

Neuropeptide Y functions as a neuroproliferative factor

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Neuropeptide Y (NPY) has a number of functions in mammalian physiology^{1–6}. Here we identify a role for NPY in promoting proliferation of postnatal neuronal precursor cells. NPY is synthesized in the postnatal olfactory epithelium by sustentacular cells, previously proposed to function only in structural support⁷. Mice with a targeted deletion of NPY⁸ contain half as many dividing olfactory neuronal precursor cells as do controls. Furthermore, NPY-deficient mice develop significantly fewer olfactory neurons by adulthood. NPY acts on multipotent neuronal precursor or basal cells to activate rapidly and transiently the extracellular signal-regulated kinase (ERK)1/2 subgroup of mitogen-activated protein kinases. The NPY Y1 receptor subtype appears to mediate this effect. The ability of NPY to induce neuronal precursor proliferation is mediated by protein kinase C (PKC), indicating an upstream PKC-dependent activation of ERK1/2. These results indicate that NPY may regulate neuronal precursor proliferation in the adult mammal.

NPY is a 36-amino-acid neuropeptide that is broadly expressed in the central and peripheral nervous system during development and adulthood^{1,2}. It regulates feeding behaviour, gastrointestinal activity and central cardiovascular function, and influences seizure threshold and alcohol intake^{3–5}. NPY messenger RNA is upregulated following peripheral axotomy and in pheochromocytoma and ganglioneuroblastoma tissue, although its role in neuronal proliferation is unknown⁶. To investigate the action of NPY in neuroproliferation, we examined the rodent olfactory epithelium. The olfactory epithelium is composed of immature and mature olfactory receptor neurons, non-neuronal sustentacular cells and proliferative basal cells, which comprise the multipotent progenitor cells of the olfactory epithelium⁷. The olfactory basal cells represent one of the few well-documented regenerating neuronal populations in the adult mammalian nervous system; the others are the stem cells of the rostral migratory stream, hippocampus and retina^{9,10}.

We used immunohistochemical methods and radioimmunoassays to investigate NPY expression in the rat olfactory system. NPY was previously identified in the ensheathing cells of olfactory axon bundles of the adult rat¹¹, but not within cells of the olfactory epithelium. We detected NPY expression in a subpopulation of neuronal cells in the olfactory epithelium at embryonic day 16. The NPY-expressing cells were identified as neurons by double-label immunofluorescence with neuron-specific tubulin (NST; Fig. 1a), a specific neuronal marker that is expressed early in the olfactory neuronal lineage in both immature neurons and dividing cells^{12,13}. In the adult, however, NPY no longer co-localized with sites of NST expression, but was found in a subpopulation of sustentacular cells (Fig. 1b). Sustentacular cells comprise the outermost layer of olfactory epithelial cells, and are proposed to maintain epithelial structure and to secrete protective detoxifying enzymes¹⁴. This is the first report, to our knowledge, of a heterogeneous expression pattern within the sustentacular cell population.

To confirm the existence of NPY in the olfactory epithelium, we quantified NPY content by radioimmunoassaying rat olfactory epithelium (0.56 ± 0.7 pmol NPY per mg protein (mean \pm s.e.m.)), olfactory bulb (1.95 ± 0.28 pmol NPY per mg), hypothalamus (6.17 ± 1.12 pmol NPY per mg) and primary

olfactory neuronal cultures (levels below the limit of detection (< 0.05 pmol NPY per mg)). Although the arcuate nucleus of the hypothalamus contains the highest reported level of NPY in the central nervous system¹ the NPY content of the olfactory system is substantial in comparison to regions such as the cerebellum or pons¹, which express very low levels of NPY.

We examined the adult olfactory epithelium of NPY-deficient mice generated by targeted gene deletion⁸ and wild-type littermate controls to identify the potential role of NPY *in vivo*. Labelling of proliferating basal cells in NPY-deficient and control animals with 5-bromo-2'-deoxyuridine (BrdU) showed a roughly 50% decrease in the number of dividing cells in the NPY-deficient mice (Fig. 2a, b). The number of cells expressing Ki67, a marker of cycling cells that are not in G₀ (ref. 15), was also decreased in NPY-deficient mice, confirming a significant reduction in neuronal precursor proliferation with the loss of NPY (Fig. 2c).

