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Cholecystokinin-8 enhances nerve growth factor synthesis and promotes recovery of capsaicin-induced sensory deficit

[1,2]Luigi Manni, [2]Thomas Lundeberg, [1,2]Paola Tirassa & *,[1]Luigi Aloe

[1]Institute of Neurobiology (CNR), V.le C. Marx, 15–43, 00137, Rome, Italy and [2]Department of Physiology and Pharmacology, Karolinska Institutet, S-17177, Stockholm, Sweden

1 Alterations of nerve growth factor (NGF) expression have been demonstrated during peripheral nerve disease and the impaired expression or synthesis and transportation of NGF has been correlated with the pathogenesis of several peripheral neuropathies. Since exogenous NGF administration seems to cause undesired side-effects, therapeutical strategies based on the regulation of endogenous synthesis of NGF could prove useful in the clinical treatment of these disorders.

2 The aim of the present study was to analyse the effects of exogenous peripheral administration of the neuropeptide cholecystokinin-8 (CCK-8) on endogenous NGF synthesis, NGF mRNA and distribution of peripheral neuropeptides which are known to be regulated by this neurotrophin.

3 To address these questions we studied the effects of capsaicin (CAPS) before and after the administration of CCK-8 on NGF levels, NGF mRNA expression and localization, and the concentration of substance P (SP) and calcitonin gene-related peptide (CGRP) in peripheral tissue.

4 These studies demonstrate that administration of the CCK-8 induces an increase of NGF protein and mRNA in peripheral tissue. NGF level in paw skin of CAPS/CCK-8-treated mice is 3 fold higher than in controls (1241 ± 110 pg gr^{-1} of tissue wet weight versus 414 ± 110 pg gr^{-1} of controls) and nearly 6 fold higher than in CAPS-treated mice (1241 ± 110 pg gr^{-1} versus 248 ± 27 pg gr^{-1}). The increase of NGF is correlated with the recovery of impaired nocifensive behaviour and with an overexpression of SP and CGRP.

5 The evidence that CCK-8 promotes the recovery of sensory deficits suggests a potential clinical use for this neuropeptide in peripheral neuropathies.

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Keywords: Sensory response; peripheral neuropathies; nerve growth factor (NGF); cholecystokinin (CCK-8); substance P (SP); calcitonin gene-related peptide (CGRP); capsaicin; mice

Abbreviations: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; CAPS, capsaicin; CCK-8, cholecystokinin-8; CGRP, calcitonin gene-related peptide; ChAT, choline-acetyltransferase; CNS, central nervous system; DAB, diamminobenzydine; DRG, dorsal root ganglia; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger ribonucleic acid; NGF, nerve growth factor; PN, peripheral neuropathies; PNS, peripheral nervous system; RT–PCR, reverse transcriptase-polymerase chain reaction; SP, substance P

Introduction

The term 'peripheral neuropathy' (PN) is used to encompass many distinct syndromes, which differ according to the aetiology and to which neuronal population is actually affected. PN may be induced by surgery or injuries or else may develop as a side effect after exposure to neurotoxic compounds, such as anti-neoplastic drugs, or as a disease-related syndrome (Barohn, 1988). PN caused in patients by anti-neoplastic drugs is a serious side effect in the therapeutical utilization of these compounds (Barohn, 1988). PN is also one of the common complications in patients with diabetes mellitus, causing impairment of sensory neurones and resulting in impaired wound healing (Younger *et al*., 1998). It is, therefore, reasonable to suggest that the identification of substances that can prevent or reduce PN be of major clinical interest. Recent studies on the utilization of nerve growth factor (NGF) in the treatment of peripheral neuropathies have attracted great attention (Tomlinson *et al*., 1997).

NGF is the best-characterized target-derived neurotrophic factor, and plays a crucial role in growth and differentiation of peripheral sensory and sympathetic neurones (Levi-Montalcini, 1987). NGF can differentially regulate peptide expression in adult sensory neurones of dorsal root ganglia (DRG) in both normal and pathological conditions (Verge *et al*., 1995). Moreover, the exogenous administration of NGF stimulates the synthesis of neuropeptides (Amann *et al*., 1996) and is able to recover the neurochemical function of both sympathetic and sensory neurones after selective chemical impairment (Donnerer, 1996; Donnerer *et al*., 1996). NGF and NGF receptor expression are profoundly altered in both humans (Sobue *et al*., 1998) and animals suffering from peripheral neuropathies (Heumann *et al*., 1987; Hellweg & Hartung, 1990). Administration of exogenous NGF greatly reduces hypersensitivity to noxious stimuli after induction of peripheral neuropathies (Ren *et al*., 1995), and a protecting role for NGF in experimental diabetic peripheral neuropathy has been demonstrated (Apfel *et al*., 1994; Tomlinson *et al*., 1997). These findings indicate that therapeutical strategies based on the modulation of NGF expression both at target and at peripheral nerve level could be useful in the clinical treatment of peripheral neuropathies. Unfortunately, major problems are caused by systemic treatment with the neurotrophin, due to its short half-life when injected into the blood circulation (Podluso *et al*., 1998) and to possible side effects of the high pharmacological doses that need

*Author for correspondence; E-mail: aloe@in.rm.cnr.it

to be used (Apfel *et al*., 1998). We recently reported that exogenous peripheral administration of cholecystokinin-8 (CCK-8), a neuropeptide widely distributed in the peripheral and central nervous system (Rehfeld, 1980; Linden *et al*., 1989), is able to induce NGF synthesis in rodent brain through a CCK-receptor mechanism (Tirassa *et al*., 1998). This observation raised the question as to whether CCK-8 is able to enhance the NGF effects in lesioned peripheral NGF-responsive neurones.

