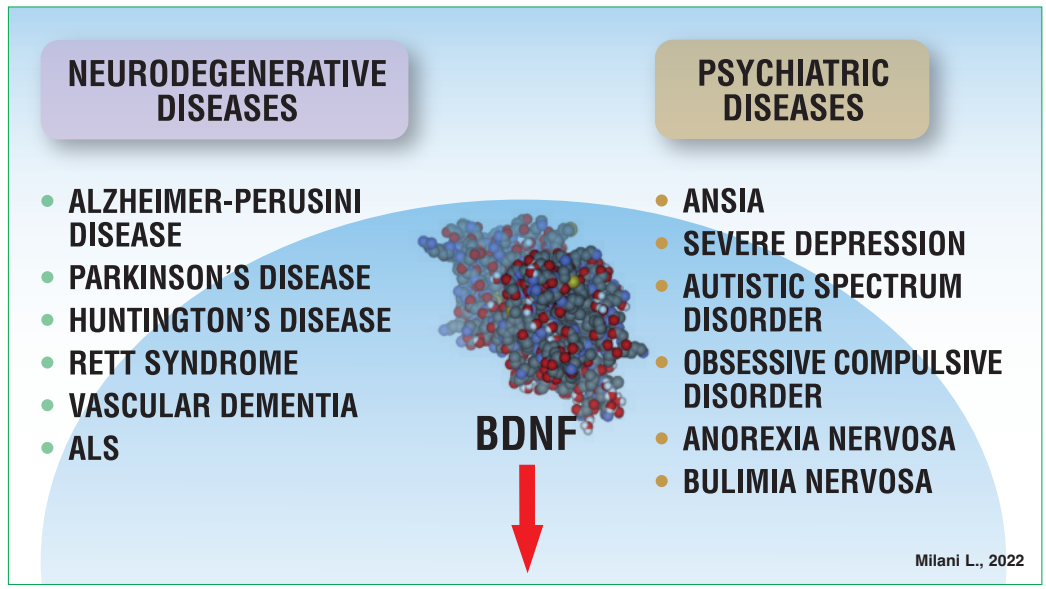
• Optimal cognitive function is dependent on dynamic, active neuronal plasticity (Herndon *et* Al., 1997; Morrison & Hof, 1997; Gonzalez *et* Al., 2016), but over the years this cracks, mainly due to the loss of dendritic spines (Morrison & Hof, 1997).

This does not take place in the entire brain, but only in some specific areas: in cerebral ageing, neuronal loss is a phenomenon localised in the **cortical**, **subcortical** (Smith *et* Al., 2004) and **hippocampal neurons**, where a high concentration of BDNF is expressed (Erickson *et* Al., 2010).

▶ This is the rationale for the progressive decline in voluntary and involuntary motor function, learning, memory and processing-critical thinking in many elderly people (Silhol *et* Al., 2008), in which a transition from **neuro-inflammation** to **neuro-degeneration** has begun (Milani, 2014, 2016; Milani &

TAB. 3

Many neurodegenerative and psychiatric diseases emerge from a decrease of BDNF in the brain.



Pelosi, 2016; Montenero & Milani 2019) and/or atherosclerosis/arteriosclerosis of the cerebral vessels (Vascular dementia).

• The *primum movens* is Low-Grade Chronic Systemic Inflammation. This is the "silent killer" to be identified and neutralised, nothing else.

BDNF – NON-PHARMACOLOGICAL THERAPY

The conventional therapies currently available to reduce the phenomenological aspects of cerebral ageing are both pharmacological and non-pharmacological; the former, which target the neurotransmitters, with no guarantee of success, have various side effects and are expensive (Uberti & Molinari, 2018).

The gene expression of BDNF can be affected by a series of stimuli:
– **Endurance motor exercise** increases BDNF levels by 200%-300% (Salfert *et* Al., 2010).

– **Strength motor exercises** [e.g.. repeated weight lifting for 5-10 min/day (Neeper *et* Al., 1995)]. The increase in BDNF in skeletal muscle is proportional to contractile intensity in aerobiosis relating to a more favourable fat/lean mass ratio, which is enhanced by the consumption of fatty acids for oxidation, and by the improvement in energy consumption of glucose (Mattheus *et* Al., 2009; Henry *et* Al., 2018).

– **Deep sleep** (Eckert *et* Al., 2017).

– **Light stimulation**: BDNF increase in the visual cortex (Castrén *et* Al., 1992).

– **Osmotic stimulation**: BDNF increase in the hypothalamus (Castrén *et* Al., 1995; Dias *et* Al., 2003).

– **Meditation, Yoga** (Cahn *et* Al., 2017), **Mindfulness** (Gomutbutra *et* Al., 2020).

– **Ketogenic diet** (Vizuete *et* Al., 2013).

– **Intermittent fasting** (Mattson *et* Al., 2020).

– **Foodstuffs**: Polyphenols (coffee, green tea, dark chocolate, blueberries) (Gravesteijn & Mensink, 2021).

– **Polyunsaturated fatty acids**: Omega 3, Omega 6 (Wu, 2004).

– **Probiotics** containing Bifidobacteria (Intestine-Brain Axis) (Tian *et* Al., 2019).

• Avoiding patho-stress, social isolation, refined sugars and ethanol: these are powerful depressants that affect the production of BDNF.

BDNF – LOW-DOSE PHARMACOLOGICAL THERAPY

A therapeutic approach using BDNF could be extremely effective in preventing and treating neurodegenerative diseases (and others) (see above), which suffer from a deficiency of this crucial NT.

Knusel *et* Al. (Aut. cit., 1992) demonstrated that the administration of recombinant human BDNF in the cerebral ventricles of rats results in the neuroprotection of damaged cholinergic neurons.
Similarly, in 2001 (Chen *et* Al., 2001) it was demonstrated that intravitreal injections of BDNF in cats increased the survival of retinal ganglion cells after partial destruction of the optic nerve.

– At the time it was believed that BDNF could not cross the **Blood-Brain Barrier (BBB)**, a functional structure located between blood and brain parenchyma that selectively regulates blood flow to and from the brain, thus protecting it from intoxication and poisoning.
For this reason, experiments were necessarily designed as *in loco* procedures (e.g., as stated above, in the cerebral ventricles and the vitreous body), directly into the CNS.

– The discoverer of BDNF himself expressed scepticism about the rational therapeutic use of NTs (Thoenen & Sendtner, 2002).

• This continued until **2010**, when Klein *et* Al. (2010) brilliantly demonstrated that the blood concentration of BDNF reflected what was found inside the brain: BDNF did cross the BBB.
– It did, however, present problems relating to the dose administered.

▶ In this respect, the **BDNF low dose** formulation **(4CH)** successfully solves the problem.
Uberti & Molinari (Aut. cit., 2018; Molinari *et* Al., 2020) demonstrate that **BDNF 4CH** (Guna Laboratories - Milan):
in vitro
– *crosses* the intestinal barrier;
– *crosses* the BEE, thereby reaching the brain tissue;
– activates the astrocytes and neurons, the 2 main types of cells involved in the ageing process;
– activates its main TrKB receptor through the recruitment of MAPK [(mitogen-activated-protein-kinase) (also defined as MAP = ERK)], a kinase that directs cellular responses to various stimuli, such as e.g. mitogens, osmotic stress, pro-inflammatory cytokines, etc. (Pearson *et* Al., 2001).

in vivo
– after the oral intake of BDNF 4CH, the drug arrives in the brain within **1 day**, reaching a peak within **2 days**;
– BDNF 4CH remains in the brain even in the absence of treatment, since it triggers the endogenous physiological systems that support the anti-ageing processes.

The authors conclude that *"the use of low-dose BDNF offers a number of benefits due to the fact that this preparation has no side effects or adverse reactions, features that are necessary if it is used without the supervision of a specialist"*.

In the section "BDNF – Non-pharmacological therapy", I have also identified – among others – **deep sleep**, **Omega 3/6 fatty acids** and **Bifidobacteria** as being stimulants of BDNF gene expression.

– Therefore, for all patients who could benefit from taking **Guna-BDNF** (Brain Derived Neurotrophic Factor - human recombinant), **15-20 drops x 2/day by sublingual route for 4-6 months** (children < 6 years of age: 10 drops x 2/day by sublingual route for 4-6 months) and, in general (TAB. 3):

1) patients with neurodegenerative diseases (vascular and non-vascular dementia, Alzheimer-Perusini disease, Parkinson's disease, multiple sclerosis, Huntington's disease, Rett syndrome and others);

2) Patients suffering from psychiatric diseases [anxiety (Malzac, 2002), severe depression, depression, autistic spectrum disorders, obsessive compulsive disorder, anorexia nervosa, bulimia nervosa, and others (Supino, 2020; Melcarne, 2021)];

3) Specific learning disorders (Supino, 2019);

4) Patients suffering from sarcopenia [BDNF is actively involved in muscle regeneration and in boosting the oxidation of fatty acids; BDNF is also produced directly in striated muscle, with a peripheral endocrine function (So *et* Al., 2014; Delezie *et* Al., 2019)];

5) Cardiovascular diseases. BDNF stimulates angiogenesis and controls the survival of adult endothelial cells, vascular smooth muscle cells, and cardiomyocytes;

6) Type 2 diabetes (Krabbe *et* Al., 2007; Meek *et* Al., 2013; Davarpanah *et* Al., 2021) ...
... my best advice is therapeutic supplement, with or without overlapping with conventional drugs, with:
– **Guna-Melatonin**, 15-20 drops x 2/day;

– **Omegaformula** – Omega 3-6-9 from micronised seeds of the Baobab fruit, 3 chewable tablets/day during or after main meals;

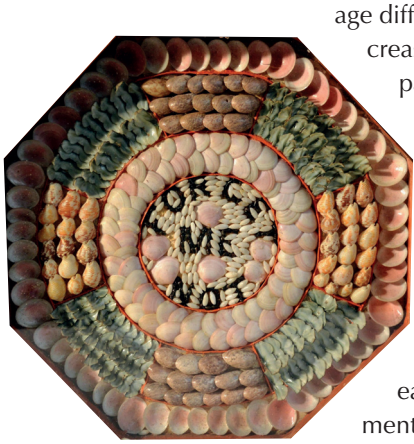
– **Proflora** (probiotic component: *Bifidobacterium* and *Lactobacillus* genera), 1 sachet/day before lunch.

Guna-BDNF is also an effective treatment for mental fatigue, chronic fatigue syndrome and neurovegetative disorders due to long-COVID (post COVID-19 brain fog, or neuro-COVID).

HI... I AM HENRY, NICE TO MEET YOU, MA'AM

All over the world, humans are living longer and seemingly healthier than just a few decades ago.

All this – however – must of course be set in the context of the basic biology of each individual, "his/her" medical history, "his/her" physiological and pathological ageing processes, "his/her" genetic programming [mitotic clock (Ehrenstein, 1998; Fossel, 2018)], "his/her" lifestyle, and the numerous, not always identifiable factors that play a part in making old age difficult – for many – and increasingly dependent on third parties.



– Adding years to life is not the same as adding life to years.

Neurodegenerative diseases, especially Alzheimer-Perusini disease and other forms of dementia that result in the progressive loss of memory, elaborate thinking and purposeful behaviour have a negative impact on learned functioning and daily activities (emotional problems, semantic and lexical difficulties, memory loss, reduced motivation).

– This entire procession depends on the progressive atrophy of the cortical and subcortical regions that underlie the factual nature of these behaviours (including neuro-ageing).

• These brain areas, which formed in humans as an accident of evolution, these 4 brains (Milani, 2018) are "orchestrated" by BDNF which *"does not marry the soma as such; it rests on that and it is from that position that it can put together the less perfect products of corporeality, turning them into Regulation"* (Turco & Turco, 2019).

– It is "ageing", impoverished BDNF that withers the hippocampus and the cortices that "communicate" with it (and ... vice versa); it is lipofucsin, neuromelanin, the Lewy and Hirano bodies, the substance β-amyloid and the Tau proteins that interrupt the neurotransmission wires.

– BDNF 4CH opens up new scenarios, new and real hopes, revitalises ageing life, breathes life into destiny, takes the edge off... *"Hi, I am Henry, nice to meet you, Ma'am"*.

I dedicate this work *in memoriam* to my dear friend Dr Marlowe Elizabeth Reynolds of Berwyn, Chicago, who recently passed away.
– Marly... this goes for you.

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Fig. p. 5
https://suzannecorkin.com/patient-h-m/
Fig. 1 (author's caption)
https://www.ilsuperuovo.it/lindimenticabile-storia-di-h-m-il-paziente-amnesico-piu-famoso-al-mondo/
Fig. 2 - ③ (insert)
https://en.wikipedia.org/wiki/Hippocampus
Fig. 3 (author's highlights)
Fig. p. 14
https://www.1stdibs.co.uk/furniture/folk-art/nautical-objects/sailors-valentine-forget-me-not-barbados-circa-1885/id-f_10642463/

– **Fig. 2** is composed of 3 Tables (①,②,③) taken from Valzelli L. – Profili di Psicofisiologia e Neurochimica. Manfredi Editore, Milano; **1971**. Author's caption.
Tables 1, **2**, and **3** were completed by the author.
In tables 2 and 3, the 3D molecule of BDNF is taken from:
https://en.wikipedia.org/wiki/Brain-derived_neurotrophic_factor

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brain sciences

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Article

The Role of BDNF on Aging-Modulation Markers

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Abstract: An important link between brain aging and a class of growth/survival factors called neurotrophins has recently been demonstrated. In particular, brain-derived neurotrophic factor (BDNF) plays a fundamental role during age-related synaptic loss, preventing cerebral atrophy and cognitive decline. The aim of the present study was to investigate whether the use of low dose BDNF sequentially kinetic activated (SKA) was able to counteract some mechanisms underlying the degeneration and aging of nervous tissue by increasing endogenous protection mechanisms. Both in vitro and in vivo experiments were performed to assess the ability of BDNF SKA to protect and regenerate survival-related molecular pathways, studying intestinal absorption in vitro and brain function in vivo. Our pioneering results show that BDNF SKA is able to induce the endogenous production of BDNF, using its receptor TrkB and influencing the apolipoprotein E expression. Moreover, BDNF SKA exerted effects on β-Amyloid and Sirtuin 1 proteins, confirming the hypothesis of a fine endogenous regulatory effect exerted by BDNF SKA in maintaining the health of both neurons and astrocytes. For this reason, a change in BDNF turnover is considered as a positive factor against brain aging.

Keywords: low dose BDNF; brain aging; astrocytes; BBB; in vivo model

1. Introduction

Research on brain aging and on neurodegenerative diseases is one of the most important challenges that the scientific community has been facing in recent years. The mechanisms underlying brain aging are many and closely related. Recently, an important role has been demonstrated for a class of cell growth and survival factors called neurotrophins [1]. Neurotrophins belong to a family of small proteins playing a fundamental role in both the central and the peripheral nervous systems. The main functions of these proteins are the regulation of axonal growth and neuronal differentiation. The neurotrophins family includes nerve growth factor, neurotrophin-3, neurotrophin-4/5 and, even more important, brain-derived neurotrophic factor (BDNF) [2]. BDNF is the most abundant neurotrophin in the brain and appears essential for neuronal survival during the development and formation of neural networks in the adult brain, exerting its biological actions through tyrosine receptor kinase B (TrkB receptors) [3]. It is produced in the brain by different types of cells and can be transported outside the brain through the blood–brain barrier (BBB). BDNF plays a fundamental role during brain development as it supports the survival and differentiation of neuronal populations of the peripheral and central nervous systems and regulates synaptogenesis, synaptic transmission and plasticity. Furthermore, BDNF plays a crucial role in learning and memory mechanisms [4]. BDNF and other neurotrophins are now considered to be growth factors with a wide spectrum of functions also outside the nervous system, including the modulation and regulation of the immune function [5]. Moreover, BDNF and its specific precursors may

have a role in sustaining thymocyte precursor survival and supporting the thymocyte differentiation process [6].

A preclinical study stated that the dysregulation of BDNF signaling is involved in several neurodegenerative disorders, including Alzheimer's disease, and leads to a deficit in age-related learning [7]. BDNF has also been shown to be able to interact with oxygen radicals (ROS) whose imbalance is involved in the mechanisms of aging, neurodegenerative diseases and some neuropsychiatric disorders [7]. In brain aging there is a decline in the normal antioxidant potential, which leads to an increase in the brain's vulnerability to the harmful effects of oxidative damage [8]. BDNF is considered one of the protective agents against oxidative stress in the central nervous system [9]. Some brain areas particularly involved in neurodegenerative diseases such as the hippocampus, the *substantia nigra*, the amygdala and the frontal cortex are particularly sensitive to oxidative stress [10]. The role of ROS as a marker of brain aging is supported by numerous studies demonstrating that an increase in these substances is related to the reduction of mitochondrial function [11,12]. Several clinical studies have shown changes in blood concentration of BDNF in patients with neuropsychiatric disorders such as major depression [13], schizophrenia [14] and Alzheimer's disease [15]. Studies conducted both in vitro and in vivo have shown that the expression of BDNF and of its specific TrkB receptor is essential to keep an appropriate number of proliferating stem cells, for the differentiation of neuronal populations and for the maturation of excitatory synapses [2].

The possibility of using exogenous BDNF as a therapeutic approach against neurodegenerative diseases has been hypothesized in recent years. However, supplementation of exogenous BDNF presents several problems. The main one depends on the amount of BDNF that reaches the brain. If such amount is too low, it might not be enough to produce the desirable effects. On the other hand, if it is too high it might paradoxically be dangerous, as it might cause, for example, TrkB receptors to be downregulated, thus reducing the intracellular machinery linked to BDNF. The possibility that BDNF can cross the BBB is rather controversial. While some authors argue that it is not clear whether BDNF can readily cross the BBB [16], others indicate that BDNF is able to [17]. In an attempt to overcome such delivery problems, different methods have been developed, which, however, did not thoroughly solve the problem [18]. Other studies have reported further problems related to the administration of neurotrophins in humans, depending on the dose and pharmacokinetics of these molecules [19]. A promising way to achieve a fine regulation of physiological mechanisms could be the use of low-dose substances, as demonstrated by numerous studies available in the literature [20,21]. Several experimental studies have also shown that the administration of low-dose bioactive molecules is effective to obtain clean biological effects with a low probability of adverse effects.

To date, there are no therapies capable of blocking the neuronal death process that triggers neurodegenerative diseases. The use of the family of neurotrophic factors BDNF is part of, whose deficiency has been found in brain aging and in various pathological conditions, could become a valid therapeutic solution. However, there are still numerous problems to be solved, such as the calculation of the dose, the protocol of administration and the crossing of the BBB. Therefore, the aim of this study was to analyze the effect of low doses of BDNF on ROS production in both cultured astrocytes and cortical neurons by observing the behavior of endogenous antioxidant mechanisms. Furthermore, experiments were carried out in order to improve the tolerability of the substance by studying its ability to exert beneficial effects on the molecular pathways linked to viability in the nervous tissue. Finally, experiments were conducted both in vitro and in vivo to evaluate the characteristics of intestinal absorption after oral intake of BDNF and to evaluate the ability of BDNF to cross the BBB.

2. Materials and Methods

2.1. Preparation of BDNF Solutions

All dilutions were prepared starting from a stock solution (0.001 ng/mL) of BDNF 4CH in 0.9% NaCl. Based on previous knowledge on activated blends [21,22], BDNF solutions were prepared

at two different concentrations: 1 pg/mL for in vitro studies and 1.20 pg/mL for in vivo studies. Each concentration was prepared using the sequential kinetic activation (SKA) method [21]. These solutions are kinetically energized by a mechanically applied force via a standardized shaking process (sequential kinetic activation named SKA), characterized by vertical shaking corresponding to 100 oscillations in 10 s. All solutions were prepared by GUNA Laboratories (GUNA S.p.a, Milan, Italy). For each treatment, the volume of each solution was calculated by comparing the volume added to the sample treated with 50 ng/mL BDNF for in vitro study [23] and with 25ng/mL BDNF [24] for in vivo experiments. The BDNF used to compare the results obtained with BDNF 1 pg/mL SKA was not subjected to SKA treatment in order to replicate the same experimental conditions as in other studies.

2.2. Astrocytes Isolation

Primary mouse astrocyte cultures were extracted from C57BL/6 mouse pups, following a classical technique [25] according to the National Guideline for the Use and Care of Laboratory Animals. Briefly, within 24 h of birth pups were euthanized, and cortices were dissected, minced, mechanically digested and left to settle for 30 min at room temperature. The cell suspension was centrifuged at 800 rpm for 5 min and pelleted cells were resuspended in Neuronal Basal Medium (Sigma-Aldrich, Milan, Italy) supplemented with 5% fetal bovine serum (FBS, Sigma-Aldrich, Milan, Italy), 1% penicillin/streptomycin (Sigma-Aldrich, Milan, Italy) and 2 mM L-glutamine (Sigma-Aldrich, Milan, Italy), plated in multi-wells and maintained in culture for 6 days before treatment. The cells were plated 4 × 104 astrocytes/cm^2 on a 24-well Transwell support to prepare the model BBB; 1 × 104 on 96-well plates for MTT and crystal violet staining; 4 × 104 cells were plated in black 96-well plates to study o × ygen consumption and mitochondrial membrane potential; 5 × 104 cells were plated on 24-well plates to analyze reactive o × ygen species (ROS) production; 2 × 105 cells were plated on 24-well plates to quantify BDNF; and 1 × 106 in 6-well plates to analyze the intracellular pathways by Western blot and ERK activity by ELISA test.

2.3. Primary Cortical Neuronal Cells

Primary mouse cortical neuronal cultures were obtained from the brains of P0 C57BL/6 mouse pups, as reported in literature [26]. All procedures used in these studies follow the guidelines in accordance with the National Institutes of Health Guidelines. Cortices were dissected from embryonic brains and the tissue was mechanically dissociated and left to settle for 30 min at room temperature. After centrifuging only, the supernatant was re-suspended in Neuronal Basal medium (Sigma-Aldrich, Milan, Italy) supplemented with 2% B27 (Sigma-Aldrich, Milan, Italy), 1% penicillin/streptomycin (Sigma-Aldrich, Milan, Italy) and 2 mM L-glutamine (Sigma-Aldrich, Milan, Italy). Cells were plated on pre-coated plates with 10 µg/mL poly-L-lysine at a density of 1 × 106 cells/mL and were maintained in incubator at 37 °C with 5% CO2 and 95% humidity. At three days from plating, the medium was changed. All experiments were performed on primary cortical neuronal cells grown for 9–10 days in vitro. The cells were plated 1 × 104 on 96-well plates for MTT; 5 × 104 cells were plated on 24-well plates to analyze reactive oxygen species (ROS) production; 2 × 105 cells were plated on 24-well plates to quantify BDNF; and 4 × 104 cells were plated in black 96-well plates to study oxygen consumption and mitochondrial membrane potential.

2.4. In Vitro Experimental Protocol

Before treatment, both primary cortical neuronal cells and astrocytes were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Milan, Italy) without red phenol and FBS, supplemented with 1% penicillin/streptomycin (Sigma-Aldrich, Milan, Italy) 2 mM L-glutamine (Sigma-Aldrich, Milan, Italy) and 1 mM sodium pyruvate (Sigma-Aldrich, Milan, Italy) at 37 °C, 5% CO2 and 95% humidity for 1 h. Cells were treated with 1 pg/mL BDNF SKA and 50 ng/mL BDNF at T0, checked every 24 h, and maintained for 6 days (named 6 days protocol). The vehicle, a saline solution, was also analyzed. Moreover, the involvement of TrkB using a specific antagonist, 1 µg/mL

ANA-12 (Sigma-Aldrich, Milan, Italy) [27] treating cells 30 min before stimulation was investigated. Additional experiments were carried out to analyze the ability of BDNF solutions to restore the damage caused by oxidative stress, a major cause of aging and neurodegeneration. Both cortical neuronal cells and astrocytes were pre-treated with 200 μM H_2O_2 (Sigma-Aldrich, Milan, Italy) [28] for 30 min and then treated with 1 pg/mL BDNF SKA and 50 ng/mL BDNF. Finally, astrocytes were used in time-course study within 24 h to mimic the human posology.

2.5. Intestinal Barrier In Vitro Model

Caco-2 cells (human epithelial colorectal adenocarcinoma cells), purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), were used as an experimental model [29] to predict the features of intestinal absorption following oral intake [30]. These cells were grown in a complete medium composed of Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM-F12, Sigma-Aldrich, Milan, Italy) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Milan, Italy), 2 mM L-glutamine (Sigma-Aldrich, Milan, Italy), 1% penicillin-streptomycin (Sigma-Aldrich, Milan, Italy) and maintained in an incubator at 37 °C with 5% CO2 and 95% humidity. Cells were used from passages 46 to 49 and seeded in 24-well polyester Corning® Costar® transwell plates (Sigma-Aldrich, Milan, Italy) in complete medium. The cells were cultured for up to 21 days in a humidified incubator maintained at 37 °C in an atmosphere of 5% CO2, changing medium every 3 days first basolaterally and then apically. The monolayer integrity was checked every 3 days (at the time of the medium change) [31]. After 21 days, 1pg/mL BDNF SKA and 50 ng/mL BDNF were added to culture medium under different pH conditions, as reported in literature [31]; pH 6.5 preparations were added to the apical side, whereas pH 7.4 was added to the basolateral side. The slightly acidic pH (pH 6.5) in the apical side represents the average pH in the lumen of the small intestine, whereas the neutral pH (pH 7.4) in the basolateral side mimics the pH of the blood. During treatments, the cells were maintained in an incubator at 5% CO2, and at the end of stimulations the BDNF quantity was measured by ELISA kit after 30 min and 1, 3, 4, 5and 6h from stimulation. This model is suitable to predict the absorption of substances after oral intake by evaluating the apparent permeability coefficient (Papp) [31,32]. Briefly, the Papp (cm/s) was calculated as [29]:

$$Papp = dQ/dt \times 1/m0 \times 1/A \times VDonor$$

dQ: amount of substance transported (nmol or μg)

dt: incubation time (sec)

m0: amount of substrate applied to donor compartment (nmol or μg)

A: surface area of transwell membrane (cm^2)

VDonor: volume of the donor compartment (cm^3)

Negative controls without cells were tested to exclude transwell membranes influence.

