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The Notch intracellular domain represses CRE-dependent transcription

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ABSTRACT

Members of the cyclic-AMP response-element binding protein (CREB) transcription factor family regulate the expression of genes needed for long-term memory formation. Loss of Notch impairs long-term, but not short-term, memory in flies and mammals. We investigated if the Notch-1 (N1) exerts an effect on CREB-dependent gene transcription. We observed that N1 inhibits CREB mediated activation of cyclic-AMP response element (CRE) containing promoters in a γ-secretase-dependent manner. We went on to find that the γ-cleaved N1 intracellular domain (N1ICD) sequesters nuclear CREB1α, inhibits cAMP/PKA-mediated neurite outgrowth and represses the expression of specific CREB regulated genes associated with learning and memory in primary cortical neurons. Similar transcriptional effects were observed with the N2ICD, N3ICD and N4ICDs. Together, these observations indicate that the effects of Notch on learning and memory are, at least in part, via an effect on CREB-regulated gene expression.

1. Introduction

The "notched" wing phenotype was first noted in the fruit fly, *Drosophila melanogaster*, 100 years ago. Subsequent molecular cloning of the gene involved, named Notch, found it to encode a large type-1 transmembrane receptor protein [1]. The gene is highly conserved across species and in mammals there are four Notch receptor homologs, Notch-1, -2, -3 and -4 [2]. The signalling pathway the Notch receptors regulate plays numerous roles during development in many tissues and is of particular importance in the nervous system where it regulates neurogenesis and other cell fate decisions [3–6]. Notch receptors are also expressed post-development in neurons of the adult CNS where they participate in contact-dependent inhibition of neurite growth [7,8].

It now emerges that Notch signalling also plays a role in memory formation across species: In *Drosophila* [9,10] and in mice the loss of Notch-1 (N1) [11,12] disrupts long-term memory formation (LTM) but not short-term memory (STM). Notch signalling activity in the postnatal mouse hippocampus was found to be dynamically regulated by ARC in response to neuronal activity, with the deletion of N1 disrupting both long-term potentiation (LTP) and long-term depression (LTD) [13], the electrophysiological correlates of memory associated processes. Most recently *Drosophila* Notch has been linked to the phosphorylation state of the fly homolog of the cyclic-AMP response element binding protein (CREB), dCreB-17a [14], a transcription factor (TF) family intimately linked with memory [15,16].

CREB TFs directly bind DNA and regulate genes involved in a range of cellular processes in multiple tissues [17]. In the CNS CREB family TFs regulate the expression of genes mediating stimulus-dependent, long-term responses underling neuronal survival and synaptic plasticity and the expression of genes required for the formation of long-term memory (LTM) [15,17,18].

In the "canonical" Notch signalling pathway, Notch receptors are cleaved by γ-secretase, a presenilin-dependent multi-protein complex that cleaves several type-1 transmembrane proteins to release the intracellular portion of the receptor from the plasma membrane [19]. γ-Secretase release of Notch receptor intracellular domains (ICDs) permits them to translocate to the nucleus, where they regulate gene transcription by binding with TFs of the CSL family. The mammalian N1ICD also has been shown to interact with TFs from other signalling

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pathways, for example Lef-1 [20], FoxO1 [21], Smad1 [22] and, as we demonstrated, p73 [23]. Given the promiscuous nature of the N1ICD and the centrality of CREB to LTM formation, we asked if N1ICD might interact with, and modulate, CREB-dependent gene transcription. Specifically, we investigated if N1ICD modulates the expression of neuronal genes involved in memory regulated by CREB.

We found that in multiple cell lines N1 antagonises CREB-regulated gene transcription in a γ-secretase-dependent manner, that CREB1α co-localises with N1ICD in the nucleus and that N1ICD and CREB directly bind as shown by GST pull-down and chromatin immunoprecipitation assays. In rodent primary neurons N1ICD also antagonises cAMP-mediated neurite outgrowth and the neuronal expression of CREB-regulated genes whose hippocampal expression is modulated by a fear-conditioning paradigm of LTM. Finally, we found that this antagonism of CREB-dependent gene transcription activity extends to all four mammalian Notch ICDs.

2. Materials and methods

2.1. Cell lines, plasmids and reagents

HEK293a cells (an adherent clone of HEK293 cells) were purchased from Quantum Biotechnologies (Montreal, QC, Canada). SH-SY5Y and NT2 neuroblastoma cells were obtained from ECACC (UK). BD3 and BD8 cells were a gift from Dorit Donoviel (Lexicon Genetics, Inc., Texas, USA). A human CREB1α cDNA, gifted by Dr Helen Hurst, was subcloned into pcDNA3.1(+) (Invitrogen, Paisley, UK) and pGEX p6.1 (Amersham Biosciences, UK). A construct encoding the C-terminal 57 amino acids of the human β-amyloid precursor protein (APP) was generated using the TOPO cloning system (Invitrogen). A human N4ICD construct was generated from the Int3 cDNA, gifted by Dr U. Lendahl (Karolinska Institute, Sweden). The CRE-Luc reporter and the α-isoform of the murine catalytic subunit of PKA (PKA-Cα), were from Stratagene (La Jolla, CA 92037, USA). The following constructs were generously gifted to us by: A-CREB, Dr David Ginty (Johns Hopkins School of Medicine, Maryland, USA); VP16-CREB, Dr Boyoung Lee (Ohio State University, USA); myc-His tagged human N1ICD, ΔTAD-N1ICD, ΔRA-N1ICD, N2ICD and N3ICD, Dr Tom Kadesch (University of Pennsylvania, USA); ΔEN1, Dr J. Aster (Brigham and Women's Hospital, USA); Human EGFP tagged N1ICD (ICN-1), Dr S. Artavanis-Tsakonas (Yale, USA); 4xwt-CBF-Luc and 4xmut-CBF-Luc, Dr Gerry Weinmaster, (UCLA Medical School, USA).

2.2. Cell culture

All cells were maintained in 5% CO_2/air in a humidified incubator at 37 °C. HEK293a and NT2 cells were cultured in low glucose Dulbecco's modified Eagle's medium (DMEM) – all culture media and supplements were obtained from Invitrogen, Paisley, UK, unless otherwise stated – supplemented with 10% (v/v) foetal bovine serum (FBS) (Sera Laboratories International, UK), 2 mM L-glutamine, 100 IU penicillin, and 100 mg/ml streptomycin. SH-SY5Y neuroblastomas were grown in a 50/50 mix of F12/EMEM supplemented with 15% (v/v) FBS, 2 mM L-glutamine, non-essential amino acids (Sigma, UK), 100 IU penicillin, and 100 mg/ml streptomycin. Rodent primary neurons were prepared and cultured in Neurobasal media plus B27 supplement as previously described [24].

