

## Brn-3c (POU4F3) regulates BDNF and NT-3 promoter activity

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### Abstract

Brn-3c is a transcription factor necessary for maturation and survival of hair cells in the inner ear. Mutations in Brn-3c are associated with deafness in mice and with hearing loss in humans. Mice lacking Brn-3c also show reduced innervation and loss of sensory neurons presumed to be an indirect effect of hair cell loss potentially through lower BDNF and NT-3 expression. Using transient transfection assays we show that Brn-3c is capable of activating both BDNF and NT-3 promoters in inner ear sensory epithelial cell lines. In vitro analysis shows that Brn-3c binds to specific elements within the promoters of both genes and these elements are sufficient to confer Brn-3c regulation on a heterologous promoter. Additionally, BDNF expression is reduced in the inner ear of a Brn-3c mutant mouse during embryogenesis. Our data suggest that Brn-3c may play a role in regulating neurotrophin gene expression in the inner ear.

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Hair cells of the inner ear are the mechanosensors of the cochlea and vestibular organs responsible for detecting sound, gravity, and acceleration. Crucially, unlike amphibians and birds, the mammalian cochlea is unable to regenerate hair cells that are lost [1]. Damage and defects to hair cells are the major cause of hearing loss and deafness [1]. Elucidation of hair cell developmental, regeneration, and survival mechanisms is therefore envisaged to have considerable therapeutic benefit. Brn-3c (also known as Brn3.1 and POU4F3) is one of the genes identified as a cause of non-syndromic hearing loss in humans (DFNA15) [2]. Brn-3c is a POU domain transcription factor expressed in sensory neurons but within the inner ear it is exclusively expressed within hair cells [3–6]. Studies in knock-out mice indicate that Brn-

3c is required for mature differentiation and survival of sensory hair cells [3–6]. Hair cells develop in the embryonic knockout mice but are progressively lost during the late gestation and early postnatal period, causing defects in hearing and balance. Other members of the Brn-3 subfamily of POU domain transcription factors, Brn-3a and Brn-3b, are known to play similar roles in the mature differentiation and survival of sensory cell populations, specifically retinal ganglion cells and sensory neurons [3,6–9]. In the mouse inner ear, Brn-3a and Brn-3b are expressed in the vestibulo-cochlear ganglion and its derivatives but not in hair cells [10]. Identification of the targets of Brn-3c in hair cells is likely to lead to elucidation of the mechanisms involved in producing the mature hair cell phenotype and survival of the vulnerable mature cell.

A critical event during hair cell maturation is the direction of innervation to hair cells of the vestibular

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and cochlear sensory epithelia. Sensory epithelium derived neurotrophins brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are required for normal innervation of hair cells of the inner ear [11–16]. The two neurotrophins do not perform the same function, although there appears to be some overlap; expression of NT-3 under the BDNF promoter does rescue some but not all of the defects in the BDNF knock-out mice [16]. NT-3 and BDNF are expressed both in hair cells and supporting cells, however NT-3 is expressed primarily in supporting cells and moves only around birth to hair cells, while BDNF is expressed primarily in hair cells [11–19].

The phenotype of Brn-3c knockout mice includes loss of sensory neurons and hair cell innervation, although this has been assumed to be secondary to the loss of mature hair cells [3–6]. Here, we have investigated whether Brn-3c may have a more direct role in the development and maintenance of inner ear innervation by regulating BDNF and NT-3 gene expression. Studies *in vivo* are hampered by the difficulty of isolating and culturing reasonable amounts of hair cells from the inner ear. In addition, studies in knock-out mice cannot distinguish between the direct effects of Brn-3c loss and the secondary effects due to loss of mature hair cells. Therefore, we have utilised two cell lines (OC-1 and OC-2) derived from mouse embryonic day 13 inner ear sensory epithelium and previously used as an *in vitro* system for studies of the auditory sensory epithelium [20,21]. These cells express many hair cell markers including the Brn-3c transcription factor. Our data reported here show that wild type Brn-3c, but not its mutant form, can directly activate expression of individual BDNF and NT-3 promoters in inner ear sensory epithelial cell lines. This activation occurs through binding of Brn-3c to newly identified regulatory elements within the promoters of these genes. These elements are sufficient in themselves to confer Brn-3c regulation on a heterologous promoter. Moreover, investigations in Brn-3c mutant mice have established reduced expression of BDNF in the inner ear of these mice at time points where hair cells still exist. Reducing Brn-3c levels in OC cell lines by antisense technology leads to reduced activity of BDNF and NT-3 promoters in these cells. These data provide strong evidence of a mechanism of direct regulation of BDNF and NT-3 gene expression by Brn-3c. Such regulation may have implications for understanding the role of Brn-3c in the inner ear and maintenance of inner ear innervation.

## Materials and methods

**Plasmid DNA.** Full-length Brn-3c and Brn-3c POU domain, wild type and mutant human Brn-3c [2], and the *ddl* mutant sequences were cloned into the SV40 expression vector pSi (Promega). BDNF CAT

