Development of Experimental Tools to Analyze the Function of the TGF-β-Activated Kinase (TAK1) in Neurons *in vivo* and *in vitro*

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Development of Experimental Tools to Analyze the Function of the TGF-β-Activated Kinase (TAK1) in Neurons *in vivo* and *in vitro*

Abstract: The development of the nervous system requires the spatial and temporally controlled elimination of supernumerary neurons and their precursors. A balance between pro-survival and pro-death signals tightly regulates this programmed cell death, termed apoptosis. These signals can be executed by the c-Jun amino-terminal kinase (JNK) and nuclear factor-κB (NF-κB) pathways, which either inhibit or activate the apoptotic machinery. An important activator of both JNK and NF-κB in the immune system is the TGF-β-activated kinase (TAK1). To test whether TAK1 plays a similar role in the developing nervous system, we developed a genetic strategy to generate embryos with a neuron-specific deletion in *Tak1*. We also developed molecular tools to suppress TAK1 function in neurons *in vitro* and *in vivo*. By immunohistochemistry (IHC), we determined that TAK1 is expressed in all neuronal layers of the postnatal day (P)9 cerebellum. We also tested and optimized IHC protocols on paraffin-embedded embryos for different antibodies that could be useful for the analysis of the nervous system of mutant embryos.

Développement d'Outils Expérimentaux pour l'Analyse du Rôle de la Kinase TGF-β-Activated Kinase (TAK1) dans les Neurones *in vivo et in vitro*

Résumé: L'élimination ciblée par apoptose de neurones excédentaires et de leurs précurseurs est nécessaire à l'organisation du système nerveux pendant le développement. Des facteurs de survie et des facteurs pro-apoptotiques influencent la survie des neurones. Ces signaux contrôlent l'activation des exécuteurs de l'apoptose à travers les voies de signalisation de la c-Jun amino-terminal kinase (JNK) et du nuclear factor-κB (NF-κB). Dans le système immunitaire, la kinase TGF-β-activated kinase (TAK1) est un activateur central de ces voies de signalisation. Nous avons développé une stratégie pour tester l'hypothèse selon laquelle TAK1 jouerait un rôle similaire dans le système nerveux. Premièrement, nous avons tenté de supprimer TAK1 par délétion *in vivo*, plus particulièrement dans les neurones durant l'embryogenèse. Deuxièmement, nous avons développé des outils moléculaires pour tenter de supprimer TAK1 dans des neurones *in vitro* et *in vivo*. Par immunohistochimie (IHC) sur cervelets imprégnés de paraffine, nous avons déterminé que TAK1 était exprimée dans toutes les régions neuronales du cervelet en développement. Finalement, nous avons testé et optimisé des protocoles d'IHC sur embryons inclus en paraffine pour plusieurs anticorps qui pourraient être utiles pour l'analyse du système nerveux d'embryons mutants.

List of Scientific Abbreviations

293T-HEK	293T human embryonic kidney
AP-1	Activating protein-1
Apaf-1	Apoptosis protease-activating factor-1
ATF2	Activating transcription factor 2
ATP	Adenosine triphosphate
AAV	Adeno-associated virus
Bcl-2	B cell lymphoma-2 protein
BDNF	Brain-derived neurotrophic factor
BH	Bcl-2 homology
Bim_{EL}	Bim extra-long
BMP	Bone morphogenic protein
Caspase	Cysteine aspartate proteases
CGN	Cerebellar granule neuron
CMV	Cytomegalovirus
CNS	Central nervous system
DLK	Dual leucine zipper-bearing kinase
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
dTAK1	*Drosophila* TAK1
E1	Embryonic day 1
EGFP	Enhanced GFP
EGL	External granule layer
ERK	Extracellular-regulated kinase
EmGFP	Emerald GFP
FasL	Fas ligand
GFP	Green fluorescent protein
HIV	Human immunodeficiency virus
IAP	Inhibitor of apoptosis protein
IGL	Internal granule layer
IHC	Immunohistochemistry
IKK	IκB kinase
IκB	Inhibitor of κB
IL	Interleukin
Imd	Immune deficiency
JNK	c-Jun amino-terminal kinase
KCl	Potassium chloride
kDa	Kilodalton
LTP	Long-term potentiation
LTR	Long terminal repeat
LV	Lentivirus
MAPK	Mitogen-activated protein kinase
MAP2K	MAPK kinase

