Specific ablation of the transcription factor CREB in sympathetic neurons surprisingly protects against developmentally regulated apoptosis

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The cyclic-AMP response element-binding (CREB) protein family of transcription factors plays a crucial role in supporting the survival of neurons. However, a cell-autonomous role has not been addressed in vivo. To investigate the cell-specific role of CREB, we used as a model developing sympathetic neurons, whose survival in vitro is dependent on CREB activity. We generated mice lacking CREB in noradrenergic (NA) and adrenergic neurons and compared them with the phenotype of the germline CREB mutant. Whereas the germline CREB mutant revealed increased apoptosis of NA neurons and misplacement of sympathetic precursors, the NA neuron-specific mutation unexpectedly led to reduced levels of caspase-3-dependent apoptosis in sympathetic ganglia during the period of naturally occurring neuronal death. A reduced level of p75 neurotrophin receptor expression in the absence of CREB was shown to be responsible. Thus, our analysis indicates that the activity of cell-autonomous pro-survival signalling is operative in developing sympathetic neurons in the absence of CREB.

KEY WORDS: CREB, CREM, Sympathetic ganglia, Apoptosis, Mouse

INTRODUCTION

Cyclic-AMP response element-binding protein (CREB) is a ubiquitously expressed transcription factor and, together with the related proteins cAMP-responsive element modulator (CREM) and ATF-1 (also known as ATF1 – Mouse Genome Informatics), is activated via cAMP and several other signalling pathways supporting the survival, growth and plasticity of neurons throughout development and in adulthood (Lonze and Ginty, 2002).

The first mutation of the Creb gene (also known as Ceb1 – Mouse Genome Informatics) realized in mice, the so-called CREB<sup>−/−</sup> mutation, results in a viable hypomorphic allele (Blendy et al., 1996; Hummler et al., 1994). In contrast to the CREB<sup>−/−</sup> mutation, a null allele is lethal at birth because of postnatal lung failure (Rudolph et al., 1998). By conditional mutagenesis the specific deletion of the Creb gene in neural and glial precursors was achieved, and massive widespread apoptosis in the embryonic brain was observed when these mutants were also Crem deficient (Mantamadiotis et al., 2002). Moreover, specific deletion of the Creb gene in the postnatal forebrain resulted in selective and progressive neurodegeneration of the striatum and part of the hippocampus in the absence of CREM (Mantamadiotis et al., 2002). Analysis of these mutants also revealed that compensatory activities exist among these members of the CREB family (Mantamadiotis et al., 2002).

However, the cell-specific role of CREB in neuronal survival has not been addressed. We have recently demonstrated by conditional ablation of CREB in dopaminergic neurons only that the survival of dopaminergic neurons is weakly affected and that CREM upregulation does not contribute to the phenotype (Parlato et al., 2006).

To investigate the cell-specific role of CREB we used as a model developing sympathetic neurons. The sympathetic neurons have been a classical model for the study of the molecular mechanisms underlying neuronal survival activated by target-derived neurotrophins (Levi-Montalcini, 1987). During embryonic development neurons are produced in excess and their survival is controlled by the availability of target-derived growth factors. In many areas of the nervous system, programmed cell death is the predominant mechanism for determining mature neuron number. The final number of neurons is thus dependent on the balance between signals leading to either cell death or cell survival. In the peripheral nervous system this process to select surviving neurons is, in part, dependent on the nerve growth factor (NGF), acting through transcriptional regulation of gene expression.