constructs (kindly provided by Dr. Tonis Timmusk, University of Helsinki) have been described before [22] and are summarised in Fig. 1A. A 900 bp *HincII*–*XbaI* fragment was subcloned from the BDNF III 0.9 CAT vector into the pGL3-Basic (Promega) luciferase vector to allow for accurate quantification of activation from this sequence. The NT-3 1A and 1B exon sequences were amplified from mouse genomic DNA and cloned into pGL3-Basic and pGL3Prom [NT-3 1A primers: 5'-gagctctccagttagaactgg-3' and 5'-agatctctcatcattatctctggctg-3'. NT-3 1B primers: 5'-gagctcaaaagcggcagtgctgggc-3' and 5'-agatctctgtggcagaagtaaccatgg-3']. Constructs used in Fig. 3A were generated by cloning the following restriction fragments of NT-3 1A into pGL3Prom. (position in NT-3 1A shown in brackets): a 340 bp *SacI*–*PvuII* fragment (1–340), a 429 bp *PvuII*–*SmaI* fragment (340–769), a 224 bp *SmaI*–*XhoII* fragment (769–993), and a 140 bp *XhoII* fragment (993–1133). Oligonucleotide sequences  $\alpha$  and  $\beta$  (see below) and to the putative Brn-3c binding site in fragment 3 of the NT-3 promoter were cloned separately into the SV40 promoter luciferase pGL3Prom vector using the overhangs generated in their synthesis. The number of binding sites present in these constructs was estimated by sizing on an agarose gel. An additional construct was made by amplifying the region spanning sequences  $\alpha$  and  $\beta$  by PCR inserted into the pGL3Prom vector ( $\alpha + \beta$ ). Antisense Brn-3c construct contains the N-terminus of mouse Brn-3c cDNA [primers: 5'-accgtttcggatgcacc-3' and 5'-tcgagggtctgactccacat-3'] cloned in the antisense orientation into the pSi expression vector. Transfection of this plasmid into OC-2 cells is sufficient to reduce Brn-3c mRNA levels as determined by semi-quantitative RT-PCR (data not shown).

**Cell culture and transient transfections.** OC-1 and OC-2 cell lines were grown at 33 °C, 5% CO<sub>2</sub> in Eagle's minimal essential medium with glutamax (Gibco-BRL), 10% foetal calf serum, and 50 U/ml  $\gamma$ -IFN. ND7 cells [23] were grown at 37 °C, 5% CO<sub>2</sub> in L15 medium (Gibco-BRL) with 10% foetal calf serum, 0.3% D-glucose, 0.37% sodium bicarbonate, and 0.2 mM L-glutamine. BHK-21 cells were grown at 37 °C, 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium with 10% foetal calf serum (Gibco-BRL). Transfections were carried out according to the calcium phosphate method [24]. Assays of chloramphenicol acetyl transferase activity were performed as Gorman [24]. Samples were equalised for differences in transfection rate as determined by co-transfection with a *Renilla* expression construct and subsequent Dual Luciferase Assay (Promega) or *Renilla* assay in CAT transfections. Graphs show mean value of at least three experiments, error bars indicate the standard error of the mean. Relative luciferase value (RLV) is the luciferase activity relative to activity level of vector alone in each experiment. In every case control transfections were performed to ensure base reporter vectors were not regulated by Brn-3c (data not shown).

**DNA footprinting assay and gel retardation assay.** A 703 bp *HincII*–*NheI* fragment of sequence from the 5' region of BDNF promoter III was DNase I footprinted using the Core Footprinting System (Promega) using *in vitro* translated Brn-3c (TNT Coupled Reticulocyte Lysate System, Promega). For the gel retardation assays, 1 ng of  $\gamma$  <sup>32</sup>P labelled double-stranded oligonucleotide was incubated in Parker buffer (8% Ficoll, 20 mM Hepes, pH 7.9, 50 mM KCl, 1 mM EDTA, and 0.5 mM DTT), 3  $\mu$ g poly(dI/dC), 5  $\mu$ l Brn-3c *in vitro* translated or T7 *in vitro* translated control or 9  $\mu$ g of cell extract and incubated at room temperature for 15 min. Supershift reactions: 1–2  $\mu$ l of antibody was added and reaction was incubated for a further 30 min (Oct-2 antibody, Santa Cruz; Brn-3c antibody, Covance Research Products). Samples were resolved on a 4% non-denaturing acrylamide at 4 °C gel, which was dried and subjected to autoradiography. Double-stranded oligonucleotides were designed to three regions (with 4 base overhang for subsequent cloning)

BDNF  $\alpha$ : 5'-ctagcatacagctaaatgaaaatagctctcca-3'

BDNF  $\beta$ : 5'-tcgacatgtaactgatctcttagccac-3'

NT3 1A: 5'-gatcggagcttgagaatgagcccca-3'

**Real-time PCR.** Total RNA was extracted from whole inner ears dissected out of E16.5 and E18.5 homozygote *ddl* and wild type mice as previously described [25]. Real-time semi-quantitative PCR was performed using an ABI Prism 7700 Sequence Detector (PE Applied Biosystems), VIC labelled *Gapdh* control primers (150 nM) and probes (PE Applied Biosystems), and the following primers and FAM labelled probes (PE Applied Biosystems): *Bdnf* (F) 5'-gcc caa cga aga aaa cca taa g-3 (600 nM), (R) 5'-agg agg ctc caa agg cac tt-3' (300 nM), and (probe) 6FAM—tac act tcc cgg gtg atg ctc agc a—TAMRA. Experiments were performed in six repeats and results were quantified using a relative standard curve. Error bars represent one standard error measured.

## Results and discussion

*Brn-3c is able to activate BDNF promoter III but not promoters I, II, and IV*

The rat BDNF gene consists of four short 5' exons (I–IV) and one 3' exon (exon V) that encodes the mature protein. The four 5' exons can each occur separately in combination with exon V and each of these exons and their flanking regions act as individual promoters. Together with two different polyadenylation sites, the four upstream exons lead to eight possible mRNA species (see Fig. 1A). Timmusk et al. [22,26] have shown that use of these exons and regulation of their individual promoters are tissue-specific; mRNAs containing exons I, II or III are expressed predominantly in the brain, exon IV transcripts predominate in the lung and heart.

We tested the ability of the Brn-3c transcription factor to regulate expression of BDNF promoter constructs in two conditionally immortalised inner ear sensory epithelial cell lines, OC-1 and OC-2. Fig. 1B shows the results of co-transfecting the mouse Brn-3c cDNA with different reporter gene constructs of various regions of the four individual BDNF promoters. The data clearly show that all three constructs which contain differing lengths of promoter III sequences are up-regulated by Brn-3c (constructs III 4.5 CAT, III 1.8 CAT, and III 0.9 CAT in Fig. 1B). In contrast, no construct containing promoters I, II or IV is activated by Brn-3c. Indeed, the 9.5 kb construct containing a combination of both promoters I + II appears to be downregulated in the presence of Brn-3c (Fig. 1B). Regulation of these promoters by Brn-3c was reproducible in the two different cell lines OC-1 and OC-2 (data not shown for OC-1).