In the present study, we used a model of peripheral sensory neuropathy induced by capsaicin (CAPS) – which results in long-term impairment of afferent sensory neurone functions (Holzer, 1991) – to investigate the effects of exogenous CCK-8 administration. The results show that this neuropeptide, acting through the regulation of endogenous NGF synthesis and the expression of sensory neuropeptides, promotes functional recovery of capsaicin-induced deficits.

Methods

Animals

Adult 3-month-old male mice of CD-1 strain, purchased from Charles River (Calco, Italy), were housed four or five per cage under a 12–12 h light–dark cycle with water and food *ad libitum*. Animal care and procedures were conducted in conformity with the intramural committee and institutional guidelines in accordance with national and international laws (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987).

Drugs

The following drugs were used: cholecystokinin-8 (CCK 26–33) was obtained from Peninsula Lab. (U.S.A.); capsaicin was obtained from Fluka AG (Germany).

Treatment with capsaicin and CCK-8

To induce sensory neuropathy, adult mice ($n=48$) were first intraperitoneally (i.p.) injected with 50 mg kg^{-1} ketamine and 15 mg kg^{-1} xylazine to induce anaesthesia and then subcutaneously (s.c.) injected with 50 mg kg^{-1} capsaicin or left untreated as controls. When given systemically to adult animals, capsaicin produces a generalized desensitization and loss of sensory nerves (Holzer, 1991). After 10 days mice were divided in four groups and treated with and without CCK-8, as previously described (Tirassa *et al*., 1998), as follows: (i) CAPS-treated, injected with CCK-8 ($n=12$); (ii) CAPS-treated, injected with vehicle ($n=12$); (iii) untreated and injected with CCK-8 ($n=12$); (iv) untreated and injected with vehicle ($n=12$). CCK-8 (8 nmol kg^{-1}) or vehicle (saline) was subcutaneously injected once a day for 10 consecutive days, starting 10 days after the last CAPS treatment; mice were then sacrificed and peripheral tissue removed, immediately frozen and used for NGF or neuropeptide determination.

Behavioural study

To evaluate the effect of sensory denervation we used the hot-plate test (Aloe *et al*., 1997). Nocifensive withdrawal responses were measured using a hot-plate apparatus (Socrel Hot-plate model DS37, Ugo Basile, Italy). Temperature was set at $50\pm0.2^{\circ}C$, cut-off time was 60 s. Nocifensive responses were measured by measuring the latency to the first episode of jumping or forepaw/hind paw licking (Aloe *et al*., 1997). Latency time was determined using a digital stopwatch. All groups of mice were tested starting 2 days after the second injection of the neurotoxic compounds and every 2 days until the last CCK-8 injection was performed.

NGF determination

All mice were sacrificed with an overdose of sodium pentobarbital, hind paw plantar skin was removed, and used for the evaluation of peripheral innervation, neuropeptide content, NGF and brain-derived neurotrophic factor (BDNF) levels. The levels of NGF were measured by a highly sensitive two-site enzyme-linked immunosorbent assay (ELISA) (Weskamp & Otten, 1987) which recognizes human and murine NGF and does not cross react with brain derived neurotrophic factor (Bracci-Laudiero *et al*., 1992; Tirassa *et al*., 1998). Briefly, polystyrene 96-well immunoplates (Nunc) were coated with monoclonal mouse anti-NGF antibody (Boehringer Mannheim, Germany) diluted in 0.05 M carbonate buffer (pH 9.6). Parallel wells were coated with purified mouse IgG (Zymed, San Francisco, CA, U.S.A.) for evaluation of the non-specific signal. After an overnight incubation at room temperature and 2 h incubation with a blocking buffer (0.05 M carbonate buffer, pH 9.5, 1% BSA), plates were washed three times with 50 mM Tris-HCl pH 7.4, 200 mM NaCl, 0.5% gelatine and 0.1% Triton X-100. Tissues were sonicated in sample buffer (mM): Triton X-100 0.1%, Tris-HCl pH 7.2 100, NaCl 400, EDTA 4, PMSF 0.2, benzethonium chloride 0.2, benzamidine 2, aprotinin 40 u ml^{-1}, sodium azide 0.05%, BSA 2% and gelatine 0.5%, centrifuged at 8500 $\times g$ for 30 min and supernatants used for the assay. After extensive washing of the plates, samples and NGF standard solutions diluted with sample buffer were distributed into the wells and left at room temperature overnight. The plates were then washed three times and incubated with 4 mu $well^{-1}$ anti-β-NGF-galactosidase (Boehringer Mannheim, Germany) for 2 h at 37°C and, after further washing, 100 μl of substrate solution (4 mg ml^{-1} of chlorophenol red, Boehringer Mannheim, Germany, substrate buffer (mM). HEPES 100, NaCl 150, $MgCl_2$ 2, sodium azide 0.1% and BSA 1% was added to each well. After an incubation of 2 h at 37°C, the optical density was measured at 575 nm using an ELISA reader (Dynatech 5000, Germany), and the values of standards and samples were corrected by taking into consideration the non-specific binding. The recovery of NGF during assay procedure was estimated by adding a known amount of highly purified NGF to the samples or to the homogenization buffer, as internal control. The yield of the exogenous NGF was calculated by subtracting the amount of endogenous NGF from the value of endogenous plus exogenous NGF. Under these conditions, the NGF recovery was over 90%. The limit of sensitivity of NGF ELISA averaged at 0.5 pg per assay. Data are represented as pg g^{-1} wet tissue weight and all assays were performed in triplicate.

