LETTERS

S-nitrosylation of histone deacetylase 2 induces chromatin remodelling in neurons

Alexi Nott[1], P. Marc Watson[1], James D. Robinson[1], Luca Crepaldi[1] & Antonella Riccio[1]

Brain-derived neurotrophic factor (BDNF) and other neurotrophins have a vital role in the development of the rat and mouse nervous system by influencing the expression of many specific genes that promote differentiation, cell survival, synapse formation and, later, synaptic plasticity[1]. Although nitric oxide (NO) is known to be an important mediator of BDNF signalling in neurons[2], the mechanisms by which neurotrophins influence gene expression during development and plasticity remain largely unknown. Here we show that BDNF triggers NO synthesis and *S*-nitrosylation of histone deacetylase 2 (HDAC2) in neurons, resulting in changes to histone modifications and gene activation. *S*-nitrosylation of HDAC2 occurs at Cys 262 and Cys 274 and does not affect deacetylase activity. In contrast, nitrosylation of HDAC2 induces its release from chromatin, which increases acetylation of histones surrounding neurotrophin-dependent gene promoters and promotes transcription. Notably, nitrosylation of HDAC2 in embryonic cortical neurons regulates dendritic growth and branching, possibly by the activation of CREB (cyclic-AMP-responsive-element-binding protein)-dependent genes. Thus, by stimulating NO production and *S*-nitrosylation of HDAC2, neurotrophic factors promote chromatin remodelling and the activation of genes that are associated with neuronal development.

Although most cells in multicellular organisms contain an identical genome, different cell types stably express different sets of genes, reflecting progressive and persistent alterations in chromatin structure that underlie cell specialization and cell memory[3,4]. The differentiation of multipotent stem cells into neurons is a quintessential example of epigenetics at work. Despite growing evidence that neurotrophic factors and other extracellular signals influence the epigenetic changes associated with gene transcription, the details of how they regulate these changes are still poorly understood. Neurotrophins are a family of peptide growth factors that exert their growth and survival-promoting effects on neurons through the activation of various intracellular signalling pathways, including the NO pathway[2,5,6]. In the nervous system, NO has been linked to many physiological and pathological functions, including neurogenesis, hippocampal long-term potentiation, neurodegeneration and neuronal survival and differentiation[7,8]. There is increasing evidence that NO can act by directly modifying cysteine residues in target proteins[9,10]. Cysteine nitrosylation (*S*-nitrosylation) is now regarded as a selective and specific signal controlled by cellular NO[9]. *S*-nitrosylation of transcription factors in the cytoplasm, for example, is one way that NO can regulate gene expression[11].

To investigate how neurotrophins regulate NO-dependent gene expression, we first tested whether BDNF stimulation induced nuclear localization of NO in rat cortical neurons. To visualize NO, we used 4-amino-5-methylamino-2′,7′-difluororescein diacetate (DAF-FM DA), a fluorescent dye that specifically binds NO[12]. In basal conditions, NO was synthesized at low levels in many neurons (Fig. 1a, b). Stimulation of cortical neurons with BDNF for 30 min resulted in a rapid accumulation of DAF-FM DA reactivity in both cytoplasmic and nuclear compartments. Pre-treatment of cortical neurons with the specific neuronal NO synthase (nNOS) inhibitor NO-propyl-L-arginine (NPA, 300 μM) consistently resulted in a lack of BDNF-stimulated DAF-FM DA reactivity (Fig. 1a, b). Notably, removal of NPA from the culture medium followed by stimulation with BDNF resulted in a pronounced increase of DAF-FM DA reactivity in both the nuclear and cytoplasmic compartments (Fig. 1a, b). To test whether the NO generated on neurotrophin treatment directly modifies cytoplasmic and nuclear proteins by means of *S*-nitrosylation, we used the biotin-switch assay[12–17]. Stimulation of cortical neurons with BDNF induced *S*-nitrosylation of several proteins detected in whole cell extracts (Fig. 1c, arrows), many of which were nitrosylated within minutes of exposure of the neurons to BDNF and remained nitrosylated 1 h after stimulation. Measurement of nuclear and cytoplasmic extracts exposed to the NO donor *S*-nitrosocysteine (SNOC) showed that several nuclear proteins are targets of NO, and are therefore potential substrates of *S*-nitrosylation *in vivo* (Fig. 1d, arrows and Supplementary Information 1). The *S*-nitrosylation pattern of nuclear proteins was clearly distinct from the cytoplasmic pattern, with prominent nuclear nitrosylation of proteins of molecular masses 90, 75, 38 and 25 kDa.

One function of NO in the nucleus may be to regulate the interaction of transcription factors with chromatin[2,18]. During transcriptional activation, the tightly compacted chromatin is modified by histone acetyltransferases, which are recruited to gene promoters, and this chemical modification is counteracted by histone deacetylases (HDACs)[19,20]. As NO influences gene expression primarily by changing the acetylation state of chromatin associated with the promoter of neurotrophin-regulated genes[2] (Supplementary Fig. 2a, b), we investigated whether NO influences histone acetylation by modifying histone deacetylase activity. Messenger RNA and protein expression analyses showed that HDAC2 is highly expressed in embryonic neurons (A.N. and A.R., unpublished observations). To test whether HDAC2 itself may be a target of BDNF-dependent *S*-nitrosylation, cortical neurons were stimulated with BDNF and nitrosylated proteins were precipitated and subjected to HDAC2 immunoblotting analysis (Fig. 2a). BDNF was found to induce a rapid and sustained *S*-nitrosylation of HDAC2. Moreover, treatment of either cortical neurons or HEK293T cells overexpressing nNOS with the Ca^{2+} ionophore A23187 also led to an increase in the *S*-nitrosylation of HDAC2 (Fig. 2a, b). BDNF and A23187 stimulation did not induce HDAC2 *S*-nitrosylation in cortical neurons lacking nNOS (Fig. 2b and Supplementary Fig. 2d). Experiments performed using the Saville–Griess assay, an alternative biochemical assay to assess protein *S*-nitrosylation[21,22], confirmed that recombinant HDAC2 is strongly nitrosylated after treatment with SNOC *in vitro* (Supplementary Fig. 2c).