A role for CREB in NGF-dependent survival has been clearly demonstrated for the sensory ganglia (Lonze et al., 2002). In vitro experiments performed on postnatal sympathetic neuronal cultures indicated that NGF-dependent survival requires CREB-mediated gene expression. These experiments also indicate that BCL-2 (also known as BCL2 – Mouse Genome Informatics) could be the pro-survival effector of CREB activity (Riccio et al., 1999). However, it remains to be established whether these in vitro observations reflect the role played by CREB in vivo in regulating the survival of sympathetic neurons. Analysis of the neurons in the superior cervical ganglia (SCG) of CREB knockout mice suggested the importance of CREB in the survival of sympathetic neurons during embryonic development (Lonze et al., 2002). However, because the number of sympathetic neurons is already affected before the acquisition of NGF-dependence for survival and the CREB protein is ubiquitously ablated, the crucial role of CREB in mediating survival of sympathetic neurons (Riccio et al., 1999) could not be unequivocally established in vivo using CREB knockout mice as a model (Lonze et al., 2002). Prior to the requirement of NGF, recent genetic evidence has indicated the importance of other extracellular...
cues, such as the glial cell line-derived neurotrophic factor (GDNF) family member artemin (Honma et al., 2002), in sympathetic axon outgrowth and directed neuronal migration via GFrα3 (Nishino et al., 1999) and RET (Enomoto et al., 2001). Thus, it is of great interest to analyze whether CREB-dependent signalling is of crucial importance not only for NGF-dependent survival, but also at earlier stages in the development and migration of sympathetic ganglia.

In order to investigate the role of CREB in developing sympathetic ganglia, we generated mouse mutants in which the Creb gene is deleted specifically in noradrenergic (NA) and adrenergic neurons of the central and peripheral nervous system (Creb<sup>fl/fl</sup>; DBHCre mice, abbreviated as Creb<sup>DBHCre</sup>) in a Crem- or Aft-I null genetic background. This specific mutation was made possible because we had developed transgenic mice faithfully expressing the Cre recombinase in cells under the control of the gene for dopamine-β-hydroxylase (DBHCre) using the PAC technology, which allows position-independent expression of the transgene (Casanova et al., 2001; Parlato et al., 2006; Wintermantel et al., 2002). This Cre line has also been used for the specific inactivation of the gp130 signalling in sympathetic neurons (Stanke et al., 2006).

The analysis of the specific ablation of CREB in comparison with the CREB knockout mice provides unexpected new insights into the cell-autonomous role of CREB, and demonstrates that loss of CREB unexpectedly results in neuroprotection.

**MATERIALS AND METHODS**

**Generation and genotyping of mutant mice**
A PAC harboring the DBH gene was isolated from the RPC121 mouse genomic library. Using ET recombination (Zhang et al., 1998), the coding sequence of the iCre, followed by the bovine growth hormone polyadenylation signal, was introduced in-frame with the ATG of the Dbh gene. The linear insert of 150 kb, carrying the transgenic construct, was released by Ncol digestion and separated by pulse field gel electrophoresis. The male pronucleus of fertilized C57Bl/6 mice was injected with the DNA purified by agarase treatment and microdialysis (Schedl et al., 1996). Transgenic offspring was identified by dot blot and hybridization with a probe specific for the iCre.

To exclude the possibility that CREM or Aft-1 may compensate CREB, double-mutant mice were also generated crossing Creb<sup>fl/fl</sup>;DBHCre<sup>+/–</sup> mice with Crem<sup>–/–</sup> or Aft-I<sup>–/–</sup> mice. The progeny of this first cross was then mated to yield F2 progeny of interest. Creb<sup>fl/fl</sup>Cre<sup>fl/fl</sup>DBHCre<sup>+/–</sup> mice were the following: forward 5‘-CTG-CCAGGGACATGGCCAG-3‘; reverse 5‘-GCACAGTCGAGGCTGA-3‘. The average number of caspase-3-positive cells per mm<sup>2</sup> was calculated for every fourth section per ganglion, spanning the entire SCGs, in at least four sections per side, because both SCGs were analyzed. Values shown are means±s.e.m. for at least four to five mice for each genotype. The volume of the SCG reported is the mean±s.e.m. for both SCGs in at least four to five mice per genotype. The total number of neurons per ganglia was determined by counting the neurons with visible nuclei in every fourth section. The total counts were quadrupled to calculate the total number of neurons. Values shown are means±s.e.m. Statistical significance was analyzed using a homoscedastic Student’s t-test. Values were considered significantly different with *P<0.05 and ***P<0.001.

The average number of caspase-3-positive cells per section was also calculated for each animal. In this case, values are means±s.e.m. for 12-15 sections per animal (both SCGs were analyzed in at least four to five mice of each genotype) (data not shown).

