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### **Relationship between the neuroprotective effects of Insulin-Like Growth Factor-1 and 17 $\beta$ -Oestradiol in human neuroblasts.**

Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

*Original Citation:*

Relationship between the neuroprotective effects of Insulin-Like Growth Factor-1 and 17 $\beta$ -Oestradiol in human neuroblasts / P.Luciani ; C.Deledda; S. Benvenuti; I.Cellai; G.Modi; B.Fibbi; G. Danza; G.B.Vannelli; A.Peri. - In: JOURNAL OF NEUROENDOCRINOLOGY. - ISSN 0953-8194. - ELETTRONICO. - .....:(2012), pp. 0-0.

*Availability:*

This version is available at: 2158/652102 since: 2019-07-23T09:49:18Z

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## ORIGINAL ARTICLE

Relationship Between the Neuroprotective Effects of Insulin-Like Growth Factor-1 and 17 $\beta$ -Oestradiol in Human NeuroblastsP. Luciani<sup>1\*</sup>, C. Deledda<sup>1\*</sup>, S. Benvenuti\*, I. Cellai\*, G. Modi\*, B. Fibbi\*, G. Danza\*, G. B. Vannelli† and A. Peri\*<sup>\*</sup>Endocrine Unit, Department of Clinical Physiopathology, Center for Research, Transfer and High Education on Chronic, Inflammatory, Degenerative and Neoplastic Disorders for the Development of Novel Therapies (DENOThe), University of Florence, Florence, Italy.<sup>†</sup>Department of Anatomy, Histology and Forensic Medicine, University of Florence, Florence, Italy.Journal of  
Neuroendocrinology

Insulin-like growth factor-1 (IGF-1) and oestrogens interact with each other as neuroprotective factors. We have previously demonstrated that 17 $\beta$ -oestradiol protects against  $\beta$ -amyloid and oxidative stress toxicity and increases the amount of cell cholesterol in human foetal neuroblasts (FNC). The present study aimed: (i) to assess the protective effects of IGF-1 in FNC cells; (ii) to investigate the relationship between IGF-1 and 17 $\beta$ -oestradiol; and (iii) to determine whether cholesterol was a major mediator of the effects of IGF-1, similarly to 17 $\beta$ -oestradiol. We found that IGF-1 effectively exerts neuroprotective effects in FNC cells. We also demonstrated that the IGF-1 receptor (IGF-1R) pathway is needed to maintain oestrogen-mediated neuroprotection. Finally, we found that, opposite to 17 $\beta$ -oestradiol, IGF-1 did not cause a significant increase in cell cholesterol. These findings indicate that a cross-talk between IGF-1 and 17 $\beta$ -oestradiol occurs in FNC cells. In particular, the activation of the IGF-1R cascade appears to be fundamental to warrant 17 $\beta$ -oestradiol-mediated neuroprotection, even though cell cholesterol does not play a major role as an effector of this pathway.

**Key words:** IGF-1, nervous system, neuroprotection, 17 $\beta$ -oestradiol, cholesterol.

doi: 10.1111/j.1365-2826.2012.02343.x

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this study.

There is strong evidence that the insulin-like growth factor (IGF) system (namely IGFs, IGF receptors, IGF binding proteins) is represented in the central nervous system (CNS) and plays an important neuroprotective role by favouring neuronal development, metabolism, survival and regeneration (1–4). Similarly, experimental evidence strongly supports a neurotrophic and neuroprotective role of oestrogens both *in vitro* and *in vivo* (5–7). Furthermore, a strong link between oestrogen receptors (ERs) and the IGF-1 receptor (IGF-1R) occurs in the brain (8). Many neurons and astrocytes express both receptors and 17 $\beta$ -oestradiol is able to activate IGF-1R and its signalling pathways, leading to the activation of Akt that, in turn, regulates synaptic plasticity (9) and favours neuronal survival (10,11). On the other hand, IGF-1 can regulate the transcriptional activity of ERs. Indeed, IGF-1 may activate ERs in the absence of oestrogens (12,13), inducing ER-mediated gene expression in different cell types including neuronal cells (14–16).

We have previously demonstrated that, in human foetal neuroblast long-term cell cultures (FNC cells), the neuroprotective effects of oestrogens are, at least in part, mediated by seladin-1 (SElective AD INdicator-1) (17,18). This protein, which has been found to be down-regulated in brain regions affected by Alzheimer's disease

(AD) (19), confers resistance against  $\beta$ -amyloid-mediated toxicity and oxidative stress by acting as an anti-apoptotic factor via the inhibition of caspase-3 activation (19). Furthermore, it was subsequently demonstrated that the *seladin-1* gene corresponds to the gene encoding 3 $\beta$ -hydroxysterol  $\Delta$ 24-reductase (DHCR24), which catalyses the reduction of the  $\Delta$ 24 double bond of desmosterol to produce cholesterol (20). There is evidence that the amount of cell cholesterol may be a critical factor for CNS homeostasis. One of the mechanisms of  $\beta$ -amyloid toxicity is the generation of membrane pores permeable to toxic calcium ions. This mechanism is enhanced in cholesterol-depleted membranes, whereas it is markedly reduced in cholesterol-enriched membranes (21,22). Accordingly, reduced membrane lipids in the cortex of AD transgenic mice have been detected (23). Furthermore, the amount of cell cholesterol may also affect amyloidogenesis. Indeed, a moderate decrease in cholesterol levels, similar to that occurring in the nerve cells membrane of AD patients, has been shown to increase  $\beta$ -amyloid production (24–26).

In a previous study, we have demonstrated that FNC cells express IGF-1R and IGF-1. These cells also secrete IGF-1 and its release is increased by 17 $\beta$ -oestradiol. Finally, we found that IGF-1 induces

cell growth and significantly increases the expression level of seldin-1 (27). The present study aimed: (i) to investigate on the neuroprotective effects of IGF-1 in FNC cells; (ii) to determine whether these effects are, at least in part, related to the amount of cell cholesterol; and (iii) to identify the presence of a cross-talk between IGF-1 and 17 $\beta$ -oestradiol in this cell model.