[1]MRC Laboratory for Molecular and Cell Biology, and Department of Neuroscience, Physiology and Pharmacology, University College London, London WC1E 6BT, UK.

HDAC2 belongs to the class I HDACs, which includes HDAC1 to HDAC3 and HDAC8. Structural analysis has shown that the conserved deacetylase domain of class I HDACs includes an active site containing a zinc-binding domain[23] (Supplementary Fig. 3a). We first tested whether the cysteine located in the deacetylase domain, Cys 152, was the acceptor site of *S*-nitrosylation. Mutation of Cys 152 to alanine did not influence HDAC2 nitrosylation (A.N. and A.R., unpublished observations). Structure-to-function analysis of HDAC2 showed that two residues, Cys 262 and Cys 274, were nitrosylated after SNOC treatment (Fig. 2c and Supplementary Fig. 3b, c). Single mutation of either Cys 262 or Cys 274 to alanine did not change HDAC2 nitrosylation, whereas double mutation of both Cys 262 and Cys 274 completely abolished *S*-nitrosylation of HDAC2 (Fig. 2c). Notably, Cys 262 and Cys 274 were also nitrosylated when cortical neurons were stimulated with BDNF (Fig. 2d, e).

As for other post-translational modifications, *S*-nitrosylation may regulate protein functions in more than one way. Nitrosylation of critical cysteine residue(s), for example, may influence their enzymatic activity[16,24,25]. Alternatively, *S*-nitrosylation may induce a conformational change that influences the interaction of the nitrosylated protein with its binding partners[12,15,17,26]. To test whether BDNF-dependent NO signalling regulates HDAC2 activity, cortical neurons were stimulated with BDNF or SNOC, cell lysates were immunoprecipitated with an HDAC2 antibody, and HDAC2 activity was assessed using a fluorimetric assay. We observed no difference in HDAC2 activity between untreated and stimulated cells (Supplementary Fig. 4a, b). Moreover, HDAC2 activity was not significantly affected in PC12 cells expressing the wild-type ($HDAC2^{WT}$) or the double mutant form of HDAC2 ($HDAC2^{C262A/C274A}$), which is not nitrosylated, after treatment with either nerve growth factor (NGF) or SNOC (Fig. 3a and Supplementary Fig. 4a, b).

To determine whether HDAC2 *S*-nitrosylation affects its ability to associate with DNA, we performed chromatin immunoprecipitation (ChIP) assays using PC12 cells expressing either $HDAC2^{WT}$ or