**RESULTS**

**Generation of CREB-deficient mice in NA neurons**
In order to study whether CREB-mediated signalling controls survival of sympathetic neurons, we used the Cre/loxP system to obtain mice with selective loss of CREB in these neurons. To faithfully drive the expression of the Cre recombinase, we used the regulatory regions of the DBH gene contained in a PAC clone (DBHCre) (Fig. 1A). To analyze Cre recombinase selectivity, DBHCre mice were crossed to the Rosa26 reporter line, in which recombination results in expression of the β-galactosidase gene (Soriano, 1999). β-galactosidase activity was revealed at E11.5 (Fig. 1B) in different regions of the developing sympathetic chain (Fig. 1B), as depicted in the areas (i), (ii) and (iii). Positive staining is also present in a region dorsolateral to the fourth ventricle where the precursors of the locus coeruleus (LC), the major NA neurons of the brain, are located (data not shown).

Mice in which exon 10 of the Creb gene is flanked by loxP sites (flxed Creb allele; Creb<sup>fl</sup>) (Mantamadiotis et al., 2002) were crossed to DBHCre mice to generate Creb<sup>fl/DBHCre</sup> mutants (Creb<sup>DBHCre</sup>). CREB immunoreactivity is already strongly reduced by E11.5 in the sympathetic chain of Creb<sup>DBHCre</sup> mutants (data not shown). Some experiments were found in the sympathetic chain at E11.5, because neurons located in the rostral part of the embryo show lower CREB expression compared with the caudal part. This
observation is consistent with the different timing in the maturation of the sympathetic chain, which follows rostro-caudal patterning (Hagedorn et al., 2000).

A more dramatic decrease in CREB immunoreactivity is evident in CrebDBHCre mice at E12.5 (Fig. 1E,H). The pattern of CREB loss is consistent with the expression of Cre (Fig. 1D,G), which reproduces the expression of DBH (Fig. 1C,F). At E17.5, CREB immunoreactivity is lost in most cells of the SCG, presumably neurons, but it is preserved in other cell types, as shown in Fig. 2A,B. The analysis of the SCG at E17.5 reveals no major alterations in size and morphology of NA neurons in the CrebDBHCre mutants (Fig. 2C,D). Unlike the CREB germline mutants (Creb−/−), the CrebDBHCre conditional mutants survive after birth without showing a reduced lifespan. We analyzed the synaptic projections of the sympathetic postganglionic axons from the SCG by whole-mount IHC with TH antibody at P2. As shown in Fig. 2E,F, the cutaneous sympathetic innervation of the eye is not impaired in the CrebDBHCre mutants.

**Germline deletion of Creb results in aberrant morphology of the sympathetic chain**

Because it is possible that the other member of the CREB family expressed in the central nervous system, CREM, compensates for CREB loss, we decided to generate mice that are CrebDBHCre; CREM−/−. These mutants also survive after birth and show no reduced lifespan or behavioural anomalies. The analysis of sympathetic ganglia, performed by IHC with an antibody against TH, at E15.5, a developmental stage independent of NGF for survival of sympathetic neurons, reveals that the SCG and the stellate ganglion in CrebDBHCre; Crem−/− (Fig. 3B,D) are properly shaped and placed in comparison to control littermates (Fig. 3A,C). At the same stage, in Creb-null mice, the overall organization of the sympathetic ganglia is severely affected, as shown in Fig. 3F. At E17.5, a smaller SCG in the Creb-null mutants, located in the cervical region (Fig. 3G,H, area circled in red), and a bigger stellate ganglion in the thoracic region (Fig. 3G,H) is seen.

**Specific ablation of Creb protects against developmentally regulated apoptosis**

To analyze the physiological relevance of the absence of CREB specifically in sympathetic neurons, we decided to analyze apoptotic neurons in the SCG and the stellate ganglia at different developmental stages in controls, Creb-null and CrebDBHCre; Crem−/− mutants (Fig. 4). Although we concentrate our studies on the SCG, which, because of its large size, its accessibility and its vascular supply has been classically used as
model system to study survival of sympathetic neurons, similar observations were also made in the stellate ganglia (data not shown).