2.3. Luciferase based reporter gene assays

Cells seeded in 48 well plates were transfected with 100 ng of firefly Luciferase based reporter DNA and 200 ng of each plasmid containing the cDNA constructs being examined, using 1 μl FuGene6 per well according to the manufacturer's instructions (Roche, Haywards Heath, UK) and with 50 ng of phTK-Renilla luciferase (Promega, Southampton, UK) to control for transfection efficiency. Empty vector DNA was included to maintain a constant DNA concentration, as needed. Firefly and Renilla luciferase activities were sequentially measured 24 h post transfection using Promega Dual-Glo reagents and a Wallac Trilux 1450 Luminometer (PerkinElmer, UK). Firefly values were divided by the Renilla values to control for non-specific effects. Data for each set of three replica transfections were averaged, the control in each set normalized to 1 and the data presented as fold increases (or percentage decreases) over control. All experiments were performed in triplicate a minimum of three times.

2.4. Immunofluorescence microscopy

Cells were transfected with ICN-1 independently or in combination with CREB1α (200 ng of each) using FuGene 6 and fixed in ice-cold methanol 24 h after transfection then stained according to standard protocols using anti-CREB 48H2 (Cell Signaling Technology NEB, Hitchin, UK) and an Alexophor 594 nm conjugate anti-rabbit secondary. Immunofluorescence was imaged using a CoolSnap digital camera (InterFocus Imaging, Linton, UK) attached to a Zeiss Axioscope (Zeiss, Hertfordshire, UK).

2.5. Immunoblotting

Notch-1 proteins were immunoblotted according to standard protocols using Santa Cruz sc-6014 goat anti-Notch-1 (1:1000) or sc-789 mouse anti-myc (1:5000) followed by incubation with the corresponding secondary antibody conjugated to 700 or 800 nM fluorophors and detected using an Odyssey infrared scanner (Li-Cor-Biosciences, Cambridge, UK). Densitometry was performed using Licor Odyssey software. Scanned blots were processed using PhotoShop (Adobe) with all manipulations being applied to entire blots before cropping for use in figures.

2.6. Chromatin Immunoprecipitation — ChIP

5×10^6 HEK293T cells, per condition, were transfected with CRE-Luc (3 μg) and myc-His-N1ICD (6 μg) or pcDNA3.1C-myc-His (6 μg) using FuGene6, fixed 24 h post transfection with 1% formaldehyde and subsequently processed for ChIP according to the online protocol at http://www.scbt.com/protocol_chromatin_immunoprecipitation_chip_assays.html (Santa Cruz biotechnology). DNA was recovered from eluted immune complexes by phenol–chloroform extraction and ethanol precipitation, then used as input for PCR (80 ng/reaction) with primers spanning the CRE sites within CRE-Luc.

2.7. Measurement of neurite growth

E19 rat cortical neurons were maintained in culture for 48 h then transfected with pDsRed-Express C1 (Clontech, Hampshire, UK) in combination with ICN-1, or empty vector, using Lipofectamine 2000 (Invitrogen). Five h post transfection media were removed and replaced with Neurobasal + B27. Neurons were treated 24 h post transfection with 10 μM forskolin or the equivalent volume of vehicle (DMSO) for 24 h and fixed with 4% (w/v) paraformaldehyde. Fluorescence microscopy was performed as above. The number and length of neurites was determined using MetaMorph (Universal Imaging Corporation, USA). Statistical significance was determined by ANOVA and post hoc t-tests.

2.8. Fear conditioning

Three-month old C57BL/6J female mice were housed in groups of four and maintained on a 12 h light/dark cycle with food and water ad libitum. Experiments were undertaken in accordance with United Kingdom Animals (Scientific Procedures) Act, 1986. Foreground contextual fear conditioning (contextual fear conditioning without a tone presentation) [25] was employed. Individual mice were placed into the conditioning chamber (Med Associates Inc., VT, USA) and after 148 s of exposure to the new environment the first 2 s foot shock of 0.7 mA

given, a second and third 2 s shocks were then administered at 90 s intervals. The mouse was then removed after 30 s and returned to the home cage. Animals were sacrificed at 0.5, 1 and 3 h after training. Naïve controls were placed in the apparatus for the same time period as the trained animals without receiving shocks (n = 6 per condition). Hippocampi and frontal cortices were rapidly dissected out, immediately frozen on dry ice and stored at −80 °C for subsequent protein and RNA extractions.

2.9. Quantitative real time-PCR (qRT-PCR)

Total RNA was extracted using TRIzol (Invitrogen, UK) according to the manufacturer's instructions, primed with random hexamers and reverse transcribed according to the manufacturer's instructions (PerkinElmer, UK). Gene specific primer sets were designed using the Roche Universal ProbeLibrary Assay Design Centre (www.roche-applied-science.com). SYBR green (Qiagen, Crawley, UK) qRT-PCR reactions were performed in 96-well plates using an Opticon Disciple PCR machine (Bio-Rad, London, UK). Succinate dehydrogenase A (*SDHA*), 18S ribosomal RNA (*18SRNA*) and actin-γ-1 (*Actg1*) were used as internal controls. Data were analyzed using Bio-Rad Chromo4 software. Data were pooled from 3 replica experiments and statistical significance determined by two-way ANOVA.

2.10. Forskolin treatment and infection of primary neurons with N1ICD adenovirus

Mixed cortical/hippocampal neuronal cultures, prepared from E16 C57BL/6J embryos were seeded into 6-well plates at 1.5×10^6 cells/well. After 5 days in culture cells were infected with an adenovirus carrying EGFP tagged human N1ICD or with a control virus carrying β-galactosidase at a MOI of 10 and treated 48 h later with 10 μM forskolin or vehicle for 30, 60 and 90 min. Total RNA was then extracted and gene expression determined by real time qRT-PCR.

3. Results

3.1. N1ICD inhibits CRE-driven transcriptional activity

To determine effects of the transcriptionally active portion of Notch-1, the N1ICD, on CREB/CRE activity we employed a Luciferase based