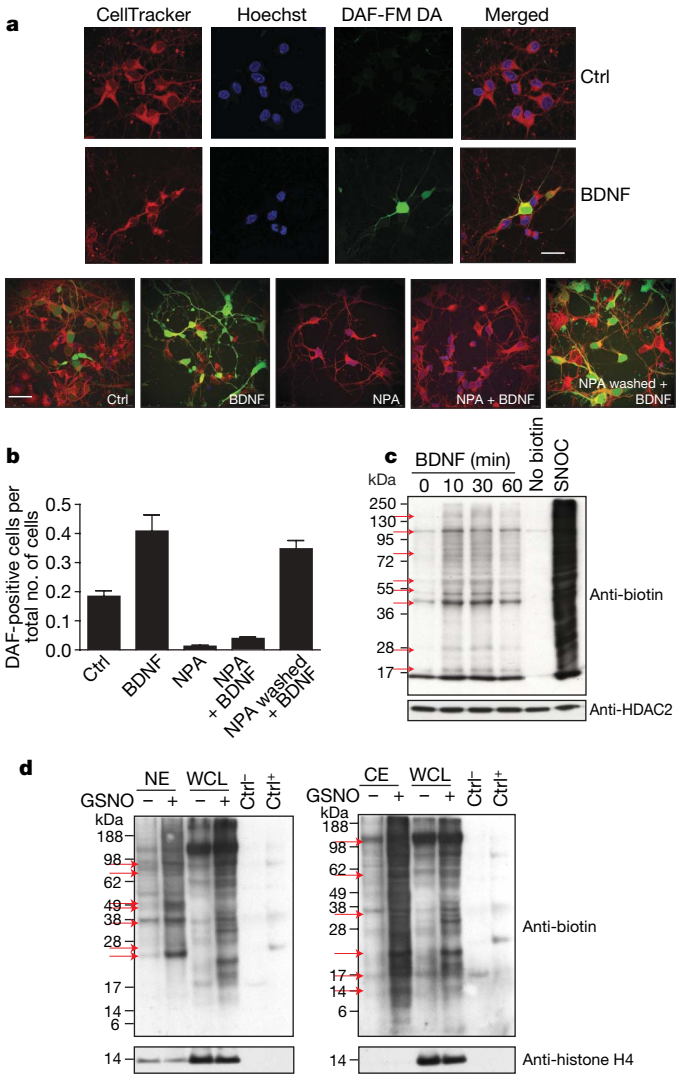

Figure 1 | BDNF induces nuclear NO synthesis and *S*-nitrosylation of nuclear and cytoplasmic proteins. **a**, Neurons were incubated for 30 min with DAF-FM DA and stimulated with BDNF (75 ng ml^{-1} for 30 min), NPA (300 μM for 30 min), BDNF in the presence of NPA (NPA + BDNF), NPA followed by BDNF (NPA washed + BDNF) or vehicle (Ctrl). Cells were stained with Hoechst and CellTracker and images were captured using a Leica DM 2500 confocal microscope. Scale bars, 25 μm (top panels) and 75 μm (bottom panel). **b**, Quantitative analysis of the data shown in **a**. Shown are the averages and the s.e.m.; $n = 3$. **c**, Biotin-switch assay of cortical neurons treated with either BDNF for the indicated times (in minutes) or 200 μM SNOC for 15 min. Neurons were subjected to the biotin-switch assay followed by anti-biotin western blot. The arrows indicate proteins that are nitrosylated after BDNF stimulation; $n = 3$. Equal loading was assessed by HDAC2 western blotting. **d**, Neuronal nuclear (left blot) and cytoplasmic (right blot) proteins were exposed to *S*-nitrosoglutathione (GSNO, 100 μM) *in vitro* and subjected to the biotin-switch assay. The purity of nuclear and cytoplasmic fractions was assessed by western blot analysis of histone H4 (bottom panels). The arrows indicate proteins that are nitrosylated after BDNF stimulation. CE, cytoplasmic extracts; Ctrl^{+}, mix of control proteins exposed to GSNO; Ctrl^{-}, mix of control proteins left untreated (the mix does not contain histones); NE, nuclear extracts; WCL, whole cell lysate; $n = 3$.

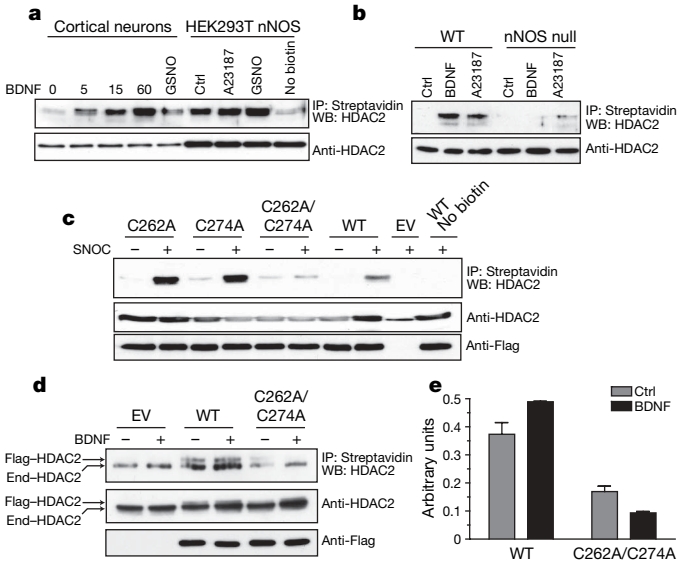

Figure 2 | BDNF induces *S*-nitrosylation of HDAC2 on Cys 262 and Cys 274. **a**, Cortical neurons and nNOS-expressing HEK293T cells were stimulated with either BDNF for the indicated times (minutes), GSNO or 50 μM A23187 for 20 min. Protein extracts were subjected to the biotin-switch assay followed by streptavidin precipitation and HDAC2 western blotting; $n = 3$. Equal loading was assessed by HDAC2 western blotting. **b**, Biotin-switch assay of BDNF-treated cortical neurons derived from wild-type (left lanes) or nNOS null (right lanes) mouse embryos. Neurons were stimulated with BDNF or A23187 for 20 min. Precipitations were performed using streptavidin followed by HDAC2 western blotting; $n = 3$. **c**, Biotin-switch assay of HEK293T cells transfected with $HDAC2^{WT}$, $HDAC2^{C262A}$, $HDAC2^{C274A}$, $HDAC2^{C262A/C274A}$ or empty vector (EV), and treated with SNOC. Streptavidin precipitation (IP) was followed by HDAC2 western blotting (WB); $n = 3$. **d**, Cortical neurons were transfected with $HDAC2^{WT}$ or $HDAC2^{C262A/C274A}$ vectors and stimulated with BDNF or unstimulated (Ctrl). *S*-nitrosylation was assessed using the biotin-switch assay. End-HDAC2, endogenous HDAC2; Flag–HDAC2, Flag-tagged HDAC2 expression vectors. **e**, Densitometric analysis of the data in **d**. Shown are the averages and the s.e.m.; $n = 3$.

HDAC2$^{C262A/C274A}$. We analysed rat *Fos*, *Egr1*, *Vgf* and *Nos1* (also known as *nNOS*) genes, as they are regulated by BDNF and contain a CREB binding site in their promoters[2]. In resting conditions, HDAC2^{WT} was associated with *Fos*, *Egr1*, *Vgf* and *Nos1* promoters. On stimulation with either NGF or SNOC, HDAC2^{WT} readily dissociated from the chromatin (Fig. 3b, c and Supplementary 5a). In contrast, HDAC2$^{C262A/C274A}$ remained strongly associated with the promoters after NGF stimulation or SNOC treatment (Fig. 3b, c and Supplementary 5a). To test whether expression of HDAC2$^{C262A/C274A}$ in PC12 cells inhibited NGF-dependent histone acetylation, we performed ChIP analysis of cells transfected with HDAC2^{WT} or the HDAC2$^{C262A/C274A}$ mutant. As expected, the treatment of PC12 cells expressing either an empty vector or HDAC2^{WT} with NGF or SNOC induced rapid acetylation of histones H3 and H4, as assessed using either acetylated histone H3 K9/K14 or pan-acetylated histone H4 antibodies (Fig. 3d and Supplementary 5b). In contrast, when PC12 cells were transfected with HDAC2$^{C262A/C274A}$, NGF failed to induce histone acetylation of the chromatin surrounding the *Fos*, *Egr1*, *Vgf* and *Nos1* promoters (Fig. 3d and Supplementary 5b). Taken together, these results indicate that neurotrophin-dependent nitrosylation of HDAC2 on the critical cysteine residues Cys 262 and Cys 274 is necessary to induce the dissociation of HDAC2 from chromatin and thereby facilitate acetylation of histones H3 and H4.