BDNF determination

The concentrations of BDNF were measured with the 'BDNF Emaxtm Immuno Assay System number G6891' following the procedure suggested by the manufacturer (Promega, Madison, WI, U.S.A. See also Angelucci *et al*., 1999).

Neuropeptide analysis

A highly specific competitive radioimmunoassay (RIA) was used (detection limit 1.5 fmol = 2 pg per incubate; detectable

concentration 15 fmol l^{-1} = 20 pg per ml) according to procedures previously described (Aloe *et al*., 1995) for evaluation of paw skin contents of substance P (SP) and calcitonin gene-related peptide (CGRP). The tissue concentration of SP-like immunoreactivity (SP-LI) was analysed using the C-terminally directed antiserum SP2 (Brodin *et al*., 1986) with ^{1251}[Tyr8]-SP as radioligand and synthetic SP as standard. The tissue concentration of CGRP-like immunoreactivity (CGRP-LI) was analysed using antiserum CGRP-8 raised in rabbit against conjugated rat CGRP with ^{1251}histidyl-rat CGRP as radioligand and rat CGRP as standard (Ahmed *et al*., 1994).

In situ *hybridization*

Fourteen-micron sections from hind paw skin were cut by cryostat and mounted on poly-L-lysine-coated slides. The slices were fixed in 4% paraformaldeyde in 0.1 M PBS (pH 7.4) for 10 min followed by repeated rinsing in 0.1 M PBS and dehydration by 70, 80, 95% ethyl alcohol. After acetylation (25% acetic anhydride in 0.1 M TEA pH 8.0), the slices were incubated at 42°C for 16 h in a hybridization mixture containing digoxigenin-labelled NGF oligonucleotide complementary to the sequences 5′-TCCTGTTGA-GAGTGGTGCCGGGGCATCGA-3′ and correspondent to position 841–871 of the mouse NGF mRNA sequence (Scott *et al*., 1983), at a final concentration of 30 ng μl^{-1} hybridization buffer (50% formamide, 2× SSC, 0.1%. SDS, 250 μg ml^{-1} denatured sheared salmon tested DNA). Hybridization using the sense orientation corresponding oligonucleotide was carried out in parallel to test the specificity of the technique. After washing, the slices were incubated for 2 h at room temperature with a 1.5 u/ml sheep anti-digoxigenin POD conjugated antibody (polyclonal Fab fragment; Boehringer Mannheim, Germany). The immunoperoxidase reaction was detected using standard diamminobenzydine (DAB) procedure (0.6 mg ml^{-1} DAB and 0.015% H_2O).

RT–PCR

Total RNA was extracted using the method of Chomczynski & Sacchi (1987) as modified in the TRIZOL® kit (GIBCO, Life Technologies, Rome, Italy). One μg of RNA was reverse transcribed into a single stranded cDNA with the reverse transcription system (Promega Italia, Milano, Italy) in a total reaction volume of 20 μl, using 250 ng Oligo $(dT)_{12-18}$ primer, 200 units of MLV-RT (Promega Italia, Milano, Italy) and 0.5 u RNasin ribonuclease inhibitor (Promega Italia, Milan, Italy). To compensate for the relative differences in sample size, integrity of the individual RNA samples and the variation in reverse transcription, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was co-amplified with murine NGF. The PCR reaction was carried out in 50 μl mixtures containing 5 μl of sample cDNA, 5 μl 10X *Taq* polymerase buffer (Promega Italia, Milan, Italy), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP (Pharmacia Biotech, C. Monzese, Italy), 12.5 pmol of the following primers: NGF: 5′-CAGGACTCACAGGAG-CAAGC-3′; 5′-GCCTTCCTGCTGAGCACACA-3′ respectively corresponding to position 660–679 and 984–1003 of the mouse NGF mRNA (Scott *et al*., 1983); GAPDH: 5′-CACCACCATGGAGAAGGCC-3′; 5′-GATGGATGCCTT-GGCCAGG-3′ respectively corresponding to position 346–365 and 517–536 of mouse GAPDH mRNA (Sabath *et al*., 1990), and 2 U *Taq* polymerase (Promega Italia, Milan, Italy) on a GeneAmp PCR System 9600 thermal cycler (Perkin Elmer Cetus, CA, U.S.A.) for 30 cycles (60 s at 95°C, 60 s at 55°C and 120 s at 72°C). The PCR products are a 343 base

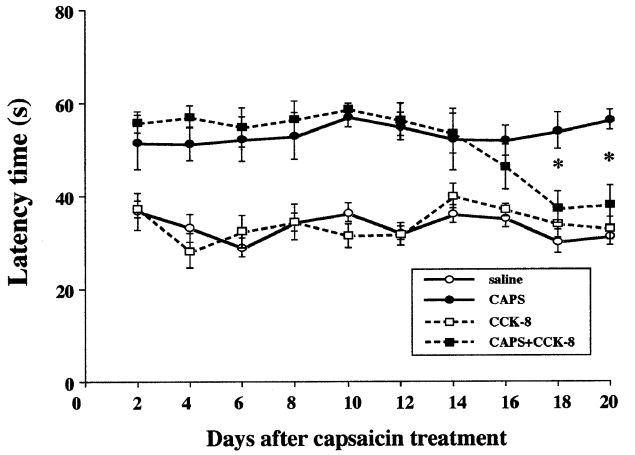

Figure 1 Hot-plate response of adult mice treated with capsaicin for 3 days. One group of CAPS-treated mice ($n=12$) and one group of control mice ($n=12$) were treated with CCK-8 for 10 days starting 10 days after the last injection of capsaicin. The latency time of the response to noxious heal in CAPS-treated mice ($n=12$) remains higher than in the controls ($n=12$) for the entire observation period, while the treatment with CCK-8 induces a decrease of the response-latency time in CAPS-treated mice, as revealed by ANOVA on the repeated measures, reaching the baseline values after 8–10 days of treatment. The vertical lines indicate pooled s.e.means derived from appropriate mean square error in the ANOVA. $*P<0.05$ when CAPS and CAPS + CCK-8 groups are compared.