Naturally occurring cell death in sympathetic ganglia starts in mice at E16-E17 (Coughlin and Collins, 1985), and indeed in mice that lack NGF, neuronal loss can be detected by E17.5 and at P0. This is associated with a 90% decrease in SCG volume, indicating that NGF action on sympathetic neurons takes place in this time window (Crowley et al., 1994; Francis and Landis, 1999). At E17.5, as revealed using IHC for activated caspase-3 in combination with the specific marker TH, the analysis of control embryos reveals the presence of apoptotic cells in the SCG. In CREB-null mice apoptotic cells are even more strongly represented (Fig. 4B,E). In CREB-null mice, it is not possible to clearly distinguish the SCG from the stellate ganglion, therefore we have measured the total volume of the SCG and stellate ganglia, identified by TH staining. Although no changes are revealed in the total volume of stellate ganglia and SCG between controls and mutants at E17.5 (data not shown), it is well possible that the increased apoptosis observed in the Creb-null mice at this stage would postnatally result in a smaller sympathetic ganglia. Surprisingly, in CrebDBHCre; Crem−/− mutants, in contrast to CREB-null mice, there is no increased apoptosis (Fig. 4C,F), rather a decrease in the number of caspase-3-positive cells in the SCG.
summarized in Fig. 4G. In order to exclude the possibility of an earlier onset of apoptosis in CrebDBHCre; Crem−/−, we searched for the presence of apoptotic cells at E15.5, but undetectable levels of apoptosis in the SCG characterize this early stage without significant differences between genotypes (data not shown). Because the CrebDBHCre; Crem−/− mutants, in contrast to the germline CREB mutation, are not postnatally lethal, we determined the number of caspase-3-positive cells at postnatal stages P0/P2 (n=4-5 per genotype). We observe a reduced level of apoptosis in the control SCG in comparison with E17.5, with no changes in the CrebDBHCre; Crem−/− mutants. When we measured the total volume of the SCG at E17.5 there were no significant differences between controls and CrebDBHCre; Crem−/− mutants (Fig. 4H). At P0/P2 we found that there is an overall increase in the size of the SCG, consistent with the reduced number of apoptotic cells occurring at E17.5 in CrebDBHCre; Crem−/− (Fig. 4H). The number of SCG neurons at P2 is significantly increased (control: 11014±1611; CrebDBHCre; Crem−/− mutants: 19661±1905, P<0.05). These results clearly indicate that the specific loss of CREB in developing sympathetic neurons not only does not lead to decreased neuronal survival, but unexpectedly has a protective effect against developmentally regulated apoptosis.

**Loss of CREB is sufficient to inhibit developmentally regulated apoptosis**

In order to establish the role of CREM or ATF-1 in survival of sympathetic neurons, we have compared the number of caspase-3-positive cells at E17.5 (n=3 per genotype) in CrebDBHCre mutants as well as in CrebDBHCre; Crem+/−, CrebDBHCre; Crem−/−, and CrebDBHCre; Atf-1−/− (Fig. 5). Independent of the presence of the CREM or ATF-1 alleles, we observed a similar decrease in the number of apoptotic cells in all the mutants in comparison with the respective controls. These data indicate that loss of CREB alone accounts for the decreased apoptosis observed in the conditional mutant embryos, as already suggested by lack of CREM and ATF-1 immunoreactivity at this developmental stage in sympathetic ganglia (data not shown). Unlike in other cell types (Bleckmann et al., 2002; Mantamadiotis et al., 2002), neither CREM nor ATF-1 play a crucial role in the survival of sympathetic neurons.

**p75NTR expression in sympathetic neurons depends on CREB**

To shed light on the molecular mechanisms underlying the reduced levels of apoptosis observed in the conditional CREB mutants, we reasoned that the expression of crucial factors promoting developmental death of sympathetic neurons might be CREB-dependent. It is well established that the p75NTR plays an important role in promoting apoptosis of sympathetic neurons lacking appropriate levels of target-derived neurotrophins, such as NGF (Bamji et al., 1998; Majdan and Miller, 1999). Interestingly, the phenotype of the CREB conditional mutants is reminiscent of the phenotype of p75NTR−/− mice, also showing an increase in the relative number of sympathetic neurons (Brennan et al., 1999).