To determine whether HDAC2 nitrosylation mediates BDNF-dependent transcriptional activation of *Fos*, *Vgf* and *Egr1* genes in cortical neurons, PC12 cells were transfected with HDAC2^{WT}, HDAC2$^{C262A/C274A}$ or an empty vector and stimulated with NGF for various time points. Northern blot analysis showed that the expression of HDAC2$^{C262A/C274A}$ significantly inhibited NGF-dependent transcription of *Fos*, *Vgf* and *Egr1*, whereas HDAC2^{WT} or empty vector did not affect *Vgf* and *Egr1* transcription (Fig. 3e). Although HDAC2^{WT} did not change *Fos* promoter activity (Supplementary Fig. 6a), it did reduce endogenous *Fos* expression, suggesting that it is also affecting chromatin regions outside the CRE-containing promoter.

We next examined the effects of HDAC2 nitrosylation on neuronal differentiation and the development of dendrites. Cortical neurons were transfected with HDAC2$^{C262A/C274A}$, HDAC2^{WT} or empty vector and the dendritic morphology was visualized by co-transfecting with a green fluorescent protein (GFP) vector. Quantitative analysis showed that in unstimulated neurons, expression of HDAC2$^{C262A/C274A}$ decreased the total and the average dendritic length, whereas dendritic branching was only slightly reduced (Fig. 4a, c and Supplementary 7a). To test whether HDAC2 nitrosylation influenced stimulus-dependent

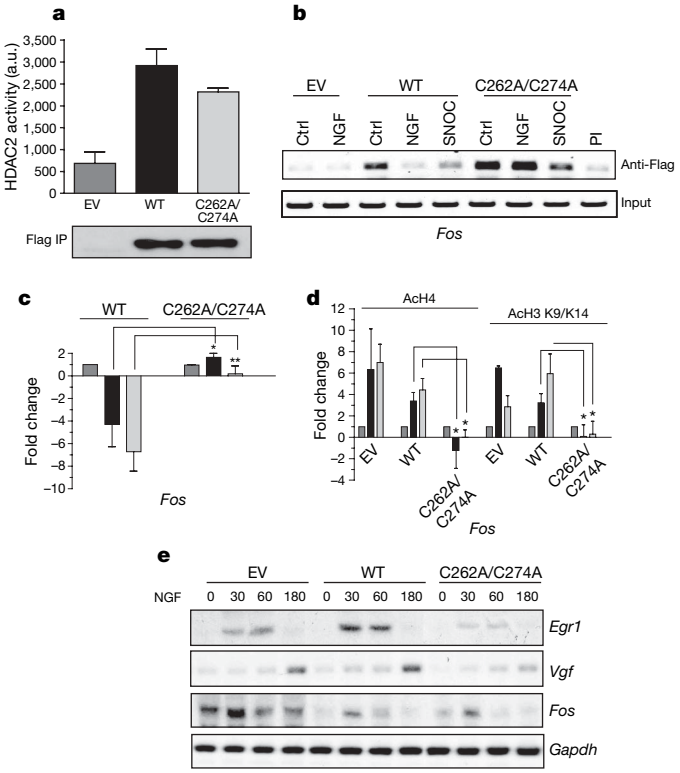

Figure 3 | *S*-nitrosylation of HDAC2 regulates its association with chromatin. **a**, PC12 cells were transfected with the indicated HDAC2 carboxy-terminal Flag-tagged vectors. Anti-Flag antibody immunoprecipitates (IP) were subjected to HDAC assay. Shown are the averages and the s.e.m.; $n = 3$; a.u., arbitrary units. **b**, ChIP analysis of PC12 cells transfected with Flag-tagged HDAC2^{WT}, HDAC2$^{C262A/C274A}$ or empty vector (EV) and stimulated with NGF (100 ng ml^{-1}) or SNOC for 20 min. Flag immunoprecipitation was followed by PCR for the *Fos* promoter. Ctrl, vehicle; PI, immunoprecipitation with pre-immune serum; $n = 3$. **c**, Real-time PCR quantification of ChIP analyses. Data were normalized by total input and represented as the fold change over unstimulated control. Shown are the averages and the s.e.m.; $n = 3$; asterisk, $P < 0.05$; double asterisk, $P < 0.005$. **d**, Real-time PCR quantification of ChIP analysis of PC12 cells transfected with HDAC2 vectors as indicated, and stimulated with NGF or SNOC. Acetylated histone H3 (AcH3 K9/K14) and pan-acetylated histone H4 (AcH4) immunoprecipitation was followed by quantitative PCR analysis of the *Fos* promoter. Data were normalized by total input and represented as fold changes over unstimulated control. Shown are the averages and the s.e.m; $n = 3$; asterisk, $P < 0.001$. **e**, Northern blot analysis of PC12 cells transfected with HDAC2 vectors and stimulated with NGF for the indicated times (minutes); $n = 3$.

