Neurotrophin-3 is required for proper cerebellar development

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Neurotrophin-3 (NT-3) is a member of the neurotrophin family, which includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5). These factors are crucial for development of the peripheral nervous system[1], but not the central nervous system (CNS), except that NT-3 and BDNF have been implicated in the postnatal development of the cerebellum[2,3]. Here we created a conditional NT-3-deficient mutant, which showed abnormal cerebellar morphology.

Postnatal cerebellar development, which involves active proliferation and migration of neuronal precursors, is complete by postnatal day 21 (P21) in the mouse[4]. During this period, the neurotrophins BDNF and NT-3 are highly expressed in the cerebellum[5,6]. BDNF-deficient mutants generally die within the first two weeks of life, but cerebellar development can be assessed during this time window[7]. NT-3 -deficient mutants, on the other hand, rarely survive past P1 (ref. 8), preventing a meaningful assessment of postnatal cerebellar development. To circumvent this problem, we used the phage P1 cre recombinase-loxP system[9] to create an NT-3 conditional mutant that was viable, yet lacked NT-3 expression in the CNS.

A targeting vector (**Fig. 1a**) designed to introduce loxP sites around the *NT-3* coding exon (exon II)[10] was transfected into embryonic stem cells. The selection cassette was removed from homologously targeted clones by transient transfection of a cre recombinase expression vector, leaving loxP sites surrounding exon II (**Fig. 1c**; data not shown). Embryonic stem cells carrying this new allele of *NT-3,* referred to as *NT-3*^{2lox}, were used to generate a mouse line (data not shown). Mice bearing the *NT-3*^{2lox} allele were intercrossed, generating viable and fertile *NT-3*$^{2lox/2lox}$ homozygotes, without behavioral abnormalities (data not shown). To delete *NT-3* in the CNS, we crossed *NT-3*$^{2lox/+}$ mice to a transgenic strain bearing the cre recombinase gene expressed under the control of the rat nestin promoter/intron 2 enhancer (A.T., unpublished data). Nestin-cre-mediated recombination resulted in the deletion of *NT-3* exon II, leaving a single loxP site remaining in the genome (**Fig 1c**). As exon II contains the entire coding sequence of the protein, this *NT-3*^{1lox} allele is equivalent to the *NT-3*-null mutation[8]. Southern blot analysis detected cre-mediated recombination in whole embryos as early as 9.5 days post coitum (E9.5). Recombination increased during embryonic development such that it was nearly complete in brain and spinal cord by E15.5, whereas other tissues showed a much smaller extent of recombination (**Fig. 2a**). In adults, incomplete recombination was detected in a number of tissues, but the *NT-3*^{2lox} allele was almost fully recombined in the CNS (**Fig. 2b**) and in the germ line of mice that carried the nestin-cre transgene (data not shown). Therefore, to generate *NT-3* conditional mutants, *NT-3*$^{2lox/+}$ males bearing the nestin-cre transgene were crossed to *NT-3*$^{2lox/+}$ nontransgenic females. Conditional mutants generated from this cross carried the transgene and had the genotype *NT-3*$^{1lox/2lox}$ at the *NT-3* locus. Conditional mutants were produced in the expected numbers and were fertile (data not shown), indicating that the conditional mutation did not lead to perinatal lethality, unlike the *NT-3*-null mutation[8]. Northern blotting revealed no NT-3 mRNA in the brain and developing cerebellum of conditional mutants (**Fig. 2c** and **d**), demonstrating that the *NT-3*^{2lox} allele was fully recombined and no NT-3 was produced.

Because NT-3 is highly expressed during postnatal cerebellar development[2,6] (see also **Fig. 2d**) and can influence the survival and differentiation of developing cerebellar neurons[2], we examined the development of the cerebellum in the NT-3 conditional mutant. Like BDNF mutants[3], NT-3 conditional mutants had defective cerebellar foliation at P8, distinguished by the absence of folium VII and the posterior superior fissure, which separates it from folium VI (**Fig. 3a** and **b**). This defect was not simply due to a delay in folding, as folium VII was also absent at P28 and later (data not shown). Formation of the characteristic layers of the cerebellum and their associated cell types seemed to be unaffected in the conditional

