The effect of the *ret-* mutation on the normal development of the central and parasympathetic nervous systems

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The *c-ret* proto-oncogene, a member of the receptor tyrosine kinase (RTK) superfamily, plays a critical role in tumour formation and embryonic organogenesis. Germ-line mutations have been identified in patients with multiple endocrine neoplasia types 2A and 2B (characterized by medullary thyroid carcinoma and pheochromocytoma) and familial medullary carcinoma (FMTC), as well as in patients with congenital megacolon (Hirschprung's disease, characterized by absence of a subset of enteric ganglia). Consistent with these studies in humans, targeted inactivation of the murine *c-ret* locus (*ret-*) results in kidney hypodysplasia and agenesis of the enteric and superior cervical ganglia (Schuchardt et al., 1994; Durbec et al, 1994a). A number of laboratories have recently established that glial cell line-derived neurotrophic factor (GDNF), a distant member of the TGF-β family of growth factors, is a functional ligand of the Ret receptor. Given the potent neurotrophic activity of GDNF on midbrain dopaminergic and motor neurons of the central nervous system (CNS), it was of interest to examine the potential *in vivo* role of *c-ret* in the development of the CNS.

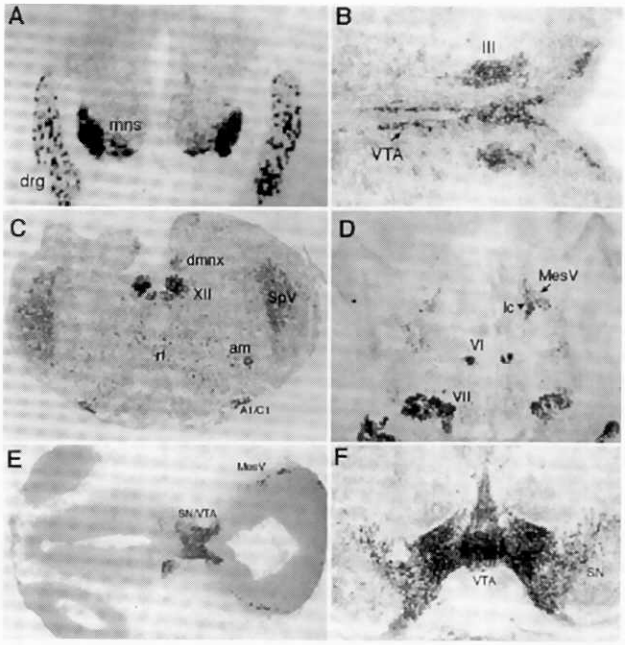

Fig 1. *c-ret* expression in transverse (A, B, E) and coronal (C, D, F) CNS sections. (A) E13.5 mouse embryo. Spinal motor neurons (mns) and dorsal root ganglia (drg) express *c-ret* transcripts. (B) Mesencephalic region of a neonate. Expression is detected in the ventral tegmental area (VTA) and the oculomotor nucleus (III). (C) Caudal brain stem region of a neonate. Expression is observed in the dorsal motor nucleus of the vagus (dmnx), hypoglossal nucleus (XII), nucleus ambiguus (am), spinal trigeminal nucleus (SpV), reticular formation (rf) and A1/C1 adrenergic cell group. (D) Pontine region of a neonate. The locus ceruleus (lc), facial (VII), abducens (VI) and mesencephalic trigeminal (MesV) nuclei express *c-ret*. (E and F) E12.5 and neonatal midbrain region respectively. *c-ret* staining is observed in the VTA and substantia nigra (SN).

c-ret expression in the CNS

To investigate the potential role of *c-ret* function in the CNS, we first characterized the distribution of *c-ret* mRNA in this system. Our results revealed that *c-ret* mRNA in the CNS is expressed predominantly in the developing spinal cord and brain stem (Fig 1).

In the spinal cord, *c-ret* expression was mainly detected in the lateral and medial somatic motor columns, and in the visceral motor column. Within the brain stem, high levels of *c-ret* expression were observed in cranial motor and sensory nuclei throughout embryonic, perinatal and adult development. In cranial motor neurons, *c-ret* is primarily expressed in branchiomotor and somatic motor nuclei and, to a lesser extent, in visceral motor nuclei. In the cranial sensory system, *c-ret* expression is restricted to the general somatic afferent column, including the neural crest-derived mesencephalic trigeminal nucleus and the spinal trigeminal nucleus.