To facilitate quantification and to further analyse Brn-3c regulation of the BDNF promoter III, we cloned the smallest BDNF promoter fragment to be activated by Brn-3c, the 900 bp fragment from construct III 0.9 CAT into a luciferase reporter vector. Subsequent transfections (Fig. 1C) confirmed our previous data, demonstrating the ability of both mouse and human Brn-3c to activate this BDNF promoter. Levels of activation

observed in OC-1 cells were greater than fivefold and greater than ninefold in OC-2 cells. Activation was dependent on a functional Brn-3c protein since transfections with a mouse dreidel (*ddl*, POU4F3<sup>ddl/ddl</sup>) mutant Brn-3c showed no activation of the BDNF promoter (Fig. 1C). Homozygotes for this spontaneous mouse mutant show a similar phenotype to targeted Brn-3c knock-out mice due to a 2 bp deletion (388del2) in the Brn-3c gene, resulting in a premature stop codon upstream of the POU domain [25].

In these transfections the isolated POU domain of Brn-3c is unable to activate BDNF promoter III. This is consistent with earlier data demonstrating that the Brn-3c N-terminal activation domain is required for the activation of the SNAP-25 gene in neuronal cells [27]. Smith et al. [27] concluded from their data that this N-terminal activation domain in Brn-3c is neuronal-specific. Our data suggest this is not the case, since activation of this BDNF construct takes place in both OC cell lines and in the fibroblast cell line BHK-21 but not in the neuronal ND-7 cell line used by Smith et al. (Fig. 2A).

*Brn-3c activates NT-3 promoter 1A but not promoter 1B in OC-1 and OC-2 cell lines*

The mouse NT-3 gene consists of two upstream exons 1A and 1B that with their flanking regions act as individual promoters and a downstream exon II which codes for the mature protein [28] in a similar organisation to the BDNF and other neurotrophin genes [22,29]. Together with three different polyadenylation sites, this generates six different possible transcripts [29] (see Fig. 2A). Results of co-transfection studies show that Brn-3c is able to activate exon 1A by approximately twofold in both OC-1 and OC-2 cells. Levels of activation were not as great as with the BDNF promoter, only twofold for NT-3 as compared to ninefold for the BDNF promoter in OC-2 cells. Although a twofold difference in expression may be physiologically significant it may also arise from experimental error. In these experiments the activation was consistent over many experiments and experimental error is greatly reduced in these assays by use of the Dual Luciferase Assay system (Promega) to equalise for different transfection rates. Control transfections established that the base reporter vectors are not regulated by Brn-3c (data not shown) and co-transfections with the *ddl* mutant establish that the regulation is dependent on a functional Brn-3c protein (Fig. 2C). Induction of the NT-3 1A promoter was increased to about threefold when the sequence was placed on a heterologous SV40 promoter, confirming that this regulation is specific to the NT-3 1A promoter sequence. Levels of 1B promoter activity were unaffected by Brn-3c, see Fig. 2B.