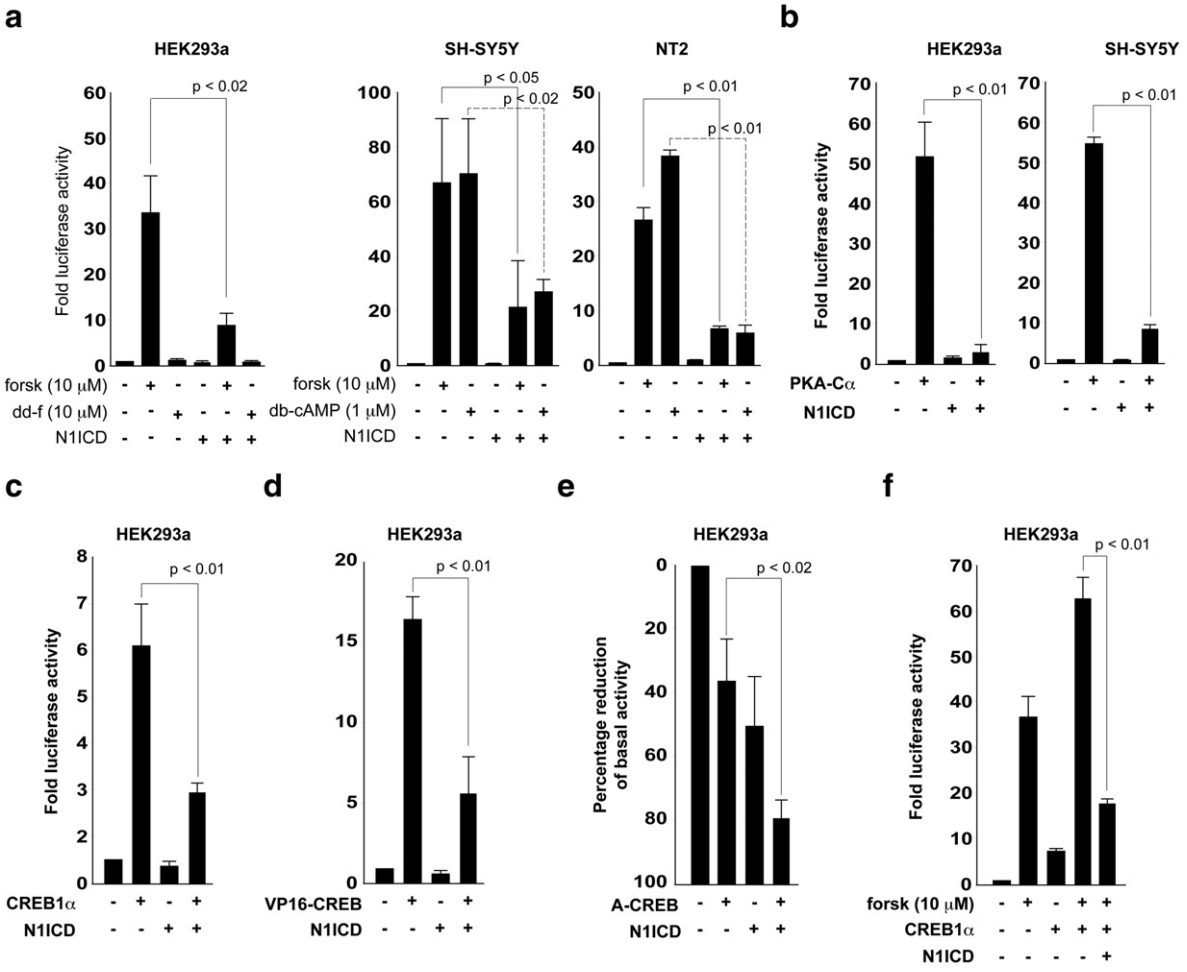


Fig. 1. N1ICD represses CREB-dependent gene transcription. a) HEK293a, SH-SY5Y and NT2 cells were transfected with a luciferase based CRE-reporter gene (CRE-Luc) and empty vector DNA or the myc-His N1ICD construct and 18 h post transfection treated with 10 μM forskolin (forsk) or 10 μM d,d-forskolin (dd-f) for 6 h, as indicated and luciferase activity subsequently measured. b) HEK293a and SH-SY5Y cells were transiently transfected with CRE-Luc and PKA-Cα and N1ICD as indicated. Luciferase activity was measured 24 h post transfection. c) HEK293a cells were transfected with CRE-Luc, N1ICD and CREB1α as indicated and luciferase activity measured 24 h post transfection. d) As c) with CREB1α being replaced by the constitutively active form of CREB, VP16-CREB. e) As c) with CREB1α replaced by a dominant negative form of CREB, A-CREB. f) HEK293a cells were transfected with CRE-Luc, N1ICD and CREB1α as indicated, 18 h post transfection cells were treated with forskolin for 6 h and luciferase activity subsequently measured. All data are pooled values from a minimum of three replica experiments. Statistical significance was determined by one-way ANOVA and post hoc t-testing.

reporter-gene construct containing tandem repeats of the human CRE sequence from the promoter region of the prototypic CREB regulated gene, somatostatin (SST). HEK293a cells were transfected with this construct, CRE-Luc, alone or in combination with myc-His-N1ICD. Next day, transfected cells were treated with 10 μM forskolin or an inactive analogue, 1,9-dideoxy-forskolin (dd-forskolin), for 6 h and Luciferase activity measured. Forskolin treatment resulted in an ~33 fold increase in CRE-Luc activity. Alone myc-His-N1ICD and dd-forskolin had minimal effect upon reporter activity. However, in cells treated with forskolin and receiving myc-His-N1ICD CRE-Luc activity was reduced by almost 70% ($p < 0.02$), Fig. 1a, left. To determine if this was cell-type dependent we conducted replica experiments in two human neuron-like lines, SH-SY5Y and NT2 cells, and also used a membrane permeable cAMP analogue, dibutyryl-cAMP (db-cAMP), to activate PKA. In both cell lines myc-His-N1ICD substantially reduced forskolin and db-cAMP induction of CRE-Luc activity, Fig. 1a, middle and right panels. Next we examined the effects of N1ICD when activating the reporter by transient exogenous expression of the PKA catalytic subunit-α (PKA-Cα), which activates endogenous CREB by phosphorylation at Serine133 [26]. N1ICD potently ($p < 0.01$) antagonised PKA-Cα activation of CRE-Luc in all three cell types, (HEK293a, SH-SY5Y), Fig. 1b, and in NT2 cells (not shown).

We next examined if N1ICD represses exogenously expressed CREB. HEK293a cells were transfected with the CRE-Luc reporter and CREB1α. In the absence of any agonistic stimulation, exogenous CREB1α activated the reporter ~6 fold, which was inhibited by N1ICD to <3 fold ($p < 0.01$ — Fig. 1c). N1ICD also significantly ($p < 0.01$) repressed the activity of a constitutively active form of CREB, VP16-CREB, Fig. 1d. We then examined the effects of N1ICD on a dominant negative form of CREB, A-CREB, which alone repressed basal reporter activity by ~40%. Here and in the above reporter assay experiments N1ICD gave slight repression of basal reporter activity alone, and with A-CREB repressed basal activity further (Fig. 1e). Finally, exogenously expressed CREB1α was activated with forskolin (10 μM for 6 h), resulting in a greater than 60 fold activation, which myc-His-N1ICD again potently antagonised ($p < 0.01$), Fig. 1f.

3.2. γ-Secretase cleavage is required for N1 inhibition of CRE-dependent gene transcription

The Notch receptors undergo sequential proteolytic processing, the final cleavage, responsible for the release of the ICD, is performed by the γ-secretase complex. The familial Alzheimer's disease (FAD) genes, presenilin-1 (PSEN1) [27] and PSEN2 [28], are necessary components of γ-secretase and inhibitors of γ-secretase have been shown to enhance LTM [29]. We sought to determine if a membrane tethered form of Notch-1, ΔEN1, which, due to the lack of the extracellular domain, is constitutively cleaved by γ-secretase would affect CRE-dependent gene transcription in the absence of γ-secretase activity either due to pharmacological blockade or deletion of the PSEN genes. For the latter genetic manipulation BD3 and BD8 mouse embryonic stem-cell cell lines were employed [30]. BD3 cells have a single copy of PSEN1 and no copies of PSEN2 ($PSEN1^{+/-}$, $PSEN2^{-/-}$). BD8 cells have no copies either PSEN genes ($PSEN1^{-/-}$, $PSEN2^{-/-}$).