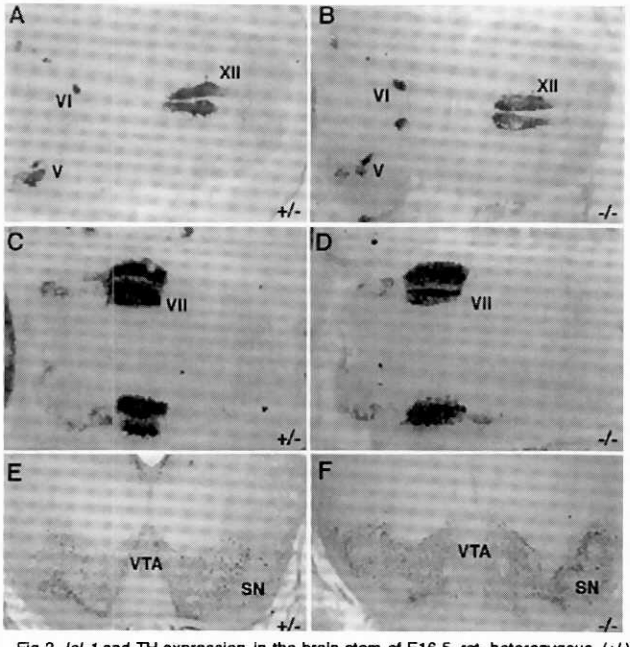

Fig 2. *Isl-1* and TH expression in the brain stem of E16.5 *ret-* heterozygous (+/-) and homozygous (-/-) mice. (A-D) The trigeminal (V), abducens (VI), facial (VII) and hypoglossal (XII) motor nuclei are not affected in -/- mice. (E, F) A normal complement of the VTA and SN is seen in -/- mice.

c-ret transcripts were also observed in the reticular formation and the main monoaminergic systems of the brain stem: serotonergic and catecholaminergic (dopaminergic, noradrenergic and adrenergic). Among these systems, the highest levels of *c-ret* expression were detected in the developing midbrain dopaminergic neurons, namely the substantia nigra (SN) and ventral tegmental area (VTA).

The CNS of *ret-* homozygous mice

To gain insight into the role of *c-ret* function in the CNS, we examined the development of *c-ret* positive cell groups in the CNS of *ret-* homozygous neonatal mice. For this purpose, we employed *in situ* hybridization and immunochemical methods using a variety of established neuronal markers (Fig 2).

To examine the development of midbrain dopaminergic neurons in *ret-* heterozygous and homozygous mice, we used tyrosine hydroxylase (TH) as a specific marker for this group of cells. As revealed by TH immunoreactivity, a normal complement of dopaminergic neurons in these cell groups was observed. Moreover, their axonal projections to the striatum also appeared normal.

To study of the effect of the *ret-* mutation on motor neurons, we examined the expression pattern of *isl-1* mRNA in the CNS by *in situ* hybridization. Our results indicate similar numbers of differentiated motor neurons are present in the brain stem and spinal cord of *ret-* heterozygous and homozygous mice.

Overall, our findings revealed that none of the CNS populations analysed are either absent or display any gross morphological

abnormalities in *ret*- homozygous mice.

Parasympathetic nervous system

Given the dramatic effect of the *ret*- mutation in the enteric nervous system (ENS), we wished to examine the function of *c-ret* in other parasympathetic ganglia. High levels of expression were detected normally in the ciliary, pterygopalatine, submandibular, otic, cardiac and pelvic ganglia. Furthermore, our findings revealed *c-ret* function is required for the development of at least a subset of parasympathetic ganglia. Using SCG10 and Phox-2 as specific cellular markers, we have observed the ciliary and pterygopalatine ganglia do not develop in *ret*- mutant mice (fig 3).