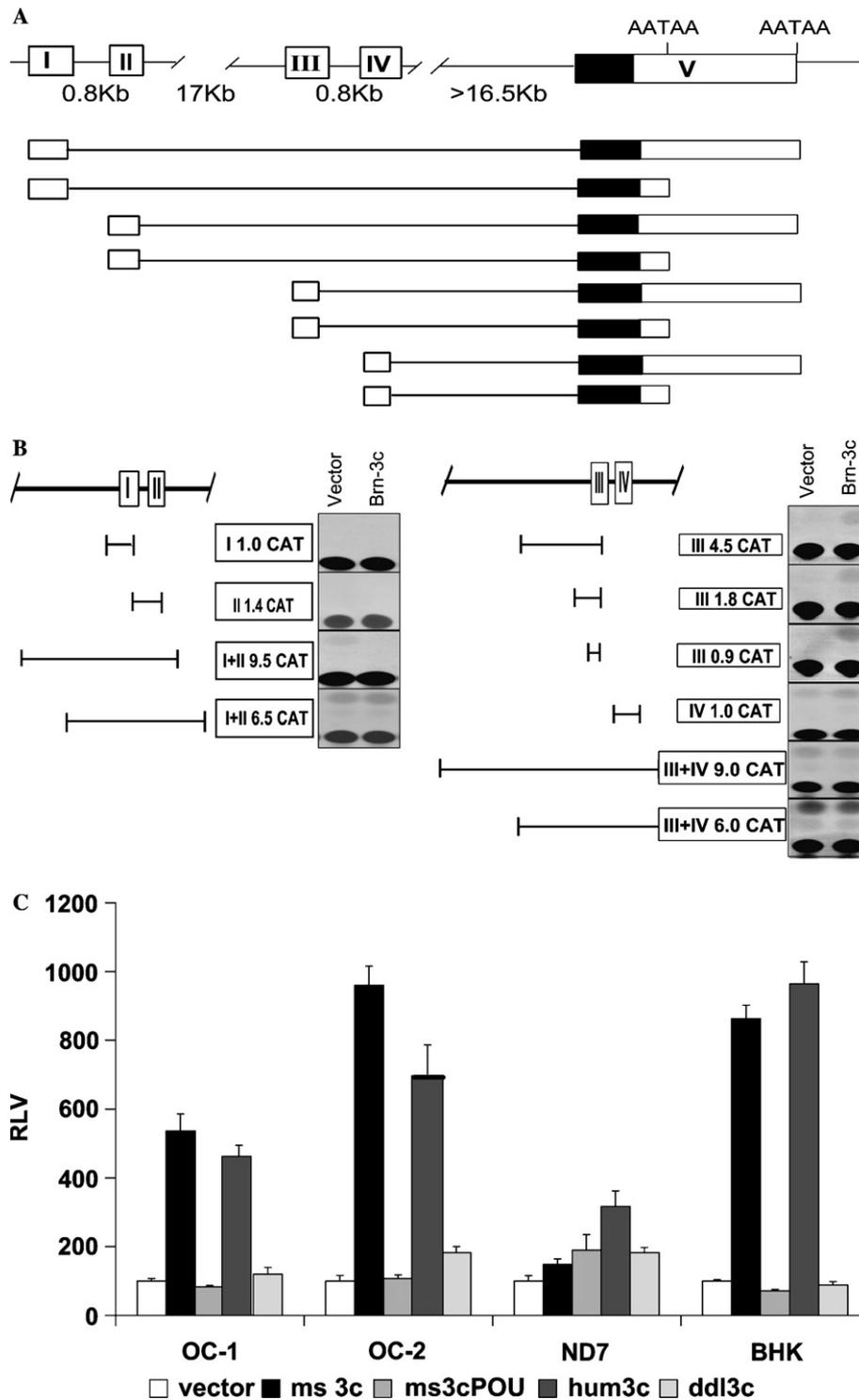


Fig. 1. Brn-3c activates the BDNF exon III promoter in OC cell lines. (A) The schematic diagram (not to scale) shows the organisation of the rat BDNF gene (top) and the eight possible mRNA species produced from the rat BDNF gene. Exons I, II, III, IV, and V are represented by open boxes. The filled portion of exon V represents the region which encodes the BDNF protein. The size of the gap between exons is marked on the gene, as are the two alternative polyadenylation sites (*AATAA*) on exon V. (B) BDNF CAT constructs co-transfected with the Brn-3c expression vector (*Brn-3c*) or with an empty vector (*Vector*) in the OC-2 cell line. The schematic diagram (not to scale) shows the genomic BDNF sequences in each CAT construct. Next to each construct is a representative CAT assay showing the result of transfecting each construct with a Brn-3c expression vector (*Brn-3c*) or with an empty vector (*Vector*) in the OC-2 cell line. (C) Activity of the BDNF exon III promoter luciferase construct in various cell lines when co-transfected with: an empty expression vector (*vector*); the same vector containing, cDNA of full-length mouse Brn-3c (*ms 3c*); only the POU domain of mouse Brn-3c (*ms3cPOU*); the full-length human Brn-3c (*hum3c*); or the mouse *ddl* Brn-3c mutation (*ddl3c*).

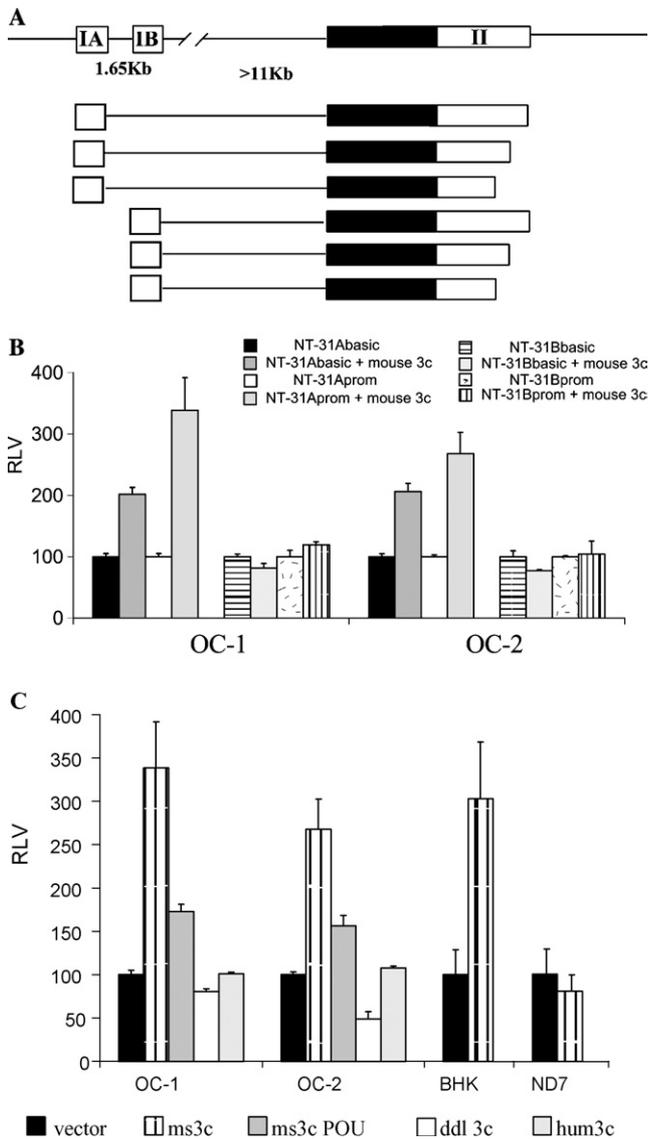


Fig. 2. Brn-3c activates the NT-3 1A promoter in OC cell lines. (A) The schematic diagram (not to scale) shows the organisation of the mouse NT-3 gene (top) and the six possible mRNA species produced from the NT-3 gene. Exons IA, IB, and II are represented by open boxes. The filled portion of exon II represents the region that encodes the NT-3 protein. The size of the gap between exons is marked on the gene. (B) Activity of the NT-3 1A and 1B promoter luciferase constructs in OC-1 and OC-2 cell lines when co-transfected with an empty expression vector or the same vector containing cDNA of full-length mouse Brn-3c. Boxes indicate which construct was transfected, either the NT-3 promoter in pGL3-Basic (NT-31Abasic and NT-31Bbasic) or pGL3Prom which contains an SV40 promoter (NT-31Aprm and NT-31Bprom). (C) Activity of the NT-3 1A promoter pGL3Prom construct in various cell lines when co-transfected with: an empty expression vector (*vector*); the same vector containing, cDNA of full-length mouse Brn-3c (*ms3c*); only the POU domain of mouse Brn-3c (*ms3cPOU*); the mouse *dll* Brn-3c mutation (*dll3c*); or the full-length human Brn-3c (*hum3c*).