First we established the effects of manipulating γ-secretase activity using a reporter gene for measuring canonical Notch transcriptional activity. This CBF-1 reporter, 4 × wtCBF-1-Luc, or its control (4 × mutCBF-1-Luc) in which the CBF-1 sites are mutated, were transfected into HEK293a cells alone or in conjunction with either N1ICD or ΔEN1. Both N1ICD or ΔEN1 activated 4 × wtCBF-1-Luc (Fig. 2a, left hand bar graph) but had little effect on the control reporter (data not shown). Treatment with a γ-secretase inhibitor, GSI-1, at 1 μM for 6 h, virtually abolished activation due to ΔEN1, but did not affect N1ICD activation.

In BD3 cells, harbouring one copy of PSEN1, both myc-His-N1ICD and ΔEN1 activated the CBF-1 reporter (Fig. 2a, centre bar graph), whilst in BD8 cells lacking both PSEN1 and PSEN2, only N1ICD activated the reporter (Fig. 2a right hand bar graph).

CRE-Luc reporter assays were then performed in HEK293a, BD3 and BD8 cells with the inclusion of N1ICD and ΔEN1. Both N1ICD and ΔEN1 antagonised PKA-Cα activation of CRE-Luc in HEK293a cells (Fig. 2b) and in the presence of the γ-secretase inhibitor N1ICD still significantly

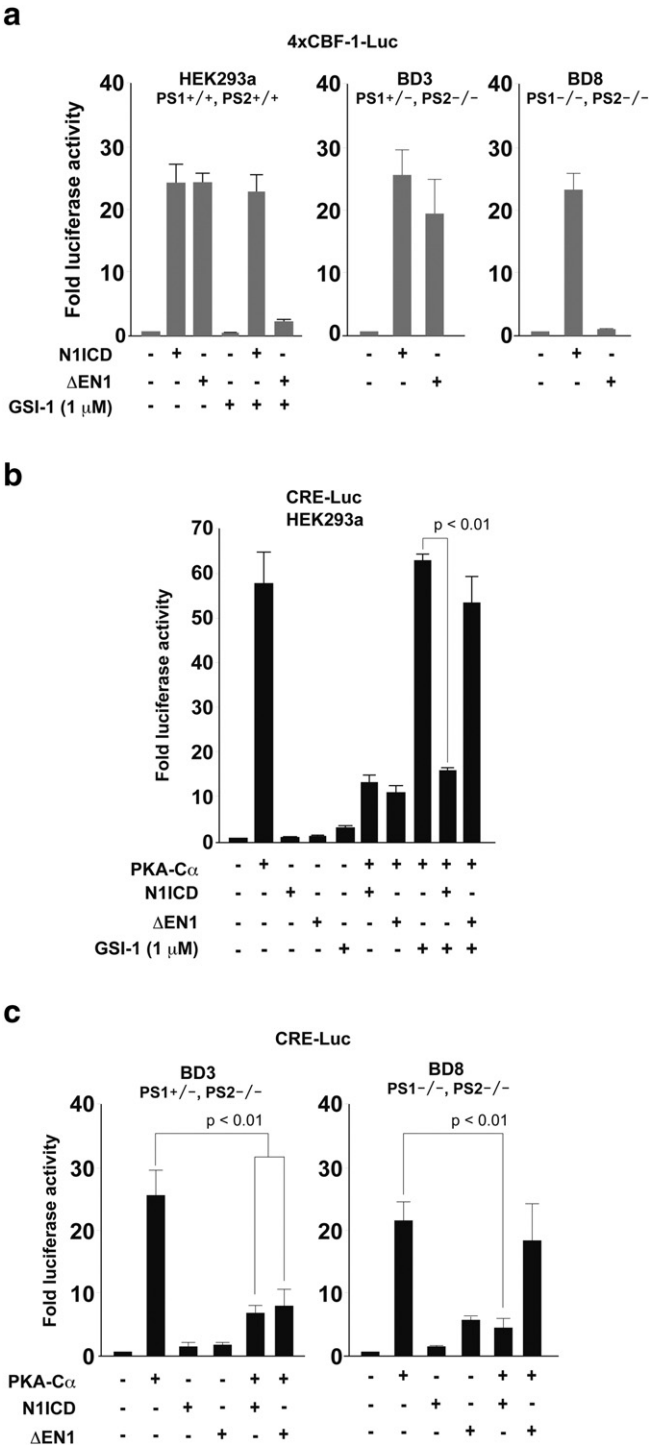
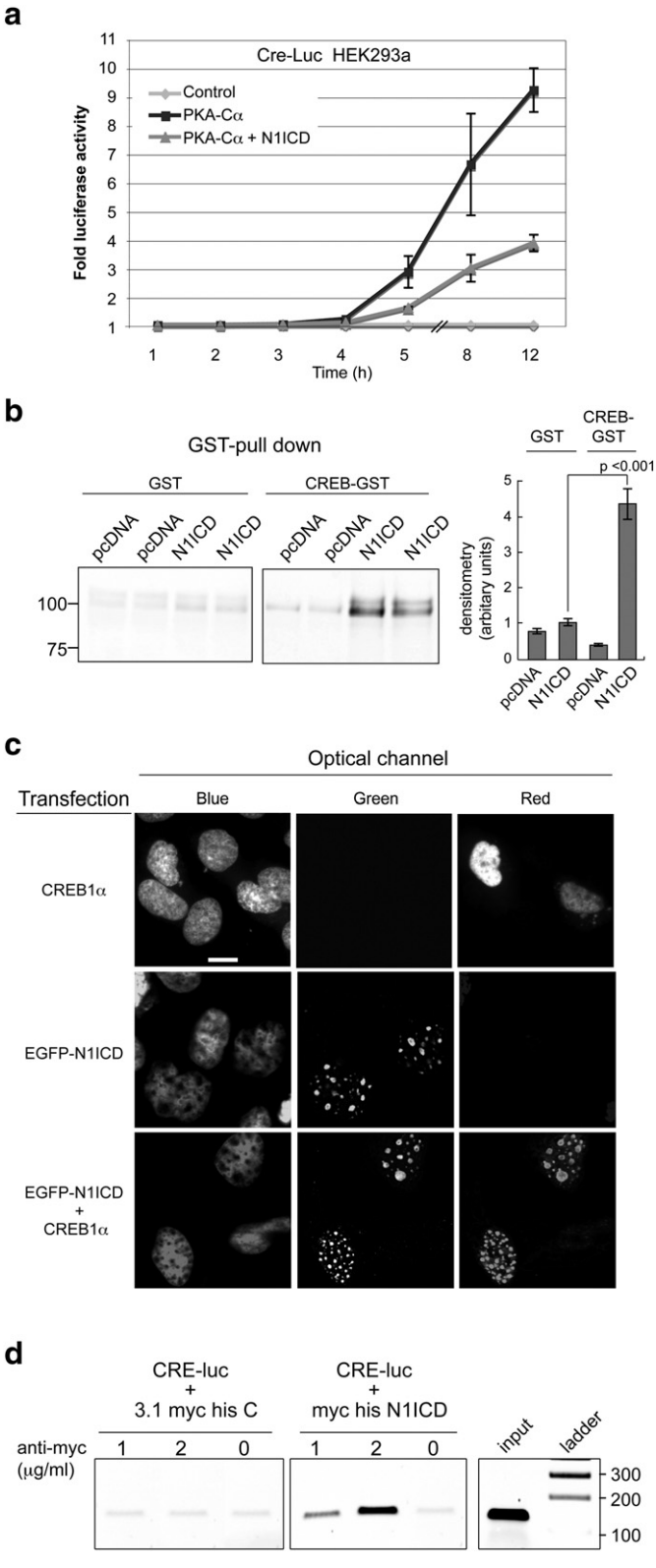