## Materials and methods

### Cell cultures

The primary neuroblast long term cell culture FNC was established, cloned and propagated *in vitro* from human foetal olfactory neuroepithelium (28). FNC cells synthesise both neuronal proteins and olfactory markers and respond to odorant stimuli, suggesting their origin from the stem cell compartment that generates mature olfactory receptor neurons (29). In addition, they display neuroendocrine features (30). FNC cell cultures were propagated at 37 °C in 5% CO<sub>2</sub> in Coon's modified Ham's F-12 medium (Sigma, St Louis, MO, USA), 10% foetal bovine serum (FBS) (HyClone, Logan, UT, USA) and antibiotic solution (penicillin 100 IU/ml; streptomycin 100  $\mu$ g/ml) (PAA, Colbe, Germany). The B4 clone, showing the highest levels of expression of neuronal and olfactory markers (28), was used in the present study.

### Viability assays

Cell viability after  $\beta$ -amyloid exposure was determined by [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS; Promega, Milan, Italy) assay, as described previously (18). Briefly, cells were seeded in 96-well plates for 24 h in growth medium. Recommended instructions for  $\beta$ -amyloid (Calbiochem, Darmstadt, Germany) preparation were observed: the lyophilised peptide was solubilised in 50 mM Tris-HCl (pH 9.0) and then diluted to the appropriate concentration with calcium- and magnesium-free PBS. Successively, the peptide was incubated at 37 °C for 48 h to allow aggregation. Thereafter, the cells were exposed to 10 nM IGF-1 or 10 nM 17 $\beta$ -oestradiol (Sigma) in the presence or in the absence of 1  $\mu$ M of the ER antagonist ICI 182 780 (Sigma) or 1  $\mu$ g/ml of the IGF-1R antagonist JB-1 (Sigma) for 48 h. During the last 20 h of incubation, cells were treated with 1  $\mu$ M  $\beta$ -amyloid or 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>. Multiwell dishes were analysed using an enzyme-linked immunosorbent assay plate reader (Seac-Radim, Moncalieri, Italy) and optical density (OD) at 490 nm was considered directly proportional to the number of living cells. Each experimental point was performed in six replicates and the results were expressed as the OD/well (mean  $\pm$  SE of three different experiments).

To assess the resistance against H<sub>2</sub>O<sub>2</sub>-mediated oxidative stress, viable cells were also determined by Trypan blue dye exclusion test, as described previously (18). Briefly, the cells were cultured in 25-cm<sup>2</sup> flasks in growth medium in the presence or in the absence of 10 nM IGF-1 for 48 h. Cells were then treated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> during the last 20 h of incubation. Subsequently, cells were stained with Trypan blue dye for 1 min. Blue-positive and white-negative cells were counted in ten  $\times$  20 fields and the results were expressed as mean  $\pm$  SE percentage of dead/total cells in three different experiments.

### Cell proliferation

FNC cell growth was evaluated by the <sup>3</sup>H-thymidine incorporation assay. Cells were cultured in 24-well plates at 5  $\times$  10<sup>4</sup> cells/well for 48 h, in the presence of 10 nM IGF-1. During the last 20 h of incubation, cells were treated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> and pulsed with 1  $\mu$ Ci <sup>3</sup>H-thymidine. Cells were then harvested and samples were analysed using a  $\beta$ -counter. Each experimental

point was performed in six replicates and results were expressed as counts per minute/well (mean  $\pm$  SE of three different experiments).

### Cell cholesterol measurement

The amount of cell cholesterol in control FNC cells and in cells treated with 10 nM IGF-1 (in the presence or in the absence of 1  $\mu$ M ICI 182 780) or 10 nM 17 $\beta$ -oestradiol (in the presence or in the absence of 1  $\mu$ g/ml JB-1) for 48 h was determined by gas chromatography-mass spectrometry (GC-MS) as described previously (22), with minor modifications. Briefly, cells were maintained in medium without FBS for 6 h, then lysed in 1 N NaOH and the cell lysate was used for cholesterol quantification or frozen for storage at -80 °C until cholesterol measurement. Aliquots corresponding to 10  $\mu$ g of protein, as determined by Bradford staining, were used. After the addition of stigmasterol (1000 ng) as an internal standard, sterols were extracted with n-hexane, derivatised with N,O-bis(trimethylsilyl)trifluoroacetamide at 10% in trimethyl-chlorosilane and automatically injected in a Hewlett-Packard GC-MS system (Hewlett-Packard, Palo Alto, CA, USA). A six-point calibration curve in the 25–1000 ng cholesterol range was used for cholesterol quantification. The peak area ratios were calculated using the signals at *m/z* 458 and 484 for cholesterol and stigmasterol, respectively. Each point was performed in duplicate and the results represent the mean  $\pm$  SE of three experiments.

### Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using Nucleospin<sup>®</sup> RNAII (Macherey-Nagel, Duren, Germany) with DNase treatment in accordance with the manufacturer's instructions. The concentration was determined spectrophotometrically with Nanodrop<sup>®</sup> ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The amount of ER $\alpha$ , ER $\beta$  and IGF-1R mRNA was determined by real-time RT-PCR using pre-developed assays (Hs00174860\_m1; Hs00230957\_m1 and Hs00609566\_m1 respectively; Applied Biosystems, Foster City, CA, USA). According to the comparative C<sub>t</sub> (threshold cycle) method, the amount of target mRNA was normalised to the endogenous reference 18S RNA (Hs99999901\_s1) and also relative to an internal control and was calculated by the 2<sup>- $\Delta\Delta$ C<sub>t</sub></sup> method. The results were expressed as the mean  $\pm$  SE mRNA fold variations compared to the control. All measurements were carried out in triplicate and three experiments were performed.