Regulation of the NT-3 1A promoter showed the same cell-specific pattern as that observed with the BDNF promoter and it was also largely dependent on

the N-terminal activation domain of Brn-3c (Fig. 2C). In summary, data presented thus far demonstrate the ability of Brn-3c to activate individual BDNF and NT-3 promoters in OC and BHK cell lines but not in neuronally derived ND7 cells. Regulation is dependent on a full length and functional Brn-3c cDNA, and is independent of vector backbone.

#### Identification of Brn-3c binding sites within the BDNF and NT-3 promoters

Confirmation of the ability of Brn-3c to directly regulate the two neurotrophin genes requires identification of the Brn-3c binding sites within these promoters. The DNA binding site for the Brn-3 transcription factor family has been identified in several neuronal target genes [30–33]. There is core similarity between the sequence of these sites and a consensus DNA binding site has been determined experimentally for the Brn-3 family [34,35]. However, there is insufficient stringency in the requirement for specific bases in the recognition site to be able to identify putative Brn-3c response elements on the basis of consensus sequence similarity alone when examining large sequences of DNA. DNase I footprinting performed on a 703 bp region of the BDNF III promoter using in vitro translated Brn-3c identified two footprinted regions designated  $\alpha$  and  $\beta$  (data not shown). The results of gel retardation assays using in vitro translated Brn-3c and labelled oligonucleotides of sequence representing footprinted regions  $\alpha$  and  $\beta$  are shown in Fig. 3B. A specific band is produced by Brn-3c binding to each of both  $\alpha$  and  $\beta$  identified in the gel retardation assay (lanes 1 and 5 in Fig. 3B), this binding is not reduced by competition with excess unlabelled oligonucleotide containing binding sites for Sp1 transcription factor and the POU domain transcription factor Oct-2 (lanes 2, 3, and 6 in Fig. 3B). When an oligonucleotide spanning both  $\alpha$  and  $\beta$  sites was incubated with OC-2 cell extracts this produced a band which was “supershifted” in the presence of the Brn-3c antibody but not by an antibody to the closely related Oct-2 POU domain transcription factor (arrow SS1 in Fig. 3B). These data show that both recombinant and OC-2 derived Brn-3c are able to bind elements in the BDNF exon III promoter, providing evidence of the mechanism through which Brn-3c regulates this promoter.

After identification of the Brn-3c binding sites within the BDNF promoter we sought to identify any similar elements within the NT-3 1A promoter. Four sequences with some degree of homology to either the BDNF elements or the Brn-3 consensus sequence or both were identified. Deletion constructs of the full-length NT-3 1A promoter were generated to map the Brn-3c binding site to one or more of these four putative elements, see Fig. 3A. Only element 3 gave values of promoter activation at similar levels to the full-length NT-3 1A