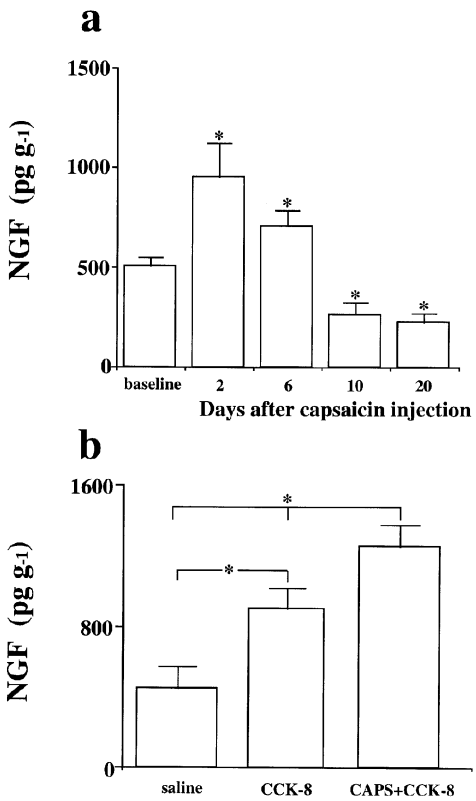

Figure 2 Effect of capsaicin and CCK-8 on NGF levels in the hind paw skin. (a) NGF, expressed as pg g^{-1} of wet weight, increases soon after CAPS treatment, reaching the highest level around 2 days from the injection of the neurotoxic compound, and then decreases to levels lower than control ($n=6$ for each group. $*P<0.05$ when compared to controls). (b) Treatment for 10 days with physiological amounts of CCK-8 increases NGF levels in normal mice and further enhances the neurotrophin expression in CAPS-lesioned mice ($n=12$ for each experimental group). Data are plotted as mean ± s.e.mean. $*P<0.05$.