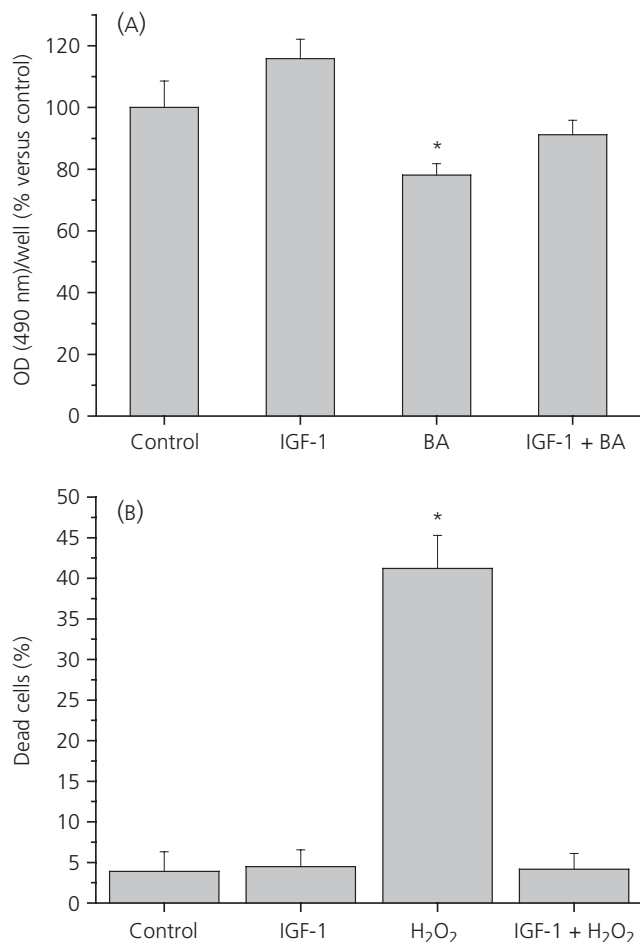
### Statistical analysis

Statistical differences were analysed using one-way ANOVA or Student's t-test. When the Student's t-test was used, significance was adjusted for multiple comparisons of means using Bonferroni's approximation.

## Results

### Effects of IGF-1 on cell viability and proliferation

The presence of the expression and secretion of IGF-1 and of the expression of IGF-1R in FNC cells was described previously (27). In the present study, to determine whether IGF-1 exerts neuroprotective effects in FNC cells, cells were exposed to toxic insults, such as  $\beta$ -amyloid and H<sub>2</sub>O<sub>2</sub>.  $\beta$ -amyloid (1  $\mu$ M) significantly decreased cell viability compared to control cells, as assessed by the MTS assay (Fig. 1A). However, in cells pre-treated with IGF-1 (10 nM), the toxic effect of  $\beta$ -amyloid was effectively counteracted. Similarly, when



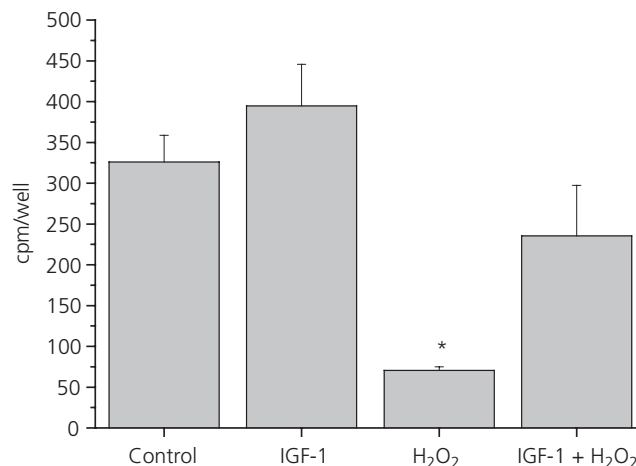
**Fig. 1.** (A) Effect of insulin-like growth factor-1 (IGF-1) (10 nM, 48 h) against  $\beta$ -amyloid (BA) (1  $\mu$ M, 20 h)-induced toxicity in human foetal neuroblasts (FNC). \* $P < 0.05$  versus control cells, cells treated with IGF-1 or with IGF-1 + BA. OD/well = optical density (absorbance at 490 nm)/well;  $n = 3$ , six replicates/experiment. (B) Effect of IGF-1 (10 nM, 48 h) against  $H_2O_2$  (100  $\mu$ M, 20 h)-induced toxicity. \* $P < 0.05$  versus control cells, cells treated with IGF-1 or IGF-1 +  $H_2O_2$ .  $n = 3$ , cells were counted in ten 20 $\times$  fields.

the cells were exposed to  $H_2O_2$  (100  $\mu$ M  $H_2O_2$ ), the number of dead cells significantly increased, as shown by Trypan blue dye exclusion test experiments, whereas pre-treatment with IGF-1 was able also to significantly prevent cell death (Fig. 1b).

With regard to cell proliferation, we observed a significant reduction, upon exposure to  $H_2O_2$ , as assessed by  $^3H$ -thymidine incorporation (77.5  $\pm$  7% reduction versus control cells; mean  $\pm$  SE). This inhibitory effect was significantly reversed in cells that were pre-exposed to IGF-1 (Fig. 2).

#### Effects of IGF-1 on cell cholesterol

We had previously demonstrated that cell cholesterol is an essential mediator of the neuroprotective effects of oestrogens, which increase the synthesis of the neuroprotective factor seladin-1 (17,18). IGF-1 is also able to increase the expression of this factor (30). To determine whether cell cholesterol may be a possible factor



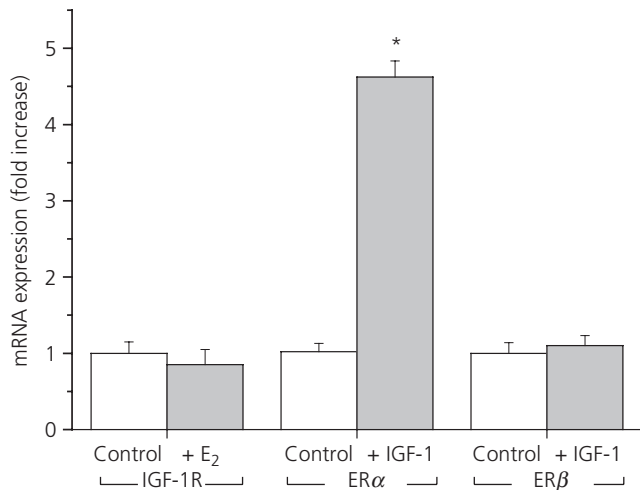
**Fig. 2.** Role of insulin-like growth factor-1 (IGF-1) (10 nM, 48 h) against the inhibitory effect of  $H_2O_2$  (100  $\mu$ M, 20 h) on cell proliferation. cpm (counts per minute)/well, as measured by a  $\beta$ -counter. \* $P < 0.05$  versus control cells, cells treated with IGF-1 or IGF-1 +  $H_2O_2$ .  $n = 3$ , six replicates/experiment.