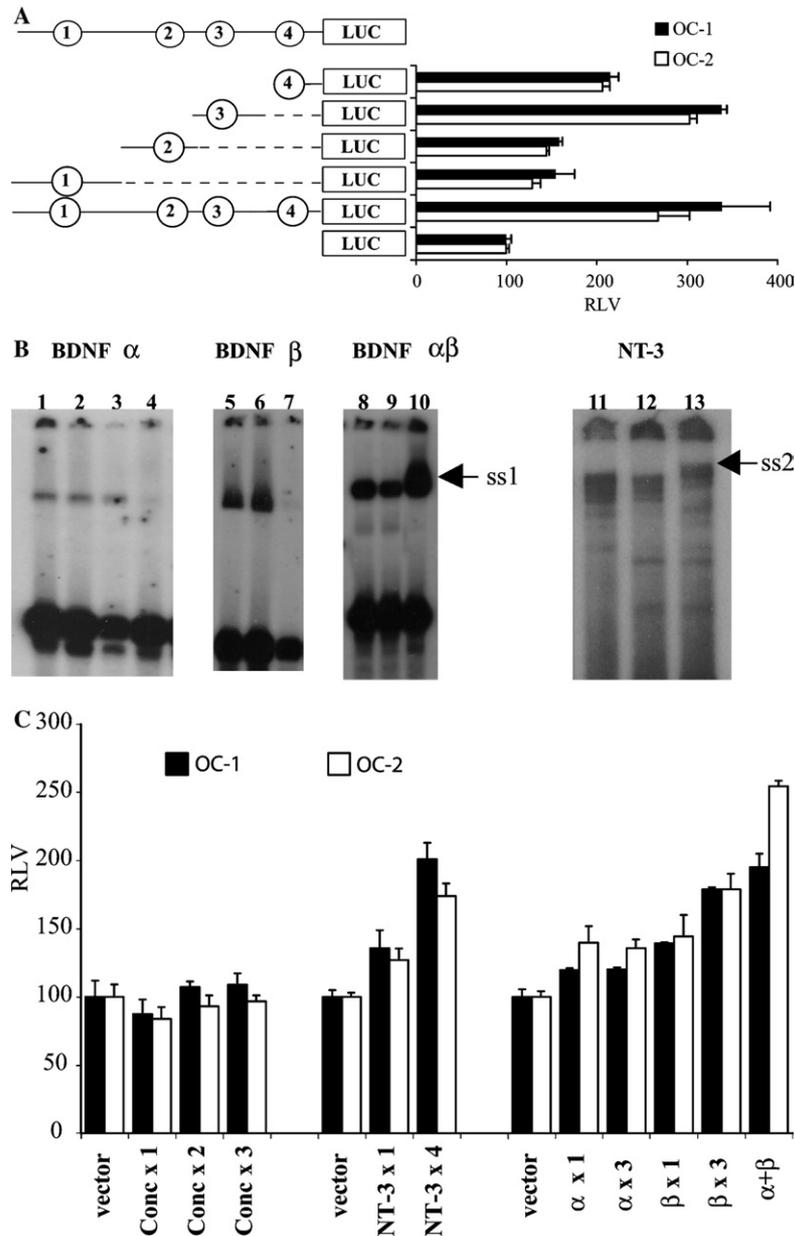


Fig. 3. Identification of Brn-3c binding sites in the BDNF III and NT-31A promoters. (A) A schematic diagram (not to scale) shows luciferase constructs created to map the Brn-3c response element in the NT-31A promoter and discriminate between four putative Brn-3c elements. The bar graph shows the relative luciferase values obtained (shown next to each construct) when co-transfected with an expression vector containing mouse Brn-3c cDNA in OC-1 and OC-2 cells. Values shown are relative to those obtained when each construct was co-transfected with an empty expression vector (set to 100 RLV for each construct). (B) Gel retardation assays on the putative Brn-3c binding sites in the BDNF and NT-3 promoters. Probes are shown above each assay and were  $^{32}\text{P}$  labelled double-stranded sequences of:  $\alpha$ ,  $\beta$  or the region spanning  $\alpha$  and  $\beta$  ( $\alpha\beta$ ) from the BDNF promoter, and the putative Brn-3c binding sites within the NT-3 promoter (for sequence see Materials and methods). Lanes 1–7, incubated with 5  $\mu\text{l}$  of in vitro translated Brn-3c (lanes 1–3 and 5 and 6) or 5  $\mu\text{l}$  of in vitro translated control (lanes 4 and 7). In the presence of 100 $\times$  molar excess of Sp1 cold competitor (lane 2) or 100 $\times$  molar excess of oct2 cold competitor (lanes 3 and 6). Lanes 8–13, incubated with 9  $\mu\text{g}$  OC-2 cell extract and in the presence of 1  $\mu\text{l}$  of oct-2 antibody (lanes 9 and 12) or Brn-3c antibody (lanes 10 and 13). Supershifts created by the Brn-3c are identified by arrows and labelled ss1 and ss2. (C) Activity of the various constructs containing individual Brn-3c binding sites on a heterologous SV40 promoter (pGL3Prom) in OC-1 and OC-2 cell lines when co-transfected with an expression vector containing a cDNA of mouse Brn-3c. Constructs contain double-stranded oligonucleotides to: the Brn-3c consensus sequence (*conc*); the Brn-3c site in the NT-3 1A promoter (*NT-3*); the  $\alpha$  and  $\beta$  ( $\alpha$  and  $\beta$ ) Brn-3c sites in the BDNF promoter and the region which spans both  $\alpha$  and  $\beta$  sites ( $\alpha + \beta$ ). The number of copies of each binding sites is indicated. *Vector* shows values obtained from the empty pGL3Prom vector in each series of experiments.

construct upon co-transfection with the Brn-3c expression vector. Gel retardation assays performed with an oligonucleotide made to this sequence incubated with

OC-2 cell extracts produced a band which was “super-shifted” by incubating with the Brn-3c antibody and not by an Oct-2 antibody (arrow SS2 in Fig. 3B).

*The Brn-3c elements identified with the BDNF and NT-3 promoter are sufficient to confer regulation on a heterologous promoter*

Our data indicate that individual BDNF and NT-3 promoters contain sequences to which Brn-3c can bind. In order to determine whether the Brn-3c binding sites identified within the BDNF promoter III and NT-31A promoter are sufficient to confer transcriptional activation by Brn-3c, the double-stranded oligonucleotides used in gel retardation assays were each cloned into the pGL3Prom vector upstream of a SV40 promoter driving luciferase expression. Fig. 3C shows the result of co-transfecting Brn-3c with these constructs in comparison with the empty pGL3Prom vector. A single copy of the Brn-3c element found within the NT-3 promoter is sufficient to confer Brn-3c activation upon the heterologous promoter, this being increased when multiple copies are present. In these assays a single copy of sequence  $\alpha$  or  $\beta$  from the BDNF III promoter only conferred a limited amount of activation on the heterologous promoter (Fig. 3C). Multiple copies of the  $\beta$  sequence do seem to confer some activation on the promoter but not at the levels one would expect. As the  $\alpha$  and  $\beta$  sequences are adjacent to each other in the BDNF III promoter, we investigated whether it was necessary to have both regions present to confer full Brn-3c responsiveness. A 69 bp region spanning both these sites was cloned into the pGL3Prom vector. This region contains only nine additional bases, between sites  $\alpha$  and  $\beta$ , compared to the oligonucleotide sequences used as probes in the gel retardation assays. A single copy of this  $\alpha + \beta$  sequence was sufficient to confer activation on the heterologous promoter with activation being at higher levels than one might expect if the effects of  $\alpha$  and  $\beta$  were purely additive. Activation of the  $\alpha + \beta$  construct was greater in OC-2 cells than OC-1 mirroring the effect observed with the BDNF promoter III. These results suggest we have identified sequences within the BDNF and NT-3 promoters that are true Brn-3c response elements, sufficient in themselves to confer Brn-3c regulation on a promoter.

Results were also compared with levels of activation conferred by cloning the Brn-3 consensus element into the same vector. Surprisingly, in OC-1 and OC-2 cell lines the experimentally derived Brn-3 consensus element [35] is not able to confer Brn-3c activation even when present in multiple copies whereas a single copy of the consensus element is sufficient to confer a twofold activation in ND-7 cells (Fig. 3C and data not shown for ND7 cells). The inability of the Brn-3c transcription factor to regulate an experimentally derived Brn-3 consensus element in OC cell lines (Fig. 3C) raises the possibility that the optimum Brn-3c binding site in these cells is slightly different to the Brn-3 consensus core CTCATTAA obtained by Xiang et al. [35]. Brn-3c bind-

ing sites we have identified in the NT-3 and BDNF  $\alpha$  elements contain a 6 bp match, AAATGA (or TCATTT on the opposite strand), with the BDNF  $\beta$  element having a 5/6 bp match to this sequence. This is not dissimilar to the Brn-3 consensus but presumably sufficiently different to discriminate between regulation or not in these cells. Our data suggest that regulation of NT-3 and BDNF promoters by Brn-3c does not occur in the ND-7 neuronal cell line and regulation of the core consensus *does* take place in ND7 cells. Therefore, it is possible that there may be subtle differences in the ability of neuronal and inner ear sensory epithelial cell Brn-3c to recognise and regulate specific sequences. Such subtle differences in DNA binding specificity and function are characteristic of POU domain transcription factors and are mediated by cell-specific co-factors [34–41].