2.6. Blood–Brain Barrier (BBB) Experimental Model

Astrocytes were co-cultured with human umbilical vein endothelial cells (HUVEC) cells according to methods reported in literature [33]. HUVEC were purchased from ATCC®. Cells were cultured in EGM Media (Lonza, Basel, Switzerland) supplemented with 10% FBS (Sigma-Aldrich, Milan, Italy), 1% penicillin/streptomycin (Sigma-Aldrich, Milan, Italy) and 2 mM Glutamine (Sigma-Aldrich, Milan, Italy) at 37 °C in a humidified atmosphere of 95% air, 5% CO2. In brief to create the BBB barrier, 4×104 astrocytes/cm^2 were plated on the basolateral side of the flipped 6.5 mm Transwells with polyester membrane with 0.4 μm pore size (Corning Costar, Sigma-Aldrich, Milan, Italy) and left to attach for 4 h. Transwells were then placed into the normal orientation and the cells left to grow for 48 h. After this time, 1×105 HUVEC cells/cm^2 were plated in the apical compartment. The inserts were then placed in a 24-well plate. After 7 days of culture, the Transwells were treated and permeability studies were performed [34]. To understand the ability of tested substances to cross the blood–brain barrier the medium at the bottom side of the Transwells was quantified over time by measuring the volume and the concentration of BDNF.

2.7. Brain-Derived Neurotrophic Factor (BDNF) Quantification

Brain-derived neurotrophic factor (BDNF) quantification was measured by Rat BDNF Elisa Kit (Thermo ScientificTM, Waltham, MA, United States) in cellular supernatants obtained from basolateral environment of BBB, primary cortical neuronal cells, astrocytes, serum and brain tissue to quantify BDNF, following the manufacturer's instructions. Tissues were washed in saline solution, weighed, cut in small pieces and homogenized 100 mg tissue/300 µL with cold lysis buffer (0.1 M Tris, 0.01 M NaCl, 0.025 M EDTA, 1% NP40, 1% Triton X-100; Sigma-Aldrich, Milan, Italy) supplemented with 2 mM sodium orthovanadate, 0.1 M sodium fluoride (Sigma-Aldrich, Milan, Italy), 1:100 mix of protease inhibitors (Sigma-Aldrich, Milan, Italy), and 1:1000 phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, Milan, Italy), using an electric potter at 1600 rpm for 2 min. The tissue extracts were centrifuged at 13000 rpm for 20 min at 4 °C.

The cellular and tissue supernatants were collected, and each sample was tested by ELISA kit. Briefly, biotinylated detection antibody was added into each well and the plate was incubated for 1 h at room temperature. Then, after 45 min of incubation with HRP-conjugated streptavidin, TMB substrate solution was added for 30 min and subsequently the reaction was stopped by adding Stop Solution. BDNF concentration was determined by measuring the absorbance through a spectrometer (VICTOR X4, multilabel plate reader) at 450 nm and calculated by comparing results to BDNF standard curve.

2.8. MTT Assay

MTT-based In Vitro Toxicology Assay Kit (Sigma-Aldrich, Milan, Italy) was performed on both cell types to determine cell viability, as previously described [35]. Briefly, at the end of each stimulation, the cells were incubated with 1% MTT dye for 2 –3 h at 37 °C in incubator, until the purple crystals were dissolved in equal volume of MTT Solubilization Solution. The relative viability (%) was based on absorbance measuring through a spectrometer (VICTOR X4, Multilabel Plate Reader) at 570 nm with correction at 690 nm. Finally, viability was calculated comparing results to control cells (defined as 100% viable).

2.9. Crystal Violet Staining

After each treatment astrocytes were fixed with 1% glutaraldehyde (Sigma-Aldrich, Milan, Italy) for 15 min at room temperature, washed, and stained with 100 µL 0.1% aqueous crystal violet (Sigma-Aldrich, Milan, Italy) for 20 min at room temperature. To multi-well plates, 100 µL of 10% acetic acid was added and mixed before reading the absorbance at 595 nm using a spectrometer (VICTOR X4, multilabel plate reader). The estimated number was calculated by comparing the results to the control cells counted at T0.

2.10. ROS Production

The rate of reactive species of oxygen (ROS) was measured using a standard protocol based on the addition of cytochrome C (Sigma-Aldrich, Milan, Italy) to the samples and to another sample of 100 µL superoxide dismutase (Sigma-Aldrich, Milan, Italy). They were added for 30 min in an incubator at 37 °C, 5% CO_2, and 95% humidity to determine the antioxidant capability of BDNF solutions on the BBB model, primary cortical neuronal cells and astrocytes. At the end of stimulations, 100 µL of supernatant were measured at 550 nm using a spectrometer (VICTOR X4, multilabel plate reader) and O_2 was expressed as the mean ± SD (%) of nanomoles per reduced cytochrome C per micrograms of protein compared to control [36].

2.11. NO Production

Nitric Oxide (NO) production was measured by Griess Assay (Promega Corporation, Madison, Wisconsin, United States). After 6 days of treatment, supernatants of basolateral BBB were mixed with equal volumes of Griess reagent and incubated in the dark at room temperature for 10 min; absorbance

was measured by a spectrometer at 570 nm. NO production corresponded to the NO (μmol) produced after each stimulation by samples, each containing 1.5 μg of protein [37].

2.12. Mitochondrial Membrane Potential

The mitochondrial membrane potential was analyzed following manufacturer's instructions of Oxygen Consumption/Mito membrane Potential Dual Assay Kit (Cayman Chemical Company, Ann Arbor, MI, United States) [38]. The mitochondrial membrane potential was measured using JC-1 aggregates at an excitation/emission of 560/590 nm and monomers at an excitation/emission of 485/535 nm in a fluorescence spectrometer (VICTOR X4, multilabel plate reader). The results are expressed as means ± SD (%) compared to control cells in both cell types.

2.13. ERK Activation Assay

ERK/MAPK activity was measured by the InstantOne™ ELISA (Thermo Fisher, Milan, Italy) on astrocytes lysates following the manufacturer's instructions [36]. Briefly, 50 μL/well of astrocytes lysed with cell lysis buffer were tested in InstantOne ELISA microplate strips after 1 h at room temperature on a microplate shaker with the antibody cocktail. At the end, the detection reagent was added for 20 min and then stopped by adding stop solution. The strips were measured by a spectrometer (VICTOR X4 multilabel plate reader) at 450 nm. The results were expressed as mean absorbance (%) compared to control.

2.14. Western Blot

To perform Western blot analysis, 1 × 106 astrocytes plated in 6-well were lysed in ice with Ripa Buffer (50 mM Hepes, 150 mM NaCl, 0,1% SDS, 1% Triton X-100, 1% deoxycholate acid, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM NaF; Sigma-Aldrich, Milan, Italy) supplemented with 2 mM sodium orthovanadate and 1:100 mix Protease Inhibitor Cocktail (Sigma-Aldrich, Milan, Italy). 30 μg proteins were resolved on 8% and 15% SDS-PAGE gels. Brain tissue was also analyzed by Western blot to verify the mechanisms observed in cell culture. At the end of treatments brain tissue was excised out, washed in ice saline solution, weighed, cut in small pieces, and homogenized 100 mg tissue/300 μL with cold lysis buffer (0.1 M Tris, 0.01 M NaCl, 0.025 M EDTA, 1% NP40, 1% Triton X-100; Sigma-Aldrich, Milan, Italy) supplemented with 2 mM sodium orthovanadate, 0.1 M sodium fluoride (Sigma-Aldrich, Milan, Italy), 1:100 mix of protease inhibitors (Sigma-Aldrich, Milan, Italy), and 1:1000 phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, Milan, Italy), using an electric potter at 1,600 rpm for 2 min. The tissue extracts were centrifuged at 13,000 rpm for 20 min at 4 °C and 40μg of each lysate were resolved on 8% and 15% SDS- PAGE gel. Polyvinylidene difluoride (PVDF) membranes (GE Healthcare Europe GmbH, Milan, Italy) obtained from cell and brain tissue lysates were incubated overnight at 4 °C with specific primary antibody: anti-phospho-tyrosine receptor kinase B (p-TrkB, Tyr515; 1:250, Santa Cruz, CA, United States), anti-tyrosine receptor kinase B (trkB; 1:250, Santa Cruz, CA, United States), anti-Apolipoprotein E (apoE, E4; 1:250, Santa Cruz, CA, United States), anti-phospho-Sirtuin1 (pSIRT1, Ser47; 1:1000, Sigma-Aldrich, Milan, Italy), anti-Phospho-p44/p42 Mitogen-activated protein kinase (pERK/MAPK, Thr202/Tyr204; 1:1000, Euroclone, Milan, Italy), anti-p44/p42 Mitogen-activated protein kinase (ERK/MAPK; 1:1000, Euroclone, Milan, Italy) and anti-Phospho-Tau (pTau, Ser262; 1:250, Thermo Fisher Scientific, Waltham, MA, United States). In addition, in brain anti-BDNF (1:500, Sigma-Aldrich, Milan, Italy) and anti-β-Amyloid (APP, B-4, 1:500, Santa Cruz, CA, United States) were also investigated. All protein expressions were normalized to the specific total protein (if possible), verified through β-actin detection (1:5000, Sigma-Aldrich, Milan, Italy) and expressed as mean ± SD (%).

2.15. Animal Model

12-month-old mice wild type C57BL/6jOlaHsd of comparable age to an elderly human (about 80 years old) [39] purchased from Envigo++++ (Bresso, Italy), were used to confirm the effects of BDNF solutions in a complex model (n = 52). Starting from a new protocol to induce a spontaneous

intake [40], we created a new rissole without bromophenol blue containing 1.2 pg/mL BDNF SKA or 25 ng/mL BDNF which is voluntary eaten by old mice. The quantity of rissoles was calculated considering the quantity of food and daily water normally taken by the animals [41]. The rissole preparation phases are summed up in Appendix A. The animals had access to food and water ad libitum and the experimental subjects were transferred to a single cage and kept in a single holding room and housed in a constant temperature of 21–22 °C, humidity of 5–55%, for 3 h [40,42]. Due to the short time taken to administer the rissole, mice showed no signs of social deprivation, such as increased aggressiveness. These signs have in fact been observed for periods of social deprivation of 6 h [43]. After this time, the rissole was added in the lower part of the cage, but the mouse had free access to food and water in the upper part. Time of stimulation started from the addition of the rissole. During the whole period of treatment, the mice were monitored to assess their health status. Serum was obtained from blood of intracardiac withdrawal after inducing anesthesia, and brain tissue was obtained after animal death. All experimental procedures on animals were reviewed and approved by the University Committee OPBA (Organismo preposto al benessere degli animali) in accordance with local ethical standards and protocols approved by national guidelines (Approval No. 41/2019-PR). Animals were randomized into four different times of treatment: untreated (12 animals, 4 for each times of treatment), 24 h (20 animals), 24 h plus 24 h (10 animals) and 6-day protocol (only one administration for 6 days, 10 animals). In particular, 24 animals were sacrificed after 24 h and 14 animals were sacrificed after 48 h (24 h with rissole plus 24 h without rissole administration but the animals had access to food and water ad libitum in the upper part of cage). Finally, to demonstrate the efficacy of treatment, 14 animals were sacrificed 6 days after the only administration of BDNF solution (6-day protocol). All groups were sacrificed at specific time points (24 h, 24 h plus 24 h and 6 days) by CO_2 asphyxiation and blood drawn at the same time. The blood was centrifuged at 3500 rpm for 15 min at room temperature and the serum was conserved at −80 °C for subsequent experiments. In addition, the brain was removed, frozen and conserved at −80 °C for successive analysis by ELISA and Western blot on whole brain tissue lysates.

2.16. Statistical Analysis

Each part of the study is supported by at least 4 independent experiments both in vitro and in vivo. All results were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test; data are expressed as mean ± SD of at least four independent experiments for each experimental protocol produced in triplicates. The percentage values were compared through Mann–Whitney U test. Comparisons between the two groups were performed using a two-tailed Student's t-test. Multiple comparisons between groups were analyzed by two-way ANOVA followed by a two-sided Dunnett post hoc testing. p-Value < 0.05 was considered statistically significant.

3. Results

The effectiveness of BDNF SKA was investigated by both in vitro and in vivo experiments, comparing 1 pg/mL and 1.2 pg/mL to BDNF 50 ng/mL and 25 ng/mL, respectively.

3.1. The Potential Intestinal Absorption as Evaluated In Vitro

Since BDNF SKA can be used by oral administration in human, the in vitro intestinal absorption was investigated. An in vitro intestinal barrier model was carried out to understand the ability of 1 pg/mL BDNF SKA compared to 50 ng/mL BDNF to cross the intestinal barrier and to become available to the body. Analyzing the volume in the basolateral compartment 1 pg/mL BDNF SKA showed a significant increase in absorption capacity compared to saline and comparable to that of 50 ng/ml of BDNF at each stimulation time (Table 1). In particular, the maximum Papp, the permeability constant value, was observed at 1 h of treatment with 1 pg/mL BDNF SKA (about 4.18 ± 0.1), supporting the hypothesis that BDNF SKA can cross the intestinal barrier.

Table 1. The Papp values obtained on intestinal barrier model and plasmatic human absorption derived from Papp value.

Stimulations	30 min	1 h	3 h	4 h	5 h	6 h
1 pg/mL BDNF SKA	2.91 ± 0.3	4.18 ± 0.1	3.36 ± 0.2	3.27 ± 0.3	2.02 ± 0.2	1.97 ± 0.3
Saline	0.11 ± 0.1	0.29 ± 0.1	0.36 ± 0.1	0.5 ± 0.1	0.57 ± 0.1	0.6 ± 0.1
50 ng/mL BDNF	3 ± 0.3	3.98 ± 0.3	4 ± 0.3	3.2 ± 0.2	1.98 ± 0.2	1.02 ± 0.1
1 pg/mL BDNF SKA	32.75	39.8	36.5	34.85	26.6	26
Saline	<20	<20	<20	<20	<20	<20
50 ng/mL BDNF	33.2	38.15	39.8	33.2	25.8	20

Saline = saline solution. Data are expressed as means ± SD (%) of four independent experiments reproduced in triplicates.

Following a standard conversion to predict the human absorption after oral intake starting from the Papp values obtained from Caco-2 cells, the BDNF bioavailability in human (Table 1) showed an increase caused by 1 pg/mL BDNF SKA compared to saline solution and 50 ng/mL BDNF at each time of treatments and confirmed a higher level at 1 h of stimulation (about 4% compared to 50 ng/mL BDNF). These data confirm that 1 pg/mL BDNF SKA is able to cross the intestinal barrier and to has a good bioavailability compared to 50 ng/mL BDNF.

Since safety is the main problem affecting human use, additional experiments on ROS production were performed on the intestinal barrier to exclude any adverse effects. Both 50 ng/mL BDNF and 1 pg/mL BDNF were able to maintain ROS at physiological levels ($p > 0.05$ vs. saline solution and control), supporting the safe use of this substance (Appendix B: Figure A2). Besides, the higher effect of 1 pg/mL BDNF compared to 50 ng/mL was observed after 3 and 4 h of treatment ($p < 0.05$; compared to saline solution, 50 ng/mL BDNF and control) demonstrating the best antioxidant action of BDNF SKA.

These data support the hypothesis that BDNF SKA is able to cross the intestinal barrier and reach the blood in the first 3–4 h after oral intake.

3.2. Permeability of BDNF SKA Through Blood–Brain Barrier (BBB)

Since the most important parameter after oral intake is the ability of BDNF to reach the brain tissue, more experiments were performed using the BBB in vitro model. The analysis on the basolateral volume of 1 pg/mL BDNF SKA and 50 ng/mL BDNF showed no significant difference between 1 pg/mL BDNF SKA and 50 ng/mL BDNF (Figure 1A), but the quantification showed a significant increase ($p < 0.05$) of both 1 pg/mL BDNF SKA and 50 ng/mL BDNF compared to control and to saline solution (about 41% and about 44%, respectively). These data suggest that only one treatment for six days appears to be more important to obtain a greater effect.

Furthermore, ROS production was analyzed in order to exclude any adverse effect caused by BDNF solutions. As shown in Figure 1B, there was no difference evident between 1 pg/mL BDNF SKA and 50 pg/mL BDNF and the effects of both solutions were not significant ($p > 0.05$) compared to control, indicating a physiological ROS production. These data confirmed the hypothesis of the higher effectiveness of administration protocol.

Since maintaining the balance of oxidative condition is an important parameter to preserve the integrity of brain cells, some additional experiments were carried out to analyze NO production within the BBB (Figure 1C). At basolateral level, the NO production induced by protocol A was significantly reduced compared to control ($p < 0.05$). In particular, there was no significant difference between 1 pg/mL BDNF SKA and 50 ng/mL BDNF treatment, suggesting that BDNF solutions were not cytotoxic.

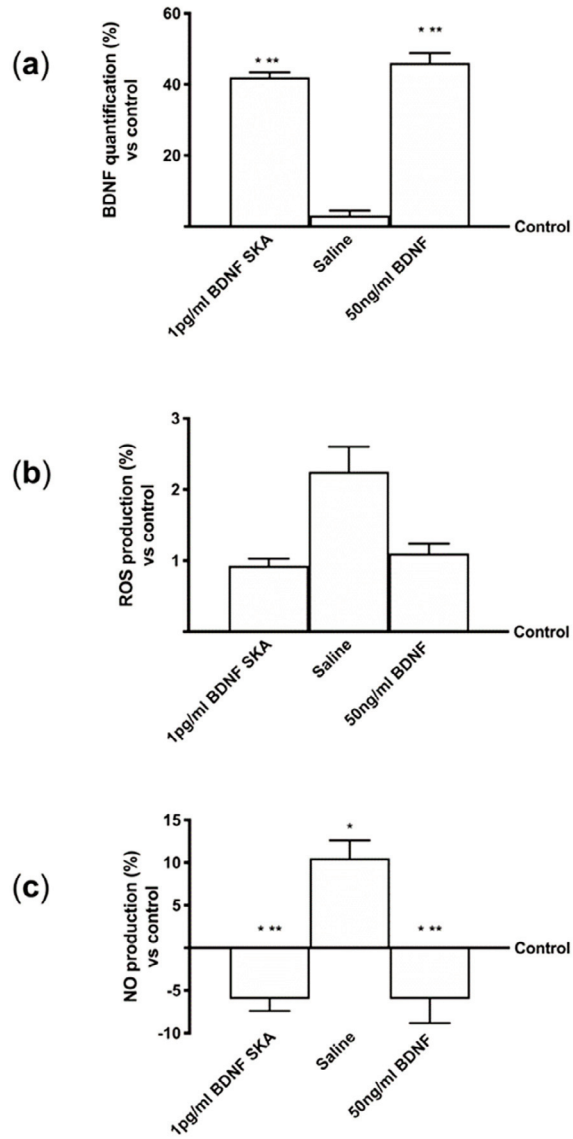

Figure 1. Analysis of the effects at blood–brain barrier (BBB) level. (**a**) Brain-derived neurotrophic factor (BDNF) quantification, (**b**) reactive oxygen species (ROS) production and (**c**) NO measurements are reported. Data are expressed as means ± SD (%) of four independent experiments performed in triplicates normalized to control (0 line as control). * $p < 0.05$ vs. control; ** $p < 0.05$ vs. saline solution.

These results suggest that 1 pg/mL BDNF SKA had a similar effect to 50 ng/mL SKA despite the different concentrations and that only one treatment is able to induce a beneficial effect. Finally, BDNF SKA is confirmed to act without any adverse effect at the neuronal level.

3.3. Topic Action of BDNF SKA on Monolayer Culture

BDNF has been demonstrated to be able to act on both cortical neuronal cells and astrocytes, additional experiments were performed in order to investigate cell viability and ROS production in these monolayer cultured cells. In particular, 1 pg/mL of BDNF SKA induced a significantly greater cell viability (Figure 2A) both in primary cortical neuronal cells and in astrocytes compared to control ($p < 0.05$) and 50 ng/mL of BDNF (in neuronal cells approximately 124% and 75%, respectively, in the astrocytes approximately 58.6% and 171%, respectively). These findings suggest that though BDNF SKA was used at lesser concentrations it was able to determine a greater effect on cell viability compared to the higher concentration; furthermore these results support the hypothesis of the effectiveness of the single administration compared to multiple administrations.

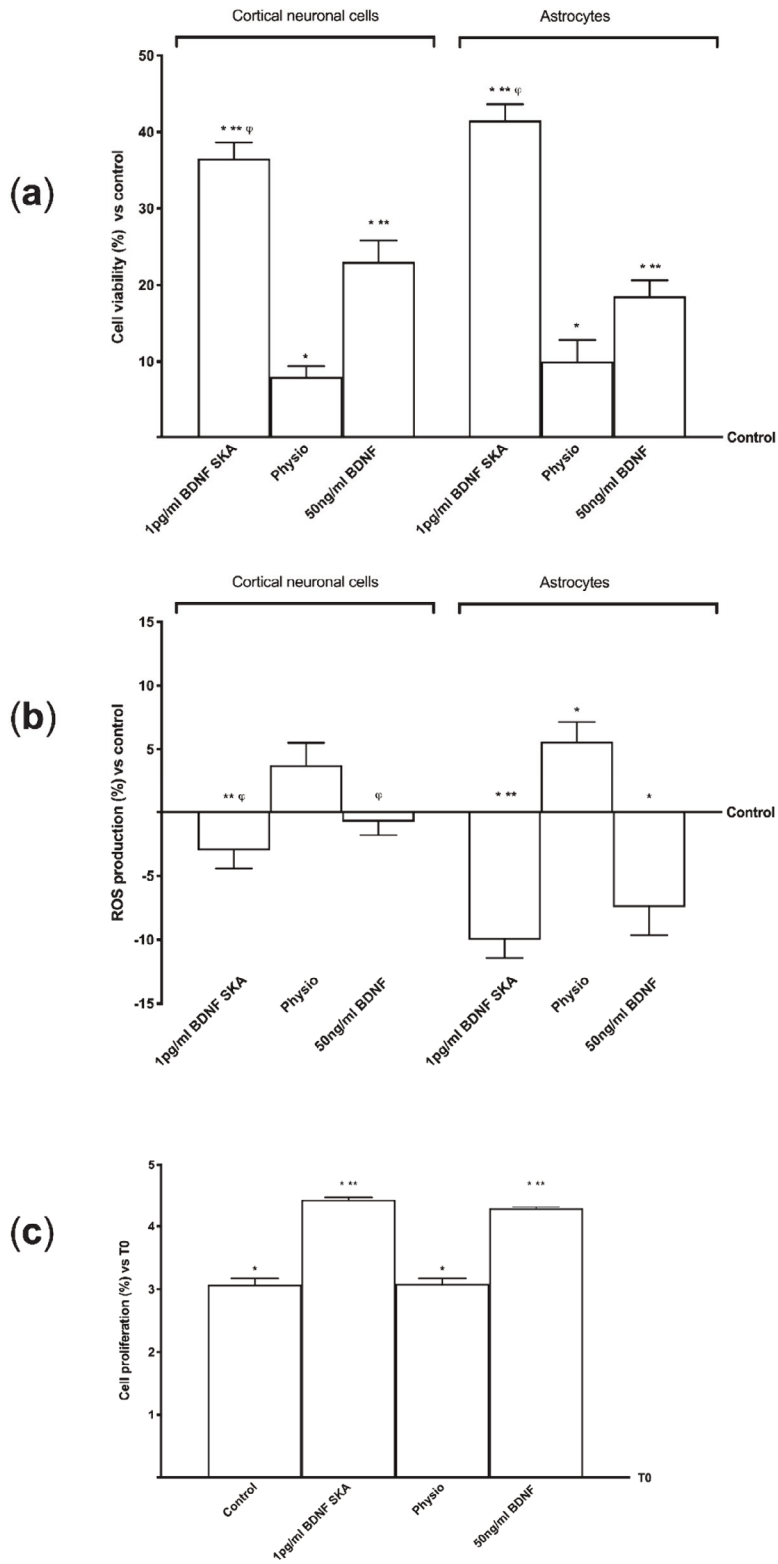

Figure 2. Effects of BDNF solutions on primary cortical neuronal cells and astrocytes. (**a**) Cell viability and (**b**) ROS production measured on both cell types. (**c**) The effects on astrocytes proliferation are shown. Data are expressed as means ± SD (%) of four independent experiments performed in triplicates normalized to control (0 line as control). * $p < 0.05$ vs. control; ** $p < 0.05$ vs. saline solution; $^{\varphi}$ $p < 0.05$ vs. the same treatments between primary cortical neuronal cells and astrocytes in the same protocol.

Since an important contributing factor to brain aging is the exaggerated ROS production, additional experiments on ROS production were performed on both cell types following both protocols of treatments (Figure 2B). Results obtained from these experiments show that 1 pg/mL BDNF SKA was able to maintain ROS production within physiological range in both cortical neuronal cells and astrocytes ($p > 0.05$ vs. control). These data suggest that BDNF SKA is able to maintain the redox balance even in monolayer cultured cells. Although 50 ng/mL BDNF also shows similar properties, the effect was significantly lower compared to 1 pg/mL BDNF SKA.