MAP3K	MAPK kinase kinase
MAP4K	MAPK kinase kinase kinase
MEF	Mouse embryonic fibroblast
MEKK	Mitogen ERK kinase kinase
miRNA	MicroRNA
MKK	MAP kinase kinase
ML	Molecular layer
MLK	Mixed-lineage kinase
MnSOD	Manganese superoxide dismutase
mRNA	Messenger RNA
Mst3b	Mammalian Ste20-like Kinase
NeuN	Neuronal nuclei
NF-κB	Nuclear factor-κB
NF-M	Neurofilament-M
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartic acid
NT	Neurotrophin
NTD	Neural tube defect
P1	Postnatal day 1
P75NTR	P75 neurotrophin receptor
PC12	Pheochromocytoma cell line 12
PCD	Programmed cell death
PI3-K	Phosphatidylinositol 3-kinase
PL	Purkinje layer
PLC	Phospholipase C
PNS	Peripheral Nervous System
Pro-NT	Pro-neurotrophin
RISC	RNA-induced silencing complexes
RNA	Ribonucleic acid
RNAi	RNA interference
RRE	Rev responsive element
SA	Splice acceptor site
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
siRNA	small interfering RNA
TAK1	TGF-β-activated kinase
TCA	Trichloroacetic acid
TCR	T-cell receptor
TGF-β	Transforming growth factor-β
TNF	Tumor necrosis factor
TNFR	TNF receptor
Trk	Tropomyosin-related kinase
TRPC	Transient receptor potential channel
VSV-G	Vesicular stomatitis virus G glycoprotein
X-Gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

Chapter 1

Introduction and Literature Review

Introduction

In patients affected by neurodegenerative conditions, such as Alzheimer's disease, the progressive and irreversible loss of neurons leads to cognitive decline. This neuronal death is thought to occur through an active mechanism of cellular suicide, termed apoptosis (Kermer et al., 2004). Although highly detrimental in pathological conditions, apoptosis plays an essential role in the development of the brain and spinal cord. A better understanding of the molecular events regulating apoptosis in the nervous system may therefore guide us in the development of more targeted and efficient treatments to halt neurodegeneration.

Apoptosis in the Developing Nervous System

Apoptosis of neurons and the cells that give rise to neurons (neuronal progenitors) is essential to the formation of the central and peripheral nervous system (CNS and PNS) (Lossi et al., 2003). Three periods of neuronal apoptosis have been described in the developing nervous system. The first period corresponds to the closure of the neural tube between embryonic day (E) 8.5 and E10 in mice (Copp, 2005). The neural tube is the embryonic structure that will give rise to the entire CNS. It originates from the neural plate, which in turn derives from the dorsal midline ectoderm. To form the neural tube, the neural plate buckles at its midline, and its edges, the neural folds, elevate and fuse. This complex process depends on the coordinated apoptosis of neuroepithelial cells in some regions of the neural folds and proliferation in others (Copp, 2005; Lawson et al., 1999). Disruption of this process can lead to an open spine, a neural tube defect (NTD) seen in newborns with spina bifida (Detrait et al., 2005).

After neural tube closure, apoptosis was observed in the ventricular zone of the neural tube, the region where neuronal progenitors divide and give rise to immature post-mitotic neurons (neuroblasts). The cells undergoing apoptosis during this second period are likely neural progenitors and neuroblasts (Blaschke et al., 1996; Blaschke et al., 1998; de la Rosa and de Pablo, 2000). Genetic disruption of this process resulted in NTDs characterized by the overproduction of neurons and severe brain overgrowth, termed exencephaly (Cecconi et al., 1998; Hakem et al., 1998; Kuida et al., 1996; Kuida et al.,

1998; Pompeiano et al., 2000; Yoshida et al., 1998). It is not clear why so many progenitors and neuroblasts are generated only to be eliminated shortly after.

A third wave of apoptosis affects more mature neurons as they project axons towards their target. This wave is thought to be important for matching the number of neurons to the size of the target tissue. It is generally assumed that about half the neurons generated during periods of neurogenesis are later lost by apoptosis, and that this process affects most neuronal populations at some point during development. For example, motorneurons of the spinal cord undergo apoptosis between E11.5 and E18.5, in a rostral to caudal wave (Yamamoto and Henderson, 1999), while dorsal root ganglia (DRG) neurons die between E11.5 and E15.5 (Farinas et al., 2002), and cerebellar granule neurons (CGNs) die within the first week after birth (Wood et al., 1995).