*BDNF and NT-3 promoter activity is reduced in response to lower levels of Brn-3c*

To determine whether Brn-3c regulation of this BDNF promoter is relevant to the expression of BDNF in vivo, we examined the level of BDNF mRNA in the inner ear of the homozygote *ddl* mutants and their wild type littermate control mice using real-time PCR. We quantified mRNA abundance of BDNF in whole inner ears of E16.5 and E18.5 Brn-3c wild type and mutant mice at time points where developing hair cells are still present. The level of BDNF expression in the mutant inner ears at both time points tested was found to be significantly different ( $p < 0.0001$ ) and measured as approximately 50% of that in the wild type (Fig. 4A). NT-3 levels in the *ddl* mice were not assayed since the relatively higher levels of NT-3 expression from supporting cells during these time points [12–19] mean any reduction of NT-3 hair cell expression due to loss of Brn-3c in hair cells would be expected to be masked in whole inner ears of wild type mice.

Although reduced levels of BDNF in *ddl* mice may be due to a loss of direct regulation by Brn-3c it is impossible to discriminate between this and the secondary effects of lack of Brn-3c activity on hair cell maturation and viability. To determine whether a reduction in Brn-3c levels may have a similar effect on the activity of the NT-3 1A promoter construct and BDNF promoter III using our in vitro system we co-transfected an antisense Brn-3c N terminal construct into the OC-1 and OC-2 cell lines. Previous studies in our laboratory show transfection of this construct into OC-2 cells is sufficient to reduce levels of Brn-3c mRNA and protein in these cells. Fig. 4B shows that levels of NT-3 1A promoter activity in antisense Brn-3c cells are reduced to approximately 70% that of normal Brn-3c levels in these cells. BDNF III promoter activity is reduced to about 60% of normal, a similar degree of reduction to BDNF mRNA levels found in vivo in *ddl* mice.

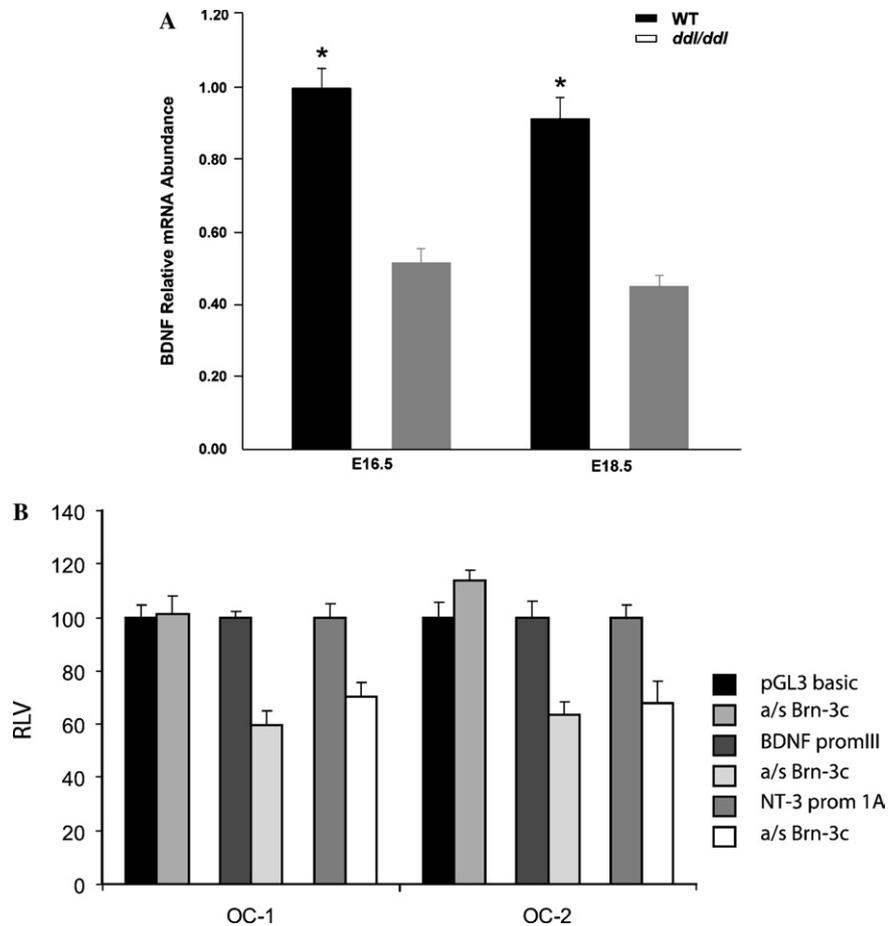


Fig. 4. BDNF and NT-3 promoter activity is reduced in response to lower levels of Brn-3c. (A) Relative abundance of BDNF mRNA transcripts in the ears of Brn-3c wild type and homozygote *ddl* mutants at E16.5 and E18.5. Y-axis: mRNA, arbitrary units; X-axis: developmental time points tested. An asterisk indicates a significant difference between wild type and mutant mRNA transcript abundance ( $p < 0.0001$ ). Error bars represent 1 SEM. (B) Activity of the various constructs containing either pGL3-Basic, the BDNF promoter III in pGL3-Basic or the NT-3 1A promoter in pGL3-Basic when co-transfected into OC-1 or OC-2 cell lines. Data are shown when co-transfected with empty pSi vector or an antisense Brn-3c construct (*a/s Brn-3c*).