Basing on previous observation of viability and ROS production in astrocytes, the involvement of BDNF solutions in cell proliferation was also investigated by crystal violet staining. As reported in Figure 2C, 1 pg/mL BDNF SKA treatment was able to increase the proliferation of astrocytes ($p < 0.05$) compared to control (about 43.9% and 13.4%, respectively) and to saline solution (about 43.5% and 29.6%, respectively). In addition, the importance of the concentration used was confirmed as well. Indeed, 1 pg/mL BDNF SKA is more effective than 50 ng/mL BDNF (about 3.1%).

3.4. Intracellular Pathways Activated by BDNF SKA on Monolayer Culture

Since all results reported above show a better influence in astrocytes compared to the same protocol on neurons, the intracellular pathways involved were investigated only on astrocytes.

In this phase of the study, the intracellular pathways involved in the previously observed effects were studied. The effects induced by 1 pg/mL BDNF SKA on ApoE expression, SIRT1 phosphorylation, ERK/MAPK pathway and the levels of activation of the BDNF receptor, TrkB, were studied.

As reported in Figure 3A, BDNF solutions seemed to act by the TrkB receptor. No significant differences were observed between 50 ng/mL BDNF and 1pg/mL BDNF SKA, indicating the ability of 1 pg/mL BDNF SKA to lead TrkB receptor to exert its effects despite the low dose used. As far as the ApoE expression is concerned, was significantly increased by 1 pg/mL BDNF SKA ($p < 0.05$) compared to 50 ng/mL BDNF (about 80%) (Figure 3B). As illustrated in Figure 3C, phosphorylation of SIRT1 induced by 1 pg/mL BDNF SKA ($p < 0.05$) was increased compared to 50ng/mL BDNF (about 30%), supporting the efficacy of the dosage to increase the presence of this molecule.

Finally, as shown in Figure 3D, BDNF has been observed to increase the ERK1/2 expression and the greater effect was obtained with 1 pg/mL BDNF SKA ($p < 0.05$) compared to 50 ng/mL BDNF (about 37%).

Additional experiments were performed to confirm the involvement of the TrkB receptor in previously observed effects, using a pre-treatment with the selective TrkB antagonist ANA-12 (1 μg/mL) on astrocytes. As reported in Figure 4, in the presence of both BDNF solutions, TrkB expression was abolished by the pre-treatment with 1 μg/mL ANA-12, confirming that both BDNF solutions acted through the TrkB receptor to explain their effects on astrocytes. These data confirm the importance of the dosage and the protocol of treatment to obtain a beneficial effect on astrocytes under physiological conditions.

3.5. Effects of BDNF Solutions Under Oxidative Conditions

Cell viability and ROS production were evaluated in cortical neuronal cells and astrocytes in order to understand the potential aging-prevention mechanism of 1 pg/mL BDNF SKA and 50 ng/mL BDNF 50 ng/mL under oxidative conditions. Exposure to 200 μM H_2O_2, in both cell types significantly reduced ($p < 0.05$) cell viability compared to control (Figure 5A), indicating cell loss caused by oxidative injury. Conversely, following post-treatment with 1 pg/mL BDNF SKA and 50 ng/mL BDNF, cell viability increased in a different manner between cell types. Indeed, only in astrocytes did both BDNF solutions significantly increase cell viability ($p < 0.05$), but the main effect was observed with 1 pg/mL BDNF SKA in both cell types ($p < 0.05$ vs. H_2O_2 alone), confirming the importance of doses and posology also under pathological conditions.

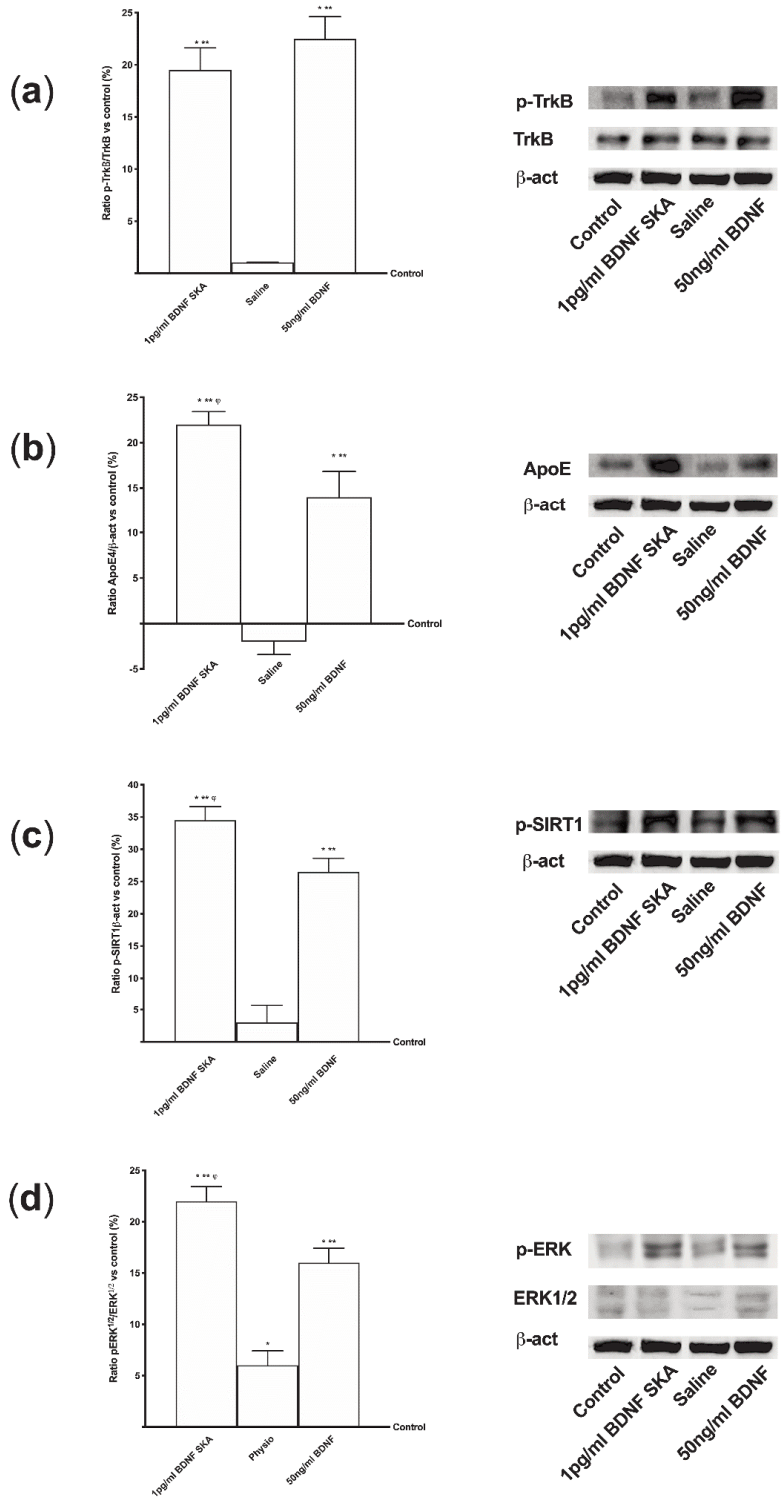

Figure 3. Analysis of intracellular pathways activated by BDNF solutions in astrocytes. In the left column densitometric analysis and in the right the examples of Western blot are reported. (**a**) TrkB receptor, (**b**) ApoE(4), (**c**) SIRT1 and (**d**) ERK/MAPK expressions are shown. Data are expressed as means ± SD (%) of five independent experiments normalized on specific total protein if possible and verified by β-actin detection. * $p < 0.05$ vs. control; ** $p < 0.05$ vs. saline solution; $^{\varphi}$ $p < 0.05$ vs. 50 ng/mL BDNF.

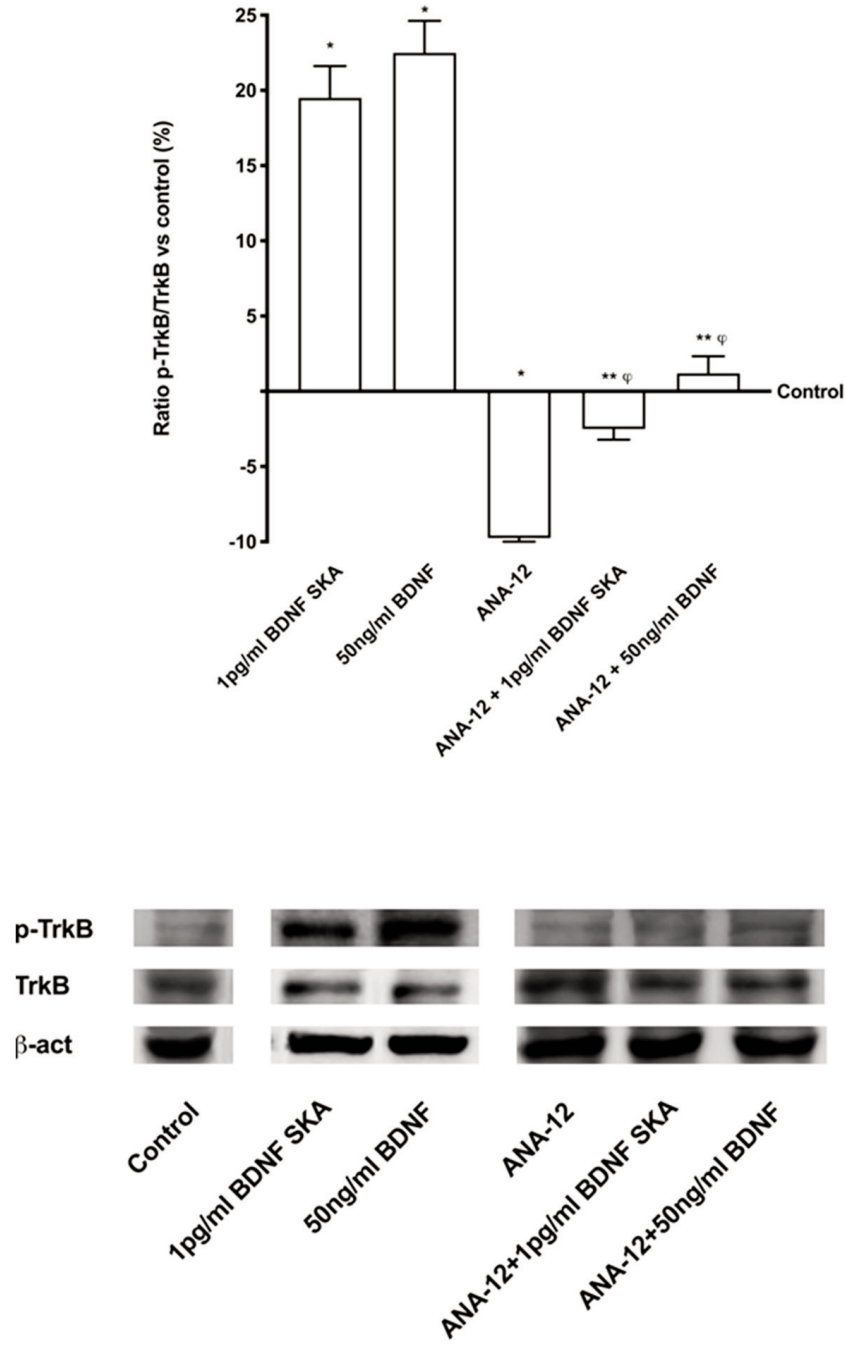

Figure 4. Analysis of TrkB receptor under blocking condition on astrocytes. In the upper panel densitometric analysis and in the lower panel an example of Western blot is reported. Data are expressed as means ± SD (%) of five independent experiments normalized on specific total protein and verified by β-actin detection. ANA-12 = 1 μg/mL ANA-12. * $p < 0.05$ vs. control; ** $p < 0.05$ ANA-12; $^{\varphi}$ $p < 0.05$ vs. the same treatments without ANA-12.

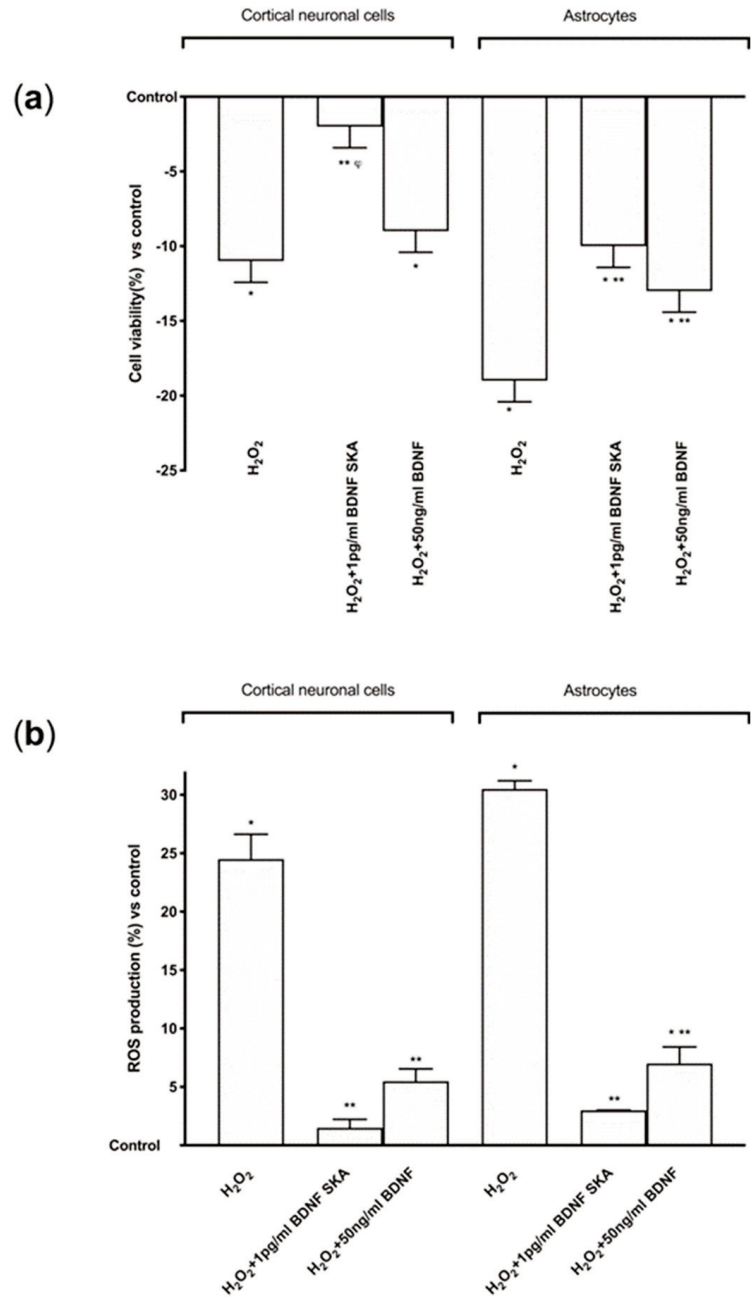

Figure 5. Cell viability and ROS production on both protocols in primary cortical neuronal cells and astrocytes under oxidative condition. (**a**) Cell viability and (**b**) ROS production measured in primary cortical neuronal cells (on the left) and astrocytes (on the right) after treatments. H_2O_2 = 200µM H_2O_2 added 30 min before stimulations. Data are expressed as means ± SD (%) of five independent experiments performed in triplicates normalized to control (0 line as control). * $p < 0.05$ vs. control; ** $p < 0.05$ vs. H_2O_2; $^{\varphi}$ $p < 0.05$ vs. H_2O_2+50 ng/mL BDNF in the same cells.

Additional experiments on ROS production were performed. Exposure of cortical neuronal cells and astrocytes to 200 µM H_2O_2 significantly increased the intracellular ROS production compared to control ($p < 0.05$), as illustrated in Figure 5B, confirming the presence of oxidative damage. Post-treatment with 1 pg/mL BDNF SKA and 50 ng/mL BDNF in both cell types caused a significant reduction of ROS production ($p < 0.05$) compared to H_2O_2 alone, supporting the hypothesis of the importance of BDNF during degeneration to prevent cell loss. These data suggest that 1 pg/mL BDNF

SKA is able to counteract the damage induced by oxidative stress with greater effectiveness compared to 50 ng/mL BDNF.

Since the data obtained from the two cell types were comparable, the analysis of intracellular pathways under oxidative conditions was conducted only on astrocytes. BDNF solutions exerted their biological actions by improving TrkB receptor expression even in the presence of H_2O_2, as shown in Figure 6A, ($p < 0.05$ versus control). No significant changes were observed between the two BDNF solutions. Moreover, the expressions of ApoE and of Tau, an important protein that modulates the stability of axonal microtubules, were analyzed. As reported in Figure 6B, the stimulation with H_2O_2 alone caused a significant decrease of ApoE expression compared to control ($p < 0.05$), indicating a loss of neuroplasticity. Conversely, the treatments with 1pg/mL BDNF SKA and 50 ng/mL BDNF repaired the damage and the expression of ApoE was increased. In particular the main effect was observed by 1 pg/mL BDNF SKA compared to 50 ng/mL BDNF ($p < 0.05$, about ten fold larger). The presence of the damage was confirmed by Tau phosphorylation, as reported in Figure 6C, where the level was even higher than control ($p < 0.05$). After treatment with 1 pg/mL BDNF SKA compared to 50 ng/mL BDNF the phosphorylation was significantly reduced ($p < 0.05$, about 56%), indicating the efficacy of 1 pg/mL BDNF SKA to restore damage. In addition, the analysis of SIRT1 confirms the protection exerted by 1 pg/mL BDNF SKA and 50 ng/mL BDNF against H_2O_2 damage (Figure 6D); 1 pg/mL BDNF SKA and 50 ng/mL BDNF were able to induce a significant increase in SIRT1 phosphorylation compared to H_2O_2 alone ($p < 0.05$) and to control ($p < 0.05$). However, the main effect was shown by 1 pg/mL BDNF SKA compared to 50ng/mL BDNF (about 85%). All these findings support the hypothesis that treatment with BDNF SKA can protect neuronal cells from the damage induced by the aging process better than high dose BDNF. Finally, as reported in Figure 6E, the activation of TrkB induced cells' survival by the involvement of ERKs/MAPK; indeed, 1 pg/mL BDNF SKA and 50 ng/mL BDNF added after the injury were able to induce a significant increase on ERK activity compared to H2O2 alone ($p < 0.05$) and to control ($p < 0.05$). However, the main effect was observed in presence of 1 pg/mL BDNF SKA compared to 50 ng/mL BDNF (about 90%).

3.6. Daily Duration of the Effects of BDNF Solutions on Neurons and Astrocytes

Since BDNF can be used as a dietary supplement in humans, some experiments were carried out to better clarify the optimal dosing schedule. The effects of 1 pg/mL BDNF SKA and 50 ng/mL BDNF during 24 h on both cell types were studied analyzing BDNF concentration, cell viability and mitochondrial potential.

As reported in Figure 7A, treatments with 1 pg/mL BDNF SKA and 50 ng/mL BDNF on both cell types caused a similar time-dependent increase in BDNF concentration. This effect showed significance from 1 h, compared to control ($p < 0.05$), and the maximum effect was observed at 24 h (23% and 22% compared to control, respectively, in neurons; about 26% and 25% compared to control, respectively, in astrocytes). No significant changes between the two BDNF solutions were observed, indicating the comparable effectiveness of low dose SKA to high-concentration BDNF.

However, 1 pg/mL BDNF had better tolerability, demonstrated by better cell viability ($p < 0.05$) compared to 50 ng/mL BDNF. This effect was significant starting from 6 h in neurons and 3 h in astrocytes, with a maximum effect on both types of cells at 24 h (about 80% and about 60% vs. 50 ng/mL, respectively), as shown in Figure 7B.

Since cell viability depends on mitochondrial activity, the analysis of mitochondrial potential variation was performed (Figure 7C). Both BDNF solutions modulated mitochondrial potential in a time-dependent manner with a significant increase from 3 h in neurons and from 30 min in astrocytes compared to control ($p < 0.05$). However, no significant changes were observed between the two BDNF solutions.

These results therefore allow us to state that low dose BDNF SKA administration has more beneficial effects on neurons and astrocytes than high dose BDNF, prolonged over time to cover 24 h.

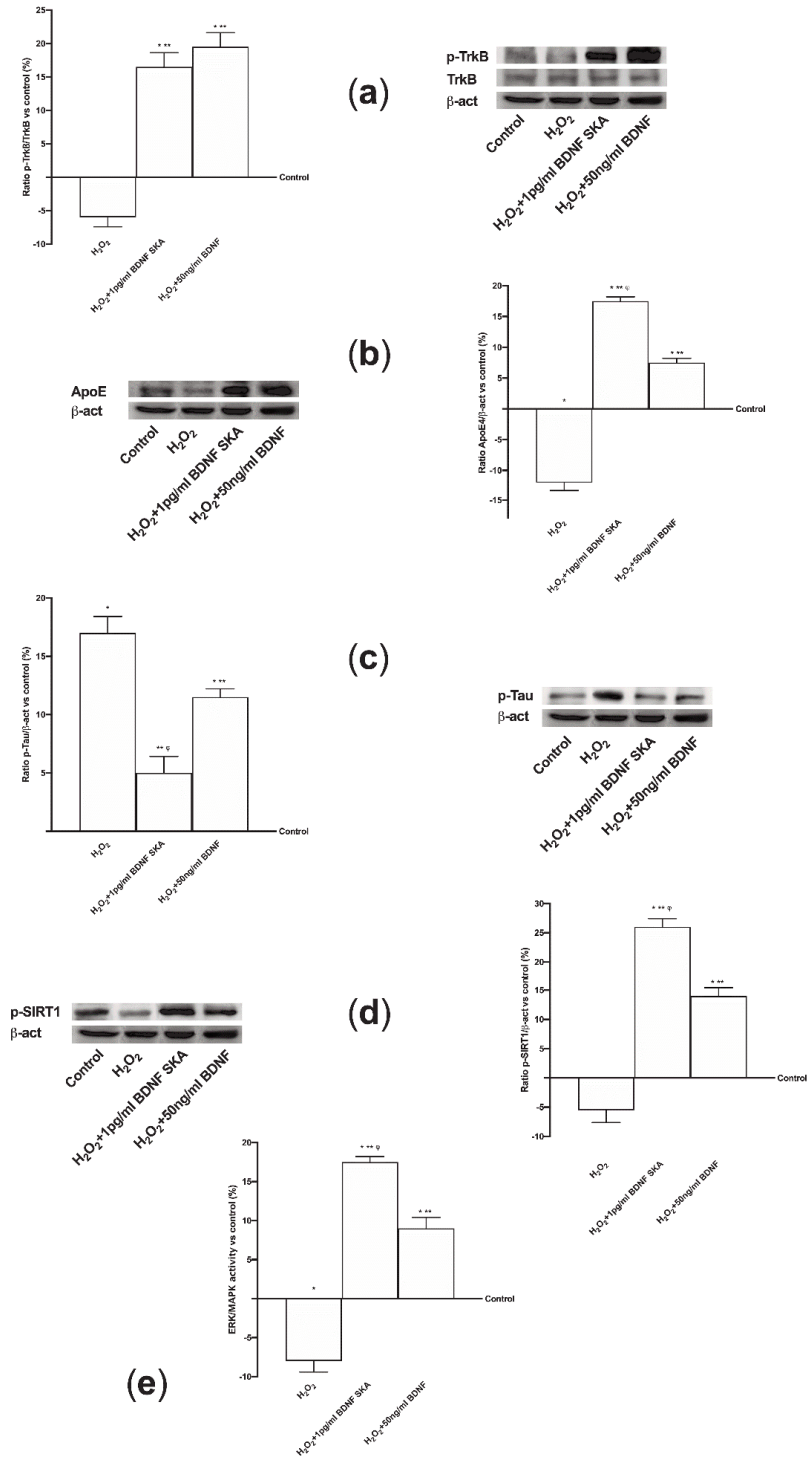

Figure 6. Analysis of intracellular pathways activated by BDNF solutions in astrocytes under oxidative condition. Kinase activity, densitometric analysis and Western blot are reported. (**a**) TrkB receptor, (**b**) ApoE(4), (**c**) Tau, and (**d**) SIRT1 expressions and (**e**) ERK/MAPK activity. Data are expressed as means ± SD (%) of five independent experiments and the densitometric analyses are normalized on specific total protein if possible and verified by β-actin detection. * $p < 0.05$ vs. control; ** $p < 0.05$ vs. H_2O_2; $^{\varphi}$ $p < 0.05$ vs. H_2O_2+50 ng/mL BDNF.