Apoptotic Versus Non-Apoptotic Neuronal Death

Apoptosis comprises a stereotyped series of cellular and molecular events that are highly conserved among eukaryotic cells. Kerr and colleagues defined morphological features that allow distinguishing apoptotic cells from cells dying by necrosis, a form of death associated with injury (Kerr et al., 1972; Lossi et al., 2003). These hallmarks include nuclear and cellular condensation, followed by nuclear fragmentation, and finally cellular fragmentation into membrane-bound apoptotic bodies that are endocytosed by nearby cells. Molecular indicators of apoptosis are the activation of cysteine aspartate proteases (caspases), the fragmentation of deoxyribonucleic acid (DNA), and the externalization of the phospholipid phosphatidylserine.

Apoptosis is essential for the normal development and homeostasis of an organism. Necrosis, on the other hand, is considered a non-physiological or "accidental" form of death, and is associated with injury and pathological conditions. Contrary to necrosis, apoptosis does not involve an inflammatory response and is therefore particularly well suited to participate in normal tissue turnover and development. However, this dichotomy between apoptosis and necrosis is an oversimplification. Alternative forms of neuronal death, sharing some morphological features of apoptosis and necrosis, have been discribed (Leist and Jäättelä, 2001; Oppenheim et al., 2001; Yuan

et al., 2003). Aberrant apoptosis can also contribute to many pathological conditions, such as Alzheimer's or Parkinson's disease (Kermer et al., 2004).

Molecular Machinery Necessary for the Execution of Apoptosis

Elegant genetic studies in the nematode worm *C. elegans* led to the discovery of the molecular machinery necessary for the execution of apoptosis (Liu and Hengartner, 1999). The caspases are responsible for the morphological and molecular changes observed during apoptosis. In particular, caspases 3, 6 and 7, the "effector" caspases, deconstruct the cell by cleaving vital proteins at aspartate residues. They are synthesized as zymogens, inactive pro-enzymes, and are activated upon cleavage by "initiator" caspases 8 or 9, also zymogens. The initiation of apoptosis can occur by two pathways (Dempsey et al., 2003). In the extrinsic apoptotic pathway, oligomerization of pro-caspase 8 in a complex with death receptors, such as the tumor necrosis factor receptor (TNFR) family members TNFR1 and Fas, leads to the auto-proteolytic activation of caspase 8, which then converts pro-caspase 6 to its active form. In the intrinsic apoptotic pathway, loss of mitochondrial integrity results in the release of cytochrome c, which aggregates pro-caspase 9 and its adaptor, the apoptotic protease activating factor-1 (Apaf-1), in a complex forcing auto-proteolytic activation of caspase 9, which can then activate caspase 3 (Yuan, 1995; Liu and Hengartner, 1999). The two apoptotic pathways are not mutually exclusive. For example, the p75 neurotrophin receptor (p75NTR), also a TNFR family member, does not activate caspase 8, but instead activates the intrinsic apoptotic pathway by the c-Jun amino-terminal kinase (JNK)–dependent phosphorylation of Bim_{EL} and Bad. These "BH-3 only" Bcl-2 proteins then render the mitochondria permeable and cytochrome c is released (Becker et al., 2004; Bhakar et al., 2003). Also, caspase 8 can directly activate the Bcl-2 family member Bid by cleavage (Gross et al., 1999; Li et al., 1998; Luo et al., 1998).

Genetic studies strongly implicate the intrinsic apoptotic pathway in developmental apoptosis in the brain, while evidence for a similar role for the extrinsic apoptotic pathway is lacking. Mutations in the genes coding for Apaf-1, caspase 3 and 9, resulted in a reduction of apoptosis in proliferative and post-mitotic regions of the

developing brain, and caused severe exencephaly (Cecconi et al., 1998; Hakem et al., 1998; Kuida et al., 1996; Kuida et al., 1998; Pompeiano et al., 2000; Yoshida et al., 1998). Surprisingly, the developing spinal cord and brainstem were not affected. The presence of a non-apoptotic form of programmed cell death (PCD) could explain this unexpected result (Leist and Jäättelä, 2001; Oppenheim et al., 2001; Yuan et al., 2003). So far, there is no genetic evidence that the extrinsic pathway contributes to apoptosis in the developing nervous system. Mice in which the gene coding for caspase 6 was knocked out apparently developed normally (Zheng et al., 1999). *Caspase 8* knockout mice showed a "wavy" neural tube phenotype, which together with several other developmental defects present in these mice, was most likely secondary to vascular and placental defects (Kang et al., 2004; Sakamaki et al., 2002).

