Neuropharmacology 117 (2017) 134–148

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Nerve growth factor derivative NGF61/100 promotes outgrowth of primary sensory neurons with reduced signs of nociceptive sensitization

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ARTICLE INFO

Article history:
Received 5 October 2016
Received in revised form
9 January 2017
Accepted 29 January 2017
Available online 2 February 2017

Keywords:
NGF
Dorsal root ganglion neurons
Substance P
CGRP
Bradykinin receptors

ABSTRACT

Nerve Growth Factor (NGF) is being considered as a therapeutic candidate for Alzheimer's disease. However, the development of an NGF-based therapy is limited by its potent pain activity. We have developed a "painless" derivative form of human NGF (NGF61/100), characterized by identical neurotrophic properties but a reduced nociceptive sensitization activity *in vivo*. Here we characterized the response of rat dorsal root ganglia neurons (DRG) to the NGF derivative NGF61/100, in comparison to that of control NGF (NGF61), analyzing the expression of noxious pro-nociceptive mediators. NGF61/100 displays a neurotrophic activity on DRG neurons comparable to that of control NGF61, despite a reduced activation of PLCγ, Akt and Erk1/2. NGF61/100 does not differ from NGF61 in its ability to up-regulate Substance P (SP) and Calcitonin Gene Related Peptide (CGRP) expression. However, upon Bradykinin (BK) stimulation, NGF61/100-treated DRG neurons release a much lower amount of SP and CGRP, compared to control NGF61 pre-treated neurons. This effect of painless NGF is explained by the reduced up-regulation of BK receptor 2 (B2R), respect to control NGF61. As a consequence, BK treatment reduced phosphorylation of the transient receptor channel subfamily V member 1 (TRPV1) in NGF61/100-treated cultures and induced a significantly lower intracellular Ca^{2+} mobilization, responsible for the lower release of noxious mediators. Transcriptomic analysis of DRG neurons treated with NGF61/100 or control NGF allowed identifying a small number of nociceptive-related genes that constitute an "NGF pain fingerprint", whose differential regulation by NGF61/100 provides a strong mechanistic basis for its selective reduced pain sensitizing actions.

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1. Introduction

Nerve Growth Factor (NGF) (Levi-Montalcini, 1987) is a neurotrophin acting on cholinergic neurons of the basal forebrain (Dreyfus, 1989) and on sympathetic and sensory neurons (McAllister, 2001), as well as on other non-neuronal cell types.

NGF represents a validated therapeutic candidate, as demonstrated in different neurodegenerative pathological conditions, including diabetic neuropathies (Apfel, 2002), ophthalmic diseases (Bonini et al., 2000; Lambiase et al., 1998, 2009) and dermatological ulcers (Bernabei et al., 1999; Landi et al., 2003). In particular, due to

Abbreviations: AD, Alzheimers' disease; BK, bradykinin; B2R, bradykinin 2 receptor; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglion neurons; Fluo-3 AM, calcium sensitive fluorescent dye indicator; NGF, nerve growth factor; NGF61, control NGF; NGF61/100, painless NGF; PPTA, preprotachykinin A; SP, substance P; TrkA, tyrosine kinase receptor 1; TRPV1, vanilloid receptor subtype 1.

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http://dx.doi.org/10.1016/j.neuropharm.2017.01.035
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its crucial actions in long-lasting cholinergic maintainance (Delcroix et al., 2004; Mufson et al., 2008), in broader neuroprotection (Chao et al., 2006; Giacobini and Becker, 2007), and as a direct anti-amyloidogenic factor (Cattaneo et al., 2008; Matrone et al., 2008), its potential for clinical applications for neurodegenerative diseases of the Central Nervous System, such as Alzheimer's disease (AD) has been well demonstrated (Eriksdotter-Jonhagen et al., 1998, 2012; Eyjolfsdottir et al., 2016; Tuszynski et al., 2005, 2015).

However, the therapeutic uses of NGF are severely hampered by its potent nociceptive activity, well demonstrated both in animals and in humans, where this pain inducing activity was responsible for the interruption of clinical trials (Apfel, 2002; Covaceuszach et al., 2010; Eriksdotter-Jonhagen et al., 1998; Petty et al., 1994; Pezet and McMahon, 2006).

Recently, a mutated form of human NGF, NGF61/100, termed “painless” NGF, has attracted a lot of attention for its ability to exert full neurotrophic and anti-amyloidogenic activities in vitro and *in vivo* (Capsoni et al., 2012), nevertheless a reduced nociceptive activity *in vivo* (Capsoni et al., 2011; Covaceuszach et al., 2010; Malerba et al., 2015). As demonstrated by mechanical allodynia and heat hyperalgesia tests, NGF61/100 injected in the hind-paw of adult mice showed a 10-fold reduced pro-nociceptive activity, as compared to that of human NGF WT and to NGF61 (Malerba et al., 2015). Painless NGF also showed a greatly reduced pain in the orofacial region, upon nasal delivery (Capsoni et al., 2016). Besides harboring the pain reducing mutation R100E, painless NGF is characterized by the replacement of the Proline residue at position 61 of NGF wild-type (WT) with a Serine residue (NGF61). This mutation, while allowing an identical biological activity to that of human NGF, allows NGF61 (and NGF61/100) to be traced, against the endogenous NGF with a specific immunoassay (Covaceuszach et al., 2010; Malerba et al., 2015).