To determine the overall consequences of NPY deficiency in the olfactory epithelium, we examined the neuronal populations of NPY-deficient and control mice. The number of neuron-specific tubulin^{12,13} immunopositive cells was reduced in NPY-deficient mice (Fig. 2d). Neuronal cell counts were performed using O/E-1, a transcription factor present in cells of the olfactory neuronal lineage¹⁶; NPY-deficient mice contained around 25% fewer neurons than control mice (Fig. 2e, f). The difference between proliferation in the epithelium (50%) and total neuronal number (25%) in NPY-deficient and control mice may be secondary to compensatory changes in the olfactory epithelium. These results indicate that loss of NPY *in vivo* reduced neuronal precursor proliferation in the olfactory epithelium, as well as the overall number of neurons.

We used primary olfactory cultures¹⁷ to examine the mechanism

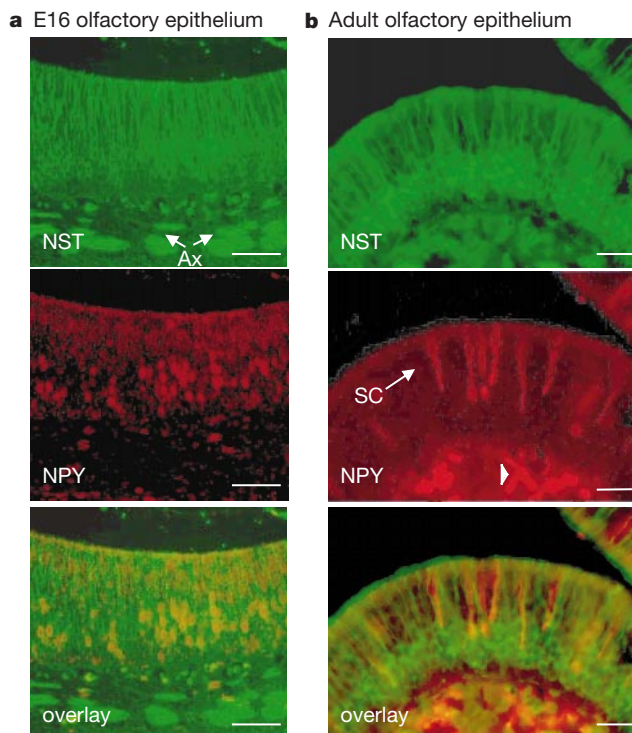


Figure 1 NPY expression in the olfactory epithelium. **a**, Expression of NPY in a subset of developing olfactory receptor neurons. All antibodies were used at a 1:1,000 dilution. Double-label immunofluorescence at embryonic day (E)16 shows NPY in cells positive for neuron-specific tubulin (NST). NPY is absent from the developing axon bundles (Ax). Scale bar, 50 μ m. **b**, Expression of NPY in non-neuronal sustentacular cells in the adult olfactory epithelium. NPY expression is detected in a subpopulation of sustentacular cells (SC) and in the underlying ensheathing cells (arrowhead). NPY in the adult was visualized using the NEN fluorescent tyramide system. Scale bar, 25 μ m.

of NPY action in olfactory receptor neurons. These cultures, which contain a predominance of basal cells and olfactory receptor neurons and a subpopulation of glial cells from the lamina propria, do not contain detectable levels of NPY by radioimmunoassay. Primary olfactory cultures contain very few sustentacular cells and, consequently, only about 2–3% of the cultured cells are immunopositive for NPY.

We verified NPY receptor binding at 4 °C and 37 °C in primary olfactory cultures (Fig. 3a). The NPY-binding cells were rounded and morphologically similar to the proliferative basal cells⁷; these cells accounted for around 1 out of 20 cells in the culture. Thus, NPY receptors are positioned to mediate an early step in olfactory neurogenesis.

NPY can mediate the proliferation of vascular smooth muscle cells and colonic lamina propria lymphocytes^{18,19}. To determine whether NPY could perform a similar function in the nervous system, we incubated primary olfactory cultures from 2-day-old rats with increasing concentrations (10⁻⁸ M to 10⁻⁶ M) of NPY (Fig. 3b). NPY increased the number of neurons (NST-positive) in a dose-dependent manner, without influencing the glial cell (GFAP-positive) subpopulation. To determine whether this increase in

neuronal number was due to increased neuronal proliferation or to increased neuronal survival, we compared results from BrdU incorporation assays and 3' terminal uridine nick end labelling (TUNEL) apoptosis assays.

NPY increased the number of basal cells undergoing cell division to produce more neurons (BrdU; Fig. 3c). The increase in BrdU labelling between control and NPY-treated cells after only 2 h of exposure to NPY indicates that NPY may promote transition of basal cells into S phase of the cell cycle²⁰. NPY did not alter the number of cells identified by TUNEL staining in the cultures (Fig. 3d), indicating that NPY does not affect the rate of apoptosis. These results indicate that NPY increases neuronal number by inducing neuroproliferation, identical to its role *in vivo*, without altering neuronal survival.