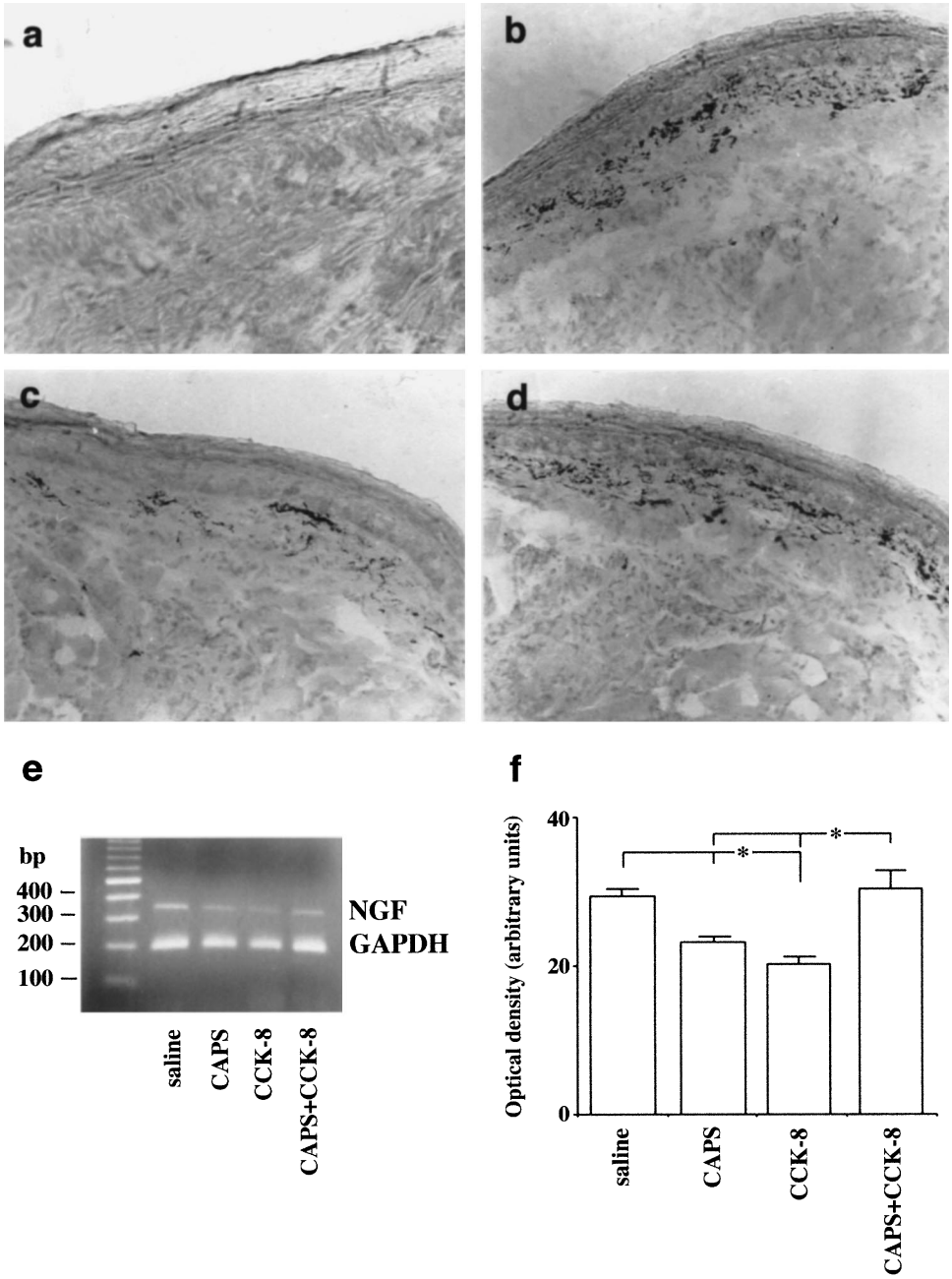

Figure 3 Effects of capsaicin and CAPS + CCK-8 treatment on NGF mRNA expression in the hind paw skin of adult mice. *In situ* hybridization (a – d) shows that NGF mRNA is normally expressed in the dermal layer of the skin (b). Specific NGF mRNA was confirmed by a specificity test including digestion of mRNA with Rnase-A before hybridization (not shown) and hybridization with sense NGF probe (a), which resulted in absence of hybridization signal. The decreased expression of NGF mRNA observed after treatment with capsaicin (c) was completely reversed by treatment with CCK-8 (d). The histological data were confirmed by quantitative evaluation of NGF mRNA performed by densitometric analysis after RT – PCR (e – f). The optical density of GAPDH bands was used as normalizing factor. The data shown in (f) represent the mean ± s.e.mean ($*P < 0.05$) of NGF-normalized densitometric values obtained from five different RT – PCR.

long fragment of NGF and a 190 base long fragment of GAPDH. After PCR, 20 μl of undiluted reaction products were loaded onto a 2% agarose MP (Boehringer Mannheim, Germany) gel containing 1 μg ml^{-1} ethidium bromide. The gel was run at 1 V cm^{-1} for 15 min and then at 5 V cm^{-1} for 3 h. The DNA-containing bands were photographed using an ultraviolet (u.v.) transilluminator. The identity of all the PCR products was confirmed by comparison with the correct size based on the known length of the DNA sequence on agarose gel and by Southern blotting (data not shown). Band densitometric evaluation – expressed as arbitrary units of grey level – was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/), which determines the optical density of the ethidium bromide stained bands using a grey scale thresholding operation. The optical density of GAPDH bands was used as normalizing factor.

Statistical analysis

Data were obtained by means of analysis of variance (ANOVA) using the SuperANOVA package for Macintosh (Abacus Concepts Inc., Berkeley, CA, U.S.A.), considering the

treatments with saline, CAPS, CCK-8 and CAPS + CCK-8 as variables. For the hot-plate response, the effect of CAPS and/or CCK-8 were analysed considering the repeated measures (ten tests) and the treatments (four levels: saline, CAPS, CCK-8, CAPS + CCK-8). Difference between groups was determined by Tukey-Kramer comparison; a $P<0.05$ was considered statistically significant.

Results

The nocifensive behaviour in mice treated with CAPS and/or CCK-8 was examined with the hot-plate test (Figure 1). ANOVA revealed a main effect of the treatments ($F_{3,35}=41.01$; $P<0.0001$) due to a longer latency response using the hot-plate in both CAPS-treated groups ($P<0.001$ in *post-hocs*). During the test, animals showed a habituation profile ($F_{9,315}=5.39$; $P<0.0001$ for the main effect of the repeated measures). In addition ANOVA revealed an interaction between treatments and the repeated measures ($F_{27,315}=6.61$; $P<0.0001$). Interestingly, *post-hoc* comparisons showed that, on both day 8 and day 10 after CCK-8 injection, CAPS animals treated with CCK-8 exhibited a normal latency response while CAPS-treated mice did not ($P<0.05$). No differences were found in the latency time between vehicle and vehicle + CCK-8 mice, and thus no hyperalgesic effect is attributable to CCK-8 in our experimental conditions.

To assess whether CAPS treatment affects NGF levels in peripheral tissues, the concentration of NGF was measured in the paw skin. As reported in Figure 2a, the concentration of NGF in the paw skin increased 2 days after CAPS treatment and then progressively decreased, reaching levels lower than in controls after 10 days. The low amount of NGF lasted for at least 20 days, the latest time point examined.

To investigate the effect of CCK-8 on NGF synthesis, 10 days after CAPS injection mice were treated with 8 nmol kg^{-1} of CCK-8 each day for ten consecutive days. As illustrated in Figure 2b, CCK-8 at these doses and under these conditions stimulated the production of NGF in the peripheral target tissue. CCK-8 is also able to enhance the constitutive level of NGF in non-lesioned animals, although to a lesser extent. The treatment with CCK-8 seems to be specific for NGF, while it has no effect on paw skin levels of other neurotrophins, such as BDNF (989 ± 69 pg g^{-1} in control group versus 935 ± 44 pg g^{-1} in CCK-8 group, $P>0.1$; 1503 ± 148 pg g^{-1} in CAPS group versus 1627 ± 113 pg g^{-1} in CAPS + CCK-8 group, $P>0.1$).

To identify the cells involved in the upregulation of NGF and to assess whether CCK-8 also affects NGF mRNA synthesis, we analysed NGF mRNA expression in the paw skin by *in situ* hybridization and RT–PCR techniques. As illustrated in Figure 3a–d, cells localized in the dermal layer of paw skin both in controls and CAPS-treated mice express NGF mRNA. However, quantitative evaluation shows that only in injured animals is CCK-8 able to upregulate the NGF mRNA in the paw skin (Figure 3e–f).