Signals Regulating Apoptosis in the Developing Nervous System

Pro-Survival Signals: the Neurotrophic Factor Hypothesis

A fine balance between pro-survival and pro-death signals is necessary to ensure time and region-specific apoptosis in the developing nervous system. Seminal work by Rita Levi-Montalcini and Victor Hamburger has provided the conceptual framework for understanding the events controlling the survival of projecting neurons (Hamburger and Levi-Montalcini, 1949). Their neurotrophic factor hypothesis presented the idea that an excess number of neurons compete for a limited supply of trophic support from their target tissue. Only neurons receiving sufficient trophic support survive. The remainder die.

The first neurotrophic factor to be identified was the neurotrophin nerve growth factor (NGF). More than a dozen neurotrophic factors have been discovered since, including the neurotrophins brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, and NT-4, as well as the transforming growth factor-β (TFG-β) family and the interleukin-6 (IL-6) family. Genetic studies elucidated some of the region- and time-specific requirements for the different neurotrophic factors. Also, neurotrophic factor receptors were identified, as well as the signaling pathways they initiate. Neurotrophins, for example, bind to the tropomyosin-related kinase (Trk) and p75NTR to activate several

survival pathways, such as the extracellular signal-regulated kinase (ERK) pathway and the phosphatidylinositol 3-kinase (PI3-K) pathway (Roux and Barker, 2002).

The apoptosis of neuronal progenitors and neuroblasts has only received attention recently. There is evidence that some neurotrophic factors also promote survival of progenitors (de la Rosa and de Pablo, 2000). So far, it is not clear how the neurotrophic factor hypothesis applies to these populations.

The neurotrophic factor hypothesis has now been broadened to include normal electrical activity, neurotransmitters and calcium as pro-survival signals for neurons and neuronal progenitors (Cameron et al., 1998; Weiss et al., 1998). In the developing cerebellum for example, post-mitotic CGNs require functional N-methyl-D-aspartic acid (NMDA) glutamate receptors and transient receptor potential channels (TRPC), both permeable to calcium when activated, for their survival and maturation (Jia et al., 2007; Monti and Contestabile, 2000). Calcium can signal neuronal survival through the ERK, PI3-K and the P38 pathways (Dolmetsch et al., 2001; Mao et al., 1999).

Candidate Pro-Death Signals

Based on the observation that in the immune system certain endogenous proteins, like the Fas ligand (FasL), can induce the death of lymphocytes by binding to death receptors, research efforts were directed towards finding proteins that would similarly act as pro-apoptotic signals in the developing nervous system. Several groups reported that FasL was up-regulated in neurons prior to apoptosis, and blocking the Fas/FasL interaction in the pheochromocytoma cell line (PC12), CGNs and motor neurons *in vitro*, prevented neurotrophin deprivation-induced apoptosis (Brunet et al., 1999; Le-Niculescu et al., 1999; Raoul et al., 1999). Several strains of *Fas* and *FasL* mutant mice were generated, none of which exhibited defects in neuronal apoptosis during developmental periods of apoptosis (Hao et al., 2004; Karray et al., 2004; Raoul et al., 2000; Senju et al., 1996).

Other candidate death-inducing ligands are pro-NGF and pro-BDNF, the uncleaved precursors of NGF and BDNF. They can induce apoptosis *in vitro* and in the injured nervous system through the p75NTR receptor (Frade and Barde, 1999; Harrington et al., 2004; Teng et al., 2005). How and to what extent pro-NGF and pro-BDNF

contribute to the development of the nervous system is still under intensive investigation. So far, it was difficult to confirm the role of p75NTR as a death receptor during development. Both strains of *p75ntr* deletion mutants have been reported to express truncated p75NTR protein products that retain signaling capabilities, making the interpretation of their phenotypes difficult (Paul et al., 2004; von Schack et al., 2001). Over-expression of the intracellular domain of p75NTR from the pan-neuronal Ta1 alpha-tubulin promoter *in vivo* only affected a subset of neurons, and suggested that the ability of p75NTR to act as a death receptor *in vivo* is spatially and temporally limited (Majdan et al., 1997).