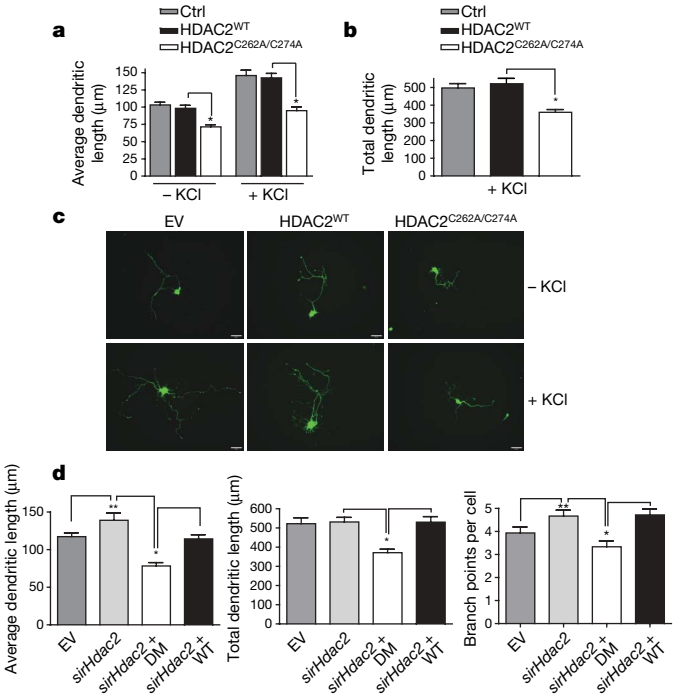

Figure 4 | Neurotrophin-dependent *S*-nitrosylation of HDAC2 regulates dendritic growth. **a**, **b**, Average (**a**) and total (**b**) dendritic length of cortical neurons transfected with HDAC2 vectors and grown under basal conditions (**a**) or stimulated with 50 mM KCl (**a**, **b**). At least 30 neurons were analysed for each condition for each experiment. Shown are the averages and the s.e.m.; $n = 3$; asterisk, $P = 0.001$. **c**, Examples of cortical neurons transfected with HDAC2 vectors and either stimulated with KCl or left untreated. Scale bar, 40 μm. **d**, Dendritic growth analysis of neurons transfected with rat *sirHdac2* and empty vector, rat *sirHdac2* and mouse HDAC2^{WT} (WT), rat *sirHdac2* and mouse double mutant HDAC2$^{C262A/C274A}$ (DM), or with empty vector alone (EV). The average dendritic length (left), the total dendritic length (middle) and the number of branch points per cell (right) were assessed. Shown are the averages and the s.e.m.; $n = 3$; asterisk, $P = 0.001$; double asterisk, $P < 0.05$.

dendritic growth, quantitative analysis was performed in cortical neurons maintained with 50 mM potassium chloride, a condition that induces neuronal depolarization and calcium influx[27]. Notably, calcium-dependent activation of CREB target genes mediates the dendritic growth observed under these conditions[27]. As previously shown, stimulation of cortical neurons with potassium chloride for 48 h significantly increased dendritic growth (Fig. 4a). Expression of HDAC2$^{C262/274A}$, but not HDAC2^{WT} or empty vector, reduced potassium-chloride-dependent axon growth and markedly affected neuronal morphology (Fig. 4a–c and Supplementary Fig. 7a). As an alternative approach, we inhibited HDAC2 by transfecting cortical cultures with a rat-specific HDAC2 short interfering RNA (siRNA; *sirHdac2*; Supplementary Fig. 7b). Cortical neurons were transfected with *sirHdac2* and mouse HDAC2^{WT}, mouse HDAC2$^{C262A/C274A}$ or empty vector alone, and the average dendritic length, the total dendritic length and the number of branches per cell was assessed. Silencing of HDAC2 increased the average dendritic length and the number of branch points per neuron (Fig. 4d), an effect that was rescued by mouse HDAC2^{WT} expression. In contrast, HDAC2$^{C262A/C274A}$ expression inhibited dendritic growth and the number of branch points per cell (Fig. 4d). Taken together, these findings indicate that HDAC2 *S*-nitrosylation regulates dendritic growth and branching, possibly by the activation of CREB-dependent genes.

In unstimulated neurons, HDAC2 is tightly associated with gene promoters and represses transcription by deacetylating histones and possibly other chromatin-associated proteins. After neurotrophin stimulation and membrane depolarization, NO is synthesized and can diffuse into the nucleus where it nitrosylates HDAC2, facilitating the dissociation of the enzyme from chromatin. The release of HDAC2 in repressor complexes, concomitant with the recruitment of nuclear activators (as histone acetyltransferases, for example), promotes histone acetylation and the onset of transcription (Supplementary Fig. 7c). nNOS is mainly responsible for NO production in the nervous system, and its expression is tightly regulated both temporally and spatially[28]. In the developing brain, nNOS reaches the highest expression level between embryonic day 16 (E16) and postnatal day 0 (P0), and this expression corresponds with the migration of neuronal precursors from the ventricular zone to the external layers of the cortex. nNOS is also expressed in sensory neurons between E12 and E15, when these neurons are highly dependent on neurotrophins for survival and growth[28]. Here we have shown that BDNF, NGF and possibly other neurotrophins, can influence chromatin remodelling in neurons by *S*-nitrosylation of HDAC2. It is therefore conceivable that NO mediates the epigenetic changes that are induced by neurotrophins and by synaptic activity, and determines the expression of genes responsible for cell-fate choice and neuronal migration. Our findings have further implications beyond the mechanisms that regulate the early development of the nervous system and may extend to various physiological functions in the adult brain. Exposing animals to a new and enriched environment, for example, can partially revert memory loss and neurodegeneration by mechanisms that involve changes in histone acetylation, and possibly NO signalling[2,29]. Notably, in a mouse model of neurodegeneration, administration of the general HDAC inhibitor sodium butyrate markedly inhibits neuronal loss and improves memory performances[29]. In such neurodegenerative disorders, manipulation of NO signalling may prove to be a more specific therapeutic approach than the use of general HDAC inhibitors.

METHODS SUMMARY

DAF-FM DA staining. Neurons were grown *in vitro* for 5 days and incubated with DMEM containing DAF-FM DA (Molecular Probes; 10 μM for 30 min at 37 °C). Cells were treated with BDNF (75 ng ml^{-1}), NPA (300 μM), BDNF in the presence of NPA, or BDNF after the removal of NPA. Neurons were stained with CellTracker and Hoechst to visualize cell bodies and nuclei, fixed with 4% paraformaldehyde and images were captured with a Leica DM 2500 confocal microscope.

Biotin-switch assay. The biotin-switch assay is a sensitive assay that has been successfully and extensively used to detect *S*-nitrosylated proteins by specifically labelling nitrosylated cysteines with a biotin moiety[12,15–17]. Biotinylated proteins can be easily detected by biotin western blot or streptavidin precipitation followed by western blotting. It is essential to perform the assay in the dark, as exposure of the samples to ultraviolet light may result in non-specific signals. We performed the biotin-switch assay as described[13,14] with minor modifications. The assay is explained in detail in the Methods.