As CAPS is known to alter not only peripheral sensory responses but also the cellular distribution of sensory neuropeptides (Gamse *et al*., 1982; Donnerer *et al*., 1996) we investigated whether the action of CCK-8 on NGF also influences neuropeptide levels in the paw skin. As reported in Figure 4, radioimmunoassay revealed that treatment of CAPS mice with CCK-8 enhances the amount of substance P (SP) and calcitonin gene-related peptide (CGRP) in the paws of the CAPS + CCK-8 group, suggesting that this treatment promotes a recovery of pharmacologically induced deficit in the peripheral field.

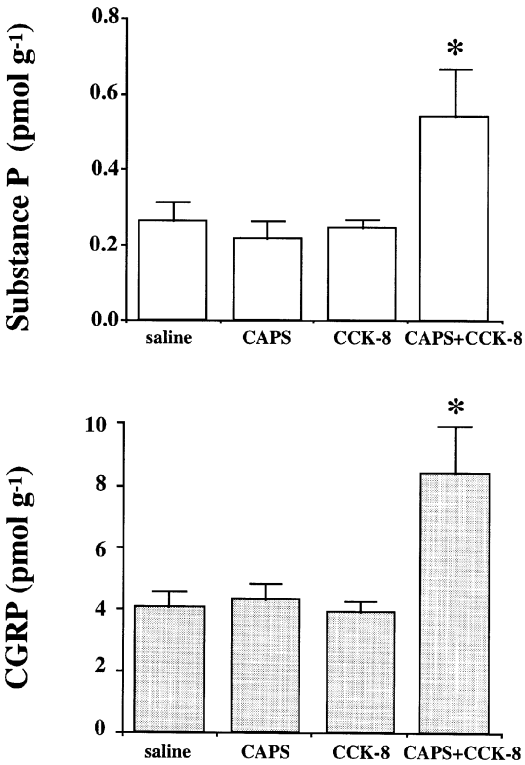

Figure 4 Capsaicin action on the level of SP and CGRP in the hind paw of adult mice before and after treatment with CCK-8, which increases the level of both sensory neuropeptides only in the paw skin of CAPS-treated mice ($n=12$ for each experimental group). Data are plotted as mean ± s.e.mean. *$P<0.05$ when compared to controls.

Discussion

CCK-8 is a gut neuropeptide widely distributed in the central (CNS) and in the peripheral nervous system (PNS) (Rehfeld, 1980). We have recently demonstrated that administration of CCK-8 enhances brain NGF levels and stimulates choline-acetyltransferase (ChAT) activity in normal and surgical injured forebrain neurones (Tirassa *et al*., 1998; 1999). Using a model of peripheral neuropathy induced by capsaicin (Holzer, 1991; Donnerer *et al*., 1996), we found that CCK-8 is also able to promote functional and biochemical recovery in CAPS-treated mice, suggesting that it exerts its effect on damaged neurones not only in the CNS (Tirassa *et al*., 1998; 1999) but also in the PNS.

Under our experimental conditions, sensory deficits, as evaluated by the hot-plate response, persist for at least 3 weeks after CAPS administration. In the present study, we provide evidence that treatment with CCK-8 induces NGF expression in peripheral tissue and the recovery of chemical-impaired sensory function. It has been demonstrated that the CAPS treatment of adult rodents induces loss of sensory endings, causing impairment of retrograde transport of NGF from the target field to the nerve cell body (Miller *et al*., 1982). The result of low or non availability of NGF protein is a downregulation of SP and CGRP, which are both regulated by NGF (Miller *et al*., 1982; Skofitsch & Jakobowitz, 1985; McMahon *et al*., 1995; Verge *et al*., 1995; Amann *et al*., 1996). Recent reports demonstrate that sensory neuropeptides

decrease in the spinal cord and in the sciatic nerve after CAPS treatment and that exogenous NGF promotes the recovery of capsaicin-induced deficits (Donnerer *et al*., 1996; Schicho *et al*., 1999). As shown in Figure 4, 20 days after CAPS treatment the level of SP and CGRP in CAPS-treated animals is comparable to the level of controls. This observation suggests that chemical peripheral sensory denervation with CAPS only transiently affect the local level of these neuropeptide (Bittner & Lahann, 1984; Maggi *et al*., 1987). Our data, showing that the increase of NGF synthesis in CAPS/CCK-8-treated mice correlates with the recovery of sensory responses and with the over-expression of SP and CGRP in the paw skin, support previous evidence indicating that sensory neurones are dependent on NGF for their survival, particularly during loss of nerve endings and/or severe neuronal deficits (Verge *et al*., 1995). Though additional correlative histological, biochemical and neurochemical studies are needed in order to understand the mechanisms through which CCK-8 regulates NGF synthesis, our study provides a clear indication that endogenous NGF can be up-regulated by this small neuropeptide and that this effect is associated with the functional recovery of biological activities of NGF target tissues.

The observation that CCK-8 treatment increases NGF protein levels in both normal and CAPS-treated mice, while NGF mRNA in paw skin was decreased in normal and increased in CAPS-treated mice following CCK-8 administration, suggests that CCK-8 is able to exert its effect on NGF synthesis only following neuronal insults. However the possibility that under normal conditions CCK-8 might affect the basal turnover of NGF protein (see Figures 2 and 3) cannot be excluded. The functional significance of the different effects of CCK-8 on injured and non-injured tissues remains therefore to be elucidated.