mediating the protective effects of IGF-1 in nerve cells, we measured the amount of cholesterol in control FNC cells and in cells treated with 10 nM IGF-1 for 48 h, by GC/MS. We found that, although in cells exposed to IGF-1 there was a trend toward an increased amount of cholesterol, there was no significant difference versus control cells (11.2  $\pm$  7.8%; mean  $\pm$  SE, percentage increase versus control).

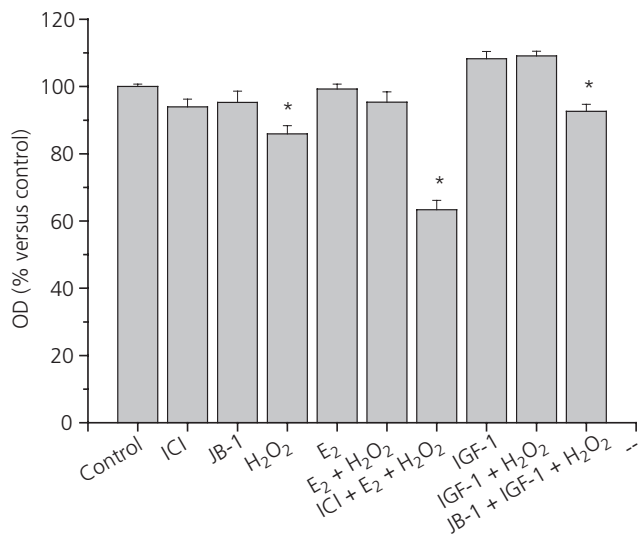
#### Cross-talk between IGF-1 and 17 $\beta$ -oestradiol in FNC cells

To determine whether a cross-talk between IGF-1 and 17 $\beta$ -oestradiol occurs in FNC cells, similar to that described in other cell models (8), we followed various experimental approaches. First, we investigated whether IGF-1 affects the expression levels of ERs and/or 17 $\beta$ -oestradiol affects the expression of IGF-1R. Accordingly, we treated the cells with IGF-1 (10 nM) or with 17 $\beta$ -oestradiol (10 nM) for 48 h. Significantly higher levels of ER $\alpha$  mRNA, but not of ER $\beta$ , were detected in cells exposed to IGF-1 compared to untreated cells (Fig. 3). Conversely, treatment with 17 $\beta$ -oestradiol did not affect the amount of IGF-1R mRNA.

Subsequently, we determined whether and how oestrogen and IGF-1 signalling cascades interact with each other by using the ER antagonist ICI 182 780 and the IGF-1R antagonist JB-1. The two antagonists did not show any effect *per se* on cell viability (Fig. 4). In a first set of experiments, we determined whether the two antagonists effectively counteracted the protective effects of the corresponding agonists against  $H_2O_2$ -induced toxicity. We confirmed that both 17 $\beta$ -oestradiol (10 nM) and IGF-1 (10 nM) were able to prevent oxidative stress toxicity, although we demonstrated that the protective effect was lost in the presence of ICI 182 780 (1  $\mu$ M) and JB-1 (1  $\mu$ g/ml), respectively (Fig. 4). Next, we investigated whether the protective effects of IGF-1 were maintained in the presence of the ER antagonist and, simultaneously, whether 17 $\beta$ -oestradiol-mediated protection was maintained in the presence

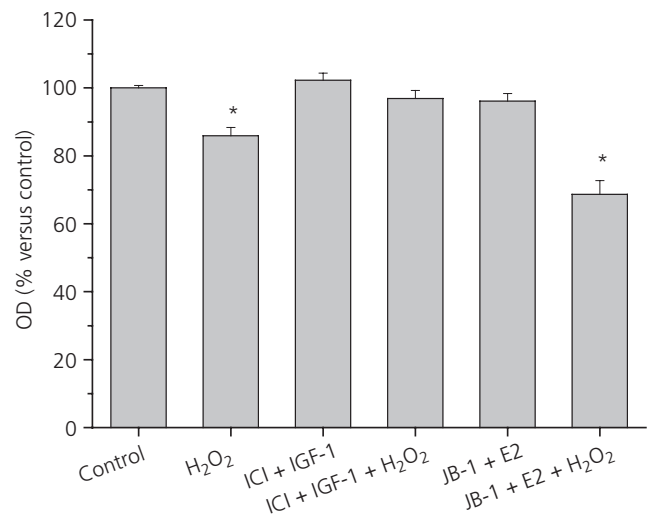


**Fig. 3.** Effect of insulin-like growth factor-1 (IGF-1) (10 nM, 48 h) on the expression of oestrogen receptor (ER) $\alpha$  and ER $\beta$  and of 17 $\beta$ -oestradiol (E<sub>2</sub>) (10 nM, 48 h) on IGF-1 receptor (IGF-1R), as assessed by quantitative reverse transcriptase-polymerase chain reaction. \*P < 0.05 versus control. n = 3, three replicates/experiment.