Analysis of dendritic growth. E17 cortical neurons were cultured on glass coverslips coated with laminin and poly-D-lysine, and transfected after 4 h *in vitro*. Transfections were carried out using 0.12 μg of GFP vector, 1.2 μg of HDAC2 vectors, 15 nM of rat *sirHdac2* oligos (Dharmacon) and 2 μl of Lipofectamine 2000 (Invitrogen). Neurons were incubated for 4 h at 37 °C and culture medium was replaced with the original plating medium with or without KCl (50 mM). Forty-eight hours after transfection, cells were fixed and quantitative analysis of dendritic growth and branching was assessed as described[27]. Neurites were confirmed to be dendrites by MAP2 staining (A.N. and A.R., unpublished observations). Rat *Hdac2* siRNA sequences are available on request.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 23 May; accepted 7 July 2008.
Published online 27 August 2008.

1. Huang, E. & Reichardt, L. Neurotrophins: roles in neuronal development and function. *Annu. Rev. Neurosci.* **24,** 677–736 (2001).
2. Riccio, A. *et al.* A nitric oxide signaling pathway controls CREB-mediated gene expression in neurons. *Mol. Cell* **21,** 283–294 (2006).
3. Reik, W. Stability and flexibility of epigenetic gene regulation in mammalian development. *Nature* **447,** 425–432 (2007).
4. Kouzarides, T. Chromatin modifications and their function. *Cell* **128,** 693–705 (2007).
5. Huang, E. J. & Reichardt, L. F. Trk receptors: roles in neuronal signal transduction. *Annu. Rev. Biochem.* **72,** 609–642 (2003).
6. Zweifel, L. S., Kuruvilla, R. & Ginty, D. D. Functions and mechanisms of retrograde neurotrophin signalling. *Nature Rev. Neurosci.* **6,** 615–625 (2005).
7. Zhang, J. & Snyder, S. H. Nitric oxide in the nervous system. *Annu. Rev. Pharmacol. Toxicol.* **35,** 213–233 (1995).
8. Boehning, D. & Snyder, S. H. Novel neural modulators. *Annu. Rev. Neurosci.* **26,** 105–131 (2003).
9. Stamler, J. S., Lamas, S. & Fang, F. C. Nitrosylation. the prototypic redox-based signaling mechanism. *Cell* **106,** 675–683 (2001).
10. Hess, D. T., Matsumoto, A., Kim, S. O., Marshall, H. E. & Stamler, J. S. Protein *S*-nitrosylation: purview and parameters. *Nature Rev. Mol. Cell Biol.* **6,** 150–166 (2005).
11. Marshall, H. E., Hess, D. T. & Stamler, J. S. *S*-nitrosylation: Physiological regulation of NF-κB. *Proc. Natl Acad. Sci. USA* **101,** 8841–8842 (2004).
12. Stroissnigg, H. *et al. S*-nitrosylation of microtubule-associated protein 1B mediates nitric-oxide-induced axon retraction. *Nature Cell Biol.* **9,** 1035–1045 (2007).
13. Jaffrey, S. R. & Snyder, S. H. The biotin switch method for the detection of *S*-nitrosylated proteins. *Sci. STKE* **2001,** PL1 (2001).
14. Forrester, M. T., Foster, M. W. & Stamler, J. S. Assessment and application of the biotin switch technique for examining protein *S*-nitrosylation under conditions of pharmacologically induced oxidative stress. *J. Biol. Chem.* **282,** 13977–13983 (2007).
15. Whalen, E. J. *et al.* Regulation of β-adrenergic receptor signaling by *S*-nitrosylation of G-protein-coupled receptor kinase 2. *Cell* **129,** 511–522 (2007).
16. Uehara, T. *et al. S*-Nitrosylated protein-disulphide isomerase links protein misfolding to neurodegeneration. *Nature* **441,** 513–517 (2006).
17. Hara, M. R. *et al. S*-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. *Nature Cell Biol.* **7,** 665–674 (2005).
18. Hausladen, A., Privalle, C. T., Keng, T., DeAngelo, J. & Stamler, J. S. Nitrosative stress: activation of the transcription factor OxyR. *Cell* **86,** 719–729 (1996).
19. Cheung, P., Allis, C. D. & Sassone-Corsi, P. Signaling to chromatin through histone modifications. *Cell* **103,** 263–271 (2000).
20. Berger, S. L. The complex language of chromatin regulation during transcription. *Nature* **447,** 407–412 (2007).
21. Wink, D. A. *et al.* Detection of *S*-nitrosothiols by fluorometric and colorimetric methods. *Methods Enzymol.* **301,** 201–211 (1999).
22. Haendeler, J. *et al.* Redox regulatory and anti-apoptotic functions of thioredoxin depend on *S*-nitrosylation at cysteine 69. *Nature Cell Biol.* **4,** 743–749 (2002).
23. Hassig, C. A. *et al.* A role for histone deacetylase activity in HDAC1-mediated transcriptional repression. *Proc. Natl Acad. Sci. USA* **95,** 3519–3524 (1998).
24. Gu, Z. *et al. S*-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. *Science* **297,** 1186–1190 (2002).
25. Mustafa, A. K. *et al.* Nitric oxide *S*-nitrosylates serine racemase, mediating feedback inhibition of D-serine formation. *Proc. Natl Acad. Sci. USA* **104,** 2950–2955 (2007).