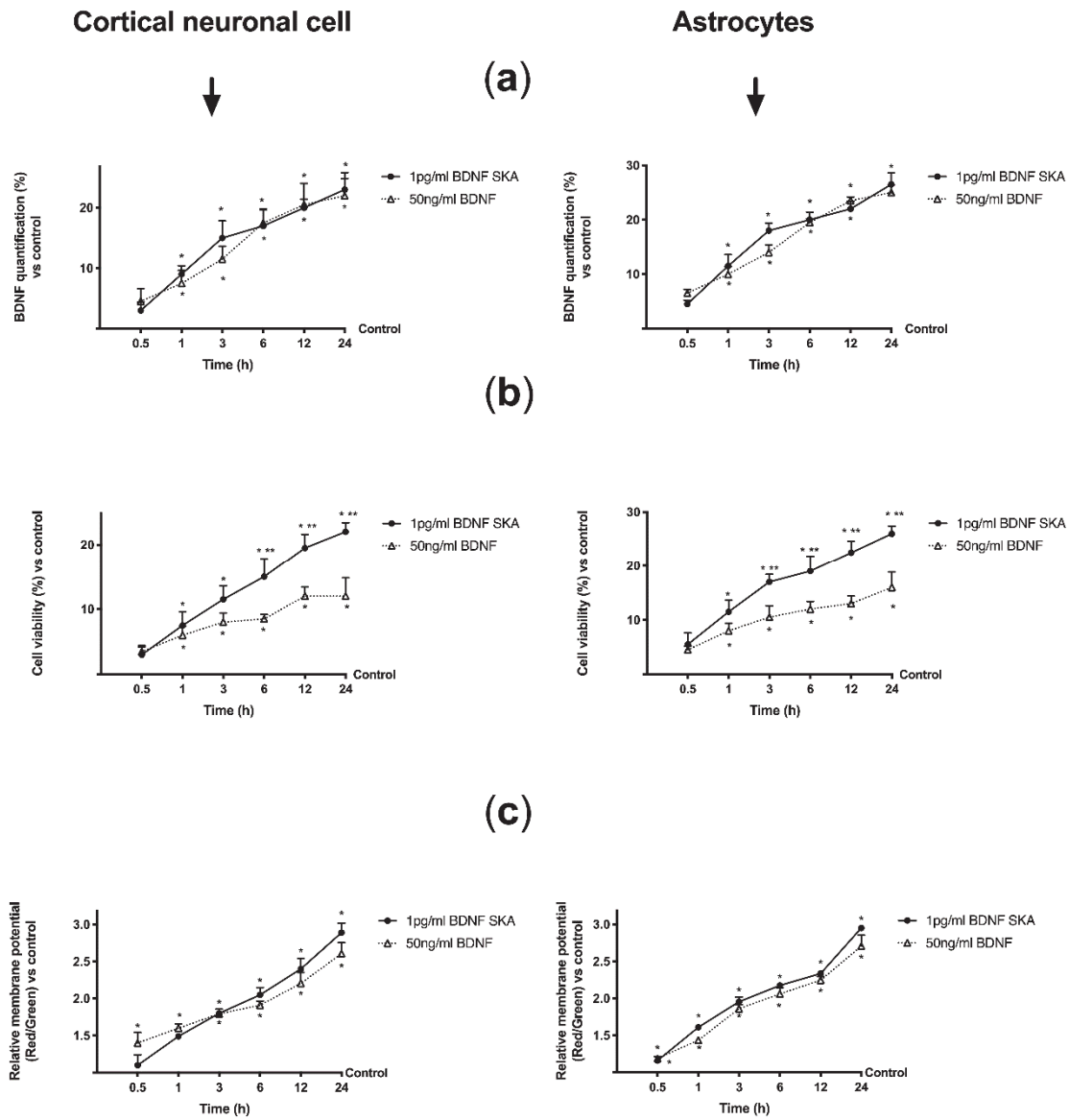

Figure 7. Effects of BDNF solutions within 24h on primary cortical neuronal cells and astrocytes. In the left column primary cortical neuronal cells and in the right column astrocytes are shown. (**a**) BDNF quantification and (**b**) cell viability measured after BDNF treatments. Data are expressed as means ± SD (%) of five independent experiments performed in triplicates normalized to control (0 line as control). (**c**) The mitochondrial membrane potential is investigated in the same condition. Data are expressed as means ± SD of five independent experiments performed in triplicates normalized to control (1 line as control). * $p < 0.05$ vs. control; ** $p < 0.05$ between 1 pg/mL BDNF SKA and 50 ng/mL BDNF.

3.7. Analysis of Bioavailability of BDNF Solutions and Their Effects in Mouse Brain

To confirm the ability of BDNF solutions to cross enterohepatic circle and the blood–brain barrier and act in brain tissue, some experiments were performed in vivo using wild type C57BL mice. Since in humans the administration would be daily, in some experiments 1.2 pg/mL BDNF SKA and 25 ng/mL BDNF were administered to animals by rissoles and BDNF concentration in both serum and brain tissues were analyzed after 24 h. In addition, to verify the stability of the effects, additional experiments were carried out adding 24h without stimulations. As reported in Figure 8A, 1.2 pg/mL BDNF SKA had a greater ability to get through the enterohepatic circle compared to 25 ng/mL BDNF (about 43%) and to control ($p < 0.05$) at 24 h. Moreover, 1.2 pg/mL BDNF SKA tended to remain in blood circulation longer (at least 24 h longer), compared to 25 ng/mL BDNF (about 68%). Since BDNF is present in blood,

it is important to verify its presence also in brain tissue (Figure 8B). In administration of both 1.2 pg/mL BDNF SKA and 25 ng/mL BDNF, it was able to enter the brain, as illustrated by BDNF quantification analysis ($p < 0.05$ vs. control). In addition, 1.2 pg/mL BDNF SKA was able to remain for a longer time (24 h plus 24 h) in brain tissue compared to 25 ng/mL BDNF (about 55%, $p < 0.05$) and 1.2 pg/mL BDNF SKA at 24 h (about 20%, $p < 0.05$). These findings demonstrate the importance of doses and posology of administration of BDNF SKA to induce a better influence on brain tissue.

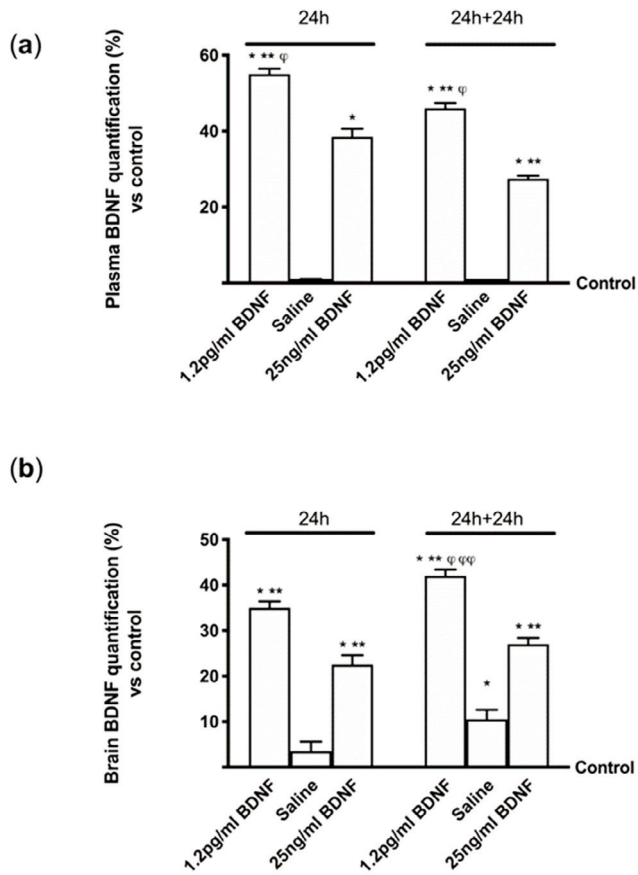

Figure 8. BDNF quantification in mice in serum and brain tissue. (**a**) Serum quantification and (**b**) BDNF quantification in brain tissue are reported. Each graph contains on the left the results obtained at 24h (12 animals) and on the right (12 animals) at 24 h plus 24 h (24 h+24 h). Data are expressed as means ± SD (%) normalized to control (0 line as control). * $p < 0.05$ vs. control; ** $p < 0.05$ vs. saline solution; $^{\varphi}$ $p < 0.05$ vs. the same treatments in the same time of administration; $^{\varphi\varphi}$ $p < 0.05$ vs. the same treatments at 24 h and 24 h plus 24 h.

To verify whether the mechanism activated by BDNF solutions is the same as the one observed in cells during in vitro experiments, the effects of 1.2 pg/mL BDNF SKA and 25 ng/mL BDNF on some main markers were investigated by Western blot. Since BDNF is necessary for survival of neurons in the brain, after encoding by this gene its expression was investigated, as reported in Figure 9A. 1.2 pg/mL BDNF SKA and 25 ng/mL BDNF both at 24 h and 24 h plus 24 h were able to induce the expression of BDNF compared to control ($p < 0.05$), indicating a better influence of stimulations. Moreover, 1.2 pg/mL BDNF SKA at 24 h and 24 h plus 24 h caused a significant increase compared to and 25 ng/mL BDNF (about 50% and about 62%, respectively), indicating the induction of endogenous production of BDNF by physiological mechanism, as shown by the significant increase induced by 1.2 pg/mL BDNF SKA at 24 h plus 24 h with respect to at 24 h ($p < 0.05$, about 24%).

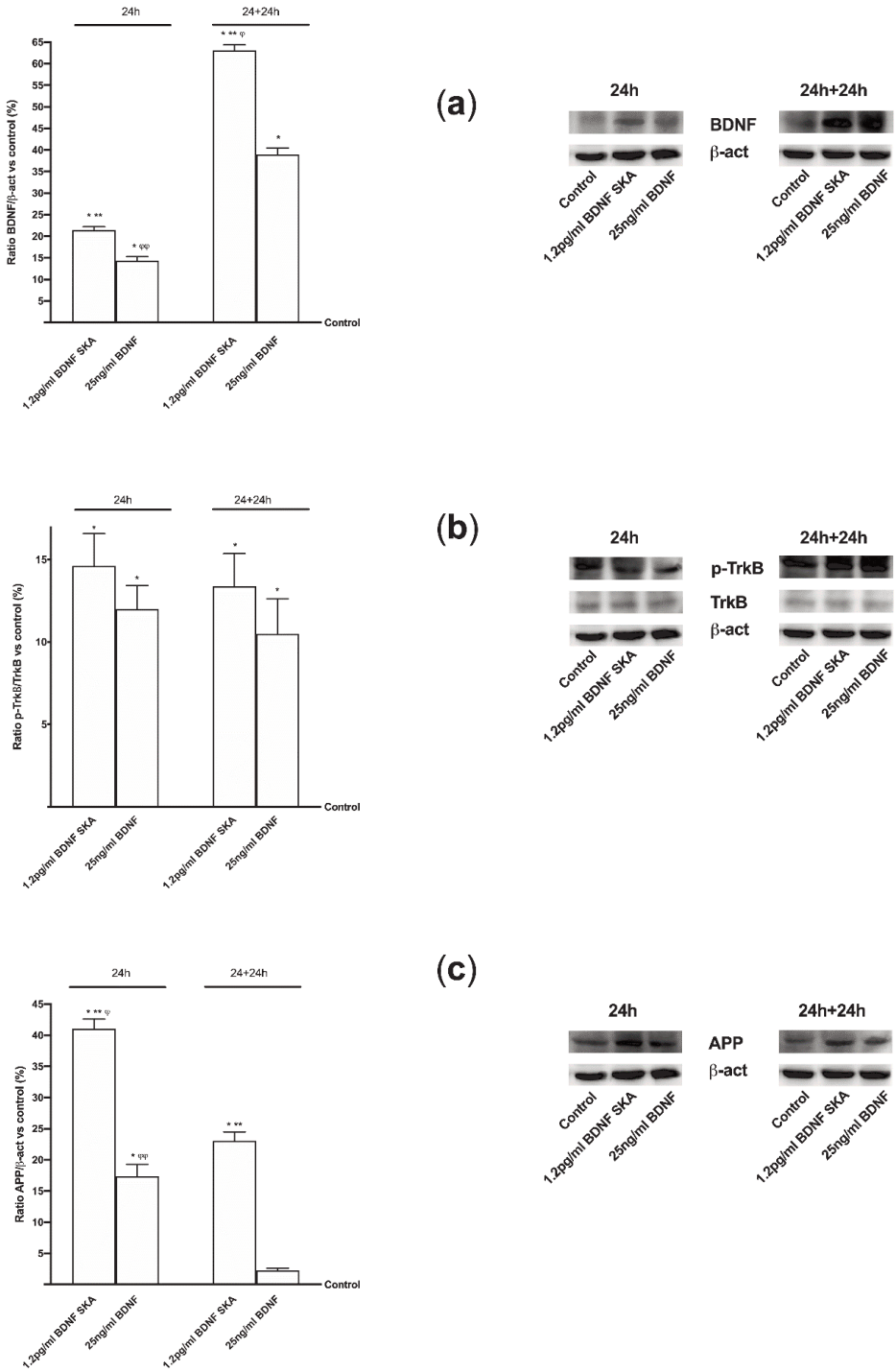

Figure 9. Western blot and densitometric analysis of BDNF protein (**a**), TrkB (**b**) receptor and (**c**) APP protein expressions in brain tissue. In the left column densitometric analysis and in the right the examples of Western blot are reported. Each graph contains on the left the results obtained at 24 h (12 animals) and on the right (12 animals) at 24 h plus 24 h (24 h+24 h). Data are expressed as means ± SD (%) of independent experiments normalized on specific total protein if possible and verified by β-actin detection. * $p < 0.05$ vs. control; ** $p < 0.05$ vs. 25 ng/mL BDNF at the same time of administration; $^{\varphi}$ $p < 0.05$ vs. 1.2 pg/mL BDNF SKA between 24 h and 24 h plus 24 h; $^{\varphi\varphi}$ $p < 0.05$ vs. 25 ng/mL BDNF between 24 h and 24 h plus 24 h.

These effects were mediated by the TrkB receptor, which was expressed in a similar manner in both times of treatment between 1.2 pg/mL BDNF SKA and 25 ng/mL BDNF ($p < 0.05$ vs. control, Figure 9B).

Since β-Amyloid precursor protein (APP) plays a central role, the beneficial effects exerted by both BDNF solutions were also assessed by the quantification of APP, as shown in Figure 9C. 1.2 pg/mL BDNF SKA and 25 ng/mL BDNF increased APP compared to control ($p < 0.05$) at 24h and 1.2 pg/mL BDNF SKA seemed to have a greater effect compared to 25 ng/mL BDNF (about 2.5 times). In addition, the APP activity at 24 h plus 24 h demonstrated the physiological action of 1.2 pg/mL BDNF SKA compared to 25 ng/mL BDNF ($p < 0.05$), indicating a better regulation exerted by 1.2 pg/mL BDNF SKA on central nervous tissue.

Moreover, since in vivo and in vitro studies suggested that ApoE may drive neurodegeneration through an Aβ-dependent mechanism, ApoE expression was assessed as well. As reported in Figure 10A, a significant increase of ApoE expression compared to control ($p < 0.05$) was observed in the presence of both 1.2 pg/mL BDNF SKA and 25 ng/mL BDNF in both time points, indicating a positive effect of BDNF on central nervous tissue. Moreover, the activation of TrkB by 1.2 pg/mL BDNF SKA and 25 ng/mL BDNF was able to induce a significant increase in ERKs expression compared to control ($p < 0.05$) in both time points. Therefore, a potential role of BDNF in tissue recovery through the involvement of ERKs/MAPK (Figure 10B) can be hypothesized. These findings support what was observed in astrocytes. However, the main effect was observed at 24 h in the presence of 1.2 pg/mL BDNF SKA compared to 25 ng/mL BDNF ($p < 0.05$, about three fold) and to 1.2 pg/mL BDNF SKA at 24 h plus 24 h ($p < 0.05$, about 30%). The last test of this series of experiments concerned the study of the expression of SIRT1. The analysis of SIRT1 confirms the beneficial effects exerted by 1.2 pg/mL BDNF SKA and 25 ng/mL BDNF (Figure 10C). In both 24 h and 24 plus 24 h, 1.2 pg/mL BDNF SKA and 25 ng/mL BDNF were able to induce a significant increase on SIRT1 phosphorylation compared to control ($p < 0.05$). However, the main effect was shown by 1.2 pg/mL BDNF SKA on both time points compared to 25 ng/mL BDNF (about 70% and 73%, respectively, $p < 0.05$). All these findings support the hypothesis that treatment with BDNF SKA can induce physiological mechanisms potentially able to slow down degeneration and protect brain during time.

3.8. Effects of BDNF Solutions in Mouse Brain During Time

To verify whether the efficacy of BDNF SKA was maintained for a long time, the mice were treated following the 6-day protocol previously used in cells experiments. As reported in Figure 11A, the administration of 1.2 pg/mL BDNF SKA and 25 ng/mL BDNF maintained the serum BDNF levels up to six days compared to control ($p < 0.05$). Besides, 1.2 pg/mL BDNF SKA was able to maintain high BDNF level compared to 25 ng/mL BDNF (about two fold higher). Similarly, the administration of 1.2 pg/mL BDNF SKA demonstrated better effectiveness ($p < 0.05$) compared to 25 ng/mL BDNF (about 80%) at brain tissue level (Figure 11B). These data suggest that BDNF SKA tends to remain present for a long time in brain tissue even in the absence of treatment, by triggering its physiological production better than BDNF at a high dose.

To confirm this, some additional experiments were performed to analyze BDNF protein (Figure 11C) and TrkB receptor by Western blot (Figure 11D). Both proteins show a significant increase at six days after both BDNF solutions, but 1.2 pg/mL BDNF SKA exerted a significant increase compared to 25 ng/mL BDNF (about two fold higher for each one, respectively, $p < 0.05$). These findings support the hypothesis of a fine endogenous regulation exerted by BDNF SKA on brain maintenance and function.

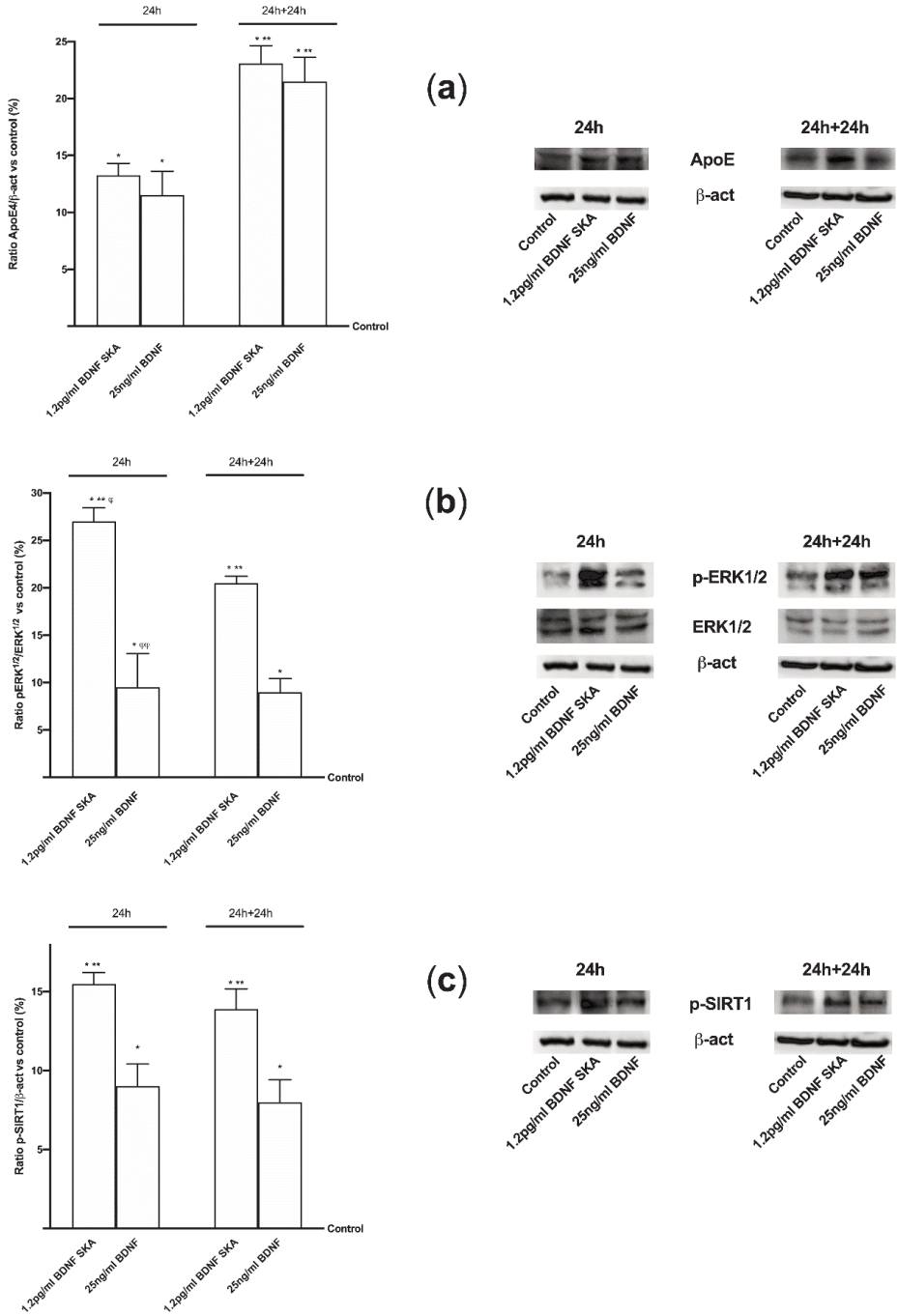

Figure 10. Western blot and densitometric analysis of ApoE (**a**), ERK/MAPK (**b**,**c**) SIRT1 expressions in brain tissue. In the left column densitometric analysis and in the right the examples of Western blot are reported. Each graph contains on the left the results obtained at 24 h (12 animals) and on the right (12 animals) at 24 h plus 24 h (24 h + 24 h). Data are expressed as means ± SD (%) of independent experiments normalized on specific total protein if possible and verified by β-actin detection. * $p < 0.05$ vs. control; ** $p < 0.05$ vs. 25 ng/mL BDNF at the same time of administration; $^{\varphi}$ $p < 0.05$ vs. 1.2 pg/mL BDNF SKA between 24 h and 24 h plus 24 h; $^{\varphi\varphi}$ $p < 0.05$ vs. 25 ng/mL BDNF between 24 h and 24 h plus 24 h.

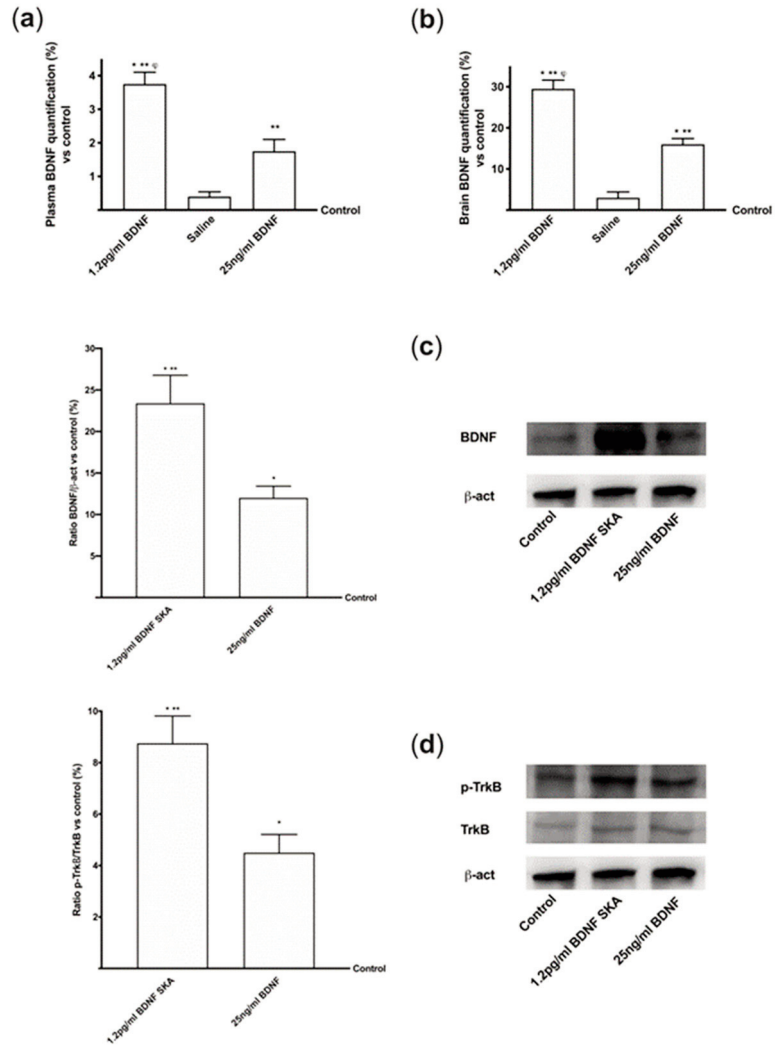

Figure 11. BDNF quantification and intracellular pathways in brain measured in the 6-dayprotocol. (**a**) Serum and (**b**) brain tissue BDNF quantification in mice. Data are expressed as means ± SD (%) of $n = 7$ independent experiments normalized to control value (0 line). * $p < 0.05$ vs. control; ** $p < 0.05$ vs. saline solution; $^{\varphi}$ $p < 0.05$ vs. 25ng/mL BDNF. (**c**) BDNF and (**d**) TrkB receptor expressions in mice brain reported as densitometric analysis (on the left) and examples of Western blot (on the right). Data are expressed as means ± SD (%) of $n = 7$ independent experiments normalized on specific total protein if possible and verified by β-actin detection. * $p < 0.05$ vs. control; ** $p < 0.05$ vs. 25 ng/mL BDNF.

4. Discussion

Today neurodegenerative disorders are considered chronic and incurable conditions, whose disabling effects can last for years or decades. This represents a huge burden of suffering for patients and costs for health organizations. Optimal cognitive functions are linked to an efficient neuronal plasticity and the ability of neurons or glial cells to improve the efficacy of the synapses through biochemical and morphological changes, both at a dendritic and axonal level [44]. However, as reported by many studies, this ability shows a marked age-related decrease [15]. At present, treatments available for these diseases are mostly symptomatic or palliative and include neurotransmitter modulators, hormonal therapies, anti-inflammatory drugs, deep brain stimulation and herbal products. Therefore, there is an urgent need to develop new solutions able to restore the physiological functions of the brain tissue. Moreover, one of the main problems concerning the administration of active ingredients into the central nervous system is the crossing of the blood–brain barrier. Modern drug delivery systems can consist both of biodegradable and non-biodegradable formulations, which offer advantages in

terms of protection, absorption, penetration and distribution of active ingredients. For this reason, the use of molecules already known for their exclusive functions within the brain and consequently physiologically predisposed to easily cross blood–brain barrier can be considered a valid option.