NPY can act through at least five receptors, including Y1, Y2, Y4, Y5 and Y6 (ref. 21). All of these receptors are G-protein-coupled and affect multiple, response-specific second messenger cascades. Evidence from studies performed in transfected cells indicates that NPY can signal through various intracellular pathways, including calcium and cyclic AMP²². The Y1 and Y2 receptors are the most prevalent NPY receptors in the mammalian brain, and subtype agonists were used to determine which receptor promotes NPY-mediated neuroproliferation²³. Incubation of cells with the Y1/Y5 agonist [Leu³¹, Pro³⁴]NPY mimicked the effect of NPY on neuroproliferation (Fig. 4a); the diminished effect of the higher dose may reflect receptor desensitization. The Y2 agonist NPY(3-36) did not affect neuronal number.

To confirm that NPY acts through the Y1 receptor, rather than the Y5 receptor, to induce neuronal precursor or basal cell proliferation, we incubated primary olfactory cultures with 10⁻⁶ M of the Y1-

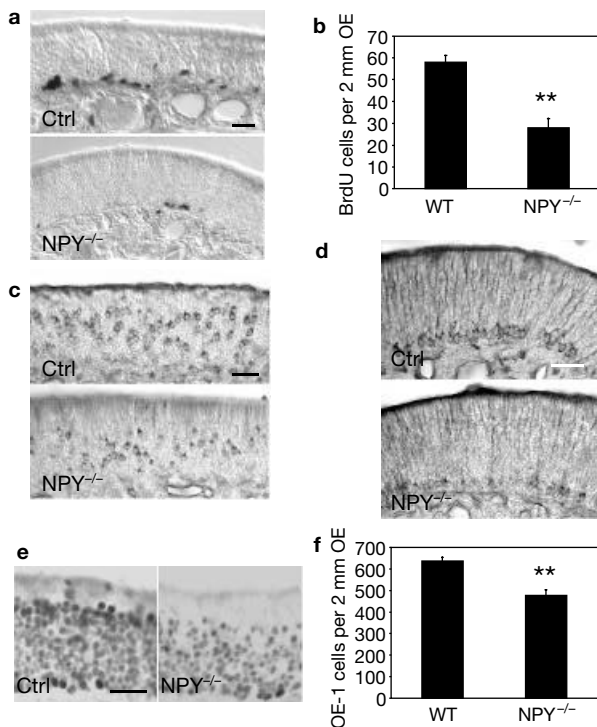


Figure 2 NPY-deficient mice⁸ show decreased precursor proliferation and decreased neuronal number. **a**, BrdU labelling of adult mice shows decreased postnatal neurogenesis in the precursor cell population of NPY^{-/-} mice. **b**, NPY-deficient mice have fewer dividing precursors. BrdU labelling of NPY^{-/-} mice and wild-type littermate controls for 24 h shows 58 BrdU-positive cells per 2 mm of olfactory epithelium (OE) in wild-type mice, as compared to 29 BrdU-positive cells per 2 mm of olfactory epithelium in NPY^{-/-} mice; six wild-type and five null mice were compared. Double asterisk, *P* < 0.001 relative to control, using student's two-tailed *t*-test with samples of unequal variance. **c**, Ki67 immunostaining of NPY^{-/-} mice and control mice. **d**, NST immunostaining of NPY^{-/-} mice and control mice suggesting decreased numbers of immunoreactive cells in NPY^{-/-} mice. **e**, O/E-1 immunostaining of NPY^{-/-} and control mice shows fewer post-mitotic neurons throughout the NPY^{-/-} olfactory epithelium. **f**, Quantification of O/E-1 immunopositive cells comparing NPY^{-/-} and control mice, examining 6–7 comparable regions of olfactory epithelium. Control animals contained 633 ± 19 cells per 2 mm epithelium, whereas NPY^{-/-} mice contained 470 ± 26 cells per 2 mm epithelium. Double asterisk, *P* < 0.001 using the student's two-tailed *t*-test with samples of unequal variance. Scale bars, 25 μM.