26. Huang, Y. *et al.* S-nitrosylation of N-ethylmaleimide sensitive factor mediates surface expression of AMPA receptors. *Neuron* **46,** 533–540 (2005).
27. Redmond, L., Kashani, A. H. & Ghosh, A. Calcium regulation of dendritic growth via CaM kinase IV and CREB-mediated transcription. *Neuron* **34,** 999–1010 (2002).
28. Bredt, D. S. & Snyder, S. H. Transient nitric oxide synthase neurons in embryonic cerebral cortical plate, sensory ganglia, and olfactory epithelium. *Neuron* **13,** 301–313 (1994).
29. Fischer, A., Sananbenesi, F., Wang, X., Dobbin, M. & Tsai, L. H. Recovery of learning and memory is associated with chromatin remodelling. *Nature* **447,** 178–182 (2007).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We are grateful to D. D. Ginty, A. Lloyd, M. Raff and A. Saiardi for comments and to all members of the Riccio laboratory for discussion. We thank E. Seto for providing HDAC plasmids and A. Chittka for help with the purification of recombinant HDAC2. We also thank C. Andreassi for help with cell lines and molecular biology techniques. This work was supported by the Medical Research Council (MRC; grant G0500792), and the European Research Council (Marie Curie International Reintegration grant MIRG-CT-2005-016501). A.R. is a recipient of an MRC Career Development Fellowship (G117/533) and P.M.W. of an MRC Career Development Award.

Author Contributions A.N. performed most of the experiments, analysed the data and helped to write the manuscript. P.M.W. performed the HDAC activity assay and some of the biotin-switch and ChIP assays. J.D.R. provided the DAF-FM DA data and contributed to the analysis of dendritic growth. L.C. designed and tested the siRNAs and helped with the HDAC2 constructs. A.R., the senior author, designed the project, performed most of the ChIP assays, analysed the data, wrote the manuscript and provided financial support.

Author Information Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to A.R. (a.riccio@ucl.ac.uk).

METHODS

Reagents. All biochemical reagents were purchased from Sigma and tissue culture reagents were purchased from Gibco (Invitrogen), unless otherwise stated. *S*-nitrosocysteine (SNOC) was prepared as described previously[30] and used within 1 h.

Cell cultures. Embryonic cortical rat and mouse neurons were cultured as previously described[2]. Before experiments, cells were placed in low serum medium (3% fetal bovine serum, FBS) containing DL-2-amino-5-phosphonovaleric acid (DL-AP5, 50 μM) for 12–16 h. HEK293T cells and PC12 cells were maintained in DMEM supplemented with 10% FBS, and 10% FBS plus 5% horse serum, respectively. Cells were transfected in Optimem containing 20 μg of DNA and 20 μl of Lipofectamine 2000 (Invitrogen) and incubated for 4–6 h before replacing the transfection medium with serum-containing media. Before experiments, PC12 cells were starved in medium containing 0.5% horse serum overnight.

Biotin-switch assay. This assay was performed in the dark. Cells were lysed in HEN buffer (250 mM HEPES, 1 mM EDTA and 100 mM neocuproine) and adjusted to contain 0.4% CHAPS. Samples were homogenized and free cysteines were blocked for 1 h at 50 °C in three volumes of blocking buffer (HEN buffer plus 2.5% SDS, HENS) containing methyl methanethiosulfonate (200 mM). Proteins were acetone precipitated at −20 °C and resuspended in 100 μl HENS solution. After adding fresh ascorbic acid (20 mM) and 1 mM biotin-HPDP (Pierce), proteins were incubated at room temperature for 1 h. After separation using an SDS–PAGE gel, biotinylated proteins were detected with streptavidin-HRP (Amersham). Alternatively, biotinylated proteins were resuspended in 100 μl HENS buffer containing 200 μl of neutralization buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) and precipitated with 50 μl of streptavidin–agarose beads at room temperature for 1 h. The beads were washed five times at 4 °C using neutralization buffer containing 600 nM NaCl. Biotinylated proteins were eluted using 50 μl elution buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 100 mM β-mercaptoethanol) and heated at 95 °C for 5 min in reducing SDS–PAGE loading buffer.

Extraction of nuclear and cytoplasmic proteins. Nuclear and cytoplasmic extracts were prepared using NE-PER nuclear and cytoplasmic extraction kit (Pierce) according to the manufacturer's instructions.

Chromatin immunoprecipitation. ChIP assays were performed mostly as described[2]. For all cell types, approximately $3–5 \times 10^6$ cells were used per ChIP. In brief, medium was removed from treated cells and replaced with PBS containing 1% formaldehyde. Cells were rinsed twice in PBS, and collected in collection buffer containing 100 mM Tris-HCl, pH 9.4, and 1 mM dithiothreitol. Cells were collected by centrifugation, rinsed with PBS and pellets were resuspended in lysis buffer containing 0.1% SDS, 0.5% Triton X-100, 20 mM Tris-HCl, pH 8.1, and 150 mM NaCl. Samples were sonicated with six 20-s pulses and a 10-s interpulse interval. Cell debris was removed by centrifugation, and supernatants were pre-cleared by incubation with Protein A–Sepharose beads (Amersham Biosciences) for 1 h at 4 °C. Beads were collected by centrifugation and supernatants were subjected to immunoprecipitation. A fraction of the supernatant was used for immunoprecipitation input control. The volume of each tube was adjusted to 500 μl with lysis buffer, and 5–10 μg of rabbit polyclonal antibody was added overnight at 4 °C. The following polyclonal antibodies were used: anti-HDAC2 (Santa Cruz), anti-acetyl histone H3 K9/K14 (Upstate), anti-acetyl histone H4 (Upstate), control IgG (Santa Cruz) and anti-Flag M2 monoclonal antibody (Sigma). Immune complexes were collected by incubation with Protein A–Sepharose beads for 1 h at 4 °C. Beads were collected and subjected to a series of seven sequential washes, as described[2]. Immune complexes were eluted from beads by vortexing in elution buffer containing 1% SDS and 0.1 M $NaHCO_3$. NaCl was added (final concentration 0.33 M), and crosslinking was reversed by incubation overnight at 65 °C. DNA fragments were purified using the QIAquick PCR purification kit (Qiagen). For PCR, specific sets of primers were designed that flank CRE regions within the upstream regulatory regions of the indicated genes. PCR conditions and cycle numbers were determined empirically for the different templates and primer pairs. Primers amplified fragments ranging in size from 200–400 bp. Primer sequences and PCR conditions are available on request.