Fig. 2. Presenilin cleavage of Notch is required for repression of CRE-dependent gene transcription. a) HEK293a, BD3 and BD8 cells were each transfected with the "Notch" reporter gene, 4xCBF-Luc and ΔEN1 or N1ICD as indicated. 18 h post transfection HEK293a cells were treated with a γ-secretase inhibitor, GSI-1, at 1 μM for 6 h as indicated. Luciferase activity was measured 24 h post transfection. b) HEK293a cells were transfected with CRE-Luc and PKA-Cα, ΔEN1 or N1ICD as indicated and treated with GSI-1 18 h post transfection for 6 h. Luciferase activity was subsequently measured. c) BD3 and BD8 cells were transfected as in b) as indicated. Luciferase activity was measured 24 h post transfection. All data are pooled values from a minimum of three replica experiments. Statistical significance was determined by one-way ANOVA and post hoc t-testing.

repressed PKA-Cα activation of CRE-Luc, whilst ΔEN1 had no significant effect on PKA-Cα activation — demonstrating that S3 (or γ-) cleavage of N1 is necessary for its ability to repress CRE activity. This observation was then confirmed in cells lacking presenilins. In BD3 cells both Notch constructs antagonised CRE-Luc activity, whilst in BD8 cells the antagonistic effect of ΔEN1 was lost (Fig. 2c). These data indicate that γ-cleavage and the subsequent nuclear translocation of N1ICD are required for N1 repression of CRE activity. However, the number of substrates of γ-secretase is growing [19], certain of which, such as $p75^{NTR}$ [31], have been shown to be associated with cAMP/PKA signalling [32] and memory [33]. As such we cannot rule out that other γ-secretase substrates contribute to the effects we observe above.



3.3. The effect of N1ICD on CREB is direct

To determine if N1ICD impacts directly upon CRE-dependent gene transcription or works through a transcriptional target gene, a time-course analysis of its effect on the CRE-Luc reporter was carried out. As can be seen in Fig. 3a the ability of myc-His-N1ICD to antagonise PKA-Cα activation of CRE-Luc occurred at the earliest time point at which an increase in reporter activity could be detected (4 h). The absence of a time lag between reporter gene activation by PKA-Cα and its antagonism by N1ICD indicates that N1ICD most likely exerts its effect directly upon the cAMP/PKA/CREB pathway and not via the induction of target genes, which in their turn antagonise CRE activity.

3.4. The N1ICD and CREB1α proteins associate in vitro

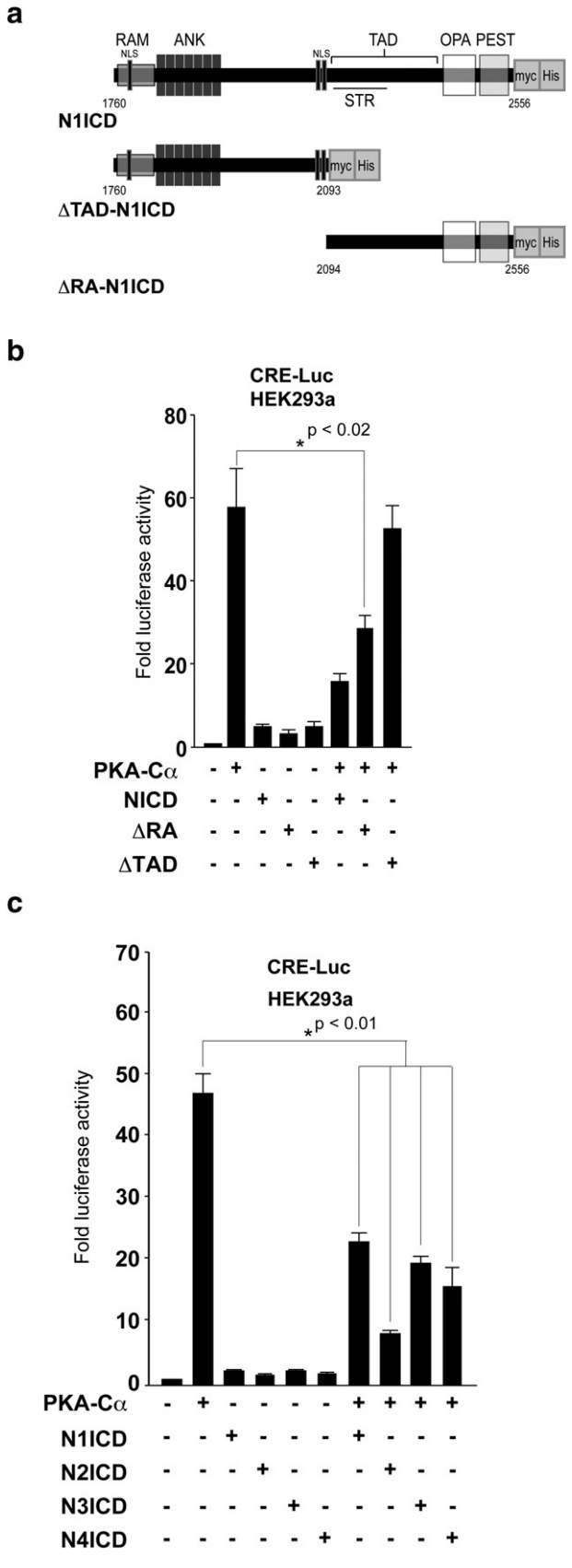
Next we sought to determine if the N1ICD and CREB proteins directly interact by performing cell-free glutathione-S-transferase (GST) pull-down binding assays. GST and GST-CREB1α recombinant proteins were generated and purified using glutathione coated magnetic beads. Cell lysates were generated from HEK293a cells transfected with myc-His-N1ICD or empty vector and incubated overnight with equal amounts of GST or GST-CREB1α bound beads. Binding proteins were subjected to SDS-PAGE, immunoblotted and probed using an antibody to the C-terminal of N1. GST-CREB1α-beads bound significantly ($p < 0.01$) greater amounts of myc-His-N1ICD compared to the control GST-beads (Fig. 3b).