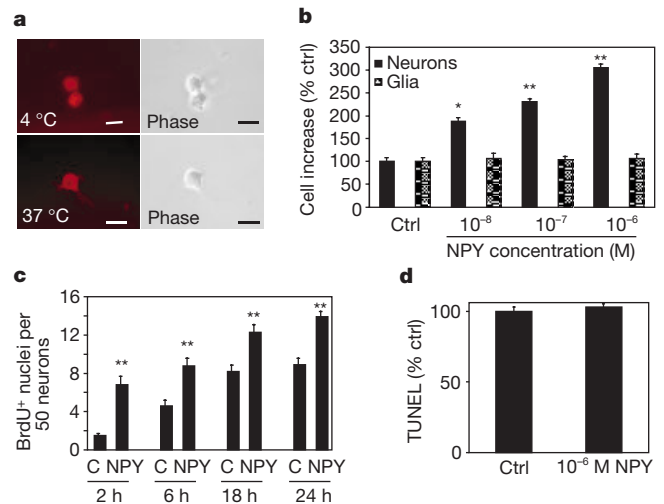


Figure 3 NPY stimulates neuroproliferation. **a**, Fluorescein-tagged NPY (30 nM; NEN) binds to the surface of a population of cells in primary neuronal cultures at 4 °C and 37 °C. Blocking control was performed by co-incubating fluorescently tagged NPY with 3 μM unlabelled peptide. Images are pseudocoloured red. Scale bar, 20 μm. **b**, Twenty-four hour incubation of 1-day-old primary olfactory neuronal cultures with NPY (Peninsula Lab) increases neuronal number, as defined by NST staining. NPY does not alter glial cell number, as defined by glial fibrillary acidic protein staining. Cell counts are reported as percentage of stained cells per high power field relative to control. 100% NST is 40 neurons per field. 100% GFAP is 25 glial cells per field. Incubation with or without NGF does not alter neuronal or glial number, or responsiveness to NPY. Error bars, s.e.m. Asterisk, *P* < 0.05; double asterisk, *P* < 0.001, relative to control (student's two-tailed *t*-test with samples of unequal variance). **c**, NPY promotes neuronal proliferation. Incubation of cells with 10⁻⁶ M NPY for the time indicated increased BrdU⁺ nuclei per 50 NST⁺ cells (neurons) at all time points. *P*-values given relative to control (C) values at each time point. **d**, TUNEL staining of primary olfactory cultures incubated with or without 10⁻⁶ M NPY indicates no significant change in the rate of apoptosis.

receptor-specific antagonist BIBP3226. Incubation of cells with NPY and BIBP3226 abolished NPY-induced neuroproliferation (Fig. 4b), indicating that the actions of NPY were probably mediated by the Y1 receptor; BIBP3226 alone did not alter neuronal numbers. These results are consistent with a role for the NPY Y1 receptor subtype in neuronal precursor proliferation, although an unknown NPY receptor, functioning in a similar manner, cannot be ruled out.

To determine whether the ability of NPY to increase neuronal precursor proliferation was a direct or an indirect effect on basal cells, we localized the NPY receptor. The proliferative neuronal precursor/basal cells in the olfactory epithelium rest along the basement membrane and undergo proliferation into adulthood to generate new neurons⁷. *In vitro* tissue binding with 50 nM of fluorescently labelled NPY identified binding of the peptide to most of the basally situated cells (Fig. 4c, d). Thus, the NPY receptor is localized to the appropriate cells to mediate directly the neuroproliferative response demonstrated in primary culture. Competition with 10⁻⁶ M unlabelled NPY blocked the ability of the labelled peptide to bind to these cells (Fig. 4e).

In other systems, the ERK subgroup of the mitogen-activated protein (MAP) kinases is important in the regulation of cellular proliferation^{24,25}. NPY increases the phosphorylation of ERK1/2 in cardiomyocytes and in a variety of clonal cell lines, although this effect has not been described in neurons²⁶⁻²⁸. ERK1/2 is activated by dual phosphorylation on threonine and tyrosine residues by MAP or ERK kinases (MEK)1/2 (refs 24, 25). We therefore examined the role of ERK1/2 in mediating NPY-induced neuronal proliferation.

Incubation of cells with 100 μM PD98059, a potent MEK inhibitor, blocked the NPY-mediated increase in neuronal number (Fig. 5a). PD98059 alone did not alter neuronal number. Incubation of cells with genistein, a general tyrosine kinase inhibitor

that blocked the activation of ERK1/2, also prevented the NPY-induced increase in neuronal number (Fig. 5b). As rapid and transient phosphorylation of ERK1/2 can lead to long-term alterations in cellular proliferation^{24,25}, we examined phosphorylation of ERK1/2 after NPY addition for times up to 30 min (Fig. 5c, d). NPY-induced phosphorylation of ERK1/2 was detected 30 s after treatment, with a sustained elevation in phosphorylation for up to 10 min, and a return to baseline levels of phosphorylation by 30 min. The maximum response was observed at 30 s, with phosphorylation increased threefold over baseline (Fig. 5d).