Quantitative real-time PCR. PCR reactions (25 μl) contained 12.5 μl of PCR Sybr Green mix (NEB) and 0.3 μM of primers. All reactions were performed in duplicate with an Opticon 2 System (MJ Research) and each experiment included a standard curve, a preimmune control and a NO template control. Standard templates consisted of gel-purified PCR products of the *Fos*, *Egr1*, *Vgf* and *Nos1* amplicons of known concentration, and each standard curve consisted of seven serial dilutions of the template. At the end of the 46 cycles of amplification, a dissociation curve was performed in which Sybr Green was measured at 1 °C intervals between 50 °C and 100 °C. Melting temperatures for *Fos*, *Egr1*, *Vgf* and *Nos1* were 83 °C, 88 °C, 92 °C and 85 °C, respectively. Results were normalized using total input DNA and expressed as fold changes over unstimulated control.

Purification of recombinant HDAC2. XL-1 Blue bacteria were transformed using a pGEX-HDAC2 construct. Cells were centrifuged and washed using a high salt buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA and protease inhibitors). Cells were resuspended in high salt buffer containing lysozyme (100 μg ml^{-1}) and incubated at room temperature for 15 min. Dithiothreitol (5 mM) was added to cell lysates and after sonication, the lysates were clarified by adding DNaseI (2.5 μg ml^{-1}, 15 min at room temperature). Glutathione beads were added and lysates were rotated for 30 min at 4 °C. Beads were washed twice with buffer A (20 mM Tris-HCl, pH 8.0, 200 mM NaCl and 10% glycerol), once with buffer B (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl and 10% glycerol), once again with buffer A, and equilibrated with 50 mM Tris-HCl, pH 9.5. Proteins were eluted with 20 mM reduced glutathione in 50 mM Tris-HCl, pH 9.5. Fractions containing proteins were dialysed in PBS.

***Nos1* mouse line.** *Nos1* homozygous null animals[31] or wild-type control animals were intercrossed to generate null or control litters, respectively. Embryos were collected at E16.5 for preparation of primary cortical cultures.

HDAC2 site-directed mutagenesis and truncations. The pME18S–HDAC2 (Flag-tagged) construct was a gift from E. Seto[32]. Point mutations were carried out using the Quickchange II site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The HDAC2 deletion constructs were generated using primers designed with 5′ SalI and 3′ NotI restriction sites to allow cloning in the pCMV–Myc plasmid (Clontech). The primers used for HDAC2 mutagenesis and deletions are available on request.

Immunoprecipitation. Lysates were prepared in immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP40) and sonicated using a BioRuptor (Diagenode). Lysates were pre-cleared with 50 μl of Protein A–Sepharose beads (Amersham) and incubated with 5 μg of primary antibody overnight on a tube rotator at 4 °C. The antibodies used for immunoprecipitation were: anti-HDAC2 rabbit polyclonal antibody (Santa Cruz) and anti-Flag M2 mouse monoclonal antibody (Sigma). Beads were washed five times in immunoprecipitation buffer and either boiled in 2× reducing SDS–PAGE loading buffer for western blotting or used in the HDAC assay.

Histone deacetylase assay. HDAC activity in whole cells lysates and immunoprecipitates was assessed using a fluorogenic HDAC assay[33]. HDAC acts on the non-fluorescent substrate peptide BOC-(acetyl)Lys-AMC (Bachem), allowing subsequent trypsin digestion of the deacetylated peptide to yield a fluorescent moiety. For whole cell lysates, 15 μl of extract was mixed with a 100 μM fluorogenic HDAC peptide in HDAC buffer (25 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$) to a final volume of 40 μl. After incubation at 37 °C for 1 h, the reaction was stopped and the peptide was digested by incubation with 1 μM trichostatin A (Tocris Bioscience) and 5 mg ml^{-1} trypsin (Sigma). The extracts were transferred to a black 96-well plate and read using a Spectramax Gemini plate-reader (Molecular Devices) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. For HDAC assay using immunoprecipitates, immunoprecipitated protein bound to Sepharose A beads was washed three times in immunoprecipitation lysis buffer and twice in HDAC buffer. Fluorogenic HDAC substrate was added to the beads at a final concentration of 100 μM in 60 μl HDAC buffer and incubated at 37 °C for 1 h, after which the reaction was stopped and fluorescence was measured as described above.

Dual luciferase assay. E17 cortical neurons were cultured in 6-well plates and transfected after 4 days *in vitro*. Transfections were carried out using 2 μg of Fos–luciferase vector (firefly), 1 μg of thymidine-kinase–luciferase (*Renilla*), 2 μg of HDAC2 vectors and 10 μl of Lipofectamine 2000 (Invitrogen). Neurons were incubated for 4 h at 37 °C and culture medium was replaced with the original plating medium. Forty-eight hours after transfection, cells were stimulated with 75 ng ml^{-1} BDNF, 200 μM SNOC, 300 μM NPA or NPA plus BDNF and samples were processed using the dual-luciferase reporter assay system (Promega), according to manufacturer's instructions.

30. Mallis, R. J., Buss, J. E. & Thomas, J. A. Oxidative modification of H-ras: *S*-thiolation and *S*-nitrosylation of reactive cysteines. *Biochem. J.* **355,** 145–153 (2001).
31. Huang, P. L., Dawson, T. M., Bredt, D. S., Snyder, S. H. & Fishman, M. C. Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* **75,** 1273–1286 (1993).
32. Laherty, C. D. *et al.* Histone deacetylases associated with the mSin3 corepressor mediate mad transcriptional repression. *Cell* **89,** 349–356 (1997).
33. Wegener, D., Wirsching, F., Riester, D. & Schwienhorst, A. A fluorogenic histone deacetylase assay well suited for high-throughput activity screening. *Chem. Biol.* **10,** 61–68 (2003).