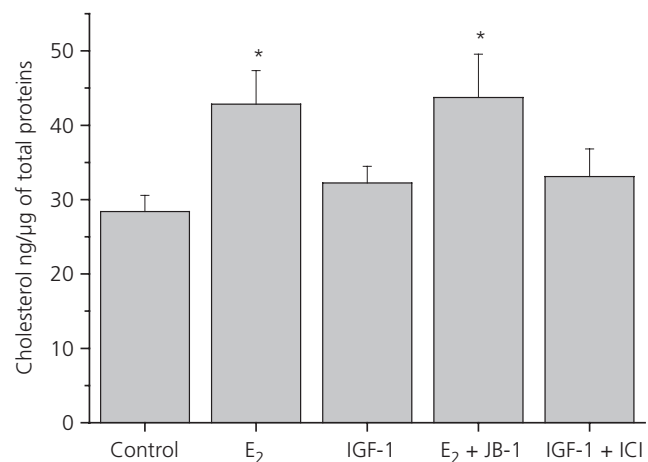


**Fig. 4.** Cell viability experiments, showing that the protective effects of 17 $\beta$ -oestradiol (E<sub>2</sub>) (10 nM, 48 h) and insulin-like growth factor-1 (IGF-1) (10 nM, 48 h) against H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M, 20 h)-induced toxicity are lost in the presence of the corresponding antagonists, ICI 182 780 (ICI) (1  $\mu$ M, 48 h) and JB-1 (1  $\mu$ g/ml, 48 h). \*P < 0.05 versus control cells. n = 3, six replicates/experiment.

of the IGF-1R antagonist. We found that the exposure of FNC cells to IGF-1 and ICI 182 780 or to 17 $\beta$ -oestradiol and JB-1 did not affect *per se* cell viability. IGF-1 readily counteracted the toxic effect of H<sub>2</sub>O<sub>2</sub> in the presence of ICI 182 780, thus indicating that the ER signalling pathway is not necessary to warrant the protective effects of IGF-1. Conversely, the protective effect of 17 $\beta$ -oestradiol against oxidative stress toxicity was lost in the presence of the IGF-1R antagonist JB-1, thus suggesting that the IGF-1R sig-



**Fig. 5.** Cell viability experiments, showing that the protective effects of insulin-like growth factor-1 (IGF-1) (10 nM, 48 h) against H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M, 20 h)-induced toxicity are maintained in the presence of ICI 182 780 (ICI) (1  $\mu$ M, 48 h), whereas the protective effects of 17 $\beta$ -oestradiol (E<sub>2</sub>) (10 nM, 48 h) are lost in the presence of JB-1 (1  $\mu$ g/ml, 48 h). \*P < 0.05 versus control cells. n = 3, six replicates/experiment.



**Fig. 6.** Cell cholesterol amount (measured by gas chromatography-mass spectrometry) in control cells, cells treated with 17 $\beta$ -oestradiol (E<sub>2</sub>) (10 nM, 48 h), insulin-like growth factor-1 (IGF-1) (10 nM, 48 h), E<sub>2</sub> + JB-1 (1  $\mu$ g/ml, 48 h) and IGF-1 + ICI 182 780 (ICI) (1  $\mu$ M, 48 h). \*P < 0.05 versus control cells. n = 3, two replicates/experiment.

nalling cascade is involved in oestrogen-mediated neuroprotection in FNC cells (Fig. 5).

To determine whether the loss of the protective effect of 17 $\beta$ -oestradiol in the presence of JB-1 and, conversely, the maintenance of the protective effect of IGF-1 in the presence of ICI 182 780 might be a result, at least in part, of a different amount of cell cholesterol, we measured cholesterol in cells subjected to different treatments. As previously demonstrated (17), we found a significant increase of cholesterol amount in cells exposed to 17 $\beta$ -oestradiol (Fig. 6). In cells treated with 17 $\beta$ -oestradiol + JB-1, the level of cell

cholesterol was not significantly different from the level measured in cells treated with  $17\beta$ -oestradiol alone. This result, together with the finding that JB-1 prevented the neuroprotective effect of  $17\beta$ -oestradiol, suggests that cholesterol *per se* is not sufficient to confer oestrogen-mediated neuroprotection. Furthermore, the amount of cholesterol in cells treated with IGF-1 or with IGF-1 + ICI 182 780 did not differ from the amount detected in control FNC cells. These data further support an important role for cell cholesterol in affecting ER-mediated, but not IGF-1R-mediated, neuroprotection in FNC cells.

## Discussion

IGF-1 is known to play an important neuroprotective role by favouring neuronal proliferation and survival (8). Accordingly, there is evidence that the IGF system may be altered in AD, the most common neurodegenerative disease in the elderly. IGF-1 gene polymorphisms have been associated with a greater risk for the onset of the disease through their effects on IGF-1 levels (31). Indeed, it has been demonstrated that lower levels of intracerebral IGF-1 in AD patients, together with the development of IGF-1 resistance, determine  $\beta$ -amyloid accumulation as a consequence of a reduced clearance (32–34). Furthermore, IGF-1 has also been associated with the preferential activation of the non-amyloidogenic  $\alpha$ -secretase processing of amyloid precursor protein (35).

In the present study, we first demonstrated that IGF-1 exerts protective effects in FNC. These cell cultures were established, cloned and propagated several years ago, starting from GnRH-secreting cells isolated from human foetal olfactory epithelium. FNC cells show unique features because they synthesise both neuronal proteins and olfactory markers and respond to odorant stimuli, suggesting their origin from the stem cell compartment that generates mature olfactory receptor neurons (28,29). These human neuronal precursors represent a more appropriate *in vitro* model for targeting studies related to neurodegenerative diseases compared to animal cell or tumoural cell lines models, which are usually employed. We have previously shown that FNC cells are a suitable cell model to study the role of IGF-1 in neuroprotection. Indeed, these cells express both IGF-1 and IGF-1R and release IGF-1 in the culture medium (27). In the present study, we observed that IGF-1 is able to prevent  $\beta$ -amyloid- and  $H_2O_2$ -induced cell toxicity, as assessed by cell viability assays. Furthermore, we demonstrated that IGF-1 effectively counteracts the inhibitory effect of  $H_2O_2$  on cell proliferation.

To determine whether the protective effects of IGF-1 in FNC cells might be related to an optimal amount of cell cholesterol, as suggested by our previous finding that IGF-1 increases the expression of the *seladin-1* gene (27), we measured cholesterol levels in control FNC cells and in cells exposed to IGF-1. We found that cell cholesterol was not significantly increased by IGF-1. This finding suggests that, in contrast to our previous experimental data showing that cell cholesterol is an essential mediator of the neuroprotective effects of oestrogens in FNC cells (17), IGF-1-mediated neuroprotection does not appear to involve cholesterol as a major factor, at least in this cell model.