*The human mutant Brn-3c which causes non-syndromic hearing loss does not interfere with activation of the BDNF promoter III construct by wild type human Brn-3c*

The 8 bp deletion (8 bp<sup>del</sup>) in human Brn-3c, which causes deafness in Israeli Family H (DFNA15), is a dominant mutation; individuals heterozygous for the mutation have non-syndromic hearing loss [2]. Recent studies suggest that this mutation is not a dominant negative mutation as had been postulated [2,42]; the mutant Brn-3c is not able to interfere with wild type Brn-3c regulation of a SNAP-25 construct in neuronal cell lines [43]. Regulation of the SNAP-25 promoter is neuronal-specific but the absence of a known target of Brn-3c regulation in hair cells meant it was the only available assay of Brn-3c activity in the study. Our data have identified BDNF and NT-3 promoters as targets of Brn-3c regulation in the OC-1 and OC-2 cell lines. We investigated whether the 8 bp<sup>del</sup> mutant Brn-3c is able to interfere with the wild type human Brn-3c activation of the BDNF promoter in these cell lines (Fig. 5). Increasing amounts of 8 bp<sup>del</sup> Brn-3c were

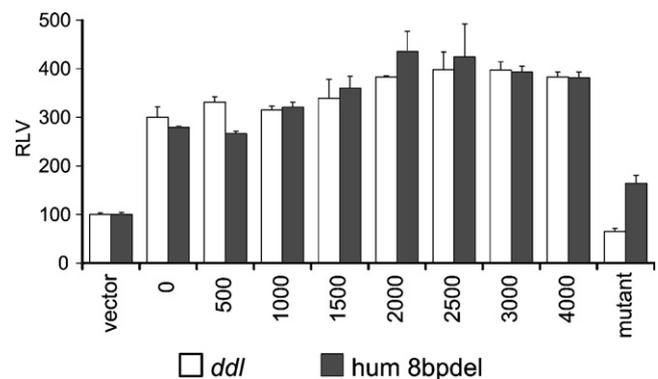


Fig. 5. Brn-3c mutants do not interfere with wild type Brn-3c activation of the BDNF promoter in OC-2 cell lines. Activity of the BDNF exon III promoter luciferase construct when co-transfected with 2000 ng of wild type mouse Brn-3c cDNA and increasing amounts of the *ddl* mutant Brn-3c cDNA (empty bars, 0–4000 ng) or the 8 bp<sup>del</sup> mutant Brn-3c against wild type human Brn-3c (hatched bars). The activity is also shown of the same BDNF exon III promoter luciferase construct when co-transfected with the empty expression vector (*vector*) or with 2000 ng of each mutant Brn-3c alone (*mutant*).

co-transfected with a constant amount of wild type Brn-3c to assay for dominant negative activity. The 8 bp<sup>del</sup> has a greatly reduced ability to activate the BDNF promoter compared to wild type Brn-3c and even when present in excess to transfected wild type Brn-3c does not interfere with its activation of the promoter construct (Fig. 5, hum 8 bp<sup>del</sup>). Similar data are obtained when the *dll* mouse mutant Brn-3c is titrated against wild type mouse Brn-3c, as would be expected for a mutation that does not have a functional DNA binding domain (Fig. 5, *dll*). These data are consistent with those obtained by Weiss et al. [43] on the SNAP-25 promoter and further suggest that another mechanism is responsible for the dominant effect of the 8 bp<sup>del</sup> mutation.

Our data presented here provide convincing evidence of the existence of Brn-3c regulatory elements in individual promoters of the BDNF and NT-3 genes. Specifically we show activation of BDNF and NT-3 promoter activity by co-transfection with a Brn-3c expression vector in two inner ear sensory epithelial lines but not in a sensory neuronal cell line (Figs. 1 and 2). Activation is dependent on the N-terminal activation domain of the Brn-3c protein and two naturally occurring mutants of Brn-3c, one in mouse and the second in human, do not activate BDNF and NT-3 promoters in the assay. Since Brn-3c expression seems to be limited to subsets of sensory neurons and hair cells of the inner ear, it is interesting to note that regulation of the neurotrophin promoters does not occur in neuronally derived ND-7 cells, perhaps implying that regulation may be restricted to inner ear sensory epithelial cells. Furthermore, we show that short sequences in the neurotrophin promoters can bind recombinant and OC-2 cell derived Brn-3c (Fig. 3B). These sequences are also capable of conferring regulation on a heterologous promoter in the OC cell lines, presumably as a consequence of Brn-3c binding (Fig. 3C). These in vitro data show that a mechanism exists through which Brn-3c could regulate expression of neurotrophin genes BDNF and NT-3. In vivo data from the *dll* mutant Brn-3c mouse show BDNF to be quantitatively reduced in the inner ear and activity of BDNF and NT-3 promoters is reduced in response to lower Brn-3c expression in sensory epithelial cell lines.

It is inconceivable that BDNF or NT-3 expression in the inner ear is *dependent* on Brn-3c since their temporal and spatial expression patterns are not exclusive and our own data show that BDNF is still present although reduced in *dll* mice. Both neurotrophins are known to play a role in patterning within the inner ear at times before the onset of Brn-3c expression and both are expressed in other cells within the inner ear whereas Brn-3c expression is limited to hair cells [12–19]. In addition, recent evidence suggests that abnormalities in innervation and axon pathfinding in Brn-3c knockout mice are much more subtle than the critical loss of innervation found in neurotrophin knockout mice [44]. A more likely role would be as a mediator of short term or local-

ised regulation either at a specific time point or in response to some stimulus to the hair cell. If Brn-3c does have a part in controlling hair cell derived neurotrophin expression as our in vitro data suggest it may be then that Brn-3 factors would be involved in controlling the expression of these neurotrophins from hair cells *and* of their receptors on the appropriate neurons [10]; Brn-3a, is involved in target field innervation and axon pathfinding in the inner ear by regulating expression of neurotrophin receptors in sensory neurons [10]. Such speculation is subjected to in vivo confirmation of our data and elucidation of the role of both Brn-3c and neurotrophin gene expression in survival of hair cells and sensory neurons in the inner ear. However, the data presented here do provide clear evidence of a molecular mechanism through which Brn-3c can regulate the expression of BDNF and NT-3.

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