It is known that CCK-8 is colocalized with SP and CGRP in DRG neurones projecting to the skin (Gibbins *et al*., 1987) and that NGF can affect CCK-8 expression inside the DRG (Verge *et al*., 1995). These observations raise the question as to whether CCK-8 acts directly on the synthesis of SP and CGRP or indirectly, through stimulation of NGF synthesis. Since these neuropeptides can be produced and stored in the same cells, another relevant question that needs to be answered is whether these effects are regulated through a paracrine and/or autocrine mechanism. As NGF regulates SP and CGRP synthesis, it would be interesting to investigate whether administration of anti-NGF antibodies influences the effect of CCK-8 on these neuropeptides.

In conclusion, using an animal model of sensory PN, the present study demonstrates for the first time that a peripheral neuropeptide may influence NGF synthesis and/or expression, and sensory neuropeptide levels in peripheral tissues. It has been reported that the effect of CCK-8 administration on brain NGF expression is dose-dependent (Tirassa *et al*., 1998) and that repeated peripheral injections with low doses of CCK-8 did not cause side effects such as alteration of food intake (Crawley & Beinfeld, 1983). Likewise, in our study we did not find hyperalgesic response after CCK-8 treatment. These findings suggest that – although the effects of a prolonged treatment with CCK-8 remain to be elucidated – a systemic treatment with low doses of CCK-8 may represent a potentially useful strategy for promoting the recovery of impaired PNS function.

References

AHMED, M., BJURHOLM, A., SRINIVASAN, G.R., THEODORSSON, E. & KREICBERGS, A. (1994). Extraction of neuropeptides from joint tissue for quantitation by radioimmunoassay. A study in the rat. *Peptides,* **15,** 317 – 322.

ALOE, L., MORONI, R., ANGELUCCI, F. & FIORE, M. (1997). Role of TNF-α but not NGF in murine hyperalgesia induced by parasitic infection. *Psychopharmacology,* **134,** 287 – 292.

ALOE, L., PROBERT, L., KOLLIAS, G., MICERA, A. & TIRASSA, P. (1995). Effect of NGF antibodies on mast cell distribution, histamine and substance P levels in the knee joint of TNF-arthritic transgenic mice. *Rheumatol. Int.,* **14,** 249 – 252.

AMANN, R., SIRINATHSINGHJI, D.J.S., DONNERER, J., LIEBMAN, I. & SCHULIGOI, R. (1996). Stimulation by nerve growth factor of neuropeptide synthesis in the adult rat in vivo: Bilateral response to unilateral intraplantar injections. *Neurosci. Lett.,* **203,** 171 – 174.

ANGELUCCI, F., FIORE, M., COZZARI, C. & ALOE, L. (1999). Effect of prenatal ethanol intake on nerve growth factor level and on the development of entorhinal cortex in postnatal mice. *Neurotoxicol. Teratol.,* **21,** 415 – 425.

APFEL, S.C., AREZZO, J.C., BROWNLEE, M., FEDEROFF, H. & KESSLER, J.A. (1994). Nerve growth factor protects against experimental diabetic sensory neuropathy. *Brain Res.,* **634,** 7 – 12.

APFEL, S.C., KESSLER, J.A., ADORNATO, B.T., LITCHY, V.J., SANDERS, C. & RASK, C.A. (1998). Recombinant human nerve growth factor in the treatment of diabetic polyneuropathy. NGF Study Group. *Neurology,* **51,** 695 – 702.

BAROHN, R.J. (1998). Approach to peripheral neuropathy and neuronopathy. *Sem. Neurol.,* **18,** 7 – 18.

BITTNER, M.A. & LAHANN, T.R. (1984). Biphasic time-course of capsaicin-induced substance P depletion: failure to correlate with thermal analgesia in the rat. *Brain Res.,* **322,** 305 – 309.

BRACCI-LAUDIERO, L., ALOE, L., LEVI-MONTALCINI, R., BUTTINELLI, C., SCHILTER, D. GILLESSEN, S. & OTTEN, U. (1992). Multiple sclerosis patients express increased levels of β-nerve growth factor in cerebrospinal fluid. *Neurosci. Lett.,* **147,** 9 – 12.

BRODIN, E., LINDEFORS, N., THEODORSSON-NORHEIM, E. & ROSELL, S. (1986). Tachykinin multiplicity in rat central nervous system as studied using antisera raised against substance P and neurokinin A. *Regul Pept.,* **13,** 253 – 272.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.,* **162,** 156 – 159.

CRAWLEY, J.N. & BEINFELD, M.C. (1983). Rapid development of tolerance to the behavioural actions of cholecystokinin. *Nature,* **302,** 703 – 706.

DONNERER, J. (1996). Improved neurochemical recovery of 6-hydroxydopamine-lesioned postganglionic neurons by nerve growth factor in the adult rat. *Neurosci. Lett.,* **221,** 33 – 36.

DONNERER, J., AMANN, R., SCHULIGOI, R. & SKOFITSCH, G. (1996). Complete recovery by nerve growth factor of neuropeptide content and function in capsaicin-impaired sensory neurons. *Brain Res.,* **741,** 103 – 108.

GAMSE, R., PETSCHE, U., LEMBECK, F. & JANCSO, G. (1982). Capsaicin applied to peripheral nerve inhibits axoplasmic transport of Substance P and somatostatin. *Brain Res.,* **239,** 447 – 462.

GIBBINS, I.L., FURNESS, J.B. & COSTA, M. (1987). Pathway-specific patterns of the co-existence of substance P, calcitonin gene-related peptide, cholecystokinin and dynorphin in neurons of the dorsal root ganglia of the guinea pig. *Cell Tissue Res.,* **248,** 417 – 437.

HELLWEG, R. & HARTUNG, H.D. (1990). Endogenous levels of nerve growth factor (NGF) are altered in experimental diabetes mellitus: a possible role for NGF in the pathogenesis of diabetic neuropathy. *J. Neurosci. Res.,* **26,** 258 – 267.