Despite the different role of intracellular cholesterol, our present and previous findings indicate that both oestrogens and IGF-1 exert neuroprotective effects in FNC cells, thus suggesting the presence of a cross-talk between these hormones. Some studies addressed this issue by using specific receptor antagonists. One study clearly showed that rat hippocampal cell death induced by *in vivo* kainic acid administration was prevented by either  $17\beta$ -oestradiol or IGF-1. However, none of these hormones was able to prevent cell death also when animals were treated with the IGF-1R antagonist JB-1 or with the ER antagonist ICI 182 780, respectively (36). Other studies showed that ICI 182 780 or JB-1 blocked the induction of neurogenesis or neurite outgrowth induced by IGF-1 or  $17\beta$ -oestradiol, respectively, both *in vitro* and *in vivo* (37,38). In an *in vivo* model of global ischaemia-induced neuronal cell death in rats, JB-1 abolished the pro-survival effects of  $17\beta$ -oestradiol (39). To assess the presence of a cross-talk between oestrogens and IGF-1 in our human neuronal cell model, we also performed additional cell viability experiments after oxidative stress, using different combinations of  $17\beta$ -oestradiol, IGF-1, ICI 182 780 and JB-1. Our results indicated that the IGF-1R signalling cascade is involved in the neuroprotective effects of oestrogens, although the ER signalling pathway is not necessary for IGF-1-mediated neuroprotection in FNC cells. This suggests that the activation of one or more factors that are associated to the IGF-1R pathway is sufficient to exert neuroprotection.

Furthermore, to determine whether variations in cell cholesterol amount might be, at least partially, responsible for the loss of the protective effect of  $17\beta$ -oestradiol in the presence of JB-1, as well as the maintenance of the protective effect of IGF-1 in the presence of ICI 182 780, we measured cholesterol levels in cells subjected to different treatments. We found that JB-1 did not blunt the stimulatory effect of  $17\beta$ -oestradiol on cell cholesterol (17), whereas IGF-1 or IGF-1 + ICI 182 780 did not cause any change compared to control cells. Overall, these findings support a pivotal role of the IGF-1R pathway in mediating the neuroprotective effects of oestrogens. However, unlike the ER pathway, cell cholesterol does not appear to play a major role in mediating the neuroprotective effects elicited by the activation of the IGF-1R cascade, which are likely the result of other mechanisms, such as the IGF-1-induced anti-apoptotic effects (1–3). Therefore, although an optimal amount of cell cholesterol appears to be essential, as previously reported (17), this is not sufficient to warrant the neuroprotective effects of oestrogens, which appear to involve the activation of multiple pathways.

Finally, in the present study, we demonstrated that IGF-1 up-regulates the expression of ER $\alpha$ , yet not of ER $\beta$ , whereas  $17\beta$ -oestradiol does not affect the expression of IGF-1R. These data, together with our previous observation that  $17\beta$ -oestradiol stimulates the release of IGF-1 in FNC cells (30), lead us to hypothesise a model in which: (i)  $17\beta$ -oestradiol and IGF-1 mutually interact in protecting nerve cells and (ii) a reinforcement mechanism is provided by the presence of an autocrine loop in which ER $\alpha$  activation stimulates the release of IGF-1, which in turn induces the expression of ER $\alpha$ .

Overall, the data reported in the present study appear to support the hypothesis that the interaction between IGF-1 and oestrogens

in neuroprotection may be relevant also in defining possible strategies against neurodegenerative diseases, and primarily against AD. With regard to this issue, although clinical trials for the prevention/treatment of AD with oestrogens have been performed (40,41), no trial so far has addressed the use of IGF-1. However, the hypothetical use of this growth factor in the treatment of CNS pathologies, including AD, has been proposed several times in the last 20 years (42,43). It is known that the cholinesterase-inhibitor donepezil increases plasma levels of GH and IGF-1 in AD patients (44) and that the lack of a response to this drug is associated with lower levels of IGF-1 (45). Accordingly, an enhancement in spatial learning and memory after peripheral administration of GH in rats displaying AD-like deficiencies has been recently reported and it is very likely to be a result of the increased release of IGF-1 (46). A phase II trial, aiming at evaluating the effects of GHRH in healthy older men and women and in those with mild cognitive impairment, is currently recruiting participants (<http://www.clinicaltrials.gov>, trial no. NCT00257712). These interventional studies, together with the basic science, are expected to provide further information and to enhance our knowledge about the neuroprotective effects of IGF-1 and its possible role against neurodegenerative diseases.

## Acknowledgements

The present study was supported by grants from Ente Cassa di Risparmio di Firenze, from the Regione Toscana 'Bando Salute 2009' and from Ministero dell'Istruzione, dell'Università e della Ricerca (PRIN 2009 n. 2009YJTBAZ). We wish to thank Dr Lucia Mancini for her help in revising the manuscript.