The ERK1/2 pathway is regulated by a variety of kinases, including phosphatidylinositol-3-OH kinase (PI(3)K), PKC and protein kinase A^{24,25}. NPY has been proposed to stimulate the phosphorylation of ERK1/2 in cardiomyocytes through a PI(3)K-dependent mechanism²⁶⁻²⁸. To determine whether NPY causes neuroproliferation through activation of PI(3)K or PKC²⁹, we incubated cells with inhibitors specific for each pathway.

Incubation of cells with either 100 nM wortmannin or 20 μM

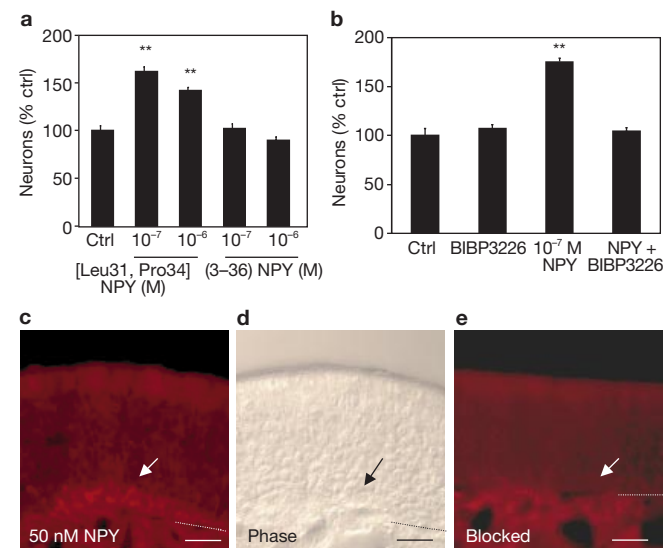


Figure 4 Y1 receptors mediate the NPY response. **a**, Incubation of cultures for 24 h with the Y1/Y5 agonist [Leu³¹, Pro³⁴]NPY (Peninsula) increases the number of NST⁺ cells. Incubation with the Y2 agonist NPY(3-36) (Peninsula) yielded no response. *P*-values given relative to control (ctrl) values. **b**, Twenty-four hour co-incubation with the Y1 receptor antagonist BIBP3226 (1 μM) and NPY blocked the ability of NPY to increase neuronal number. BIBP3226 alone had no effect. **c**, NPY binds to neuronal precursor cells. Incubation of 50 nM fluorescently tagged NPY (NEN) with Bouin's fixed rat olfactory epithelium for 2 h showed binding of NPY to the basal cell layer (arrow). Dotted line, separation between olfactory epithelium and underlying lamina propria. Scale bar, 25 μm. **d**, Phase microscopy view of **c**; arrow indicates location of stained cells (basal cell layer). **e**, Co-incubation with 50 nM fluorescent NPY and 5 μM unlabelled peptide (Peninsula) abolished binding to most of the basal cells (arrow). Nonspecific binding in the underlying lamina propria was evident in both bound and control conditions.