HEUMANN, R., KORSCHING, S., BANDTLOW, C. & THOENEN, H. (1987). Changes of nerve growth factor synthesis in non-neuronal cells in response to sciatic nerve transection. *J. Cell. Biol.,* **104,** 1623 – 1631.

HOLZER, P. (1991). Capsaicin: cellular targets, mechanism of action, and selectivity for thin sensory neurons. *Pharmacol. Rev.,* **43,** 143–201.

LEVI-MONTALCINI, R. (1987). The nerve growth factor 35 years later. *Science,* **237,** 1154–1162.

LINDEN, A., UVNAS-MOBERG, K., FORSBERG, G., BEDNAR, I. & SODERSTEN, P. (1989). Plasma concentrations of cholecystokinin octapeptide and food intake in male rats treated with cholecystokinin octapeptide. *J. Endocrinol.,* **121,** 59–65.

MAGGI, C.A., SANTICIOLI, P., GEPPETTI, P., FURIO, M., FRILLI, S., CONTE, B., FANCIULLACCI, M., GIULIANI, S. & MELI, A. (1987). The contribution of capsaicin-sensitive innervation to activation of the spinal vescico-vescical reflex in rats: relationship between substance P levels in the urinary bladder and the sensory-efferent function of capsaicin-sensitive sensory neurons. *Brain Res.,* **415,** 1–13.

MCMAHON, S.B., BENNETT, D.L., PRIESTLEY, J.V. & SHELTON, D.L. (1995). The biological effects of endogenous nerve growth factor on adult sensory neurons revealed by a trkA-IgG fusion molecule. *Nat. Med.,* **1,** 774–780.

MILLER, M.S., BUCK, S.H., SIPES, I.G., YAMAMURA, H.I. & BURKS, T.F. (1982). Regulation of substance P by nerve growth factor: disruption by capsaicin. *Brain Res.,* **250,** 193–196.

PODLUSO, J.F., CURRAN, G.L. & GILL, J.S. (1998). Putrescine-modified nerve growth factor: bioactivity, plasma pharmacokinetics, blood-brain/nerve barrier permeability, and nervous system biodistribution. *J. Neurochem.,* **71,** 1651–1660.

REHFELD, J.F. (1980). Cholecystokinin. *Trends Neurosci.* **3,** 65–67.

REN, K., THOMAS, D.A. & DUBNER, R. (1995). Nerve growth factor alleviates a painful peripheral neuropathy in rats. *Brain Res.,* **699,** 286–292.

SABATH, D.E., BROOME, H.E. & PRYSTOWSKY, M.B. (1990). Glyceraldeyde-3-phosphate dehydrogenase mRNA is a major interleukin 2-induced transcript in a cloned T-helper lymphocyte. *Gene,* **91,** 185–191.

SCHICHO, R., SKOFITSCH, G. & DONNERER, J. (1999). Regenerative effect of human recombinant NGF on capsaicin-lesioned sensory neurons in the adult rat. *Brain Res.,* **815,** 60–69.

SCOTT, J., SELBY, M., URDEA, M., QUIROGA, M., BELL, G.I. & RUTTER, W.J. (1983). Isolation and nucleotide sequence of a cDNA encoding the precursor of mouse nerve growth factor. *Nature,* **302,** 538–540.

SKOFITSCH, G. & JACOBOWITZ, D.M. (1985). Calcitonin gene-related peptide coexists with substance P in capsaicin sensitive neurons and sensory ganglia of the rat. *Peptides,* **6,** 747–754

SOBUE, G., YAMAMOTO, M., DOYU, M., LI, M., YASUDA, T. & MITSUMA, T. (1998). Expression of mRNAs for neurotrophin (NGF, BDNF, and NT-3) and their receptors ($p75^{NGFR}$, Trk, TrkB and TrkC) in human peripheral neuropathies. *Neurochem. Res.,* **23,** 821–829.

TIRASSA, P., ALOE, L., STENFORS, C., TURRINI, P. & LUNDEBERG, T. (1999). Cholecystokinin-8 protects central cholinergic neurons against fimbria-fornix lesion through the up-regulation of nerve growth factor synthesis. *Proc. Natl. Acad. Sci. U.S.A.,* **96,** 6473–6477.

TIRASSA, P., STENFORS, C., LUNDEBERG, T. & ALOE, L. (1998). Cholecystokinin-8 regulates NGF concentrations in adult mouse brain through a mechanism involving CCKA and CCKB receptors. *Br. J. Pharmacol.,* **123,** 1230–1236.

TOMLINSON, D.R., FERNYHOUGH, P. & DIEMEL, L.T. (1997). Role of neurotrophins in diabetic neuropathy and treatment with nerve growth factors. *Diabetes,* **46,** S43–S49.

VERGE, V.M.K., RICHARDSON, P.M., WIESENFELD-HALLIN, Z. & HÖKFELT, T. (1995). Differential influence of nerve growth factor on neuropeptide expression in vivo: a novel role in peptide suppression in adult sensory neurons. *J. Neurosci.,* **15,** 2081–2096.

WESKAMP, J. & OTTEN, U. (1987). An enzyme-linked immunoassay for nerve growth factor (NGF): a tool for studying regulatory mechanisms involved in NGF production in brain and peripheral tissues. *J. Neurochem.,* **48,** 1779–1786.

YOUNGER, D.S., ROSOKLIJA, G. & HAYS, A.P. (1998). Diabetic peripheral neuropathy. *Semin. Neurol.,* **18,** 95–104.

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