3.5. N1ICD sequesters CREB1α into subnuclear foci

We [34], and others [35], have previously shown that exogenously expressed N1ICD protein forms discrete nuclear foci, which, it has been suggested, may be the sites of "nuclear transcription factories" [35]. If the N1ICD and CREB1α proteins associate in vivo, we predicted that in the presence of exogenous N1ICD, CREB, which normally has a diffuse nuclear staining pattern, would also be detectable in these foci or alternatively that CREB might draw N1ICD out of the foci.

Fig. 3. N1ICD associates with CREB.a) Transcription time course analysis. HEK293a cells were transfected with the CRE-Luc reporter and PKA-Cα or the reporter and PKA-Cα and N1ICD as indicated. Luciferase activity was measured hourly over 12 h. No time lag between activation by PKA-Cα and its repression by N1ICD was observed. b) Cell free glutathione-S-transferase (GST) pull-down binding assay. HEK293a cells were transfected with myc-His N1ICD or empty vector. Cell lysates were collected 24 h post transfection and incubated with GST or a GST-CREB1α fusion protein coupled to agarose beads. Bound proteins detected by immunoblotting with an anti-Notch-1 antibody. GST-CREB1α bound significantly more N1ICD than did GST, $p < 0.01$. Statistical significance was determined by one-way ANOVA and post hoc t-testing. c) Sub-cellular co-localisation. HEK293a cells were transfected with CREB1α and EGFP-tagged N1ICD separately and together as indicated and examined by immunofluorescence microscopy. Immuno-labelled CREB1α was detected with an anti-rabbit 594 nm fluorophore conjugated antibody. Nuclei were labelled with Hoescht 33342. Cells were imaged through blue, red and green filters. Scale bar = 10 μm. CREB1α, which is normally diffuse throughout the nucleus, was found in N1ICD positive foci when co-expressed with N1ICD. d) Chromatin immunoprecipitation. HEK293a cells were transfected with CRE-Luc and myc-His N1ICD or CRE-Luc and empty vector. 24 h post transfection cells were washed and fixed and processed for chromatin immunoprecipitation using an anti myc-tag antibody as described in the methods section. PCR, using primers spanning the CRE elements of CRE-Luc, was performed on the immunoprecipitated chromatin. An amplicon of the predicted size was generated from the anti-myc immunoprecipitated chromatin in a dose dependent manner indicating N1ICD interacts with the transcriptional complex bound to CRE sites.

HEK293a cells were transfected with the EGFP-tagged N1ICD construct and CREB1α, separately and in combination, and treated with 10 μM forskolin for 1 h prior to fixation. When expressed individually exogenous CREB1α immunostaining gave a diffuse staining pattern throughout the nucleus (Fig. 3c top row, red channel) and EGFP-N1ICD formed punctate nuclear foci (Fig. 3c centre row, green channel). An identical staining pattern for N1ICD was seen with N1ICD-myc-His when labelled with an anti-N1 C-terminal antibody or an anti-myc antibody (data not shown), as previously reported [34]. When N1ICD and CREB1α were co-expressed in cells, CREB immunostaining co-localised with that of EGFP-N1ICD, CREB1α appearing to be sequestered into the N1ICD positive foci (Fig. 3c bottom row, red channel). These data further indicate that there is a protein–protein interaction between N1ICD and CREB1α.



3.6. N1ICD interacts at CRE sites

To verify the interaction between N1ICD and CREB, and to determine if it occurs at CRE sites, we performed chromatin immunoprecipitation (ChIP). HEK293a cells were transfected with linearised CRE-Luc DNA and myc-His-N1ICD or the empty pcDNA3.1 myc-His C vector, fixed 24 h post transfection and processed. Fragmented chromatin was immunoprecipitated with an anti-myc tag antibody and PCR performed with primers spanning the CRE sites of the CRE-Luc reporter. PCR products of the correct size were generated in cells receiving the myc-tagged N1ICD construct but not in cells receiving the empty vector, dose dependently to amount of anti-myc antibody added (Fig. 3d). This observation indicates that N1ICD interacts with CREB when bound to CRE containing DNA. This interaction may be direct, or via other mutual binding partners such as CBP or p300.

3.7. The TAD domain of Notch is needed for antagonism of CRE-dependent gene transcription

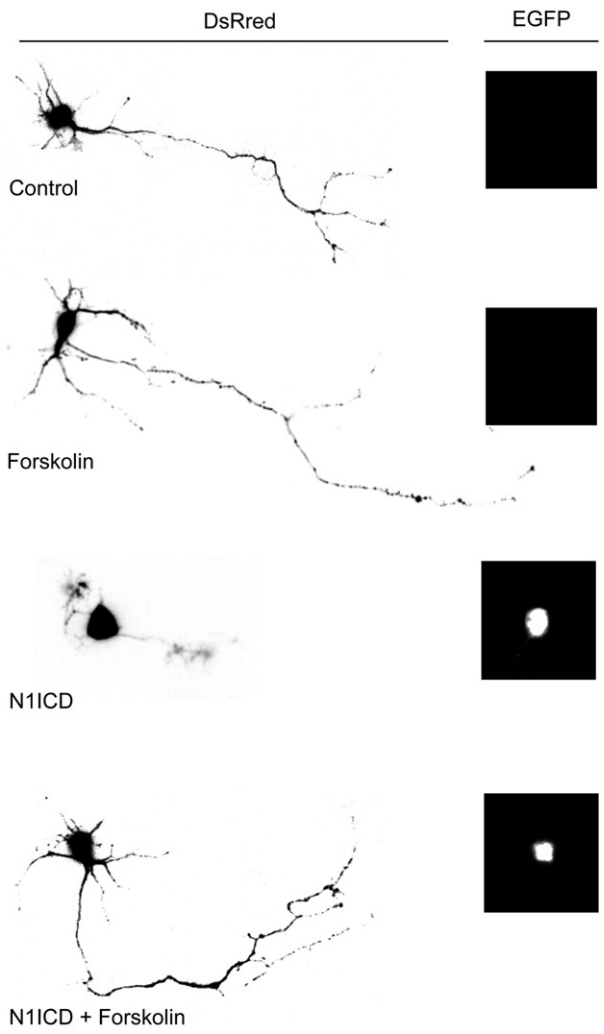
We investigated which regions of N1ICD are involved in its interaction with CREB by examining the effects of full-length and two myc-His-N1ICD deletion constructs on CRE-Luc activity. The deletion constructs, ΔRA-N1ICD and ΔTAD-N1ICD, shown schematically in Fig. 4a, were transfected alone and in combination with PKA-Cα and CRE-Luc activity measured. ΔRA-N1ICD significantly inhibited PKA-Cα activation ($p < 0.01$) whilst ΔTAD-N1ICD had little effect (Fig. 4b), indicating that it is the portion of N1ICD containing the TAD domain and the C-terminal region beyond that is necessary for antagonism of CREB.

3.8. All four Notch isoforms antagonise CREB

To determine if the antagonistic effect upon CRE activity was specific to Notch1 (N1) we examined the effects of constructs encoding the ICDs of N2, N3 and N4 upon PKA-Cα activation of the CRE-Luc reporter. N2ICD, N3ICD and N4ICD were each able to significantly ($p < 0.01$) inhibit CRE reporter activity. The most potent inhibition was consistently observed with N2ICD (Fig. 4c).