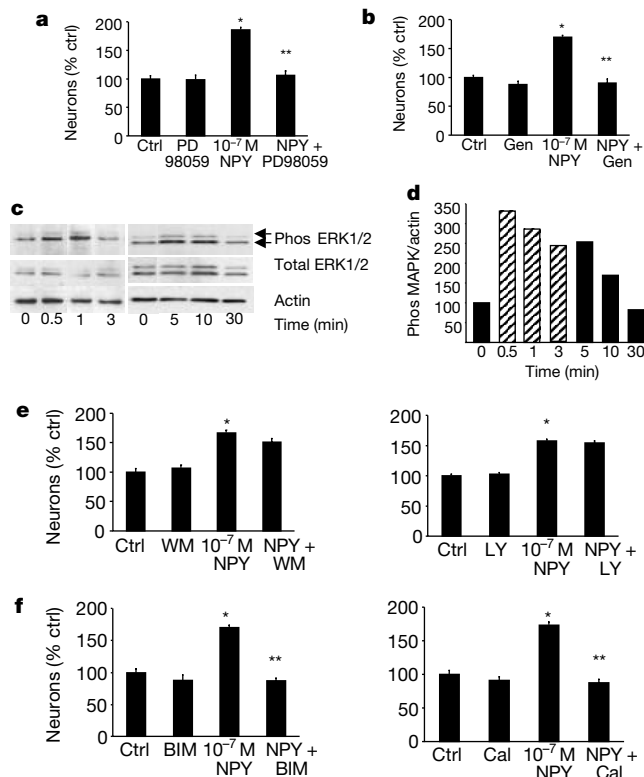


Figure 5 NPY induces neuroproliferation through ERK1/2 phosphorylation and activation. **a**, MEK blockade abolishes NPY-induced neuroproliferation. Twenty-four hour incubation of cells with NPY increases neuronal number. Incubation with PD98059 (100 μM; Calbiochem), an inhibitor of MEK activation, does not alter NST⁺ cells, but co-incubation with PD98059 and NPY blocks the ability of NPY to induce neuronal proliferation. Asterisk, *P* < 0.001 between NPY and control. Double asterisk, *P* < 0.001 between NPY and NPY plus PD98059. **b**, Tyrosine kinase inhibition blocks the NPY-induced increase in neuronal number. Twenty-four hour co-incubation of cells with NPY and 100 μM genistein (Gen; Calbiochem, concentration within manufacturer's recommended range) prevented the NPY effect. **c**, NPY promotes rapid phosphorylation of ERK1/2. Western blots from four experiments were densitized using the Scion Image Program. Ratios represent the increase over control (0 min) levels of phosphorylated ERK1/2 normalized to levels of actin. **d**, NPY does not induce neuroproliferation through the PI(3)K pathway. Co-incubation of cultures for 24 h with NPY and either 100 nM wortmannin (WM; Calbiochem) or 20 μM LY294002 (LY; Calbiochem) did not alter the NPY-induced increase in neuronal number. **e**, PKC mediates the NPY effect. Co-incubation of cells for 24 h with NPY and either 10 μM BIM-1 (Calbiochem) or 0.5 μM calphostin (Cal; Calbiochem) blocked the ability of NPY to induce neuroproliferation. Asterisk, *P* < 0.001 between NPY and control. Double asterisk, *P* < 0.001 between NPY and NPY plus drug.

LY294002, which specifically inhibit PI(3)K, did not block the ability of NPY to increase neuronal number (Fig. 5f). However, incubation of cells with the PKC inhibitors bisindolylmaleimide I (BIM-1, 10 μ M) or calphostin (Cal, 0.5 μ M) abolished the effect of NPY on neuroproliferation (Fig. 5g). These results indicate that NPY regulates upstream activation of ERK1/2 by a PKC-dependent pathway.

This is the first report, to our knowledge, of NPY-induced neuroproliferation. NPY, expressed by a subset of non-neuronal sustentacular cells in the adult olfactory epithelium, acts on neuronal precursor cells (basal cells) to induce cell-cycle activation and division. NPY acts through the Y1 receptor to increase neuronal precursor proliferation; this effect is mediated downstream through a kinase cascade involving PKC and ERK1/2. NPY can induce cardiomyocyte hypertrophy by a similar mechanism, although the upstream regulator of ERK1/2 activation in cardiomyocytes involves a PI(3)K-dependent pathway^{26–28}.

Although the olfactory system has been considered unusual in its ability to maintain multipotent stem-cell-like populations, the recent discovery of stem cells in the retina, hippocampus and rostral migratory stream^{9,10} indicates that postnatal neurogenesis could be a potentially widespread mechanism by which the nervous system repopulates certain neuronal lineages. NPY is broadly expressed in the developing nervous system and is maintained at moderately high levels in the adult, although its actions vary according to the target cell^{1,2}. The ability of NPY to stimulate neuroproliferation of the olfactory neuronal precursor cell could imply a similar function on central nervous system stem cells, and may be useful for fields such as stem cell therapy. □

Methods

Antibodies and immunofluorescent immunostaining

All experimental protocols were approved by the Johns Hopkins University Institutional Animal Care and Use Committee, and all applicable guidelines from the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* were followed. Five-week-old Sprague–Dawley rats (Harlan) were anaesthetized with Xylaket and perfused with phosphate-buffered saline (PBS) followed by Bouin's fixative. Tissue was dissected, post-fixed overnight in Bouin's fixative at 4 °C, washed in PBS, placed in 20% sucrose and embedded in Tissue-Tek (Sakura Finetek). Paraffin-embedded paraformaldehyde fixed embryos were obtained from Novagen. Rabbit polyclonal NPY antibody JH3 (5 ml) was affinity-purified using NPY peptide (1-36)NH₂ (1 mg; Peninsula) linked to Affigel 10 resin (2 ml; Bio-Rad). For double-label immunofluorescence, sections were washed in PBS, permeabilized with 0.05% SDS in PBS, incubated with 1% H₂O₂, blocked in TNB buffer (NEN) and then incubated overnight at 4 °C with monoclonal antibody against NST at 1:1,000 (BabCo). The following day, sections were incubated with affinity purified NPY antibody at 1:1,000 using the tyramide-direct protocol (NEN).