Recently, researchers' attention has focused on the involvement of the neurotrophic factors in the development of neuronal decay. Currently, it is common knowledge that there are three main neurotrophic factors: the brain-derived neurotrophic factor (BDNF), the nerve growth factor and the glial cell-derived neurotrophic factor [45]. BDNF in particular is associated with the modulation of neuroplasticity, which promotes the health of nervous tissue and also has the ability to counteract the effects of pro-inflammatory cytokines, which are key factors in neurodegenerative processes [46].

In recent years, the possibility of using a growth factor therapy has been hypothesized by exploiting the growing information that research has accumulated, mainly on BDNF. Indeed, BDNF seems to have a real therapeutic potential, based on the observation that in many disorders of the nervous system serum BDNF levels are altered [47]. However, a major problem is the delivery of the molecule to the affected cells. Although numerous studies have explored the possibility of administering BDNF through several approaches, such as gene therapy vectors, the development of mimetic peptides or even through direct administration into the nervous system, the results are still penalized by the lack of ease of use [47]. Attempts to orally administer BDNF have so far yielded poor results due to the fact that BDNF is a moderately sized and charged protein and its transport through the intestinal barrier and BBB is not clear [47].

The key idea of this work is to use a low dose BDNF solution to avoid the possible side effects of current therapies (such as sensitization and allergic reactions), supporting with experimental data a new potential therapeutic approach to treat or prevent neurodegenerative diseases. The concept of low-dose is an important and innovative aspect and has been shown to be effective in many studies. For example, in vivo treatment experiments with low-dose interferon were performed in many animal species [48]. This treatment has been shown to induce dramatic clinical improvement in models of both infectious and chronic inflammatory diseases [49].

This work demonstrated, in in vitro experimental models, that BDNF is able to cross both the intestinal and BBB barrier, thus demonstrating the safety of its use.

This study also demonstrates for the first time the efficacy of low-dose BDNF SKA in counteracting oxidative damage, which is one of the mechanisms underlying age-related neurodegeneration. The possibility of administering BDNF in very small amounts is a great advantage, both for the low risk of adverse effects and for the lower cost of treatment.

In the design of the study, it was decided to use a particular solution preparation technique, which is called SKA. It has been hypothesized that the mechanism of bioactive molecules, such as hormones, neuropeptides and growth factors, subjected to SKA and administered at low dosage, consists in the sensitization or activation of some cellular (or plasma) receptor units by virtue of their high dilution, and practically in their physiological working in the order of micrograms for hormones [50] and picograms for the other messenger molecules [51]. The SKA method has been used in previous works, which showed that SKA solutions have better biological effects than corresponding solutions that did not receive the same treatment [20,21,29]. In the present study, it has been shown that BDNF SKA does not induce neuronal stress and it is able to counteract the formation of ROS.

During brain aging, the cells that show the first signs of degeneration are the astrocytes, despite the fact that subsequently the most important site of damage is represented by cortical neurons [52]. BDNF SKA is able to increase cell viability in both neuronal cells and astrocytes, representing an important resource for the health of the nervous system.

Furthermore, the aim of this study was to explore the timing of administration, to see if it was possible to identify an effective protocol that could be used in humans in the future. It can be hypothesized that the six-day protocol, consisting of a single administration followed by six days of measurements, stimulates cells without overloading normal physiological regulation. In this way a greater crossing of BDNF is achieved through the BBB, also inducing a low concentration of ROS

and a reduced neuronal stress. In this way the normal cellular physiological processes are activated. The in vivo part of this research has allowed us to demonstrate that BDNF SKA has a high capacity to cross the enterohepatic circle and is able to remain in the bloodstream for at least 24 h. This makes the BDNF SKA a potential candidate for use as a food supplement. Since BDNF, necessary for the survival of neurons, is synthesized after the encoding of its specific gene, its expression was studied in this research. We observed that BDNF SKA at 24 h plus 24 h was able to induce the expression of endogenous BDNF, indicating a better effect of stimulation and induction of endogenous BDNF production. The beneficial effects of BDNF SKA have also been confirmed by the analysis of the amyloid protein precursor. Taken together, our results suggest that the simultaneous activation of at least ERK/MAPK is necessary to mediate a complete BDNF-dependent activation of the APP promoter [53].

APP protein plays a central role in the development of Alzheimer's disease; its expression, metabolism, splicing and secretion have been demonstrated to be regulated by ligands of the membrane tyrosine kinase receptors like BDNF [54]. Furthermore, the study of the intracellular pathways demonstrated a significant increase in the expression of ApoE, which is a member of the low-density lipoprotein receptor gene family, mainly produced by the astrocytes in the brain. ApoE has been identified as the receptor that mediates amyloid β (Aβ) uptake and clearance by astrocytes, thus increasing glial LDLR levels, which may promote Aβ degradation within the brain [55–57]; these data indicate a positive effect on brain trophism exerted by BDNF SKA and an increase in SIRT1 phosphorylation, confirming a potential role in counteracting the known mechanisms that lead to brain aging. Indeed, SIRT1 has recently been shown to play a role in normal cognitive function and synaptic plasticity, counteracting cognitive decline and neurodegenerative disease in aging [58,59]. The effectiveness of BDNF SKA has been carefully observed, allowing the serum BDNF levels to be maintained for up to six days after a single administration; these data suggest that BDNF SKA is able to remain in the nervous tissue for a long time even in the absence of treatment, triggering its own physiological production by cells better than high-dose BDNF. All our results support the hypothesis that treatment with BDNF SKA can protect the brain over time by inducing a physiological mechanism capable of slowing cell degeneration and may be a possible therapeutic strategy for the elderly population, in order to improve cognitive function. BDNF is also able to exert effects outside the nervous system, for example on the immune system [60]. Therefore, interactions between the immune system and the effects described in this work could be hypothesized. The lack of this part could be a weak point in our research, which will be filled in subsequent research. On the other hand, one of the strengths of this study is the confirmation of the efficacy and safety of low-dose BDNF. The low-dose administration reduces the side effects of active molecules, without reducing their effectiveness is an important and innovative aspect. Indeed, the common clinical practice has long understood the essential importance of small stimuli compared to strong ones, which trigger self-regulatory and self-repairing mechanisms in the organism [61]. In brain aging there is a decline in normal antioxidant defense mechanisms leading to increased brain vulnerability and finally to the deleterious effects of oxidative damage [8]. Indeed, a large body of experimental research indicates that the brain is very susceptible to oxidative damage due to a high concentration of polyunsaturated fatty acids and transition metals that are involved in the generation of the hydroxyl radical [62,63]. Members of the ROS family, if not properly detoxified, start the process of oxidative damage, which can be defined as a chain reaction leading to sequential damage of all cellular components, in particular of lipids and proteins [64]. Oxidative stress is considered one of the main mechanisms of cellular aging due to the ease of amplification of the damage and to the large number of target molecules [8]. Cognitive deficits are the most common consequences of the aging process and they are characterized by massive neuronal loss, cognitive dysfunction and memory loss. Their incidence and prevalence continuously increase with advancing age [65]. Indeed, it has been shown that low serum BDNF levels are linked to increased cognitive impairment [66]. Moreover, BDNF helps to protect neurons from damage caused by infection or injury [45] and participates in neuronal growth and maintenance and in different aspects of activity-dependent synaptic physiology by acting across different spatial and

temporal domains [67]. However, because of the difficulties associated with the administration of exogenous proteins into the central nervous system (CNS), it is important to consider the possibility of using endogenous sources of BDNF, for example, inducing increased glial cell activity. It is well known that glial cells increase expression of a variety of growth factors, including BDNF. In particular, in the adult brain, astrocytes are the cells responsible for maintaining neuronal and synaptic function [68].

5. Conclusions

In conclusion, this study demonstrates clear signs of the effectiveness of BDNF in cell models. Of course, it will be necessary, as a next step, to carry out a study on behavioral effects. If in this second phase the beneficial effects are also confirmed, it can be hypothesized that BDNF may in the future be a useful agent for modulating a damaged brain environment to improve recovery during demyelinating diseases and possibly other degenerative conditions.

Author Contributions: All authors have read and agree to the published version of the manuscript. Conceptualization, F.U. and V.M.; methodology, S.R. and F.N.; formal analysis, V.M., S.R. and R.G.; investigation, V.M.; resources, F.N.; data curation, R.S., F.U. and M.F.; writing—original draft preparation, F.U.; writing—review and editing, C.M. and F.U.; visualization, F.U.; supervision, C.M. and F.U.; project administration, F.U.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Method: Key Phases of Rissole Preparation

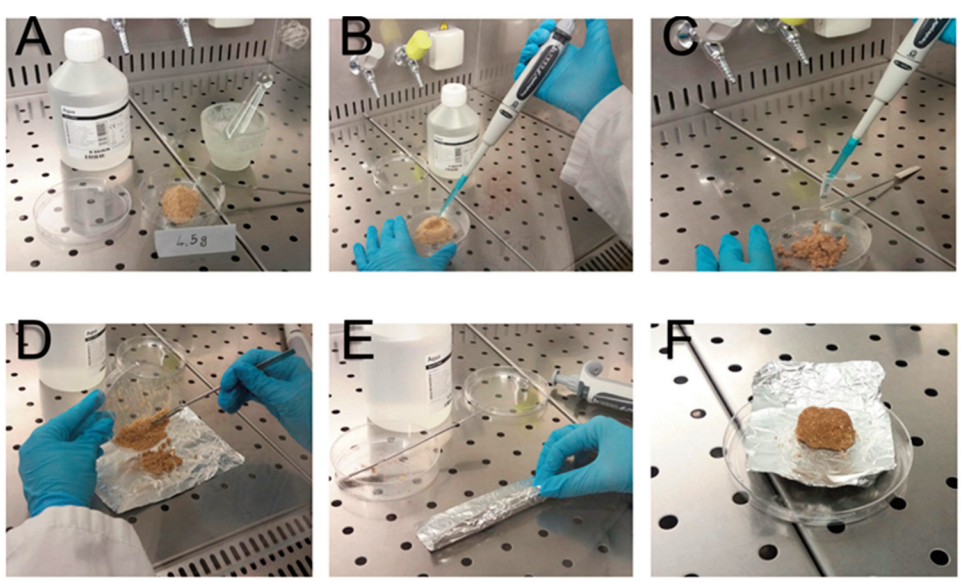

Figure A1. The rissoles were prepared in a sterile environment by pulverizing kibble food (Global Diet, Envigo plus) (**A**). Powder (4.5 g) of was mixed with 2.5 mL of sterile water (**B**) and 450 µl of BDNF solutions (**C**). Feed was prepared and kneaded under a hood (**D**) to form a rissole that was wrapped in a foil sterilized by heat (**E**). Soon after, the rissole was dried under the hood to maintain sterility for 18–24 h. At the end, the rissole (**F**) was placed in a cage with the mouse, which quickly ate it.

Appendix B

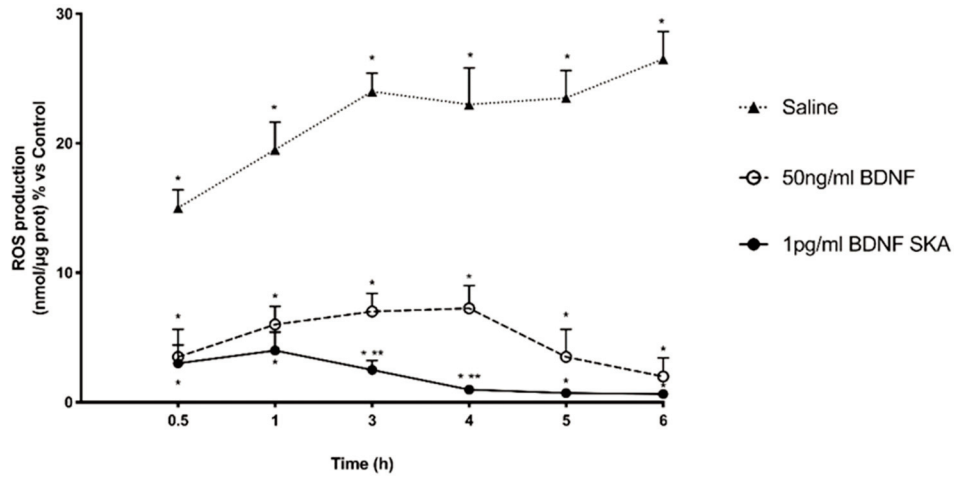

Figure A2. ROS production at intestinal barrier. Data are expressed as means ± SD (%) of five independent experiments performed in triplicates (0 line as control). Saline = saline solution. * $p < 0.05$ vs. control, ** $p < 0.05$ vs. 50 ng/mL BDNF.

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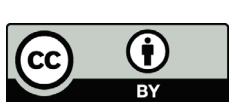


CLINICAL

C. Supino

LOW-DOSE BDNF AND SPECIFIC LEARNING DISABILITIES – A POSSIBLE INDICATION

SUMMARY

Specific Learning Disabilities (SLD) are conditions that present a discrepancy between the levels of academic performance and the potential deduced from the subject's actual intellectual abilities.
Learning disorders involve difficulty in concentration or attention, in language development, or in processing visual and auditory information.
Diagnosis includes intellectual, educational and language assessments as well as medical and psychological assessments.
Treatment consists, first of all, in educational management and in medical, behavioral and psychological therapy.
– In a group of 9 patients Guna-BDNF was added to these treatments with an evident improvement (+50%) vs the control group in test performance and an increase in self-esteem.

https://d2m3czf6fvb8bh.cloudfront.net/site_content/files/images/categories/children/dyslexia_in_children_750.jpeg

INTRODUCTION

The acronym **SLD** (Specific Learning Disabilities) refers to a diagnostic category regarding specific developmental learning difficulties pertaining to neurodevelopmental disorders according to the DSM 5 (1,2).

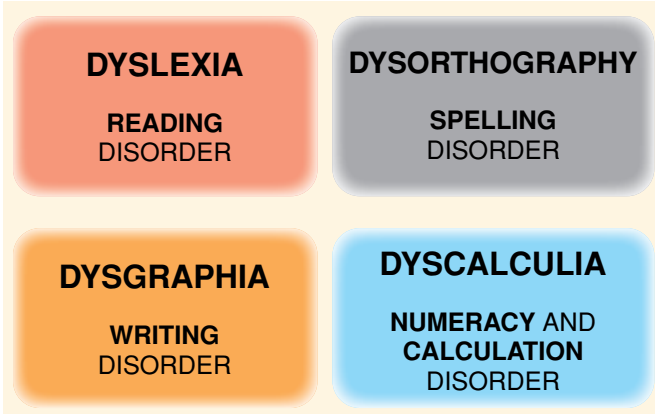
– Neurodevelopmental disorders are neurological conditions that present in early infancy, usually before the start of primary school.

SLDs impair personal, social, scholastic and/or professional development and entail difficulties in the acquisition, retention and application of skills or specific sets of information.
Although these disorders affect children and teenagers who do not usually present particular disabilities or difficulties, without adequate support, they can make scholastic activities difficult.

SLDs are, therefore, a series of disabilities that are relatively common during the developmental age, that can be attributed to a primarily constitutional neurobiological origin, and that regard the acquisition of scholastic skills, intended as tools that make it possible to obtain the formal knowledge proposed through educational processes.

– Each of these disorders concerns different functions and abilities: speech, motor skills, reading, writing, and arithmetic.
The characteristic that is common to them all is the specificity of the deficit, which can be attributed to consistent

FIG. 1



and recognisable areas that are independent of the subject's cognitive level.

– The term 'specific' regards the fact that the disability presents in an individual who does not present neurological conditions (e.g. epilepsy), or secondary defects (hearing or sight impairments), who is of adequate intelligence and does not present any particular cultural disadvantage conditions.

Depending on the type of difficulty, different conditions are identified regarding the specific skills of reading, intended as the ability to decode a text **(dyslexia)**, writing, intended as the ability of phonographic encoding and spelling **(dysorthography)**, graphomotor skills **(dysgraphia)** and arithmetic, disorder affecting numeracy and calculation skills, intended as the ability to understand and work with numbers **(dyscalculia)** (FIG. 1).

The Istituto Superiore di Sanità [Italian National Institute of Health] Consensus Conference (Cc-ISS, 2011) defines SLDs *"disorders that affect a specific area of abilities, without affecting general intellectual functioning. They involve the instrumental skills of scholastic learning"* (TAB. 1).

It is important to stress that children with SLDs are of normal or higher than normal intelligence and they find it easy to obtain an overview, to see the bigger picture.
They are able to grasp the fundamental elements of a discussion or situation, they reason in a dynamic manner and create unusual associations that others find it difficult to develop.
They learn readily from experience and tend to remember facts not in an abstract way but as life experiences, stories and examples.
They think primarily in images, visualising words and concepts in a three-dimensional manner, and memorise things far more readily by pictures.

– They are able *to see things* from different perspectives and they process information in a global manner rather than sequentially.

TAB. 1

SLDs involve a specific area of skills	Reading Spelling Writing Calculation
Intact general intellectual function	QI >70
SLDs involve the instrumental skills of scholastic learning	Compensatory tools

This matter has a considerable social importance, as SLDs are disorders that, from an epidemiological point of view, have an incidence in the general population of **2-3%** of all scholastic difficulties, in most subjects with non-specific learning disabilities or difficulties (about **20%**) (TABS. 2, 3).

At the current time, children and teenagers with SLDs are not entitled to a special needs teacher.
Pursuant to Law 170/2010 (TAB. 3), they are entitled to compensatory learning and technological aids (speech synthesis, recorders, word processing software and programmes with spelling correction functions, calculators) and dispensatory measures that allow them to replace certain types of assessment with equivalent, more suitable ones.

– An analysis of the available literature reveals that the disorders most commonly associated with LSDs are attention deficit and hyperactivity disorder (ADHD) and specific language impairment (SLI).
The 2007 Consensus Conference revealed that in clinical practice, there is a high presence of comorbidities among SLDs and between SLDs and other disorders (dyspraxia, behavioural and mood disorders, anxiety disorders, etc.).
– This high comorbidity results in a great diversity in the functional and expressive profiles with which SLDs present, which has considerable implications on the diagnostic investigation front (CC-2007) (FIG. 2).

DIAGNOSIS

Diagnosis can be difficult.
– As a matter of fact, until the diagnosis is clearly defined, the children, their parents and the school are confused regarding the poor scholastic performance, without understanding the reason for it.

In this initial stage, teachers tend to question the child's effort, and family

conditions, they complain of laziness and lack of commitment, and frequent problems regarding conduct in the classroom.
Teachers also encounter difficulties in understanding why the child, who does not appear to have any particular difficulties within his/her peer group, objects, refuses or is reluctant when asked to read and/or write (3).
– This generates confusion and disarray in the parents, who tend to alternate between strict and punitive behaviour with continuous encouragements to make a greater effort and long periods of waiting, hoping the situation will improve spontaneously.

During this phase, the child feels misunderstood by everyone and starts to question his/her own abilities, which in turn results in lower self-esteem, psycho-affective problems, a feeling of inferiority and even guilt, especially if he/she feels that judged to be lazy and unwilling.
– In these cases, the interpretations and actions of the adults tend to make matters worse.

When a SLD is diagnosed and if the disorder is not adequately treated, the psychological symptoms of the distress can take various, and sometimes opposing, forms: on the one hand the child may have a withdrawn attitude, be introverted and avoid confrontation; this set of reactions can be defined of a depressive or inhibitory type.
On the other hand, the child may demonstrate feelings of anger resulting in disruptive behaviour, challenging teachers and showing aggression towards academic staff and their peers, which inevitably triggers a vicious cycle within the class.

The same child can often present both types of behaviour at different times (4).

Statistically, the diagnosis is most often formulated by teachers at the end of the **second year** of **primary school**, due to the greater exposure to reading and writing; they then notify the parents and a diagnostic pathway is undertaken with the involvement of paediatric neuropsychiatry facilities (FIG. 3).
Primary-care paediatricians can also play a role in identifying a child with SLD by administering a checklist (TABS. 4, 5, 6).
SLDs affect males more commonly than females, with a ratio of **5:1**.

SLDs have a neurobiological origin.

In infants, the symptoms are practically inexistent, as SLDs affect cognitive areas that infants have not yet developed; warning signs may be observed in preschool children (e.g. speech problems or difficulties learning nursery rhymes).
– The disorder becomes fully evident in school-age children.

Although it is recognised that SLDs have a genetic cause, the cerebral processes involved are yet to be clearly defined, despite the active research in this field.
– The genetic origin is demonstrated by the high familiarity of SLDs; children of parents with SLDs are more likely to have the same disorder than children whose parents do not have SLDs.

Indeed, it is not uncommon for the parents of children with SLDs to report encountering the same difficulties as their child, although it is likely that no specific diagnosis was formulated at the time.
– The neurobiological origin underlying cognitive abnormalities is associated with behavioural symptoms of the disorder, which include an interaction between genetic, epigenetic and environ-

DISABILITIES law 104/1992
«Framework law regarding the assistance, social integration and rights of persons with disabilities»
Assessments are usually carried out by the Local Health Authorities, through medical commissions including a social worker and an expert on the cases to be reviewed, established within the various Local Health Authorities.

TAB. 2

SLDs law 170/2010
«New regulations for specific learning disabilities in the scholastic setting»
SLDs are diagnosed within the specialist care provided by the Italian National Health Service or accredited specialists or facilities.
Use of individualised and personalised teaching, with effective and flexible forms of schoolwork that also take into account the particular characteristics of the subjects involved.
– Incidence 2-3% of all disabilities.

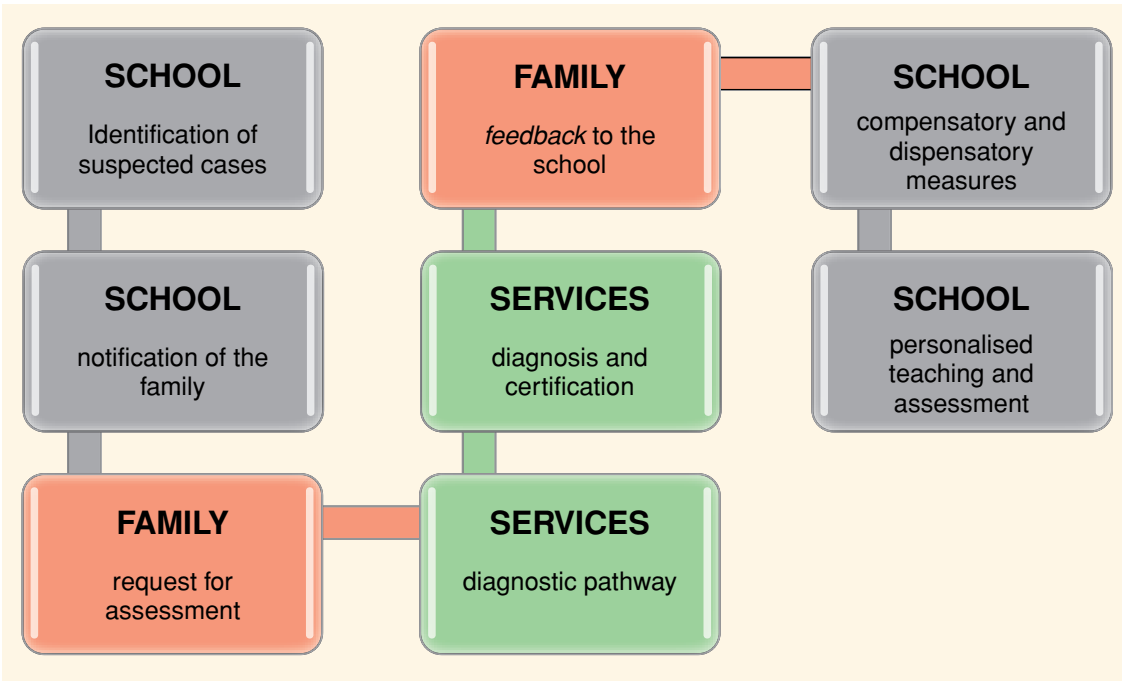
TAB. 3

FIG. 2

SLDs – COMORBIDITIES

OTHER NEUROPSYCHOLOGICAL DISORDERS (e.g. ADHD – Attention Deficit and Hyperactivity Disorder)	SEVERAL CONCOMITANT SLDs	OTHER MENTAL HEALTH DISORDERS (anxiety, depression, behavioural disorders)

FIG. 3



mental factors involving the cerebral capacity to perceive or process verbal or nonverbal information effectively and precisely (DSM-5, 2014).

Other potential causes include:

- maternal illness or substance abuse during pregnancy
- complications during pregnancy or delivery (e.g. blood loss or spotting, septicaemia, prolonged delivery or emergency delivery)
- neonatal problems (prematurity, low birth weight, severe jaundice, perinatal asphyxia, post-term birth, breathing difficulties).

Postnatal risk factors include **1)** exposure to environmental toxins (e.g. lead, heavy metals, pesticides, endocrine-disrupting chemicals), **2)** Central Nervous System infections, **3)** cancers and their treatments, **4)** trauma, **5)** malnourishment, **6)** severe social isolation, and **7)** affection deprivation.

It is important to remember that certain studies have identified a relationship between SLDs and **the dysfunctional aspects** of cerebral mechanisms that **do not** in any way affect intelligence.

When a psychological trauma interferes with normal pyschobiological development in children and adolescents, there is a shift from a brain (and body) focused on learning and a brain (and body) focused on survival.