Received 27 January 2012,

revised 17 May 2012,

accepted 17 May 2012

## References

- Russo VC, Gluckman PD, Feldman EL, Werther GA. The insulin-like growth factor system and its pleiotropic functions in brain. *Endocr Rev* 2005; **26**: 916–943.
- Mendez P, Azcoitia I, Garcia-Segura LM. Interdependence of oestrogen and insulin-like growth factor-I in the brain: potential for analyzing neuroprotective mechanisms. *J Endocrinol* 2005; **185**: 11–17.
- Zou CG, Cao XZ, Zhao YS, Gao SY, Li SD, Liu XY, Zhang Y, Zhang KQ. The molecular mechanism of endoplasmic reticulum stress-induced apoptosis in PC-12 neuronal cells: the protective effect of insulin-like growth factor I. *Endocrinology* 2009; **150**: 277–285.
- Guan J, Gluckman PD. IGF-1 derived small neuropeptides and analogues: a novel strategy for the development of pharmaceuticals for neurological conditions. *Br J Pharmacol* 2009; **157**: 881–891.
- Turgeon JL, Carr MC, Maki PM, Mendelsohn ME, Wise P. Complex actions of sex steroids in adipose tissue, the cardiovascular system, and brain: insights from basic science and clinical studies. *Endocr Rev* 2006; **27**: 576–605.
- Correia SC, Santos RX, Cardoso S, Carvalho C, Santos MS, Oliveira CR, Moreira PI. Effects of estrogen in the brain: is it a neuroprotective agent in Alzheimer's disease? *Curr Aging Sci* 2010; **3**: 113–126.
- Arevalo MA, Santos-Galindo M, Bellini MJ, Azcoitia I, Garcia-Segura LM. Actions of estrogens on glial cells: implications for neuroprotection. *Biochim Biophys Acta* 2010; **1800**: 1106–1112.
- Garcia-Segura LM, Arévalo MA, Azcoitia I. Interactions of estradiol and insulin-like growth factor-I signalling in the nervous system: new advances. *Prog Brain Res* 2010; **181**: 251–272.
- Znamensky V, Akama KT, McEwen BS, Milner TA. Estrogen levels regulate the subcellular distribution of phosphorylated Akt in hippocampal CA1 dendrites. *J Neurosci* 2003; **23**: 2340–2347.
- Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to cell-intrinsic machinery. *Cell* 1997; **91**: 231–241.
- Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME. Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science* 1997; **1**: 661–665.
- Ma ZQ, Santagati S, Patrone C, Pollio G, Vegeto E, Maggi A. Insulin-like growth factors activate estrogen receptor to control the growth and differentiation of the human neuroblastoma cell line SK-ER3. *Mol Endocrinol* 1994; **8**: 910–918.
- Patrone C, Gianazza E, Santagati S, Agrati P, Maggi A. Divergent pathways regulate ligand-independent activation of ER alpha in SK-N-BE neuroblastoma and COS-1 renal carcinoma cells. *Mol Endocrinol* 1998; **12**: 835–841.
- Font de Mora J, Brown M. IB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. *Mol Cell Biol* 2000; **20**: 5041–5047.
- Frago LM, Pañeda C, Dickson SL, Hewson AK, Argente J, Chowen JA. Growth hormone (GH) and GH-releasing peptide-6 increase brain insulin-like growth factor-I expression and activate intracellular signaling pathways involved in neuroprotection. *Endocrinology* 2002; **143**: 4113–4122.
- Mendez P, Garcia-Segura LM. Phosphatidylinositol 3 kinase (PI3K) and glycogen synthase kinase 3 (GSK3) regulate estrogen receptor-mediated transcription in neuronal cells. *Endocrinology* 2006; **147**: 3027–3039.
- Luciani P, Deledda C, Rosati F, Benvenuti S, Cellai I, Dichiarà F, Morello M, Vannelli GB, Danza G, Serio M, Peri A. Seladin-1 is a fundamental mediator of the neuroprotective effects of estrogen in human neuroblast long-term cell cultures. *Endocrinology* 2008; **149**: 4256–4266.
- Benvenuti S, Luciani P, Vannelli GB, Gelmini S, Franceschi E, Serio M, Peri A. Estrogen and selective estrogen receptor modulators exert neuroprotective effects and stimulate the expression of selective Alzheimer's disease indicator-1, a recently discovered antiapoptotic gene, in human neuroblast long-term cell cultures. *J Clin Endocrinol Metab* 2005; **90**: 1775–1782.
- Greeve I, Hermans-Borgmeyer I, Brellinger C, Kasper D, Gomez-Isla T, Behl C, Levkau B, Nitsch RM. The human DIMINUTO/DWARF-1 homolog Seladin 1 confers resistance to Alzheimer's disease-associated neurodegeneration and oxidative stress. *J Neurosci* 2001; **20**: 7345–7352.
- Waterham HR, Koster J, Romeijn GJ, Hennekam RC, Vreken P, Andersson HC, FitzPatrick DR, Kelley RI, Wanders RJ. Mutations in the 3 $\beta$ -hydroxysteroid  $\Delta$ 24-reductase gene cause desmosterolosis, an autosomal recessive disorder of cholesterol biosynthesis. *Am J Hum Genet* 2001; **69**: 685–694.
- Arispe N, Doh M. Plasma membrane cholesterol controls the cytotoxicity of Alzheimer's disease A $\beta$ P (1–40) and (1–42) peptides. *FASEB J* 2000; **216**: 1526–1536.
- Cecchi C, Rosati F, Pensalfini A, Formigli L, Nosi D, Liguri G, Dichiarà F, Morello M, Danza G, Pieraccini G, Peri A, Serio M, Stefani M. Seladin-1/DHCR24 protects neuroblastoma cells against amyloid toxicity by