DAB immunostaining

Four-week-old male NPY-deficient⁸ and wild-type littermate controls were used. Tissue was dissected and sectioned as described. Sections were hydrated in PBS, permeabilized in 0.05% SDS in PBS, blocked in appropriate 4% normal serum with 1% BSA (Sigma) and incubated overnight at 4 °C with NST monoclonal antibody at 1:1,000 (BabCo), Ki67 monoclonal antibody at 1:100 (Immunotech) and O/E-1 polyclonal antibody at 1:100 (a gift from the laboratory of R. Reed). The following day, sections were incubated in 1% H₂O₂ and developed according to the ABC protocol (Vector Laboratories). Six or seven fields were counted per animal for O/E-1.

Radioimmunoassay

NPY levels were quantified by radioimmunoassay³⁰ in acetic acid extracts of olfactory epithelium, olfactory bulb, hypothalamus and primary olfactory neuron cultures. Radioimmunoassay was performed using [¹²⁵I]-labelled NPY (NEN) and anti-NPY JH3 antibody diluted 1:40,000.

BrdU labelling

One-day-old cultures were incubated with BrdU reagent (Boehringer Mannheim) according to instructions with or without NPY. After incubation, cells were rinsed in PBS, fixed in ice-cold 70% EtOH in 50 mM glycine, pH 2.0, and processed for BrdU staining. Cells were then washed in PBS, blocked with 4% normal horse serum (Vector Laboratories), incubated overnight at 4 °C with NST antibody 1:1,000, rinsed in PBS and processed with the Elite ABC Kit for DAB staining (Vector). Cells were counted per high power field using 9–10 fields per well. For animal experiments, mice were injected with 50 mg kg⁻¹ BrdU labelling reagent, killed 24 h after injection and perfused with Bouin's fixative.

TUNEL staining

One-day-old cultures were treated with or without peptide for 48 h, rinsed in PBS and fixed in MeOH:acetone (1:1). Cells were then treated with 3% H₂O₂, incubated in TdT buffer (33 mM Tris, pH 7.2, 140 mM NaCacodylate, 1 mM CoCl₂), treated with 0.12 U μ l⁻¹ terminal transferase (Sigma) and a 1:200 dilution of biotin-16-2'-deoxyuridine-5'-triphosphate (Boehringer Mannheim), washed in \times 2 SSC, washed in PBS, blocked with 3% normal horse serum (Vector) and developed using the Elite ABC Kit (Vector).

Cell culture

We used around fifty 0–1-day-old rat pups per experiment for culture. Cells were grown for one day after plating in 12-well plates. After incubation with 10⁻⁶ M NPY for the appropriate time, cells were lysed with boiling sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 1% β -mercaptoethanol, 10% glycerol, 0.1% bromophenol blue) containing 0.3 mg ml⁻¹ PMSF, 50 μ g ml⁻¹ lima bean trypsin inhibitor, 2 μ g ml⁻¹ leupeptidin, 16 μ g ml⁻¹ benzamide, 2 μ g ml⁻¹ pepstatin, 5 mM EGTA, 5 mM Na₂EDTA, 1 mM sodium orthovanadate, 10 mM sodium phosphate and 50 mM sodium fluoride. Incubation media included 0.5% dialysed fetal bovine serum (Gibco BRL) and 2.5 ng NGF per ml medium. Samples were sonicated for 30 s, boiled for 5 min, cooled on ice and spun at 14,000 r.p.m. at 4 °C for 10 min, aliquoted, and stored at -80 °C.

Western blotting

Olfactory cultures were incubated with 10⁻⁶ M NPY for times from 30 s to 30 min, then harvested for western blotting. Blots were probed using an antibody specific for phosphorylated ERK1/2 (1:1,000; NEB). Standardization was performed by re-probing the blots with antibody for ERK1/2 (1:1,000; NEB) and actin (1:1,000; Sigma).

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Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10

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The stimulation of glucose uptake by insulin in muscle and adipose tissue requires translocation of the GLUT4 glucose transporter protein from intracellular storage sites to the cell surface^{1–6}. Although the cellular dynamics of GLUT4 vesicle trafficking are well described, the signalling pathways that link the insulin receptor to GLUT4 translocation remain poorly understood. Activation of phosphatidylinositol-3-OH kinase (PI(3)K) is required for this trafficking event, but it is not sufficient to produce GLUT4 translocation⁷. We previously described a pathway involving the insulin-stimulated tyrosine phosphorylation of Cbl, which is recruited to the insulin receptor by the adapter protein CAP^{8,9}. On phosphorylation, Cbl is translocated to lipid rafts. Blocking this step completely inhibits the stimulation of GLUT4 translocation by insulin¹⁰. Here we show that phosphorylated Cbl recruits the CrkII–C3G complex to lipid rafts, where C3G specifically activates the small GTP-binding protein TC10. This process is independent of PI(3)K, but requires the translocation of Cbl, Crk and C3G to the lipid raft. The activation of TC10 is essential for insulin-stimulated glucose uptake and GLUT4 translocation. The TC10 pathway functions in parallel with PI(3)K to stimulate fully GLUT4 translocation in response to insulin.