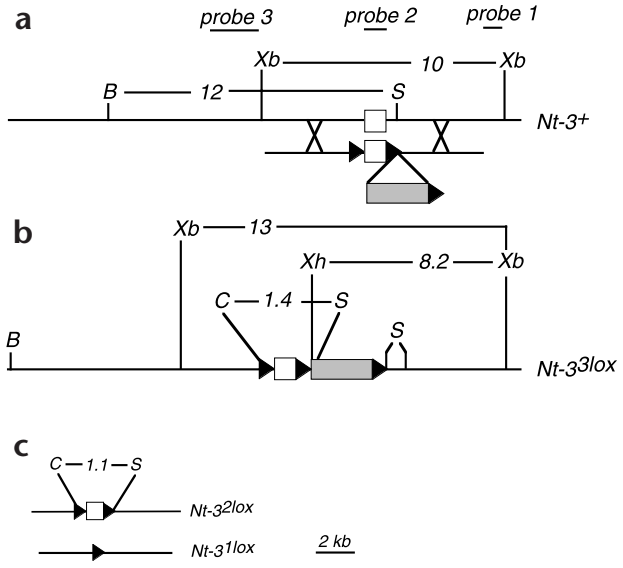

Fig. 1. Targeting strategy and Cre mediated recombination. **(a)** *NT-3* genomic locus (*NT-3*$^{+}$), with the homologous targeting vector depicted below. Filled triangles indicate loxP sites. Hatched rectangle indicates the cytomegalovirus-promoter-driven hygro^{r}/thymidine kinase fusion gene used for positive/negative selection. Probes used for detection of integration and recombination indicated above. **(b)** Structure of *NT-3* genomic locus after homologous integration of the targeting vector. This new allele is referred to as *NT-3*^{3lox}. **(c)** Top, *NT-3* allele resulting from cre-mediated recombination removing the selection cassette, referred to as *NT-3*^{2lox}. Bottom, the result of cre-mediated recombination of *NT-3*^{2lox}, referred to as *NT-3*^{1lox}. For (a–c), restriction sites (B, *Bam*HI; S, *Spe* I; Xb, *Xba* I; C, *Cla* I; Xh, *Xho* I) are shown, with the resulting fragment sizes given in kilobases (kb).

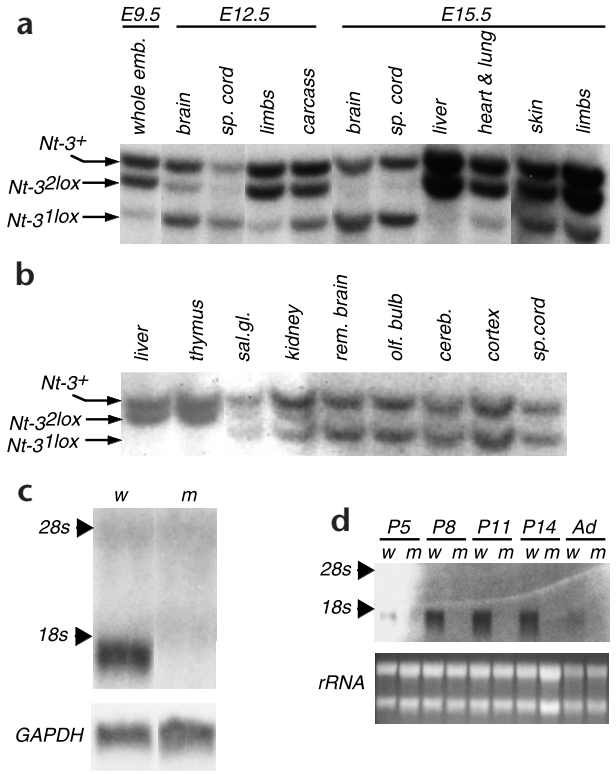

Fig. 2. Cre-mediated recombination and expression of *NT-3* in the conditional mutant. **(a)** Southern blot of embryonically derived samples that carried the nestin-cre transgene and had the genotype *NT-3*$^{2lox/+}$ at the *NT-3* locus. *NT-3*$^{+}$, *NT-3*^{2lox} and *NT-3*^{1lox} bands are indicated. Note the near-complete conversion of *NT-3*^{2lox} into *NT-3*^{1lox} by E15.5 in the brain and spinal cord. Whole emb., whole embryo; sp.cord, spinal cord; carcass refers to whole embryo minus head, liver, heart and limbs. **(b)** Southern blot showing recombination in an *NT-3*$^{2lox/+}$ adult. Rem. brain is the portion of the brain remaining after dissection into the other brain regions shown. Sal. gl., salivary gland; olf. bulb, olfactory bulb; cereb., cerebellum; cortex, cerebral cortex; sp. cord, spinal cord. **(c)** Northern blot comparing NT-3 mRNA in the whole brain of wild-type (w) and conditional mutant (m) mice. 28s and 18s indicate the position of the rRNA markers. Blot stripped and reprobed with GAPDH shown below. **(d)** Northern blot comparing NT-3 mRNA in cerebella of wild-type (w) and conditional mutant (m) mice at various postnatal time points. Ad, adult. rRNA loading control shown below.

mutant (**Fig 3c** and **d**). The external germinal layer, site of granule cell precursor proliferation, was present at P8. Cells in the proliferative zone of this layer incorporated 5-bromodeoxyuridine at P8 in wild-type mice and in conditional mutants (**Fig. 3e** and **f**). NT-3 has been implicated in differentiation of developing Purkinje neurons[2]. Purkinje neurons were present in the vermis of the conditional mutant, and although not quantified, they did not appear to be reduced in number. The elaboration of the Purkinje dendritic tree, as revealed by staining with an anti-calbindin D28 antibody, also appeared qualitatively normal (**Fig. 3g** and **h**).