Conclusions

The spatiotemporal distribution of *c-ret* transcripts in the developing mouse CNS is complex and comprises a variety of cell populations of distinct embryonic origins. Overall, the highest levels of *c-ret* mRNA are detected in two cell populations highly responsive to GDNF: midbrain dopaminergic neurons and motor neurons. *c-ret* expression in GDNF-responsive tissues suggests the GDNF-ret signalling mechanism is involved in the specification and/or maintenance of these neuronal populations.

Analysis of *ret*- homozygous mice revealed that *c-ret* function is not required for the development of catecholaminergic, motor or serotonergic neurons during embryonic development. Similarly, GDNF-deficient embryonic mice also show no apparent morphological abnormalities in motor or midbrain dopaminergic neurons (Moore et al, 1996; Pichel et al, 1996; Sanchez et al, 1996;).

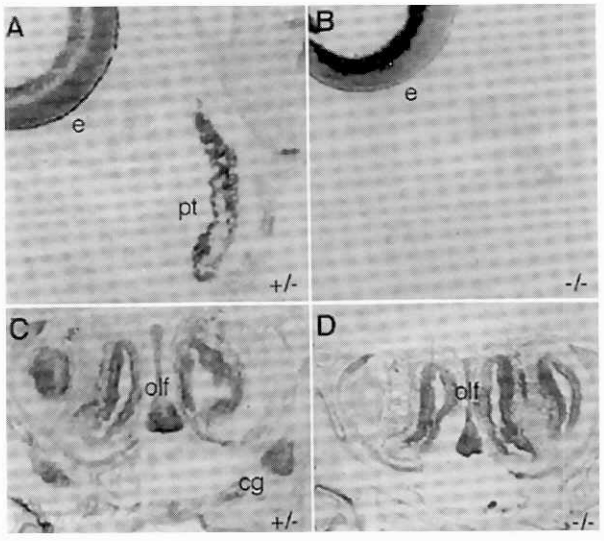

Fig 3. SCG10 expression in cranial parasympathetic ganglia of *ret*-heterozygous (+/-) and homozygous neonatal mice. (A-D) As indicated by SCG10 staining, *ret*-homozygous mice lack a normal complement of pterygopalatine (pt) and ciliary (cg) ganglia. Abbreviations: e, eye; olf, olfactory epithelium.

These findings strongly contradict the effects of exogenously supplied GDNF on midbrain dopaminergic neurons motor neurons *in vivo* and *in vitro*. To reconcile these apparently contradictory findings, two possibilities are being suggested. First, the GDNF-Ret ligand-receptor signalling system is required for the development of specific CNS populations after birth and not during embryogenesis. Given that GDNF and *c-ret* null mice die in their first day of life, the functional analysis of these genes thus far is limited to embryonic development. To study the role of GDNF and/or *c-ret* in the postnatal and adult CNS, the cre/loxP system could be used to disrupt these loci with precise spatiotemporal control. A second alternative possibility for the lack of a CNS phenotype in *ret*- mutants is that compensation occurs by means of other neurotrophin-neurotrophin receptor signalling system(s). It is possible that the GDNF-Ret signalling system acts synergistically with another ligand-receptor complex on neuronal survival in the CNS during normal development. Such complex could compensate for the loss of GDNF and *c-ret* function. For instance, known neurotrophic factors, such as CNTF, BDNF, NT-3 or NT-4/5, and their receptors, may play a role in such functional redundancy. Also, it is possible that other GDNF-like or *Ret*-like molecule might compensate for the loss of the GDNF-Ret signalling pathway. However, no such molecules have been reported so far.

We have shown that *c-ret* function is required for the neural development of a subset of parasympathetic neurons, namely the ciliary and pterygopalatine ganglia. These ganglia are derived from cranial neural crest. Interestingly, the other two main groups of autonomic neurons also affected by the *ret*- mutation, the ENS and superior cervical ganglia (SCG), are also cranial neural crest derivatives (Schuchardt et al., 1994; Durbec et al., 1996a). These findings suggest *c-ret* function is specifically required for the survival and/or differentiation of autonomic neurons of cranial neural crest origin and that the development of autonomic neurons derived from trunk neural crest is independent of *c-ret* function.

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