Fig. 4. The TAD of N1ICD is needed for repression of CREB and N2, N3 and N4 ICDs also repress CREB.a) Schematic of myc-His tagged, S3 cleaved, N1ICD and the two deletion constructs, ΔRA-N1ICD and ΔTAD-N1ICD. RAM, Rbp-associated molecule domain; ANK, ankyrin repeats; NLS, nuclear localisation signal; TAD, transactivation domain; OPA, glutamine-rich region; PEST, proline (P), glutamic acid (E), serine (S) and threonine (T) rich region; STR, serine/threnonine-rich region. Numbering corresponds to amino acids in the full-length human N1 protein. b) HEK293a cells were transfected with CRE-Luc and PKA-Cα (PKA), N1ICD, ΔRA-N1ICD and ΔTAD-N1ICD as indicated. Luciferase activity was measured 24 h post transfection. c) HEK293a cells were transfected with CRE-Luc and PKA-Cα, N1ICD, N2ICD, N3ICD and N4ICD as indicated. Luciferase activity was measured 24 h post transfection. All data are pooled values from a minimum of three replica experiments. Statistical significance was determined by one-way ANOVA and post hoc t-testing.

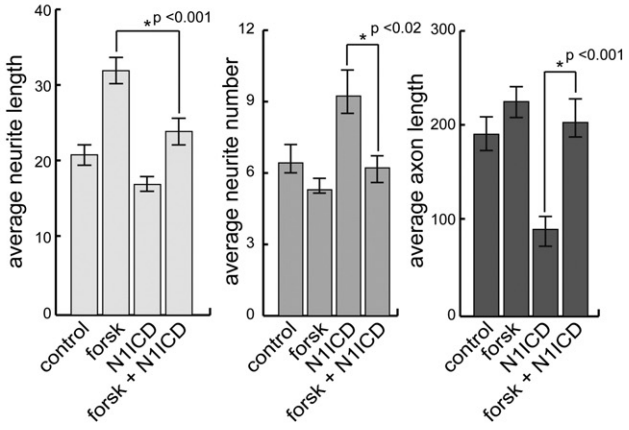
a



b

Treatment	Av. neurite number	Av. neurite length	Axon length
DS Red	6.50 n = 35 n* = 228	20.67	194.1
DS Red + Forskolin	5.53 n = 53 n* = 285	31.57	226.9
DS Red + N1ICD	9.25 n = 33 n* = 296	17.17	92.90
DS Red + Forskolin + N1ICD	6.38 n = 43 n* = 261	24.14	207.0

n = neurons measured n* = neurites measured



3.9. Notch represses PKA-dependent neurite growth

Notch1 and Notch2 influence the dendritic morphology of adult neurons through CBF-1 and the HES family target genes [7,8]. This does not rule out the possibility that the Notch ICDs might also exert effects on other signalling pathways, such as the cAMP/PKA/CREB or CaM-Kinase/CREB pathways, known to promote dendritic outgrowth in neurons and neuronal-like cell lines [36–38]. As such we examined the effects of N1ICD on forskolin stimulated neurite outgrowth.

To do this primary rat cortical neurons were cultured for 1 day on poly-D-lysine coated dishes, transfected with pDsRed and EGFP-N1ICD or pDsRed and empty EGFP-vector. Cells were then treated with 10 μM forskolin or the inactive homolog for 24 h, fixed and morphology examined by fluorescence microscopy. Treatment of neurons with the PKA inhibitor H-89 (20 μM) prevented forskolin induced increases in neurite growth indicating that the effects of forskolin on neurite outgrowth are PKA dependent, data not shown. However, some caution needs to be taken when interpreting these data as H-89 has also been shown to inhibit RhoA/ROCK [39], a regulator of actin cytoskeletal dynamics and neuronal process formation.

Neurite length and number were measured. The longest process, taken to be the axon, was measured independently. Forskolin treatment increased neurite and axonal length and caused a small reduction in neurite number. Exogenously expressed N1ICD had the opposite effects, increasing neurite number and decreasing neurite and axonal length. In the presence of forskolin treatment N1ICD reduced neurite and axonal length and neurite number to levels not significantly different from those of control values. Representative cells for each condition are shown in Fig. 5a, data on process length and number are shown in Fig. 5b. These observations demonstrate that cAMP/PKA-mediated neurite outgrowth is antagonised by N1ICD. It also indicates that the effects of N1ICD on neuronal morphology, increased neurite number and reduced axonal length, are antagonised by PKA activation.

3.10. N1ICD inhibits the expression of genes associated with LTM

To determine if the effects of Notch on LTM formation in brain may be due to inhibition of CRE activity we examined if N1ICD would antagonise the transcription of CRE regulated genes whose expression is modulated during memory formation. Three such genes were examined, the well-characterised CREB target FOS [40], the orphan nuclear receptor, NR4A1 [41], and serum/glucocorticoid regulated kinase-1, SGK1 [42,43]. We verified that the expression of these three genes is modulated during memory formation in mouse hippocampus using a hippocampal-dependent paradigm of LTM, contextual fear conditioning (CFC) [25]. Wild type C57/Black6 mice (n = 6) were subject to CFC and hippocampal tissue collected from naïve mice and conditioned mice at 30, 60 and 180 min post conditioning. Total RNA was extracted and real time qRT-PCR performed. The hippocampal expression of all three genes increased following CFC. The expression of FOS and NR4A1 peaked at 30 min and SGK1 at 60 post conditioning (data not shown). Next time-course (30 min to 3 h) and dose–response (1 to 20 μM) studies with forskolin were performed on E16 mouse primary hippocampal/cortical neurons maintained in vitro for 7 d (7 d.i.v.). Total RNA was extracted and real time qRT-PCR performed. Maximal FOS expression occurred at 30 min with a 10 μM dose of forskolin (Fig. 6a).

Fig. 5. N1ICD represses forskolin induced neurite growth. a) E18 rat primary cortical neurons were cultured for 24 h and transfected with pDsRed in combination with EGFP-tagged N1ICD or empty vector. Post transfection, cells were treated with 10 μM forskolin or vehicle for 24 h, then fixed. DsRed labelled neurons were imaged and neurite length, neurite number and axon length measured. Only cells with EGFP positive nuclei were considered transfected by the EGFP-N1ICD construct. Representative cells from each condition are shown. b) Data obtained on neurite length, axon length and neurite number are presented in tabular form and as bar charts. Statistical significance was determined by one-way ANOVA and post hoc t-testing.