- increasing membrane cholesterol content. *J Cell Mol Med* 2008; **10**: 1582–4934.
- 23 Yao JK, Wengenack TM, Curran GL, Poduslo JF. Reduced membrane lipids in the cortex of Alzheimer's disease transgenic mice. *Neurochem Res* 2009; **34**: 102–108.
  - 24 Cramer A, Biondi E, Kuehnle K, Lütjohann D, Thelen KM, Perga S, Dotti CG, Nitsch RM, Ledesma MD, Mohajeri MH. The role of seladin-1/DHCR24 in cholesterol biosynthesis, APP processing and Abeta generation in vivo. *EMBO J* 2006; **25**: 432–443.
  - 25 Abad-Rodriguez J, Ledesma MD, Craessaerts K, Perga S, Medina M, Delacourte A, Dingwall C, De Strooper B, Dotti C. Neuronal membrane cholesterol loss enhances amyloid peptide generation. *J Cell Biol* 2004; **167**: 953–960.
  - 26 Kaether C, Haass C. A lipid boundary separates APP and secretases and limits amyloid beta-peptide generation. *J Cell Biol* 2004; **167**: 809–812.
  - 27 Giannini S, Benvenuti S, Luciani P, Manuelli C, Cellai I, Deledda C, Pezzatini A, Vannelli GB, Maneschi E, Rotella CM, Serio M, Peri A. Intermittent high glucose concentrations reduce neuronal precursor survival by altering the IGF system: the involvement of the neuroprotective factor Seladin-1. *J Endocrinol* 2008; **198**: 523–532.
  - 28 Vannelli GB, Ensoli F, Zonefrati R, Kubota Y, Arcangeli A, Becchetti A, Camici G, Barni T, Thiele CJ, Balboni GC. Neuroblast longterm cell cultures from human foetal olfactory epithelium respond to odors. *J Neurosci* 1995; **15**: 4282–4294.
  - 29 Barni T, Maggi M, Fantoni G, Granchi S, Mancina R, Gulisano M, Marra F, Macorsini E, Luconi M, Rotella C, Serio M, Balboni GC, Vannelli GB. Sex steroids and odorants modulate gonadotropin-releasing hormone secretion in primary cultures of human olfactory cells. *J Clin Endocrinol Metab* 1999; **84**: 4266–4273.
  - 30 Maggi M, Barni T, Fantoni G, Mancina R, Pupilli C, Luconi M, Crescioli C, Serio M, Vannelli GB. Expression and biological effects of endothelin-1 in human gonadotropin-releasing hormone-secreting neurons. *J Clin Endocrinol Metab* 2000; **85**: 1658–1665.
  - 31 Vargas T, Martinez-Garcia A, Antequera D, Vilella E, Clarimon J, Mateo I, Sanchez-Juan P, Rodriguez-Rodriguez E, Frank A, Rosich-Estrago M, Lleo A, Molina-Porcel L, Blesa R, Gomez-Isla T, Combarros O, Bermejo-Pareja F, Valdivieso F, Bullido MJ, Carro E. IGF-I gene variability is associated with an increased risk for AD. *Neurobiol Aging* 2011; **32**: 556, e3–556.
  - 32 Carro E, Torres-Aleman I. Insulin-like growth factor I and Alzheimer's disease: therapeutic prospects? *Expert Rev Neurother* 2004; **4**: 79–86.
  - 33 Steen E, Terry BM, Rivera EJ, Cannon JL, Neely TR, Tavares R, Xu XJ, Wands JR, de la Monte SM. Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease—is this type 3 diabetes? *J Alzheimers Dis* 2005; **7**: 63–80.
  - 34 Moloney AM, Griffin RJ, Timmons S, O'Connor R, Ravid R, O'Neill C. Defects in IGF-1 receptor, insulin receptor and IRS-1/2 in Alzheimer's disease indicate possible resistance to IGF-1 and insulin signalling. *Neurobiol Aging* 2010; **31**: 224–243.
  - 35 Adlerz L, Holback S, Multhaup G, Iverfeldt K. IGF-1-induced processing of the amyloid precursor protein family is mediated by different signaling pathways. *J Biol Chem* 2007; **282**: 10203–10209.
  - 36 Azcoitia I, Sierra A, Garcia-Segura LM. Neuroprotective effects of estradiol in the adult rat hippocampus: interaction with insulin-like growth factor-I signalling. *J Neurosci Res* 1999; **58**: 815–822.
  - 37 Perez-Martin M, Azcoitia I, Trejo JL, Sierra A, Garcia-Segura LM. An antagonist of estrogen receptors blocks the induction of adult neurogenesis by insulin-like growth factor-I in the dentate gyrus of adult female rat. *Eur J Neurosci* 2003; **18**: 923–930.
  - 38 Topalli I, Etgen AM. Insulin-like growth factor-I receptor and estrogen receptor crosstalk mediates hormone-induced neurite outgrowth in PC12 cells. *Brain Res* 2004; **1030**: 116–124.
  - 39 Jover-Mengual T, Zukin RS, Etgen AM. MAPK signaling is critical to estradiol protection of CA1 neurons in global ischemia. *Endocrinology* 2007; **148**: 1131–1143.
  - 40 Rapp SR, Espeland MA, Shumaker SA, Henderson VW, Brunner RL, Manson JE, Gass ML, Stefanick ML, Lane DS, Hays J, Johnson KC, Coker LH, Dailey M, Bowen D. Effect of estrogen plus progestin on global cognitive function in postmenopausal women: the Women's Health Initiative Memory Study: a randomized controlled trial. *JAMA* 2003; **289**: 2663–2672.
  - 41 Shumaker SA, Legault C, Rapp SR, Thal L, Wallace RB, Ockene JK, Hendrix SL, Jones BN 3rd, Assaf AR, Jackson RD, Kotchen JM, Wassertheil-Smoller S, Wactawski-Wende J, WHIMS Investigators. Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: the Women's Health Initiative Memory Study: a randomized controlled trial. *JAMA* 2003; **289**: 2651–2662.
  - 42 Olson L. Grafts and growth factors in CNS. Basic science with clinical promise. *Stereotact Funct Neurosurg* 1990; **54**: 250–267.
  - 43 Doré S, Kar S, Zheng WH, Quirion R. Rediscovering good old friend IGF-I in the new millenium: possible usefulness in Alzheimer's disease and stroke. *Pharm Acta Helv* 2000; **74**: 273–280.
  - 44 Obermayr RP, Mayerhofer L, Knechtelsdorfer M, Mersich N, Huber ER, Geyer G, Tragl KH. The age-related down-regulation of the growth hormone/insulin-like growth factor-1 axis in the elderly male is reversed considerably by donepezil, a drug for Alzheimer's disease. *Exp Gerontol* 2005; **40**: 157–163.
  - 45 Tei E, Yamamoto H, Watanabe T, Miyazaki A, Nakadate T, Kato N, Mimura M. Use of serum insulin-like growth factor-I levels to predict psychiatric non-response to donepezil in patients with Alzheimer's disease. *Growth Horm IGF Res* 2008; **18**: 47–54.
  - 46 Doulah AH, Rohani AH, Khaksari Haddad M, Motamedi F, Farbood Y, Badavi M, Malek M, Sarkaki A. The effect of peripheral administration of growth hormone on AD-like cognitive deficiency in NBM-lesioned rats. *Neurosci Lett* 2009; **466**: 47–51.