Many receptor tyrosine kinases use small GTP-binding proteins as molecular switches to convert proximal tyrosine phosphorylation into the activation of serine/threonine kinase cascades¹¹. In addition, small GTP-binding proteins are critical for intracellular

vesicle trafficking¹². We have examined the modulation of insulin-stimulated glucose uptake and GLUT4 translocation by several small GTP-binding proteins¹. Expression of wild-type, dominant-interfering and constitutively active mutants of Ras, Rap1, Rac1, TC21, RhoA and RhoD in 3T3L1 adipocytes had no discernible effect on this biological response (data not shown). In contrast, expression of wild-type TC10 (TC10/WT) and a dominant-interfering TC10 mutant (TC10/T31N) significantly inhibited insulin-stimulated glucose uptake (Fig. 1a).

As the transfection efficiency was about 50%, it is likely that expression of the TC10/T31N mutant almost completely inhibited glucose uptake in the transfected cell population. Out of the known GTP-binding proteins, Cdc42 has the greatest degree of sequence similarity to TC10, with an overall sequence identity of 69% and similarity of 83%. However, expression of wild-type Cdc42 (Cdc42/WT) or the dominant-interfering Cdc42 mutant (Cdc42/T17N) had no statistically significant effect on insulin-stimulated glucose uptake (Fig. 1a).

To determine whether TC10 inhibits insulin-stimulated glucose uptake through a blockade of GLUT4 translocation, we microinjected 3T3L1 adipocytes along with expression plasmids for TC10 or Cdc42 (Fig. 1b). The cells were microinjected with the carboxy-terminal domain of Ras fused to the maltose-binding protein (MBP–Ras) as a marker for plasma-membrane in injected cells^{2–4}. As GLUT4 is localized predominantly in intracellular vesicles in the basal state, there is only a low level of GLUT4 immunofluorescence in the plasma-membrane sheets derived from both the microinjected and non-microinjected cells. As typically observed, insulin produced the robust appearance of plasma-membrane GLUT4 immunofluorescence in both the microinjected and surrounding non-injected cells. Microinjection of either TC10/WT or TC10/T31N inhibited insulin-stimulated translocation of GLUT4, in

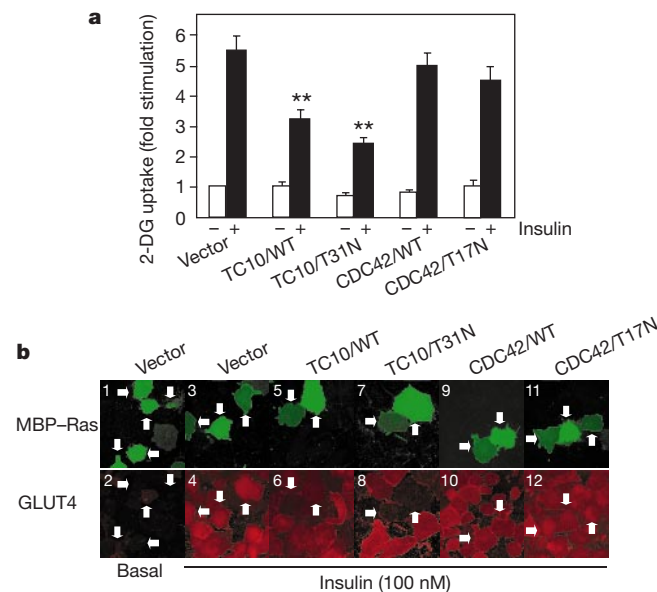


Figure 1 Expression of TC10 inhibits insulin-stimulated glucose uptake and GLUT4 translocation in 3T3L1 adipocytes. **a**, Differentiated 3T3L1 adipocytes were transfected with TC10 and Cdc42 mutants. The cells were left untreated or were stimulated with 100 nM insulin for 30 min. The rate of [³H]2-deoxyglucose (2-DG) uptake was determined. Results are the mean ± s.e. of 3–5 independent experiments from individual experiments performed in triplicate with a transfection efficiency of ~50%. Two asterisks, *P* < 0.01. **b**, Differentiated 3T3L1 adipocyte nuclei were microinjected with 0.2 mg ml⁻¹ of MBP–Ras plus either the empty vector or the cDNAs encoding TC10/WT, TC10/T31N, CDC42/WT or CDC42/T17N. The cells were allowed to recover for 24 h, and left untreated or treated with 100 nM insulin for 30 min.