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# In vivo role for CREB signaling in the noradrenergic differentiation of sympathetic neurons

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ABSTRACT

Signaling pathways involving cAMP and CREB have been implicated in several aspects of sympathetic neuron differentiation. Here, we used in vivo loss-of-function approaches in both mouse and chick embryos to characterize the physiological role of cAMP/CREB. Whereas sympathetic neuron development proceeds normally in CREB-deficient mouse embryos, a decrease in noradrenergic differentiation (TH, DBH) was observed in chick sympathetic ganglia in response to ACREB, a dominant-negative CREB variant which interferes with the function of all CREB family members. In contrast, expression of the generic neuronal marker SCG10 was not affected by ACREB. As the decrease in noradrenergic gene expression is compensated at later stages of development and TH expression in differentiated neurons is not CREB-dependent, a transient role for CREB is proposed, accelerating noradrenergic but not generic neuronal differentiation of sympathetic neurons.



## Introduction

The development of noradrenergic peripheral neurons is elicited by bone morphogenetic proteins (BMPs) which act on neural crest progenitors to induce a group of transcription factors (Ascl1, Phox2b, Phox2a, Hand2, Gata2/3) that directly or indirectly control the expression of the subtype-specific genes tyrosine hydroxylase (*TH*) and dopamine-β-hydroxylase (*DBH*) and of pan-neuronal genes (*SCG10*, *neurofilament*, *βIII*-tubulin) (Goridis and Rohrer, 2002; Howard, 2005; Huber, 2006). Phox2b expression is observed at embryonic day 3 (E3) in the chick and E10.5 in the mouse, followed closely by TH and DBH expression (Tsarovina et al., 2004; Pattyn et al., 1999). During neurogenesis, sympathetic neuron number increases by progenitor differentiation and proliferation of immature neurons (Rohrer and Thoenen, 1987; Tsarovina et al., 2008; Hendershot et al., 2008; Schmidt et al., 2009). Subsequently, postmitotic sympathetic neuron number becomes dependent on neurotrophic support (Glebova and Ginty, 2005; Ernsberger, 2009). Interestingly, signaling by cyclic 3′-5′ adenosine monophosphate (cAMP) has been implicated both in sympathetic neuron differentiation and survival (Dupin et al., 1993; Lo et al., 1999; Maxwell and Forbes, 1990; Lonze et al., 2002). The canonical effects of cAMP on gene expression involve the binding of cAMP and consecutive activation of protein kinase A (PKA), which subsequently phosphorylates and activates the transcription factor CREB (cyclic AMP response element-binding protein) (Mayr and Montminy, 2001). In neural crest cultures, treatments to increase both cAMP levels and PKA activity potentiate the effect of BMPs and of undefined noradrenergic differentiation factors present in chick embryo extract (Bilodeau et al., 2000; Chen et al., 2005b; Dupin et al., 1993; Liu et al., 2005; Lo et al., 1999). Several regulatory mechanisms seem to mediate this effect, as cAMP/PKA signaling affects the expression of the transcriptional regulators *Phox2a* (Benjanirut et al., 2006; Bilodeau et al., 2000; Chen et al., 2005b; Liu et al., 2005) and *Hand2* (Liu et al., 2005) and controls the expression of marker genes like *TH* and *DBH* that characterize the noradrenergic transmitter phenotype (Adachi and Lewis, 2002; Bilodeau et al., 2000; Chen et al., 2005b; Dupin et al., 1993; Lo et al., 1999; Swanson et al., 1997; Swanson et al., 2000). The proposed role of cAMP signaling in the control of *Phox2a* expression is supported by chromatin immunoprecipitation analysis, which identified *Phox2a* as a target gene for CREB and CBP (Benjanirut et al., 2006). In line with these results, *Phox2a* and *DBH* expression are reduced by the expression of a dominant-negative CREB variant, acidic-CREB (ACREB) (Chen et al., 2005b; Swanson et al., 1997). ACREB contains an acidic amphipathic extension fused to the N-terminal of the CREB leucine zipper and acts as a dominant-negative factor for all CREB family members by interfering with their binding to the CRE site (Shaywitz and Greenberg, 1999). The activation of the *DBH* promotor by Phox2a is potentiated by cAMP/PKA and this effect is mediated by a CRE/AP-1 site, essential for the Phox2a-mediated reporter activity, as well as its

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potentiation by Hand2 (Adachi and Lewis, 2002; Swanson et al., 2000; Swanson et al., 1997; Xu et al., 2003). In addition to effects on gene transcription, cAMP/PKA signaling was also shown to control the phosphorylation of the transcription factors Phox2a and Hand2. Whereas Phox2a activity is increased by PKA-dependent dephosphorylation (Adachi and Lewis, 2002; Chen et al., 2005b), Hand2 activity is stimulated by PKA-dependent phosphorylation (Firulli et al., 2003; Liu et al., 2005).

The effects on *DBH* and *TH* expression of neural crest progenitors suggest that noradrenergic differentiation is a major target of cAMP/PKA. However, there is conflicting evidence to which extent also generic differentiation is affected or dependent on cAMP/PKA. In neural crest cultures, BMPs simultaneously elicit the expression of noradrenergic and generic neuronal genes (Reissmann et al., 1996; Shah et al., 1996; Varley and Maxwell, 1996; Varley et al., 1995). Interestingly, these effects not only involve canonical Smad1/5/8 signaling downstream of BMPs but also the stimulation of PKA activity which is essential in neural crest cultures for the development of both catecholaminergic and generic neuronal traits, i.e. βIII-tubulin (TuJ1) expression (Liu et al., 2005). In rat neural crest cultures, however, cAMP elicited TH expression in collaboration with Phox2a but was unable to induce also generic neuronal gene expression (Lo et al., 1999).