Therefore, we have analyzed by in situ hybridization the expression of several potential CREB target genes involved in the p75NTR-mediated signalling in sympathetic ganglia of control and conditional mutants.
CREbDBHCre; Crem–/– mutants at E17.5 (Fig. 6). At this developmental stage, we found that in the conditional mutants (Fig. 6B) the levels of p75 NTR mRNA are much lower than in control embryos (Fig. 6A), whereas they are unaltered in other regions not affected by the mutation, such as the cholinergic neurons of the basal forebrain (Fig. 6C,D and data not shown). The reduced level of p75 NTR protein in the CREbDBHCre; Crem–/– mutants (Fig. 6F) in comparison with control littersmates (Fig. 6E) probably accounts for the inhibition of developmentally regulated apoptosis observed in sympathetic ganglia of the conditional CREb mutant.

**DISCUSSION**

The specific role of CREB-dependent gene expression in survival of specific populations of neurons is so far poorly understood. Using the CREb germline mutation along with the conditional line lacking CREB only in NA and adrenergic neurons, we addressed the question of whether CREB-mediated transcriptional activity is necessary for the survival of restricted neuronal subtypes, using developing sympathetic ganglia as a model system.

This analysis revealed that increased cell death is associated with misplacement of sympathetic ganglia in the CREb germline mutant. Because both effects are not observed in the conditional mutant, CREB expression in cells other than sympathetic neurons is required for neuronal survival and migration. The characterization of the conditional mutant reveals a novel distinct effect of neuronal survival upon loss of CREB. Indeed, reduced levels of developmentally regulated apoptosis are found in the sympathetic ganglia, resulting in an increased number of sympathetic neurons. This effect is correlated with reduced activation of caspase-3 and downregulation of the p75 NTR, a signal mediator necessary for apoptosis in sympathetic neurons.

**Germline loss of CREB results in misplacement and reduced survival of sympathetic neurons**

The results presented in our study are summarized in Fig. 7 by a schematic model of the sympathetic neuron development in wild type and in the different CREB mutants analyzed here. Developing wild-type neurons successfully reaching their targets survive in the presence of an optimal supply of pro-survival factors. Here, we oversimplify considering that NGF, secreted by the targets of sympathetic ganglia during the period of target competition, plays a major role in defining the final number of surviving sympathetic neurons. In CREb–/– mutants, CREB expression is lost in developing neurons, as well as in target cells. Loss of CREB in target cells may be primarily responsible for the migrational deficit of the SCG neurons, and hence responsible for the increased apoptosis in sympathetic neurons in the absence of proper survival signals originating in target cells. Developing sympathetic neurons, prior to NGF action, require migrational cues to establish their final position (Glebova and Ginty, 2005). Interestingly, gene targeting studies revealed that mice deficient in RET (Enomoto et al., 2001) or its cofactor GFRα3 (Nishino et al., 1999) or the ligand artemin exhibit severe defects in the SCG (Honma et al., 2002) during the period from E12.5 to E13.5. As in CREb-null mice, the SCG is found caudal to its normal location in all these mutants, because neuronal precursors of the sympathetic system fail to migrate and to project axons properly. These primary deficits lead to mis-routing of sympathetic nerve trunks and accelerated cell death of sympathetic neurons later in development. Because CREB phosphorylation is not only driven by NGF, but also by GDNF via its receptor tyrosine kinase RET involving the Ras/ERK pathway for activation (Hayashi et al., 2000), this signalling pathway could be affected by loss of CREB. The fact that in the conditional mutant, the SCG is correctly positioned, despite early loss of CREB, strongly suggests that CREB expression is required in cells other than neurons for proper development of sympathetic ganglia. Because in null mutants CREB ablation also occurs in cells other than neurons, extraneuronal cues may depend on CREB for their expression.