- The **learning brain** is engaged in exploring (acquisition of new knowledge and of new neuronal synaptic connections), the **survival brain** tries to anticipate, prevent and protect against the damage caused by potential or actual dangers; it identifies threats and activates bodily resources to achieve hyperalertness and deploy defensive adjustments.
- The **survival brain** surrenders to rapid automatic processes that involve the more primitive parts of the brain [brainstem (especially the midbrain), limbic system structures, such as the amygdala], largely bypassing the areas of the brain involved in more complex adjust-

TAB. 4

PAEDIATRICIAN CHECKLIST
– PRE-SCHOOL AGE AND YEAR 1 OF PRIMARY SCHOOL

The **child**:

- struggles to understand verbal instructions and messages
- has difficulties expressing him/herself clearly when recounting an episode he/she was involved in or witnessed
- has difficulties making him/herself understood to strangers
- has difficulties holding pencils or pens
- struggles to draw a person whose head, body, arms and legs are recognisable
- is clumsy and lacks dexterity
- has difficulties perceiving new words and repeating them immediately after hearing them
- has difficulties understanding the quantities the numbers 1 to 4 correspond to; counting to 5; recognising which of two sets of objects (maximum of five objects) is larger and which is smaller.

Source: C. Toso, **2009**. Associazione Culturale Pediatri.

ments to the environment (anterior cingulate cortex, insula, prefrontal cortex, etc.) (5,6).

Prolonged corticosteroid activation and the hyperadrenergic state induced by constant alarm states result in the inhibition of neurogenesis, thereby hindering dendritic development and the formation of synapses, they induce "pruning" actions on existing nervous connections and induce cell death processes that result in the shrinking of the hippocampus (7).

As mentioned previously, the neurobiological bases of SLDs have been consolidated.
In the case of **dyslexia**, the best known of these disorders, the neurobiological origin was already suspected more than a century ago.
– In 1891-2, the French neurologist Jules Déjerine (8,9) suggested that the reading problems, defined by Hinshelwood (10) as "reading blindness", were due to anatomical lesions present in the left posterior region of the brain, which plays a critical role in reading processes.
The decisive turning point in the knowledge of the pathogenetic bases of dyslexia came with the advent of dynamic neuroimaging techniques such as PET or Functional Magnetic Resonance.
– These methods are able to show changes in the activation of cerebral areas as a consequence of given tasks and – therefore – make it possible to define the differences in functioning of areas of the cerebral cortex in dyslexic individuals.

It is known that both acute and chronic stress reduce the production of **BDNF** - Brain Derived Neurotrophic Factor in the hippocampus, where they also cause structural changes and neuronal damage, (11,12) and that BDNF tends to diminish in old age (13).

– Furthermore, BDNF protects against the toxicity of certain substances, by increasing the production of **glutathione reductase** (14).

PAEDIATRICIAN CHECKLIST
– HALFWAY THROUGH YEAR 1 AND YEAR 2 OF PRIMARY SCHOOL

The **child**:
- has not yet learned to read simple words (year 1) or sentences and short passages (year 2)
- has not yet learned to write simple words (year 1); makes a lot of mistakes when writing (year 2)
- has handwriting that is not legible to strangers
- has difficulties counting forwards to 20
- is unable to establish whether a number up to 20 is greater than another
- is poorly motivated in his/her schoolwork and frequently presents avoidance behaviour in relation to studying.

Source: C. Toso, **2009**. Associazione Culturale Pediatri.

TAB. 5

PAEDIATRICIAN CHECKLIST
– FROM YEAR 3 OF PRIMARY SCHOOL ONWARDS

The **child**:
- has obvious difficulties reading and writing correctly
- has difficulties writing in joined-up writing
- has difficulties reading books or other material on his/her own (e.g. toy assembly instructions)
- has difficulties reading to him/herself (is still only able to read out loud or whispering)
- has difficulties understanding what he/she is reading
- has difficulties learning the multiplication tables
- has difficulties arranging numbers in columns correctly.

Source: C. Toso, **2009**. Associazione Culturale Pediatri.

TAB. 6

It is therefore reasonable to presume that patients with SLDs have a **deficiency** of **BDNF** due to exposure to acute and/or chronic, psychological and physical stress.

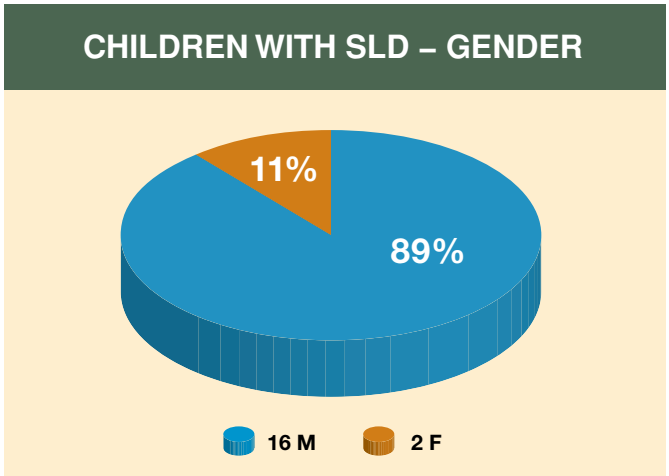
CURRENT TREATMENT

Although the treatment of Learning Disorders hinges primarily on scholastic management, it may also include medical, behavioural and psychological treatment.

– The effectiveness of teaching programmes may require a curative, compensatory, rehabilitative or strategic approach (i.e. teaching the child how to learn).
Some children require special teaching in just one subject and can continue to attend their classes normally.
Others require individual and intensive educational programmes.

– Medicinal products have a mild effect on scholastic performance, intelligence and learning abilities in general, although certain psychostimulants, like methylphenidate and certain amphetamine preparations, can improve the degree of attention and concentration, and therefore allow the child to perform assignments more effectively.

FIG. 4



MATERIALS AND METHODS

This study enrolled a total of **18 patients** (16 M; 2 F) (FIG. 4) aged between 6 and 9 years diagnosed with SLD in paediatric neuropsychiatry hub centres who were administered:
– the WISC-IV (Wechsler Intelligence Scale for Children), the gold-standard clinical tool for assessing cognitive abilities in children of between 6 years and 16 years and 11 months of age.
The WISC-IV makes it possible to calculate 5 composite scores: total intelligence quotient (TIQ) representing the overall cognitive abilities of the child and 4 additional scores, namely 4 Verbal Comprehension Index (VCI), Perceptual Reasoning Index (PRI), Working Memory Index (WMI) and the Processing Speed Index (PSI)
– writing and spelling tests pertaining to the assessment battery for writing and spelling skills (BVSCO)
– MT reading tests.

Lastly, in order to obtain a personality profile, each parent was separately administered the *Child Behaviour Checklist* (FIG. 5).

Patients who, at the time of diagnosis, also presented other types of current or known comorbidities were excluded from the study.
– The children were split *random* into 2 groups (A, B).
Both groups were assigned compensatory learning and technological aids and dispensatory measures at school and speech therapy in an outpatient setting.

Patients in **Group A** were also administered **Guna-BDNF**, 20 drops via the sublingual route, morning and evening before meals (TAB. 7).

Patients in **Group B** were not administered Guna-BDNF.

• BDNF, which was isolated in 1982 by Yves- Alain Barde *et* Al. (15), is a 25 kDa homodimeric protein produced by the Central and Peripheral Nervous System, and particularly in the hypothalamus, hippocampus, cerebral cortex (frontal lobe, occipital lobe, insula, sensory and motor cortex), amygdala, salivary glands, kidney, prostate, retina, endothelial cells and in the follicular fluid.

BDNF acts by activating the receptors p75 and Trk.

During development, the neuropeptide plays a key role in neuronal survival, migration and phenotypical differentiation, as well as in axonal and dendritic growth and in the formation of synapses.
In adult life, its main function is to regulate synaptic plasticity and it is involved in learning, memory and behavioural processes.
BDNF has also been detected in serum with concentrations 10 time greater than those of plasma.

– BDNF is the most active of all neurotrophins in terms of neo-neurogenesis.

It has a protective action against injuries involving the dopaminergic brain structures.
It exerts its effect primarily on the serotonergic neurons (16).
Furthermore, in one *in vivo* study it was also demonstrated that Guna-BDNF reaches the brain within 24 hours of oral administration and reaches peak levels after 48 hours. It remains in the cerebral tissue for a long time even in the absence of further treatment , as it triggers the physiological production systems underlying good endogenous anti-ageing functioning (17).

During the treatment, no side effects were reported and there were no dropouts.

FIG. 5

CHILD BEHAVIOUR CHECKLIST

The first part acquires information on the various areas of personal and social functioning.

The second part consists of 118 items taking the form of statements regarding behaviour in various areas and emotional problems.

The scores are compared with reference value, to obtain two overall scores, one for skills (activity, social life, school) and one for behavioural and emotional problems, and two separate profiles; a skills profile and psychological and/or mental disease profile.

MEDICINAL PRODUCT	POSOLOGY
GUNA-BDNF	20 drops morning and evening

TAB. 7

– At the 1-year follow-up visit conducted at the paediatric neuropsychiatry centres, the same tests indicated above were re-administered and an improvement in performance was observed in both groups (A, B).

▶ **Group A** achieved a **50%** ≈ higher score than Group B in the various items.

DISCUSSION AND CONCLUSIONS

SLDs are changes in normal development with a neurobiological origin and they affect the acquisition of certain scholastic skills. The characteristic common to this group of disorders is the specificity of the deficit.
SLDs cannot be cured, but they are susceptible to appropriate compensatory measures and neuropsychological training, especially as regards speech, memory, and attention.

At the current time, there is no specific pharmacological therapy that has a significant impact on these functions.

▶ The considerable result obtained by administering Guna-BDNF on the performance of subjects with SLDs allowed these young patients to obtain better scholastic performance and a more effective inclusion in their peer groups, thereby sparing them detrimental feelings of inadequacy and isolation.

– It would be appropriate to enrol further patients and for other studies to be conducted in order to confirm the validity of the low-dose treatment proposed. ■

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CLINICAL

CHEWING, NEUROPLASTICITY, COGNITIVE FUNCTION, CLINICAL EFFICACY AND REHABILITATION SYNERGIES – ROLE OF LOW DOSE BDNF

ABSTRACT

Chronic inflammation is among the main causes of progressive degeneration of health in the elderly; the more intense the problem, the more the advancement of ageing deviates from its normal course (healthy ageing) and evolves into a pathological course (inflamm-ageing).
Progressive impairment of health among the elderly manifests itself in the onset of frailty, a condition characterised by a gradual reduction in the ability to respond to stress, accompanied by the impairment of multiple physiological systems. Frailty in the elderly is directly correlated with comorbidity, disability, risk of institutionalisation and mortality.
One factor that is often overlooked when analysing factors that lead to frailty in the elderly is oral health. The loss of teeth and the resulting reduction in the ability to chew among the elderly can be directly correlated with the onset of sarcopenia, a condition that can act as a trigger in the cycle of frailty.
There is a strong correlation between oral health, general psychological and physical well-being, cognitive function, quality of life and longevity; this evidence presents the dental practitioner with new therapeutic challenges, which can only be confronted using an integrated, multidisciplinary approach that makes a decisive impact on general well-being and cognitive functionality, particularly in the elderly.
– The following report describes a clinical experiment on the combined use of implantology methods and a pharmacological intervention based on the systemic oral administration of low-dose BDNF.
Systemic administration of BDNF improves the prosthetic incorporation process in frail patients, accelerating the recovery of effective masticatory function and thereby improving nutrition; all this leads to improved social integration, reducing the risk of depressive disorders. From a biological point of view, the recovery of masticatory function supports the endogenous production of BDNF, slowing down cognitive deficit.

INTRODUCTION

With advancing age, chronic inflammatory events increase in intensity, giving rise to the phenomenon of ***inflamm-ageing***. As long as its progression remains within normal limits for the specific age group, this is considered *healthy ageing*, a process where inflamm-ageing accompanies the natural progression of life, without necessarily manifesting itself in acute or chronic diseases that can suddenly jeopardise the state of health of an elderly person.

– Physiological *inflamm-ageing* is mediated by IL-6 as a result of the effective adaptation to stressors. If it is disturbed by pathological inflammatory stimuli, it degenerates into the phenomenon of **frailty** (FIG. 1), i.e. increasing the susceptibility of the individual to diseases typical of advanced age (multimorbidity), leading to non-self-sufficiency (1-3).
The boundary between the normal and pathological ageing process can be blurred, especially among older age groups; ageing can therefore, to a large extent, be assessed by studying inflammatory parameters; the temporal trend of these parameters reflects that of life expectancy under normal conditions of good health (4).

Frailty in the elderly and the role of Low Grade Chronic Inflammation (LGCI) in cognitive decline

There is a growing interest in frailty and, although this issue has been widely ad-

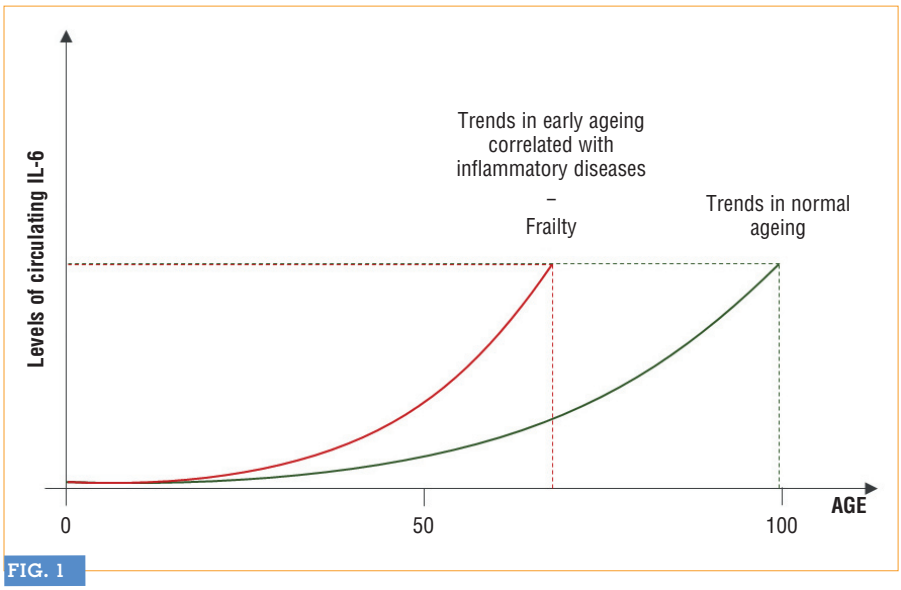


FIG. 1

Correlation between progressive increase in levels of IL-6 (*MARKER of inflamm-ageing*) under normal and pathological conditions related to inflammatory events and the development of frailty.

dressed in scientific literature, the definition and most accurate criteria for its identification have not yet been fully agreed. There is, however, agreement that it is considered an age-dependent biological state characterised by reduced resistance to stress, secondary to the cumulative decline in multiple physiological systems and related to comorbidity, disability, risk of institutionalisation and mortality.

– The main stressor (or variable) responsible for the progressive increase in fragility in the elderly is **inflammation** (5).
– The correct interpretation of inflammatory events in relation to the age of the individual is essential in preventing and managing frailty among the elderly, which leads to the coexistence of several diseases in which, among the aetiological agents, we can often recognise **Low Grade Chronic Inflammation - LGCI**, a condition that manifests itself with a characteristic phenotype described by specific changes in markers such as **IL-6** and **CRP** (C-Reactive Protein).

From the point of view of physiological homeostasis in the elderly, frailty is characterised by profound multi-system dysregulation, leading to a reduction in the self-regulation capacity of the systems involved.

– Consistent literature data indicate that LGCI is characterised by:
- elevated levels of IL-6 and CRP and dysregulation of the immune response;
- increased numbers of white blood cells and related subpopulations;
- progressive cognitive decline.

At a central level, disorders of the immune homeostasis contribute to neuroinflammatory problems responsible for the progressive impairment of neuronal activity; this is because the neuron, like other cells if not more so, is sensitive to inflammatory stressors that gradually reduce its activity, ultimately leading to death.
A progressive reduction in the number of neurons and the resulting reduction in CNS connectivity are key events in the cognitive decline of the elderly. The fight against chronic inflammation and the support of cognitive function are key aspects of the management of ageing in the elderly, particularly when normal ageing is replaced by frailty.
– From a traditional therapeutic point of view, there has so far been no possible targeted intervention in the management of inflamm-ageing related to LGCI; on the contrary, the drug burden associated with the inevitable multiple therapy regimen to which the majority of over-65s are subjected helps aggravate the condition of the precarious systemic balance, increasing the possibility of declining into fragility.

Frailty and reduced masticatory capacity in the elderly: a link between ageing and cognitive decline

The percentage of elderly people in the world is continuously increasing, with increased life expectancy, often in chronic and disabled conditions that involve a considerable healthcare burden. The health of an elderly patient depends on the mutual contribution of many factors, involving physical efficiency, the cognitive and affective condition, social and family support.

– In this context, oral health has an essential role and an increasing number of studies (6,7) have found indisputable evidence for a relationship between oral health, general well-being, improvement in the quality of life and longevity.
– A state of oral health has recently been defined not only as the absence of disease, but also an oral condition that effectively satisfies the functional, aesthetic, psychological and social needs of the individual in the absence of pain and discomfort.
Oral health as a factor that regulates quality of life and life expectancy has been widely investigated in recent years, with the aim of defining the relationships and mechanisms underlying this interconnection.

– We refer to ***"Oral Health-Related Quality of Life"*** **(OHRQoL)** (8), .e. quality of life correlated with the oral health and well-being of the individual, to describe the impact of oral health on the personal experiences of the individual in everyday life, for example speaking fluently and correctly, smiling and laughing without embarrassment when showing the teeth, eating properly and

satisfactorily, preserving and maintaining an adequate emotional state, continuing to perform activities, and maintaining rewarding family and social relationships.
– OHRQoL is a meaningful predictor of overall health, particularly in medically compromised and/or institutionalised elderly patients.
The condition of frailty and the healthcare setting (9) have a considerable effect on oral health, because oral hygiene and the maintenance of prosthetic devices are still not considered priority aspects of routine care.

– Tooth loss in the elderly is responsible for a reduction in masticatory function, with the ensuing changes in eating habits, in both a quantitative and qualitative sense.
This increases the risk of malnutrition and loss of lean body mass, with an evolution towards sarcopenia.
The role of nutrition in triggering the cycle of frailty is well documented. It contributes to some negative outcomes such as increased comorbidity and rapid loss of autonomy.

Sarcopenia in the elderly (10) is generally defined as the loss of muscle mass and muscular strength. The term derives from the literal translation of the Greek terms *sarx* (flesh) and *penia* (loss).
The *European Working Group on Sarcopenia in Older People* (EWGSOP) identifies Sarcopenia as *"reduced muscle mass associated with reduced muscular strength or reduced physical* performance*"*.
– The values of muscular strength, assessed by "handgrip strength", below which the individual can be defined as sarcopenic and at risk of developing motor disability are: 26 kg in males and 16 kg in females.
Recent scientific evidence highlights the link between oral health status and frailty, although difficulties remain in interpreting the data, mainly due to the heterogeneity of methods used in the measurement of masticatory function (11, 12).
A recent systematic review in fact concluded that there is currently no method of measuring masticatory function with all the characteristics of ease of handling, reliability and repeatability, which could be rolled out as a universal instrument.
However, since the oral musculature is the first stage of the swallowing process, it is likely that age-related oral frailty (13, 14) is also involved in the determinism and onset of presbyphagia.

The oral cavity opens into the digestive and respiratory systems. Its structure and function are finely regulated at various levels and ageing is accompanied by significant changes that affect dental components, periodontium, oral mucous membranes, major and minor salivary glands and the musculoskeletal system responsible for chewing and the articulation of speech [muscles and TMJ (Temporomandibular Joint)].

– The hygiene of the oral cavity is certainly one of the key factors in the correct management of an elderly patient in order to avoid changes in the oral microbiota in the first place. The oral microbiota is the group of endogenous, symbiotic microorganisms that live in the oral cavity. This highly complex ecosystem is made up of a rich, diverse microbial community containing several different species of bacteria. The oral microbiota plays an important role in maintaining homeostasis, protecting the oral cavity and preventing the development of disease. In a healthy patient who observes good hygiene standards, the Immune System is boosted by the numerous bacterial species that populate the oral flora and live in balance. In the elderly, on the other hand, changes in the bacterial species that populate the oral cavity facilitate the establishment of immunosenescence, which in turn is related to fragility and Sarcopenia.
There is a dynamic balance between dental plaque bacteria and the host's innate defence system; if this balance is upset it can lead to periodontal disease. This evidence has suggested a situation in which oral dysbiosis is closely related to the degree of fragility.

NEUROLOGICAL REFLEXES OF REDUCTION IN MASTICATORY CAPACITY AND INFLAMMATION – ALTERATION OF THE BRAIN-STOMATOGNATHIC AXIS

Periodontal disease typically involves a chronic inflammatory process in the tissues that support the tooth (gums, periodontal ligament, root cement and alveolar bone). The most recent research is focusing on the study of inflammatory biomarkers related to oral fragility, poor hygiene and periodontopathic disorders. The aetiology of periodontal disease is bacterial, particularly involving Gram-anaerobic species which, as well as invading the deeper periodontal tissues, stimulate the inflammatory and immune response by interfering with the coagulation system and enhancing the production of pro-inflammatory cytokines such as Il-1, IL-6, TNF-α and PGE2 (Prostaglandin E2).
The main difficulty in interpreting these biomarkers stems from the fact that they are also considered markers of the ageing process, causing an overlap that makes it difficult to understand the cause-effect relationship. However this highlights the profound interconnection between the oral cavity and many chronic diseases (high blood pressure, type 2 diabetes mellitus, obesity and dyslipidemia) probably in a relationship of mutual interdependence.
– The lack of stimuli from the oral cavity due to the loss of dental components and poor oral hygiene decreases cerebral blood flow and the activation of the prefrontal cortex.
Chewing increases blood flow in the somato-sensory pathways, motor areas, insular cortex, thalamus, cerebellum and pyramidal cells of the hippocampus, regions all associated with memory and learning.
Experimental studies conducted on animals [SAM *(Senescence Accelerate Mouse)*] (15-18) have highlighted that disabling the molars by applying resin platforms leads to the impairment of memory and learning after 2 months from the start of the experiment, with

greater impairment in groups of older specimens and an improvement in the results once masticatory function is restored (19, 20).
Moreover, there is a relationship between stress and active chewing, the latter having a protective effect against the endocrine and nervous responses caused by excessive activation of the hypothalamic-pituitary-adrenal axis.
A number of studies have been carried out to cast light on the relationship between stress, cognitive function and chewing, in which rodents were subjected to stress stimuli such as immobilisation, cold, painful stimulation applied to the tail, etc.
– If the mice were placed in conditions where they could chew actively, for example by gnawing on wooden sticks, the systemic response to stress was inhibited (21). They also showed greater ability related to cyto-architectural changes of the synapses in the neurons of the hippocampus, therefore better learning capacity and mnemic process functionality.
Despite the multiple relationships that exist between masticatory function and cognitive function, between oral cavity and hippocampus (18, 22, 23), the possible interactions have not yet been clearly identified.
There are probably several factors involved in these mechanisms, and others become important when some of these fail, making the picture difficult to interpret.
– The cause-effect mechanisms are therefore still theoretical; research in recent years has focussed on the possibility of bridging this gap using neuro-imaging methods, such as Functional Magnetic Resonance Imaging.

Neuro-imaging studies are an aid to understanding brain-stomatognathic axis function through the identification of neuronal circuits associated with masticatory function, analysis of the interactions between masticatory function and circuits involved in cognitive and affective processes, and functional study of the mechanisms underlying the long-term changes in masticatory function related to various types of prosthetic treatment.

In the hippocampus, impaired mastication is associated with a significant reduction in the number of pyramidal neurons, reduced neurogenesis, glial proliferation and decreased expression of BDNF (Brain Derived Neurotrophic Factor) (24).

• **BDNF** is the most abundant neurotrophin in the CNS; it plays an essential role in **neuronal survival** during the development and **formation of the neural network** in the adult brain, performing its biological action by binding to its low affinity **p75** and high affinity **TrkB** (*Tro-pomyosin kinase* B) receptors.

– BDNF plays a fundamental role in the development of the CNS, in that it supports the survival and differentiation of the neuronal population and regulates synaptogenesis, synaptic transmission and plasticity, in addition to stimulating and controlling neurogenesis by its own action on the pluripotent neuronal stem cells in the brain.

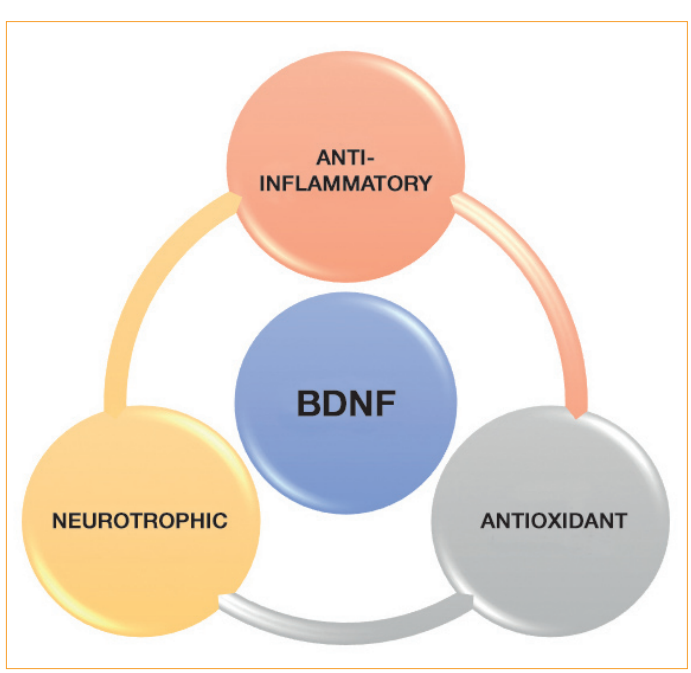
BDNF also plays a crucial role in the mechanisms of learning and memory.
BDNF has a broad range of functions, even outside the nervous system, including the modulation and regulation of immune function (FIG. 2).