Although in vitro cAMP, PKA and CREB have been shown to play an important role in the development of noradrenergic neurons from neural crest progenitors, their in vivo function is still unclear. Previous analysis of the $CREB^{-/-}$ phenotype in the mouse peripheral nervous system focussed on survival and maintenance of differentiated postmitotic sympathetic neurons at later embryonic stages (Lonze et al., 2002; Parlato et al., 2007). The initial analysis of $CREB^{-/-}$ mouse embryos showed a reduction in the size of the superior cervical ganglia (SCG) at E15.5, suggesting CREB-mediated effects in sympathetic neuron survival or in neurogenesis (Lonze et al., 2002). A recent re-evaluation of this mouse line confirmed the reduction in SCG size but found the combined total size of SCG and stellate ganglia unaltered (Parlato et al., 2007). As the SCG is formed by rostral migration of sympathetic progenitors from the stellate ganglion primordia, a function of CREB in cell migration seems to underlie this phenotype (Parlato et al., 2007). The same study investigated the effects of a conditional *CREB* deletion in sympathetic neurons, using a

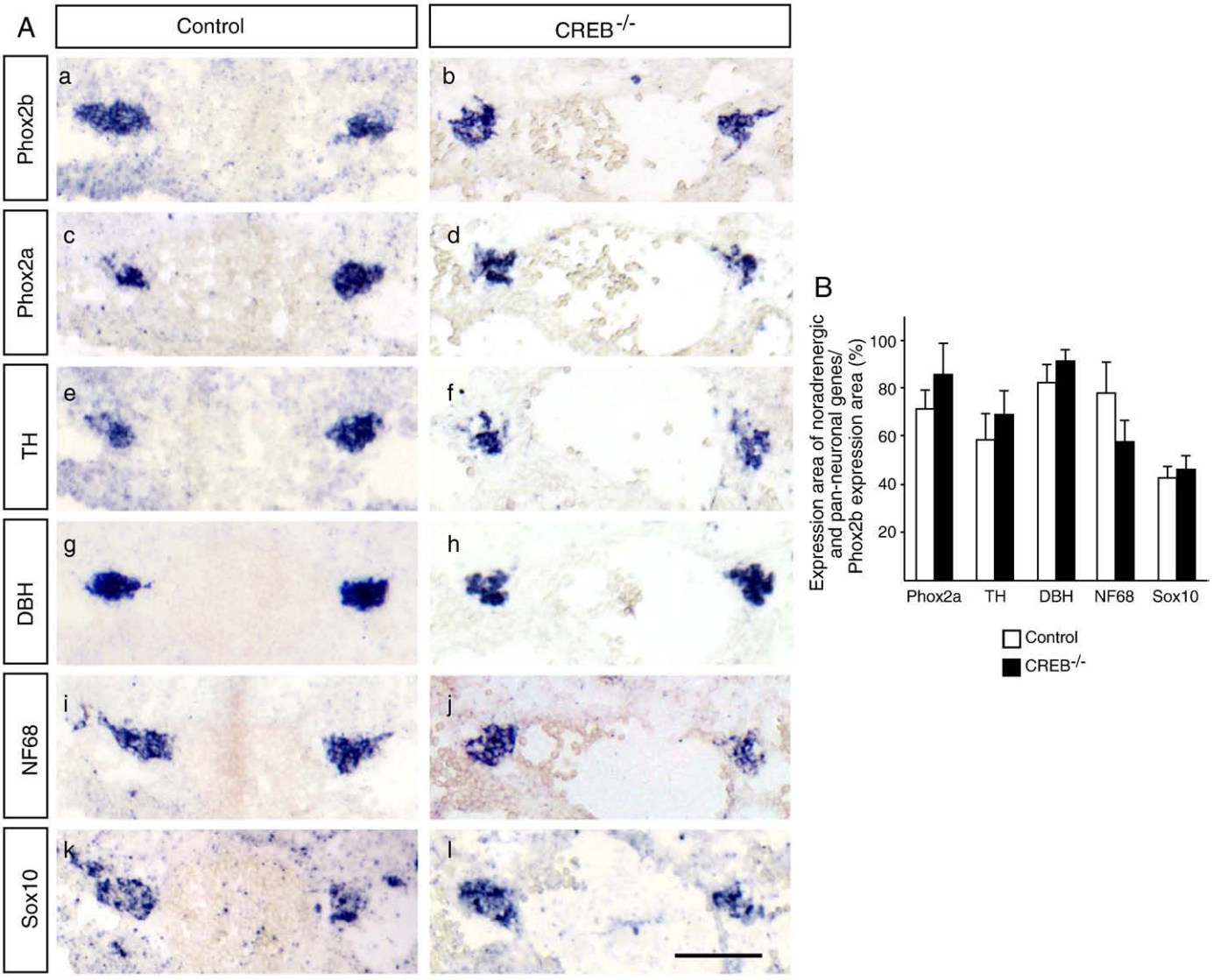


**Fig. 1.** Expression of noradrenergic and pan-neuronal marker genes is not affected in *CREB* null mice. (A) In situ hybridisation signals for *Phox2b*, *Phox2a*, *TH*, *DBH*, *NF68* and *Sox10* in sympathetic ganglia on sections from E10.5 wt and *CREB* null mouse embryos. (B) Morphometric quantification of the in situ hybridisation areas for the markers shown in (A). The values for *Phox2a*, *TH*, *DBH*, *NF68* and *Sox10* in wt and *CREB* null mice are compared to *Phox2b* (%). At least 4 embryos were analyzed for each parameter. Marker gene expression is unaltered in the absence of *CREB*. Scale bar: 200 μm.