**A cell-autonomous role of CREB in survival of sympathetic neurons is indicated by the conditional mutant**

In the sympathetic ganglia of the conditional mutants lacking CREB only in NA neurons, we evaluate the cell-autonomous role of CREB activity in pro-survival and pro-apoptotic pathways, because the neurotrophic supply from target organs is probably unaffected. Previous work performed on postnatal sympathetic neuronal cultures in which CREB-mediated gene expression is abolished by the use of a dominant-negative protein indicates that all three CREB family members contribute to the survival of sympathetic neurons (Riccio et al., 1999).

In the present study we focused on developmentally regulated cell death and we observed that in the conditional CREB mutants lacking either CREM or ATF-1, this process is inhibited, as evidenced by the reduced number of activated caspase-3-positive cells in sympathetic ganglia. Despite what has been previously shown in other CREB mutants regarding the possibility of compensation by other family members (Bleckmann et al., 2002; Mantamadiotis et al., 2002), loss of CREB and CREM or ATF-1 does not result in a more severe impairment of cell survival (Fig. 5). Although neither CREM nor ATF-1 is expressed in sympathetic ganglia from controls and conditional mutants at E17.5 (data not shown), we cannot rule out the possibility that inactivation of all three transcription factors
would result in increased cell death. The generation of transgenic mice expressing a dominant-negative CREB protein exclusively in sympathetic neurons could represent a valuable tool to address this issue.

To our knowledge, this is the first example of mutants in which CREB ablation leads to protection against cell death. Several hypotheses can be taken into account to explain this observation. An imbalance between pro-apoptotic signals and pro-survival signals during such a crucial time-window could result in an increased number of surviving sympathetic neurons. It has been indicated that CREB may regulate a pro-survival factor, such as Bcl-2 (Lonze and Ginty, 2002; Riccio et al., 1999). However, although in vitro experiments established that Bcl-2 is an important regulator of survival of sympathetic neurons after NGF deprivation (Greenlund et al., 1995), inactivation of Bcl-2 itself in mouse mutants (Michaelidis et al., 1996) did not result in increased death of sympathetic neurons during naturally occurring cell death starting at E16-E17 in mice (Coughlin and Collins, 1985).

Interestingly, the phenotype of the CREB conditional mutants is reminiscent of the phenotype of Bdnf−/− mice (Bamji et al., 1998), showing an increase in the relative number of sympathetic neurons. The brain-derived neurotrophic factor (BDNF) is a well-characterized CREB target (Shieh and Ghosh, 1999; Shieh et al., 1998; Tao et al., 1998). This neurotrophin may play a dual role in the fate of developing neurons, either promoting their survival or their apoptotic death. In sympathetic neurons, BDNF signalling may inhibit axonal growth and neuronal survival through the p75NTR, because its receptor TrkB is not expressed in sympathetic neurons (Bibel and Barde, 2000). In vitro experiments indicate that, when sympathetic neurons are exposed to suboptimal survival signals, activation of p75NTR by BDNF leads to neuronal apoptosis. Although p75 signalling mechanisms remain poorly understood, loss of such a mechanism during the period of cell death could explain the increased number of sympathetic neurons observed in the Bdnf−/− mice (Bamji et al., 1998) and in p75−/− mice (Brennan et al., 1999). The similarity between phenotypic alterations suggests a correlation between CREB and BDNF/p75NTR signalling. Interestingly, the p75NTR gene is included, among other signalling molecules, in a comprehensive study aiming to identify CREB targets by an approach based on chromatin immunoprecipitation and a modification of SAGE (Impey et al., 2004). Although the functional role of CREB in regulating p75NTR gene expression has not been demonstrated, our finding that lower levels of p75NTR expression are shown in developing sympathetic neurons of the conditional CREB mutants, indicates that CREB is required for p75NTR expression. Consequently, lower levels of p75NTR expression leads to decreased apoptosis, resulting in enlarged sympathetic ganglia after birth.

In summary, we conclude that CREB expression in cells other than sympathetic neurons is required for proper shaping of the sympathetic chain and for controlling neuronal survival, as illustrated by the comparison between germline and conditional Creb mutants. Indeed, loss of CREB in developing sympathetic neurons neither affects their position nor impairs their survival. Unexpectedly, loss of CREB exclusively in developing sympathetic neurons results in a protective effect against developmentally regulated apoptosis because of downregulation of p75NTR expression.

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