The molecular mechanisms through which this “painless” NGF exerts a reduced nociceptive activity have not been investigated.

NGF is well known to modulate the nociceptive properties of a subset of dorsal root ganglion sensory neurons (DRG) (small-diameter sensory neurons) (Pezet et al., 2001; Skoff and Adler, 2006; Sofroniew et al., 2001; Verge et al., 1989, 1995). The nociceptive activity of NGF is mediated by the activation of the tyrosine kinase receptor A (TrkA) and p75(NTR) (p75NTR) receptors on sensory nerve endings (Pezet and McMahon, 2006) and profoundly up-regulates gene expression of several proteins involved in synaptic transmission and neuronal excitability (McAllister, 2001; Pezet and McMahon, 2006).

Specifically, NGF positively regulates the mRNA and secretion levels of the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP), normally expressed in DRG neurons (Bowles et al., 2006; Lindsay and Harmar, 1989; Malcangio et al., 1997).

Acute exposure to NGF enhances excitability of sensory neurons and induces hyperalgesia (Lewin et al., 1993), while long term exposure, occurring during inflammation, increases the content of neuropeptide transmitters, and their release from sensory nerve endings (Sun et al., 2004; Yang et al., 2007).

In inflammatory conditions, NGF itself is up-regulated and plays an important role in inflammatory pain by driving peripheral sensitization, acting directly on the peripheral terminal to produce hyperalgesia (Ji and Woolf, 2001; Lewin et al., 1993; Thompson et al., 1995).

The aim of the present study was to compare and characterize the response of rat DRG neurons to the painless NGF61/100, with respect to the control NGF61, molecule exhibiting an identical biological activity to that of human wild type NGF (WT) (Covaceuszach et al., 2010; Malerba et al., 2015).

In particular, it is not known by which mechanisms painless NGF exerts or reduces pain perception and sensitization with respect to WT NGF and whether such mechanism involves the release of noxious mediators such as SP and CGRP. The results reveal a new role of painless NGF in regulating the release of SP and CGRP, induced by bradykinin (BK) via BK receptors 2 (B2R).

2. Materials and methods

2.1. Expression and purification of human NGF

Human NGF and its mutants NGF61/100 and NGF61 were expressed as recombinant proteins in E. coli in the proNGF form, refolded from inclusion bodies, purified, and proteolytically processed to NGF, as previously described (Malerba et al., 2015). All reagents were from Sigma (St. Louis, MO, Missouri), if not specifically reported.

2.2. Dorsal root ganglion (DRG) primary cultures and cell viability

All procedures were approved by the Italian Ministry of Health (Rome, Italy) and performed in compliance with the guidelines of the US National Institutes of Health and the Italian Ministry of Health (D.L.116/92). DRG neurons were prepared from neonatal (5 days, P5) Wistar rats (Charles River) from both sex, as reported (Bonnington and McNaughton, 2003; Taneda et al., 2010). Briefly, DRG were collected, incubated for 1 h at 37 °C with 0.125% collagenase (Sigma, St. Louis, MO, USA), mechanically dissociated and plated onto coverslips or Petri dishes pretreated with 10 μg/ml poly-L-lysine (Sigma-Aldrich, St Louis, MO), at a density of 50000 cells/well of a 48 well tissue culture plate. The DRG cultures were maintained in serum-free medium, consisting of Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 87.5 ng/mL 5-fluoro-2′-deoxyuridine, 37.5 ng/mL uridine, 50 U/mL penicillin and 50 μg/mL streptomycin (all from Sigma) and 0.05% N2 supplement (Invitrogen, Carlsbad, CA, USA) at 37 °C in 5% CO_2. The treatment with N2 supplement allows the presence of a physiological level of growth factors able to prevent the onset of a neurotrophin withdrawal state. After 2–3 days in vitro, DRG cultures were stimulated for experimental procedures using either control NGF61 or NGF61/100 (100 ng/ml) for 5 days, or maintained in basal medium conditions (CTRL). At the end of this incubation period, media and cells were collected for further processing as described below. Cell viability was assessed by counting the number of neuronal intact nuclei as described (Severini et al., 2015). Briefly, DRG neurons were fixed and permeabilized in 4% paraformaldehyde with 0.2% Triton X-100 in Tris HCl 0.1 M pH 7.4 for 5 min, incubated overnight at 4 °C with mouse anti NeuN (Sigma, 1:200), followed by secondary goat anti mouse antibody (Sigma, 1:400) and Hoechst 33258 (0.25 μg/ml) for 5 min at room temperature. After washing with PBS, the NeuN positive nuclei were visualized by a Leica fluorescent photomicroscope and scored by counting 12 microscopic fields per coverslip in 2 coverslips from 4 experiments.

2.3. RNA isolation, real-time RT-PCR and microarray analysis

Total RNA was extracted from DRG neurons treated with different NGF species (as described above) using the TRIzol solution Invitrogen (Carlsbad, CA, USA), according to the manufacturer's instructions. RNA quantity was determined on a NanoDrop UV-VIS. Only samples that showed a ratio of absorbance $1.8 < OD260/OD280 < 2.0$ were further analyzed.

For real-time RT-PCR analysis, cDNA was synthetized as follows: 2 μg total RNA were reverse transcribed in MLV reverse

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