At the peripheral level, BDNF is also synthesised by striated muscles and performs autocrine and paracrine functions, increasing fatty acid oxidation, mitochondrial respiration and glucose uptake in the muscles.
– Its production is increased by muscular exercise, with a positive impact on the muscle-brain *cross-talk* (25).
The interconnection between muscle function, in this particular case masticatory, and the action of BDNF at a central level involves two highly important functional axes: **1)** the Hypothalamic-Pituitary Adrenal (HPA) Axis and **2)** the Cerebro-Stomatognathic Axis, functionally included in the Muscle-Brain Axis (26, 27) (FIG. 3).

– From a physiopathological point of view, impaired masticatory muscle activity reduces the somato-sensory stimuli and causes excessive activation of the HPA axis, leading to an up-regulation of corticosterone release.
In the hippocampus, the response to elevated corticosterone levels results in **1)** reduced cell proliferation, **2)** lowering of the survival capacity of new neurons, and a resulting increase in apoptosis, **3)** reduced BDNF production, a

FIG. 2

Main physiological functions of BDNF *(Brain Derived Neurotrophic Factor)*.



key factor of progressive changes in brain morphology and cognitive decline.

– From a conceptual point of view, a therapeutic approach aimed at supporting the biological function of endogenous BDNF is currently one of the most promising prospects for pharmacological intervention within the context described.

LOW-DOSE THERAPY FOR THE TREATMENT OF COGNITIVE DECLINE AND ITS APPLICATION IN DENTISTRY

In the last 10 years, the results of Italian biotechnological research in the field of low-dose pharmacology has highlighted new possibilities for the treatment of many diseases, and the attention of the scientific community has been drawn to new, ground-breaking drugs and a new medical paradigm: Low-Dose Medicine.

Low-Dose Medicine originates from the meeting of Molecular Biology and Psycho-Neuro-Endocrine-Immunology (P.N.E.I.) (28-33); it has developed as the fruit of research in the field of low-dose pharmacology.

– More recently, **Systems Medicine** has changed the perspective in the interpretation of the biological functions of the human body and its diseases. There has been a shift from a reductionist vision (each disease affects a single organ or tissue) to a systemic view of cellular network, arriving at a recognition of the importance of continuous dialogue (cross-talk) between cells, organs and systems in both normal and pathological conditions (34-37).

– Based on these premises, pharmacological research has focused on the role of signal molecules (neurotransmitters, cytokines, hormones and growth factors), thus opening up the way to a new solution in the therapeutic field: the use of these organic molecules as drugs to restore normal conditions in a diseased organism.

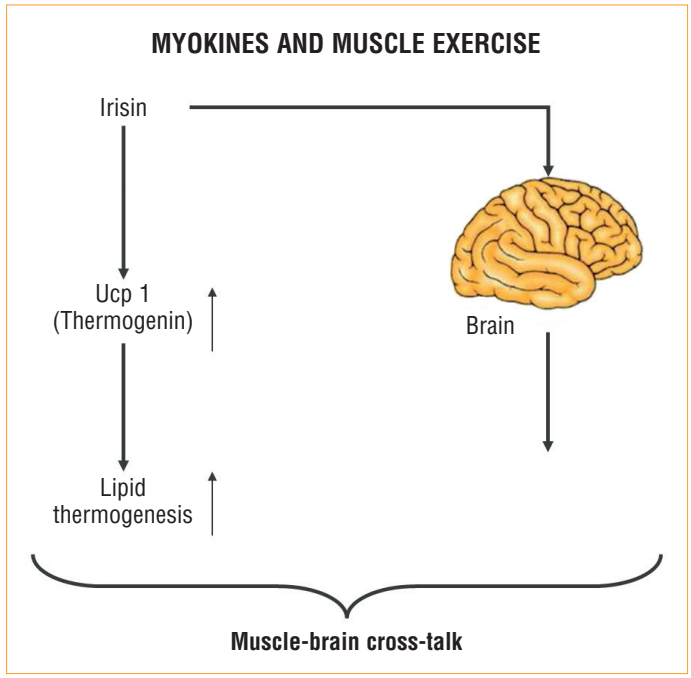


FIG. 3

Interaction between muscular activity and BDNF synthesis.

– Contractile muscle activity and related thermogenesis are key factors in the induction of BDNF release at a central level.

– While modern Molecular Biology has discovered and made us aware of the meaning of the "words" that cells use to communicate, and helped us understand how diseases are the expression of a fault (or even interruption) in the communication between cells, it had not yet explained **what** "volume" was used by the cells to communicate.

• And this is precisely where research in the field of Low-Dose Medicine comes in.

Pharmacology based on the activity of signal molecules such as cytokines is one of the most interesting frontiers of medical science.

– The possibility of using these molecules at low doses (sub-nanomolar) is enriching the pharmacology of cytokines with even greater interest and fascination: anyone involved in inflammatory diseases and autoimmune diseases has "dreamed" of obtaining these molecules in the form of drugs, but the dream has vanished every time they noticed the side effects experienced at the dosages normally tested.

This new pharmacological and clinical paradigm, defined as Low-Dose Medicine, currently suggests that the history of therapeutic use of cytokines has yet to be written, most likely in **low-dose** form.

– Scientific research has corroborated the hypothesis of Low-Dose Medicine: over fifteen years of scientific research in the field of Low-Dose Medicine have demonstrated the validity of the conceptual approach and the efficacy and safety of therapy based on the oral administration of low doses of activated signal molecules (38).

We can now confirm that scientific literature **supports** the Low-Dose Medicine therapy, and that it is no longer just a scientific theory, but may be the basis of a new medical paradigm.

• The critical mass of published work has, above all, made it clear that the safety of these dosages is very high, and this is no small matter.

– It has also highlighted that low-dose drugs are particularly useful in keeping disease activity low, even in patients with highly challenging diseases in remission: this is the second very important aspect of low-dose pharmacology.

– It has helped us understand that low-dose therapies are ideal for long-term treatment because they cause no adverse events or issues associated with overdose.

– It has also thrown light on certain limitations of low-dose medicine: in some stages of disease, in which the homoeostatic and biological regulation systems are highly compromised, low-dose pharmacology does not work on its own

(we need to ask ourselves whether this is a true limitation or whether it opens up new frontiers in the combined use of synthetic and low-dose drugs...).

Low-dose medicine will help understand how to act on the most obscure causes of many diseases – especially those of an inflammatory and degenerative nature – which recognise their profound origin by a change in communication between self-regulating networks.

– In May 2020, *Brain Sciences* published the article *"The Role of BDNF on Aging-Modulation Markers"* (39) presenting the results of research conducted at the Institute of Human Physiology at the University of Eastern Piedmont on the effects of low doses of *Brain-Derived Neurotrophic Factor* **(Guna-BDNF)** on primary cell cultures of mouse cortical neurons and astrocytes, and an animal model (12-month-old mice, approximately corresponding to human beings aged 80 years, in whom a cerebral stress had been induced that mimicked a pathological condition attributable to some forms of dementia).

– This study falls within the scope of research in the field of low-dose pharmacology that has been taking place for over 15 years, but it is the first that involves nerve growth factors.

• This aspect is also of considerable interest in dentistry, given the growth of the elderly population and increase in the incidence of neurodegenerative diseases.

The percentage of the general population aged 60 or over with dementia is currently estimated at 5 to 8%, but no complete epidemiological data that relates specifically to dementia is available. What is most alarming is the fact that dementia, along with Alzheimer's, will become an emergency in the future: in 2010, there were 36 million individuals worldwide suffering from dementia, increasing to 44 million in 2013, and by 2030 it is expected to be 76 million, rising to 135 million in 2050.

– Dementia affects over 1,200,000 people in Italy, of which approximately 600,000 are Alzheimer patients; numbers are constantly increasing and expected to double over the next 20 years. These are diseases for which no cure has yet been identified: sufferers depend entirely on treatment to relieve the symptoms.

– The results presented in the aforementioned article are extremely encouraging; this led us to adopt a Guna-BDNF-based protocol in patients with reduced masticatory activity at a rate of 20 drops twice a day for prolonged periods (at least 3 consecutive months).

CONCLUSIONS

The most recent scientific evidence reveals therapeutic challenges that the dental world must address using an integrated, multidisciplinary, multidimensional approach, in order to come up with specific and appropriate solutions.

New roles are emerging for dentists, who were hitherto required merely to perform a clinical procedure aimed at treating one part of the body by applying a prosthetic product; dentists may become "sentinels" in this field, working fully to achieve and maintain a state of oral health as understood in the broad term we have described.

– In particular, the recovery by dental treatment of edentulous patients who have never been rehabilitated or who have "abandoned" rehabilitation (for anatomical, clinical or social reasons), affecting not only their nutrition and social and family integration but also the cognitive decline and the course of the chronic degenerative diseases characteristic of ageing, would have a significant effect on general well-being.

With this in mind, dental literature has recently been concerned with the development of simplified prosthetic methods [SET *(Simplified Edentulous Treatment)*] (40), the use of dental components that aid prosthetic integration [WFA *(Wide Functional Area)*], and the refining of occlusal procedures that do not require the formation of complex neuromuscular circuits [LO *(Lingualized Occlusion)*] (41, 42).

– In our experience the combined use of simplified methods with the use of elements with a large occlusal pit in lingualised occlusion, in conjunction with the systemic oral administration of BDNF at a rate of 20 drops twice a day for cycles of at least 3 months, **helps patients through the process of prosthetic incorporation**.

This makes it possible to restore effective masticatory function to a large slice of the population, avoiding improper planning that is in fact unworkable or destined for clinical failure, in a hazardous, unwanted short circuit with negative outcomes of frailty and disability.

– Viceversa, effective, appropriate rehabilitation can trigger and maintain a virtuous circle of "anti-frailty", improving nutrition, facilitating social integration, reducing the risk of depression, increasing the endogenous production of BDNF and slowing down cognitive decline.

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THERAPEUTICS

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LOW DOSE INTEGRATED THERAPY IN PARKINSON'S DISEASE

ABSTRACT

This study, of 6 months' duration, was carried out on a sample of 10 patients suffering from Parkinson's disease in conventional treatment with Levodopa or Sinemet at doses ranging from 100 to 600 mg/day. The study is based on the introduction of a support of low-dose Neurotrophins (NGF and BDNF), Extrabios 1 and Extrabios 2 into the usual therapy, with the aim of promoting greater control of symptoms and a better quality of life. To assess the efficacy of the proposed therapy, the Barthel Index, Tinetti Scale and Mini-Mental State Examination (MMSe) were used: the measurements were made during a bimonthly consultation, when information was collected on the 3 main symptoms (tremors, bradykinesia and rigidity).
From the data obtained during the 6 months of therapy it is evident that this therapeutic overlapping facilitates a significant reduction in rigidity, moderate control of accessory symptoms and a slight improvement in memory and cognitive function. Age and comorbidities affected the timing and level of efficacy of the treatment.

INTRODUCTION

Parkinson's disease **(PD)** is a degenerative disease of the central nervous system due to the depletion and ultimate disappearance of the dopaminergic neurons in a finite area of the midbrain, the *Substantia nigra* (FIG. 1).

Dopamine activity has a fundamental role in controlling movement and posture.
– Its deficiency/absence causes hypoactivation of the so-called Direct Pathway (medial striatum-pallidus) with the onset of **bradykinesia** and excitation of the Indirect Pathway (Medial striatum-pal-

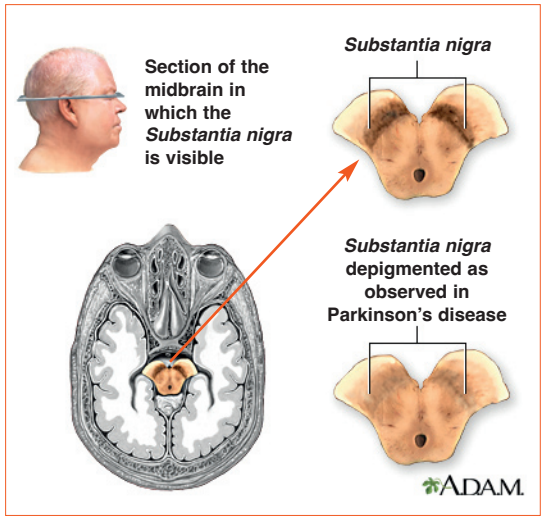


FIG. 1

The darkest area corresponds to the *Substantia nigra*. In patients with Parkinson's this area is significantly depigmented.
The cells of the *Substantia nigra* contain a pigment, "neuronal melanin", which is responsible for the typical black colour.
– Microscopically the cells that constitute it are degenerated and replaced by glial cells.

– Source: https://medlineplus.gov/medlineplus.html.

lidus, passing through the lateral pallidus and the subthalamic nucleus of Luys), with the onset of **rigidity**.
Tremors are thought to be caused by reverberating activities, a type of short circuit between the two Pathways, Direct and Indirect (FIG. 2).

AETIOPATHOGENESIS

The aetiology of PD is still a subject of debate and detailed study.

Recent evidence has demonstrated a direct, fundamental transition from neuroinflammation to neurodegeneration, a transition which is "obligatory" even in PD (Milani, 2016).

The biomolecular disorders that characterise PD are not limited to the downregulation of dopamine and the upregulation of acetylcholine and other neurotransmitters; they are also involved in the reduction of neurotrophins such as NGF (Lorigados *et* Al., 1996) and BDNF (Montenero, 2018).

The neurotrophins are a family of 4 molecules. **NGF**, **BDNF**, **NT3** and **NT4**, responsible for some important trophic and survival activities of the nervous tissue.

The number of neurons that innervate the muscles and organs is destined to decrease over time due to programmed neuronal death: neurotrophic factors protect the neurons from apoptosis (Kursching, 1993; Lewin *and* Barde, 1996).

– NGF is a protein that stimulates the differentiation and survival of specific neurons (neuronal anti-apoptotic activity); it is responsible for the differentiation and maintenance of sensory and sympathetic neuron function.
This neurotrophin ensures the development and maintenance of neurons in the hypothalamus, pituitary gland, hippocampus and cerebral cortex [Neocortex and Subcortical Limbic sottocorticale (Lorigados *et* Al., 2002)].

– BDNF is a protein that supports the survival of existing neurons and facilitates the growth and differentiation of new neurons and synapses, especially in the cortex, hippocampus and basal ganglia, crucial areas for learning, memory and the formulation of associative thought.

It is the neurotrophin that is most active on **neo-neurogenesis**; it has a protective effect against traumas involving dopaminergic brain structures and acts especially on serotonergic neurons.

In PD there is a **sharp reduction** in serum BDNF (Howells *et* Al., 2000).
BDNF deficiency is also associated with the emergence of cognitive deficit (Angelucci *et* Al., 2015).

BDNF – consequently – plays a key role in the survival of dopaminergic neurons and the regulation of synaptic connectivity.
– Overall, BDNF takes on a dual role in the dopaminergic system:
- it is a protective agent of the Nigro-Striatal circuit (survival action);
- it modulates cognitive processes, regulating synaptic plasticity in the hippocampal and cortical pathway.

– This dual role is particularly relevant in PD.

PURPOSE OF THE STUDY

In light of what has been concisely illustrated above, the aim of this study was to improve the quality of life for patients with Parkinson's through the administration of an integrated therapy consisting of low-dose neurotrophins, Extrabios 1, Extrabios 2 and Levodopa.

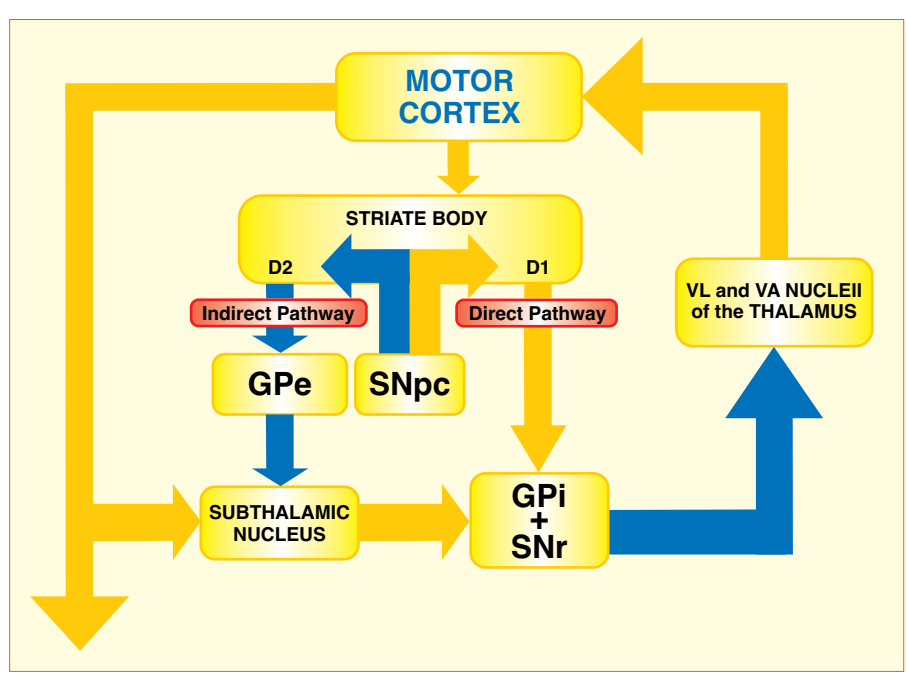
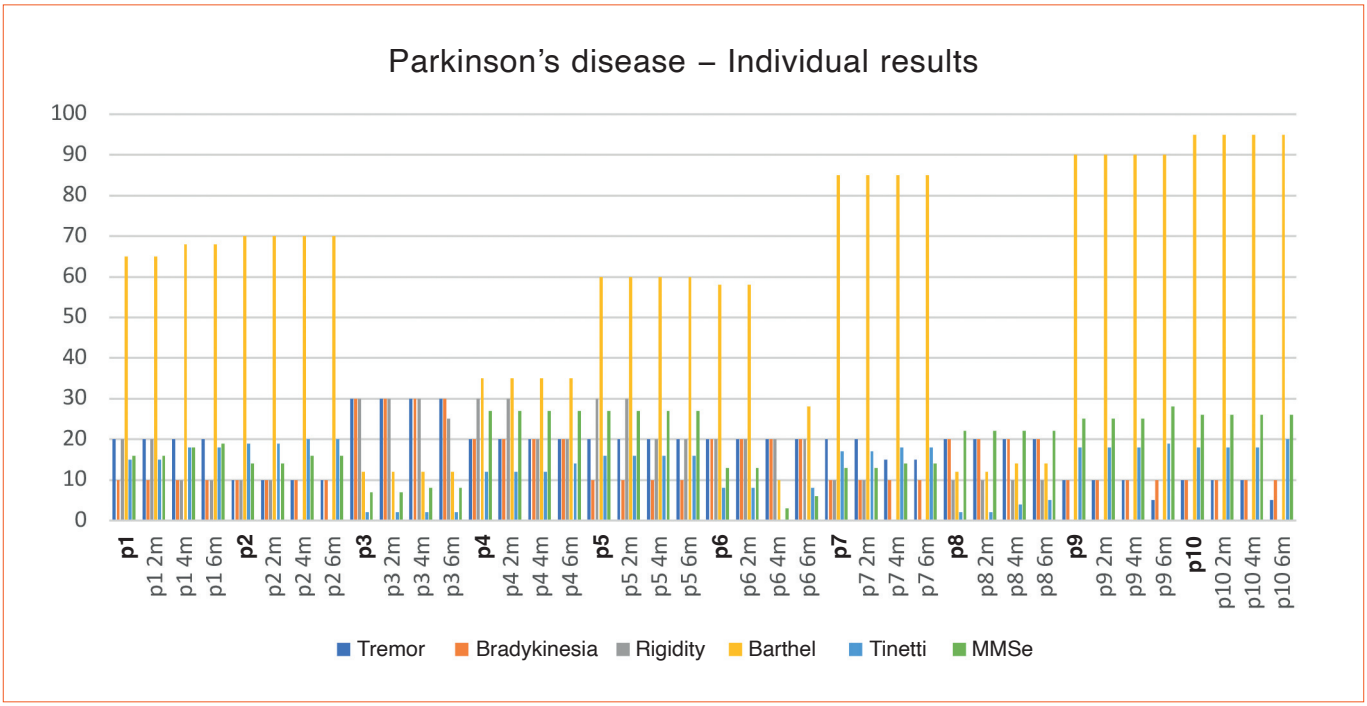


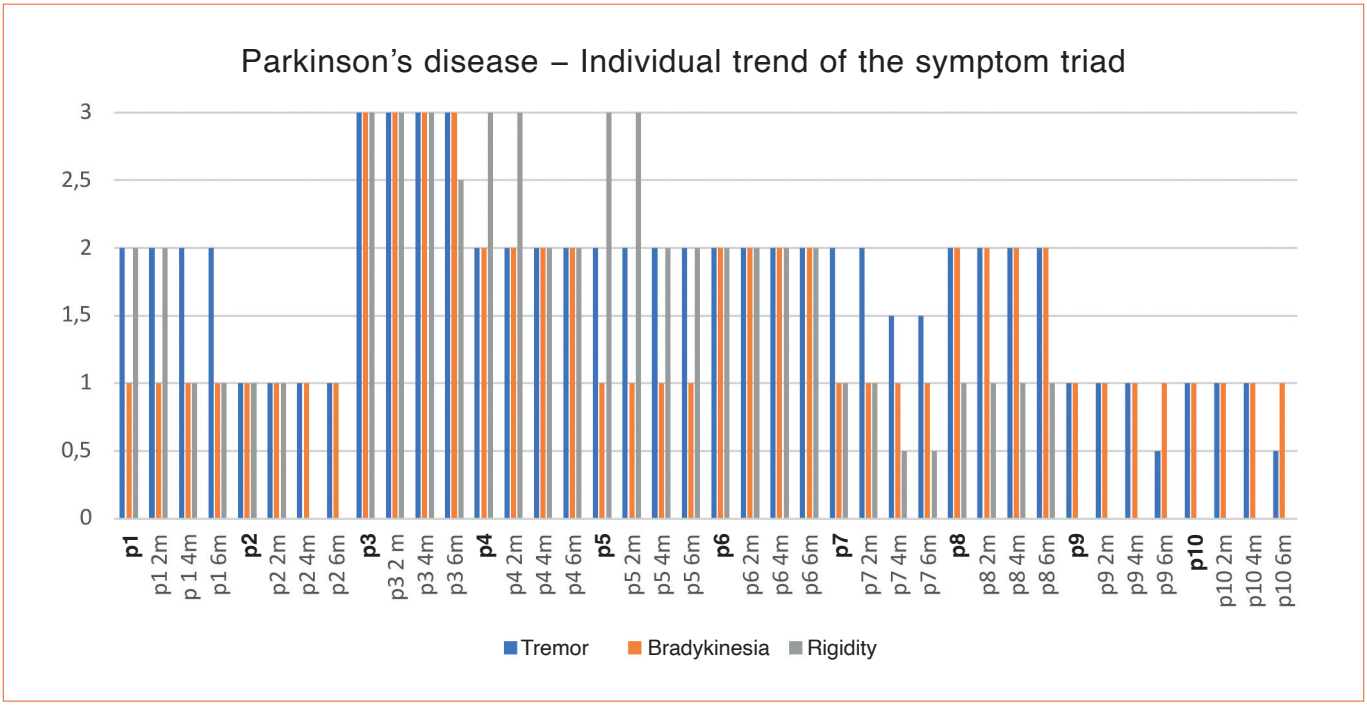
FIG. 2

Exemplary diagram showing the Direct Pathway and Indirect Pathway.

– Source: http://medmedicine.it/sito/wp-content/uploads/2013/05/circuit11.jpg



TAB. 1



TAB. 2

MATERIALS AND METHODS

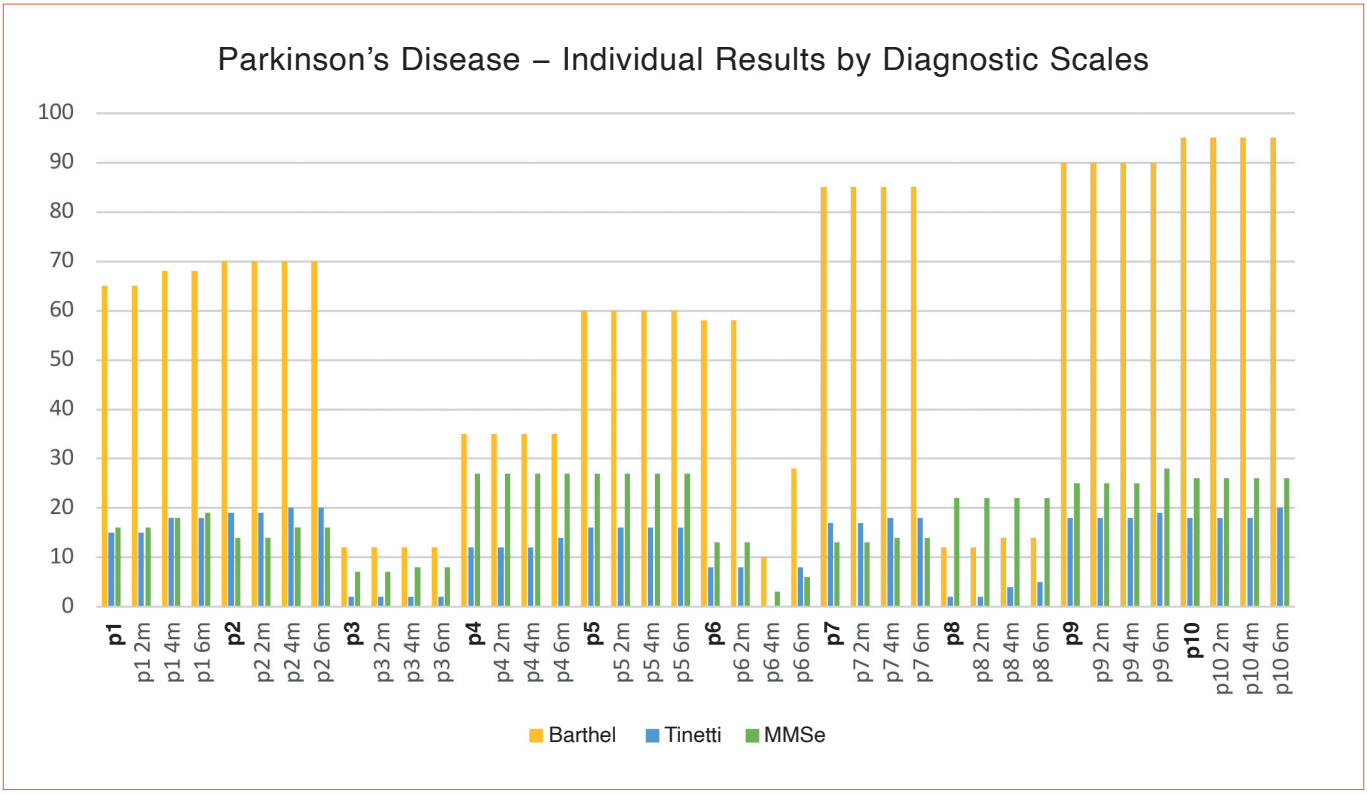
– POPULATION OF STUDY

Ten patients aged 72 to 94 years, 8F and 2M, were enrolled; all patients were taking Levodopa at the time of enrolment and had poor symptom control (primarily rigidity).