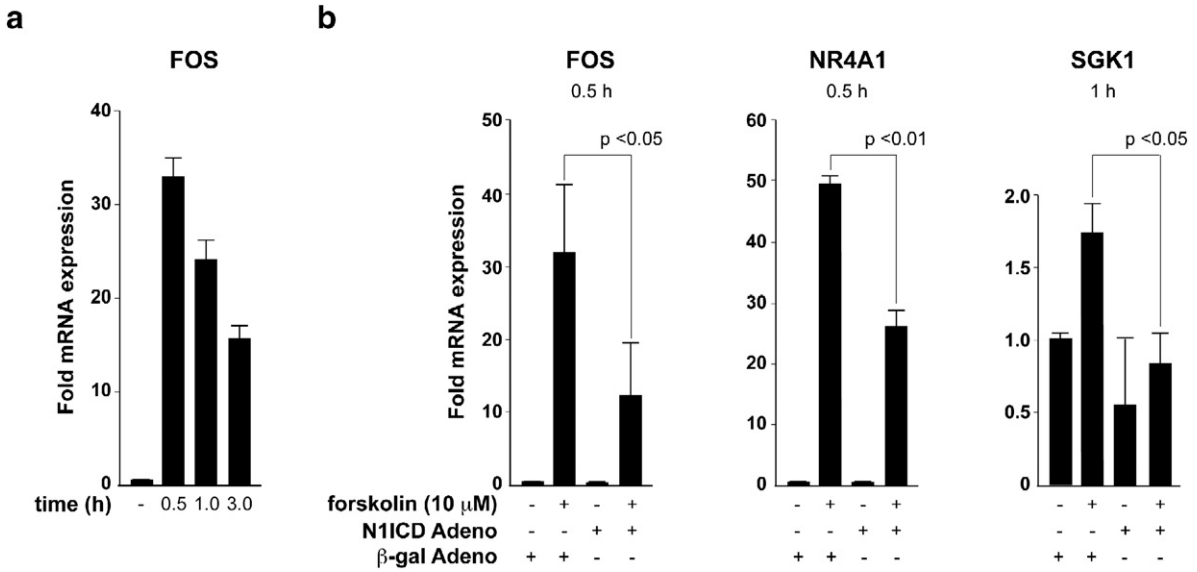


Fig. 6. N1ICD represses the expression of endogenous CRE containing genes. Real time quantitative-PCR (qRT-PCR) for endogenous CRE containing genes in mouse primary neurons. a) A forskolin time course was performed to determine the optimal time of FOS induction. b) Cultured neurons were infected with EGFP-N1ICD adenovirus (N1ICD-AD) or control β-galactosidase (β-gal-AD) virus and treated with 10 μM forskolin as indicated. FOS, NR4A1 and SGK1 expression were determined by qRT-PCR at the time points indicated, corresponding to their peak expression in hippocampus following CFC. N1ICD significantly repressed the expression of each gene. Data shown are pooled values from three replica experiments. Statistical significance was determined by one-way ANOVA and post hoc t-testing.

To examine the effects of N1ICD on neuronal gene expression we used an EGFP-N1ICD adenovirus and a β-galactosidase adenovirus as control. Transformation of cultured neurons with the control β-galactosidase adenovirus at a multiplicity of infection (MOI) of 10 had little effect on basal, or on forskolin induced expression levels of FOS (data not shown). Cultured neurons were infected at 5 d.i.v. with either EGFP-N1ICD or control adenovirus at a MOI of 10 and subsequently treated 48 h later with 10 μM forskolin for 30 min, 1 h and 3 h. The expression of FOS, NR4A1 and SGK1 at the time points corresponding to their peak induction post CFC was significantly reduced in cells infected with EGFP-N1ICD compared to cells infected with the control virus (Fig. 6b). N1ICD thus represses the neuronal expression of endogenous CREB regulated genes that have been implicated in LTM formation.

4. Discussion

Although *Drosophila* Notch [9,10] and murine Notch-1 [11,12] have been found to be necessary for the proper establishment of LTM, the molecular mechanism(s) through which Notch impacts on LTM formation have not been determined. Here we present data obtained through several different approaches that together strongly indicate that the transcriptionally active portion of mammalian Notch 1, the N1ICD, binds with CREB1α and exerts a repressive effect on CREB-dependent gene transcription. The region of N1ICD involved in this interaction lies between amino acids 2094 and 2556 of human Notch-1, which contains the transactivation, OPA and PEST domains. Importantly, in respect to LTM, we found that in primary neurons N1ICD represses the transcription of hippocampal genes whose expression levels are modulated during memory formation. Furthermore, we found that this repressive effect of Notch extends to the whole mammalian Notch family, i.e., Notch-2, -3 and -4 ICDs.

Heterozygous null mutations of CBF1, the canonical Notch pathway TF, have similar effects on LTM as heterozygous null mutations of Notch-1 [12], implying that the effects of Notch-1 upon LTM are through its canonical signalling pathway. However, such studies do not rule out the possibility that the Notch ICDs might also exert effects via other signalling pathways, in particular the cAMP/PKA/CREB pathway, which is known to drive neurite growth [44,45] and the expression of genes required for LTM [17].

In support of N1ICD exerting effects on CREB an interaction between Notch-2 and CREB1 has been reported in T-cells, which regulates the differentiation of CD8(+) cytotoxic T lymphocytes [46]. N2ICD was found to interact with and integrate CREB1 activity in a co-operative manner [47], which contradicts our observations here. However, in this report Maekawa et al. (2008) show that the N2ICD and CREB1 interaction is mediated through adjacent CRE and CBF-1 DNA binding sites in the promoter of the one gene examined, granzyme B. Our observations suggest that N1ICD can interact with CREB at CRE sites without the need for adjacent CBF-1 DNA binding motifs, as such elements are not present in the CRE-Luc promoter used for transcriptional and chromatin immunoprecipitation experiments. Thus, the effects of Notch-2 and CREB on granzyme B expression may be a special case, and the antagonistic effects we observe here may be more generally applicable.

Most recently, with respect to Notch and memory, it has been reported that in *Drosophila* full-length Notch acts in conjunction with PKC to regulate the phosphorylation of dCREB to trigger ultradian oscillations in dCREB protein levels. This cyclical accumulation of dCREB is proposed to be important for repetitive aspects of LTM formation, such as memory consolidation [14].

We found that the inhibitory effect of Notch-1 on CREB is dependent upon N1 γ-secretase cleavage, and release of the ICD from the membrane bound receptor. This may speak to the report from Dash et al. (2005), showing that the application of γ-secretase inhibitors enhanced LTM [29], leading these authors to suggest that a signalling molecule(s) generated by γ-secretase activity may have a negative influence on long-term memory formation.

There have been consistent but incompletely understood reports indicating there is a link between Notch signalling, the presenilins and Alzheimer's disease, the most prevalent disorder of memory dysfunction [48]. The familial Alzheimer's disease gene, PSEN1, is required for γ-secretase cleavage of the Notch receptors, a process we show is necessary for the antagonistic effect of Notch-1 upon CRE-dependent gene transcription. Familial mutations in PSEN1 impair Notch cleavage and the nuclear translocation of NICDs [49], which, given NICDs regulate CREB activity may account, at least in part, for the memory deficits

observed in mutant PSEN1 transgenic mice [49,50] and be a contributory factor to the cognitive deficits associated with Alzheimer's disease in man.

Conflict of interest

The authors declare that they have no conflicting interests.

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