During cerebellar development, excess granule neurons are produced and subsequently lost via apoptotic cell death. In the mouse, the peak of apoptosis in the cerebellum occurs around P8 (ref. 11). At this age, TUNEL staining showed that conditional mutants had 75% higher density of apoptotic cells in the granule cell layer (mutant mean ± standard error, 37.3 ± 11.4 cells/mm^2; wild-type, 21.3 ± 6.98 cells/mm^2; $p < 0.0001$, unpaired student's *t*-test) During this time, both astrocytes and granule neurons are undergoing apoptosis in the granule cell layer[12]. However, granule neurons express the NT-3 receptor TrkC[5], and their survival *in vivo* is enhanced by NT-3 (ref. 2), suggesting that this increase in apoptosis is due to granule neuron death. We observed no increase in TUNEL-positive cells in the external germinal layer, suggesting that granule neuron precursors did not require NT-3 for survival (mutant, 4.1 ± 0.31 cells/mm; wild type, 3.8 ± 0.26 cells/mm; $p = 0.38$, unpaired student's *t*-test).

We have used the cre/loxP system to create a conditional mutation that eliminated NT-3 expression in the CNS but did not cause early postnatal death. The absence of NT-3 in the developing CNS caused abnormal cerebellar development, providing evidence that it is required for the correct forma-

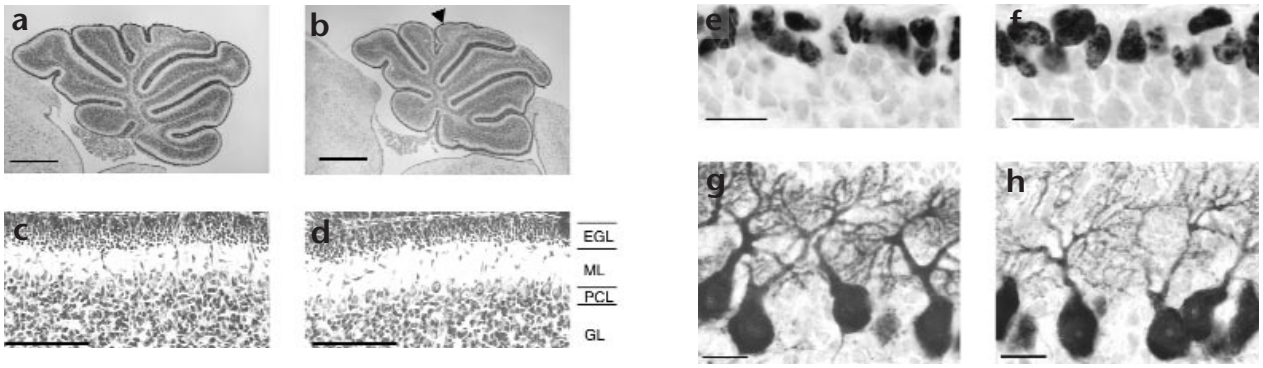

Fig. 3. Conditional mutants have aberrant cerebellar foliation but normal layering, external germinal layer proliferation and Purkinje arborization. **(a–d)** Paraffin-embedded, cresyl-violet-stained midsagittal sections through the cerebellar vermis of wild-type (a, c) and mutant mice (b, d) at P8. Triangle in (b) marks the area of the absent folium VII in the mutant. (c, d) Higher magnification showing the characteristic layers of the cerebellum at P8. EGL, external germinal layer; ML, molecular layer; PCL, Purkinje cell layer; GL, granule cell layer. Scale bars in (a) and (b), 500 µm; in (c) and (d), 40 µm. **(e, f)** Granule cell precursor proliferation assessed by injection of 5-bromodeoxyuridine (BrdU). Incorporation of BrdU is detected by immunohistochemistry using an antibody to BrdU in mutant (f) and wild-type mice (e). Scale bars, 20 µm. **(g, h)** Purkinje cells detected with an antibody to Calbindin D28. The mutant (h) appears qualitatively similar to wild-type (g) at P8.

tion of at least some components of the CNS. Indeed, this mutation was also associated with behavioral abnormalities, such as increased gait width and impaired balance (data not shown), consistent with a role for NT-3 in the development and maintainence of the cerebellar system. The finding that cell death was increased in the granule cell layer further suggests that NT-3 may act as a survival factor for a subset of granule neurons at P8. Because granule neurons express both NT-3 and TrkC[2,5,6], the factor may be acting in an autocrine or paracrine fashion. NT-3 is also expressed in the adult cerebellum[6](see also **Fig. 2d**), suggesting that it may be involved in adult cerebellar function and long-term neuronal survival. Apart from the well characterized activity of NT-3 as a neuronal survival factor, it has other effects on CNS neurons, including dendritic arborization[2,13] and synaptic plasticity[14]. This conditional mutant therefore will allow the exploration of other NT-3 functions in the adult CNS, as well as the efficacy of NT-3 substitution therapy.

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