Diagnostic instruments: Semeiology, Barthel Index, Tinetti Scale, **MMSe** (Mini-Mental State Examination).

All patients were assessed bimonthly (2m, 4m, 6m).
The consultation involved medical, care support, physiotherapy and educational/social assessments; more specifically, the following were taken into consideration:

1. Symptoms (tremor, bradykinesia, rigidity);
2. Barthel Index (assessment of daily activities);
3. Tinetti Scale (walking and balance);
4. MMSe (cognitive state).



TAB. 3

– THERAPEUTIC PROTOCOL

The patients included in the study were treated using a therapeutic combination of Levodopa + NGF + BDNF + Extrabios 1 + Extrabios 2, and re-assessed every 2 months according to the respective ATS protocol.

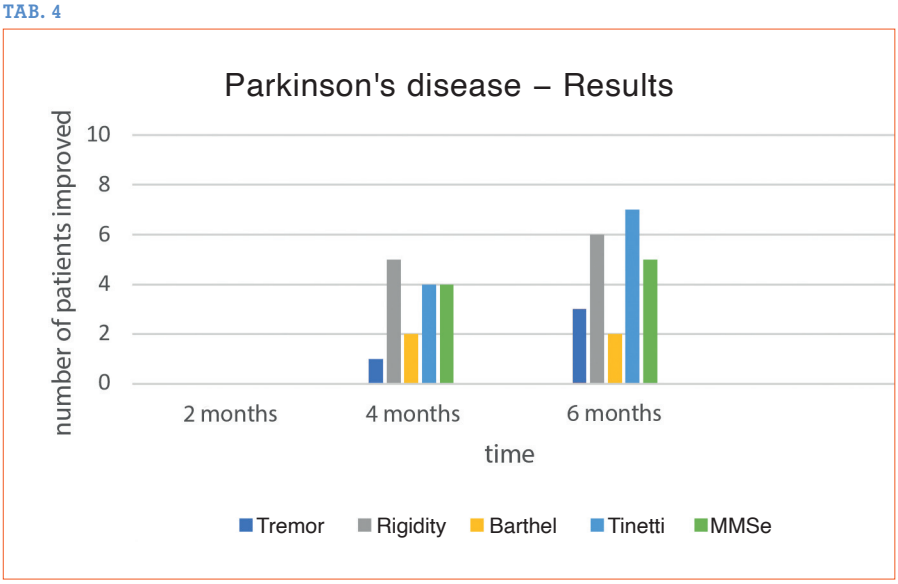
– PHARMACOLOGICAL DOSAGES

- **Levodopa** or **Sinemet** [according to the current dosage (100-600 mg/*day*)] in multiple administrations/*day* (dosage monitored by the treating doctor)
- **Guna-NGF**, 20 drops x 2 times/*day*
- **Guna-BDNF**, 20 drops x 2 times/*day*
- **Extrabios 1** (Mon-Wed-Fri), 20 drops x 2 times/*day*
- **Extrabios 2** (Tues-Thurs-Sat), 20 drops x 2 times/*day*.

At each bimonthly consultation the patient was assessed for the parameters in question by the ward doctor (symptomatic triad - **Tremor**, **Bradykinesia**, **Rigidity**), by nursing/assistant practitioners **(Barthel Index)**, by a Physiotherapist **(Tinetti Scale)** and by an educator **(MMSe)**.

The low-dose therapy continued for **6 months** at constant dosage.

TAB. 4



RESULTS

From the analysis of the data obtained it is clear that (TAB. 1-4):
– at **2 months** from the start of the combined therapy there were no significant changes;
– at **4 months** from the start of the combined therapy: One patient showed a slight reduction in tremors; 5 patients showed a reduction in rigidity; 2 patients showed an increase, albeit slight, in the Barthel Index; 4 patients an increase in the Tinetti Scale; 4 patients an

increase in cognitive ability (MMSe).
– at **6 months** of combined therapy: another 2 patients further demonstrated a reduction in tremors; 1 patient showed a reduction in rigidity; a further 3 patients increased the value on the Tinetti Scale; 1 patient increased the MMSe value; finally 3 patients were put forward for assessment regarding a reduction in the dosage of Levodopa or Sinemet.

▶ In total, on completion of 6 months of therapy:
– **2 patients/10** increased, albeit slightly, the **Barthel Index**.
– **7 patients/10** increased, albeit slightly, the **Tinetti Scale**.
– **5 patients/10** achieved a mild-moderate increase in **MMSe**.
– **3 patients/10** achieved a reduction in **tremors**.
– **0 patients/10** achieved a reduction in **bradykinesia**.
– **6 patients/10** achieved a reduction in **rigidity**.

DISCUSSION AND CONCLUSIONS

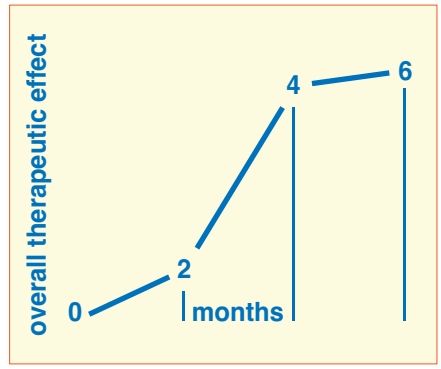
The results obtained in this study provoke some thoughts:

1. the combined therapy proposed here enables better control of symptoms, facilitating an increase, albeit slight, in the patients' quality of life;

2. the greatest effect was recorded between the 2nd and 4th month of therapy (TAB. 5);

3. The combined therapy had no effect on bradykinesia.
   – This result opens up new investigations into possible interactions with the Direct and Indirect Pathways of the Dopamine System;

4. one outstanding impact on the overall therapeutic effect was the continuous stimulation by professional practitioners such as educators and physiotherapists, who offered personalised cognitive and motor rehabilitation courses to each patient;



TAB. 5

5. combined therapy should be the first step taken in any Neurology or General Medicine clinic from the initial diagnosis of PD, so as to slow down or attempt to block neurodegeneration and thereby the unstoppable progression of PD.

   – Moreover, such use could lead to a lower prescription of dopaminergic drugs and thus avoid disuse atrophy of nigro-striatal neurons. ■

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The author thanks the editors of the websites from which the diagrams were taken 1 and 2.

Editor's note:
The bibliographic entries of Milani L., **2016** and Montenero P., **2018** can be consulted on **www.medibio.it** → **La Medicina Biologica**.

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CLINICAL

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LOW DOSE BDNF IN THE BEHAVIOURAL REHABILITATION OF DOGS – CORRELATION BETWEEN OBSERVED EFFECTS AND MECHANISMS OF ACTION OF THE MEDICINE

ABSTRACT

Behavioural diseases in dogs are increasingly common and can present risks, both for the adopted family and for third parties and dogs, as well as causing significant suffering to the patient.
– The objective of this clinical study is to list and catalogue behavioural changes observed in canine patients following a course of rehabilitation and taking low-dose BDNF as part of an integrated medicinal therapy to support practical work, and to correlate the listed changes with the mechanism of action of low-dose BDNF that could have induced them.

KEY WORDS

BDNF, LOW DOSE, BEHAVIOURAL PROBLEM, BEHAVIOUR, DOG, BEHAVIOURAL MEDICINE

INTRODUCTION

The plan to carry out this study arose by observing hundreds of dogs within their family systems and the ways in which they changed, evolved and overcame difficulties, in the knowledge that, when appropriately accompanied, the patient can transform a behavioural disease into a "characteristic", learning to stop being defined and guided by their pathology.

On the other hand, in order to accompany the patient on this journey, a veterinarian expert in canine behaviour must be able to "see" them for who they are, and look **beyond** the disease, to know what this patient can achieve and where to work in guiding the path towards rehabilitation.

– In line with this approach to behavioural rehabilitation through the use of therapeutic devices capable of activating the patient's own resources, it seemed natural to use **BDNF** which – as with all low-dose medicines and our way of approaching a rehabilitation process – stimulates patients to regain their own balance and synthesise their own resources.

In this sense the therapist becomes a "prompter", someone who provides an input which the patient, together with their family group, can process in their own way and their own "style".

– This type of approach requires strong component of practical work in the field, and indeed is derived from this: it is by working alongside patients and their families that we have been able to develop strategies increasingly intended, not to add pre-structured external elements, but to provide flexible, multifunctional tools that can be used by patients and their families in their daily lives.

As part of a PNEI and Systems Medicine approach, low-dose BDNF is not the only therapeutic strategy we are using and have prescribed to our patients.

Each patient undergoing treatment and rehabilitation receives an average of 3-4 different therapies, including low-dose BDNF, but the field work and daily observation of videos that each participating family sends us, enabling us to monitor patients more effectively, has made it possible to identify obvious differences in patients taking BDNF compared with those who are not, and changes in patients not taking BDNF when they begin their therapy with this important medicine.

– This work represents a proposal to catalogue the changes induced by low-dose BDNF observed in rehabilitation patients and an attempt to correlate them with the hitherto known mechanisms of action by which this neurotrophin interacts with organic systems.

MECHANISMS OF ACTION OF BDNF AND RELATIONSHIPS WITH THE BODY'S SYSTEMS

Mature BDNF is a soluble protein combined into dimers by non-covalent bonds. Its biological action occurs mainly through TrkB receptors (Tropomyosin receptor Kinase B); TrkB is expressed by a single gene, but it exists in four distinct isoforms: the full-length receptor and its 3 truncated versions, TrkB-T1, TrkB-T2 and TrkB-T4. The exact function of truncated TrkB receptors is currently still poorly understood.

The binding of BDNF to TrkB triggers its dimerisation and the subsequent autophosphorylation of tyrosine residues, with the resulting recruitment of adapter proteins to activate 3 intracellular signalling pathways: MAPK, Phosphatidylinositol-3-kinase and Phospholipase C.
– These signalling pathways mediate a variety of functions, including **learning and memory** mechanisms.
BDNF is crucial to multiple processes within the nervous system, both during development and in the mature brain.

These processes are concerned with the survival and differentiation of neuronal stem cells, axon and dendrite differentiation, axonal growth, the formation and maturation of the synapses and the refinement of developing circuits.

In vitro and *in vivo* studies conducted on mice demonstrate that the expression of BDNF and its receptor TrkB are essential to increasing the number of proliferating stem cells, the differentiation of neuron populations (especially in the hippocampus) and the maturing of excitatory synapses.

Although the functions of BDNF in development are widely understood, the question of whether the source of BDNF is pre- or postsynaptic has not yet been clarified.

The most widely accepted opinion is that BDNF acts as a retrograde factor synthesised by target cells in the central nervous system.

– In addition to its roles in the development of neural circuits, BDNF performs a more important action in mature circuits, for example the modulation of synaptic plasticity and the formation of memory, particularly in the cortex and hippocampus (Uberti & Molinari, 2018; Milani, 2022).

BDNF is the most active among the neurotrophins with regards to neo-neurogenesis (true brain amplifier).
It has a protective effect against trauma involving the dopaminergic brain structures; it acts mainly on the serotonergic neurons.

Some very recent studies suggest that the cellular receptors for BDNF function as dependence receptors: for optimal performance they must all be saturated.

If the BDNF receptors in a cell are not completely saturated, the metabolic pathways it induces are not activated.
– Once bound to the brain cells or other structures (retina, kidney, prostate, motor neuron, skeletal muscle, or heart), BDNF induces various effects at cellular level (Molinari *et* Al., 2020):

1) inhibition of the nuclear translocation of NFKb, a nuclear transcription factor which, once activated, enters the nucleus and activates the expression of inflammatory mediators. This is the first direct effect relating to inflammation control.
2) downregulation of phosphorylation of the Tau protein, responsible for the organisation of microtubules in the cerebral intercellular matrix.
If the Tau protein is phosphorylated, the microtubules become disorganised, forming "tangles" which prevent interneuronal synaptic dialogue.
3) increased synthesis of Interleukin 10 with a resulting downregulation of Interleukin 6.
Interleukin 6 is mainly responsible for activation of Tau protein kinase, which brings about the phosphorylation of the Tau protein.
– This is the second mechanism that acts on the inflammatory cascade, albeit indirectly.
4) activation of NRF2, a nuclear transcription factor that triggers the pro-

duction of superoxide dismutase and glutathione reductase, both of which have an antioxidant function.
– Since the presence of oxidising substances would seem to sustain inflammation as well as being a consequence of it, the elimination of these substances is another mechanism of indirect inflammation control.

5) increase in the viability of cortical neurons and induction of proliferation of cerebral glial astrocytes.

6) stimulation of neurogenesis in the brain (Hippocampus, Cortex and Forebrain).

7) increase in apolipoprotein E2, which has a neuroprotective effect. Apolipoproteins, in fact, play a fundamental role in the catabolism of cholesterol and triglycerides.

8) stimulation of the expression and/or release of VEGF, vascular endothelial growth factor, a mechanism also implicated in the antidepressant effect (Deyama *et* Al., 2019).

BDNF is able to modulate various neurotransmitter receptors, particularly alpha 7-type nicotinic receptors: these are receptors for calcium channels that have both a pre- and post-synaptic excitatory effect on the central neurons.

It would seem that BDNF also develops a potent antidepressant effect (Palma & Brugnoli, 2007); some studies have in fact shown that, when administered in overlap with SSRI type antidepressants (selective serotonin reuptake inhibitors, NdR), it shows a booster effect, in that it increases the ratio between serotonin and its catabolite: it therefore decreases the catabolism of tryptophan, a serotonin precursor.

BDNF and Cortisol show a diurnal rhythm in women with similar fluctuations; the amplitude of the fluctuations is modulated by ovarian function (Quirici, 2009).

Dogs, especially female dogs, are very frequently sterilised and therefore do not have the normal series of sexual hormones.

Since daytime levels of BDNF are significantly lower in post-menopausal women than in women with active hormonal activity, it is reasonable to ask whether sterilised female dogs also have reduced levels of BDNF.

– On the other hand, it has been demonstrated that sterilised dogs have a greater probability of showing senile cognitive decline.

Physical activity increases plasma BDNF levels (Dinoff *et* Al., 2017; Milani, 2022); it follows that its production could be stimulated by a rehabilitation programme which **1)** takes the patient's body movement into consideration, **2)** includes daily physical activity appropriate to the patient's age and race and **3)** is based on rehabilitation activities that involve movement.

MATERIALS AND METHODS

Dogs whose families joined the rehabilitation proposal from 1 January 2016 were selected; a total of **257 dogs** were enrolled.

The patients were observed and/or filmed during field work with an instructor and in their daily lives, noting any behavioural changes observed after the initiation of therapy that included low-dose BDNF.

We then proceeded to catalogue these changes, grouping them into **6 types**.
– The study considered the following types of behavioural changes:
1) ability to calm down: the ability to reduce one's level of emotion and achieve an appropriate level of relaxation, partly due to a sense of satisfaction at the end of any type of activity. Achieving a state of calm is possible due to a reduction in the level of alertness and environmental control, successful self-management of emotions by the dog, and the ability to complete an activity, finding satisfaction in it and finishing it off.

2) change of behaviour after a break: during a work session, or during walks or daily activities, the dog is not constantly engaged in activities; breaks are provided to allow the dog to reflect on the experience.
Behaviour changes significantly after a pause that allows the dog to reflect. This enables a quicker, more effective growth of skills.

3) appropriate response to communication with a conspecific: the capacity to send coherent communication signals consistent with those sent by another dog.

For example, stopping or leaving space rather than invading the space of a conspecific if requested, including the ability to discontinue communication when requested by the interlocutor.

4) problem solving capability: to approach a problem with a suitable emotional attitude and solve it effectively in a way that is repeatable in the future. A dog that faces and solves a problem effectively and adequately is able, if the problem reappears, to immediately implement the appropriate functional behaviour to resolve it, without resorting to trial and error.

5) disappearance of the defeatist attitude: the occurrence of behaviours relating to adherence to proposals made to the dog, regarding both interaction with conspecifics and other activities.

6) learning and respecting rules: the ability to understand, or rather to adopt, rules that define a situation, be it work or everyday life.
For example, don't put your paws on the table, don't climb on the couch, sleep in the dog house, etc. This does not include dogs that need to be told the rule every time in order to adhere to it.

Finally, certain hypotheses regarding the correlation between the various mechanisms of action of BDNF and each type of behavioural change were put forward.

RESULTS

The study involved the observation and collection of data on a total of **257 dogs** (118 F and 139 M).
– The dogs examined presented a wide variety of behavioural problems and associated functional disorders; the majority of the dogs had comorbidities involving several diseases and/or functional disorders.
The classification of diseases and functional disorders refers to the model described in the reference text (Colangeli *et* Al., 2015).

The patients who showed a single nosological entity were:
- **66** with hypersensitivity-hyperactivity syndrome
- **5** with sensory deprivation syndrome
- **4** with social phobias
- **3** with competitive relationship syndrome
- **2** with cognitive impairment
- **1** with attachment disorder.

A total of 81 dogs out of 257 (31.5%) presented a single disease or functional disorder.
– A more complex and detailed statistical study is therefore required in order to analyse the data of patients with comorbidities.

– Out of **257** dogs:
247 **(96%)**learned and respected rules;
245 **(93.3%)** showed evidence of the ability to calm down;

TAB. 1

TYPE OF PROGRAMME	No. DOGS	LEARNING AND RESPECTING RULES	ABILITY TO CALM DOWN	CHANGING BEHAVIOR AFTER THE BREAK	PROBLEM SOLVING CAPABILITY	APPROPRIATE RESPONSES TO COMMUNICATION WITH A CONSPECIFIC	DISAPPEARANCE OF THE DEFEATIST ATTITUDE
COMPLETE	148	146	148	144	139	140	20
IN PROGRESS	49	45	44	40	31	20	5
INCOMPLETE	60	56	53	50	43	25	2
TOTAL	**257**	**247**	**245**	**234**	**213**	**185**	**27**

234 **(91%)** changed their behaviour after a break;
213 **(82.9%)** were able to solve a problem effectively;
185 **(72%)** demonstrated an adequate response to communication with conspecifics;
27 **(10.5%)** showed evidence of the disappearance of a defeatist attitude.

• The most common behavioural change is undoubtedly **learning** and **respecting rules**.

The data referring to the number of dogs that demonstrated disappearance of a defeatist attitude is very limited when compared with the other groups.
However, this result should be considered in light of the number of dogs that showed a defeatist attitude at the start of the programme, which was only 30 patients; 27 out of 30 **(90%)** changed this behaviour.

– To check whether some behavioural changes that are more complicated to achieve than others could occur later in the rehabilitation process and require greater commitment from the family group, the dogs were classified into 3 groups:
Group 1, rehabilitation programme completed;
Group 2, programme in progress;
Group 3, programme not completed.

TAB. 1 shows the different frequencies of presentation of behavioural changes in the 3 groups of dogs.

In TAB. 1 there is a clear difference between the frequency of presentation of behavioural changes between dogs that have not, or have not yet, completed the programme and those that have.

• Completion of the programme is defined as the achievement of a functional, stable balance in the dog and the family system to which it belongs.

If we exclude the disappearance of the defeatist attitude, related to a specific initial condition in order to occur, the least frequent change observed is the adequate response to communication with conspecifics; this in fact represents an advanced step in the rehabilitation process that also requires a great commitment on the part of the family group.
– The hypothesis is that the ability to calm down (change 1) is, in the case of dogs, preparatory to the possibility of adapting its behaviour following a break (change 2); in this respect, out of 245 dogs that demonstrated change 1, 234 also demonstrated 2, while out of the 12 dogs that did not demonstrate change 1, none demonstrated 2.

DISCUSSION

We believe that the amount of data collected is especially interesting because there are no similar collections in the literature; this enables us to analyse information regarding a fairly extensive series of cases for veterinary medicine.
The method we have structured contains key steps in the growth of dogs in a rehabilitation course; the behavioural changes observed in dogs taking low-dose BDNF represent the key points on a programme.

It is interesting to note that, even in the context of a discontinued programme, behavioural changes are still quite frequent.
– Although some programmes were discontinued even after a short time and with little work carried out in the field, even the directions for management, safety measures, therapies and outings in the natural environment produced significant changes.

• This indicates that low-dose BDNF

therapy leads to significant change in patients, even if the family is not committed to the long, complex work.
Some changes can only be achieved through intense, demanding field work that involves the dogs working in groups with other dogs and people; in fact, the change that concerns communication with conspecifics is much less common in dogs that have discontinued the programme, or those for whom it is still in progress, than in those that have completed it.
It is no accident that this stage of the rehabilitation programme is offered at a later time, when the dogs have already acquired other skills that enable them to process information provided by their conspecifics during the sessions.

Moreover, it often takes multiple sessions for a dog to learn to communicate effectively and adequately with conspecifics: like all languages, it requires time and study to learn.

– It is amazing that the number of dogs that manage to overcome a defeatist attitude they had previously is very high indeed: only 3 out of 30 dogs failed to change their defeatist attitude.
In rehabilitation, changing this attitude is one of the most complex challenges of all; this number of successes is highly satisfying.

The preparatory nature of certain skills compared with others is also apparent in the relationship between changes 1 and 2, as already stated: in order to be able to develop the concept of pause and use it to reflect effectively on the lived experience, a dog must first have acquired the ability to manage it in the best possible way from an emotional point of view, achieving calm and satisfaction, and therefore being able to actually close the activity that has just ended.

It is also apparent that low-dose BDNF promotes change in dogs: only 2 out of the 257 subjects failed to demonstrate any behavioural changes; these are both dogs whose programme is still in progress.

Furthermore, it seems that cognitive plasticity is at the basis of these changes; we therefore propose to correlate these changes with effects of BDNF relating to neurogenesis, and also to the fact that it acts predominantly on the dopaminergic and serotonergic pathways, which are the most degraded in patients with behavioural disorders.
– Another effect that can be correlated with the increase in cognitive plasticity, and to greater emotional of awareness in dogs, is the downregulation of phosphorylation of the Tau protein, which inhibits the formation of tangles that result in a more difficult, less effective diffusion of neurotransmitters in the intercellular matrix and therefore of interneuronal dialogue.
It seems clear that the increase in dendritic projections can also be correlated with greater ability to direct information, leading patients to a true functional restructuring of memory: patients suffering from hypersensitivity-hyperactivity syndrome in particular, whose storage of experience in the long-term memory is fragmented, finally manage to correlate information and "classify" it, making it functionally accessible such that it can be used in future experience.

The change in the ability to adequately address and solve a problem is proof of this.
– The effect of proliferation on the glia is probably correlated with these changes.
With regard to the changes in patients suffering from cognitive impairment, the observations and considerations regarding Alzheimer's in humans are confirmed (Molinari, 2020).
There is no doubt that, due to the "physical" nature of the proposed rehabilitation activities, even the chance for the dog to perform physical activity further enhances the endogenous production of BDNF.

CONCLUSIONS

The behavioural changes indicated in this study are essential to a successful outcome on a rehabilitation programme, as well as being the focal points for achieving the most common therapeutic objectives in canine rehabilitation.
The fact that the changes occurred at an extremely high frequency highlights the effectiveness of BDNF in promoting these changes.
In many cases we were able to explore how these changes, which would have previously been impossible in the same patient, could be achieved shortly after the initiation of BDNF administration.
– Since this is an initial data collection, a detailed analysis of the data was not performed; however, the high number of cases studied and the quantity of data collected could generate analyses that enable us to highlight possible correlations between different disorders and the progress of the rehabilitation programmes, along with analyses of the percentages of comorbidity between the various behavioural disorders.
We therefore propose to explore this topic in more detail in subsequent studies. ■

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The editorial team thanks the editor of the website from which the image is taken:

Fig. page 69
https://www.ageco.co.uk/viewpoint/insurance/how-to-stop-your-pet-from-destroying-your-home/

– The photographs on pages 71, 72 are by the second author.

Editor's note:
The bibliographic entries Uberti F. & Molinari C., **2018** and Milani L., **2022** can be found on **www.medibio.it** → **La Medicina Biologica**.

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GUNA-BDNF

HOMEOPATHIC MEDICINE

Oral drops, solution

INGREDIENTS

30 ml contain: Brain Derived Neurotrophic Factor C4 30 mL.
Inactive ingredient: ethyl alcohol 30%.

DIRECTIONS

Take the drops diluted in a little water or directly in the mouth. Oral use.

WARNINGS

Read the package leaflet before use.

PACKAGING

30 ml / 1.0 fl.oz. bottle with dropper. Oral drops, solution.

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