Bone morphogenic proteins (BMPs) could induce neuronal progenitor apoptosis *in vitro* and overexpression of a BMP receptor induced apoptosis in proliferative regions of the neural tube *in vivo* (Kendall et al., 2005; Panchision et al., 2001). However, the physiological relevance of BMPs as pro-death signals for neuronal progenitors still needs to be confirmed. The P38 pathway could mediate apoptotic signaling by BMPs (Kendall et al., 2005).

Signaling Pathways Linking Pro-Survival and Pro-Death Signals to the Apoptotic Machinery in the Developing Nervous System

The MAPK Signaling Pathways and Their Roles in Neuronal Survival

The JNK, P38 and ERK mitogen-activated protein kinases (MAPKs) form intracellular signaling pathways, which serve as links between a diversity of signals and decisions concerning the cell's fate. Each of them has been suggested to play a role in neuronal survival. The JNK and P38 pathways are generally associated with cell death and neurodegeneration in response to stress or injury, but are sometimes associated with survival. They are activated by trophic factor deprivation, ischemia and osmotic shock (Mielke and Herdegen, 2000). ERKs, on the other hand, are associated with cell division, differentiation and survival, and their activators are trophic factors, serum and phorbol esters (Aouadi et al., 2006). The MAPKs phosphorylate transcription factors, which in turn affect the expression of genes favoring different cell fates. MAPKs can also directly

phosphorylate important regulators of apoptosis, like the BH-3 only, pro-apoptotic Bcl-2 family members.

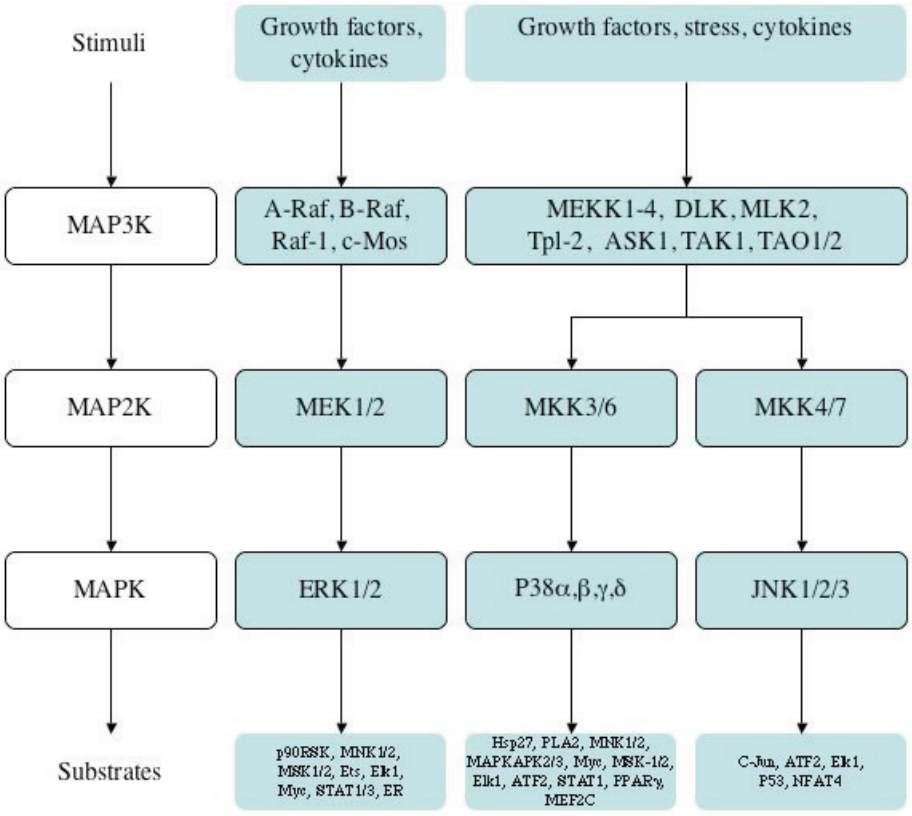


Figure A. MAPK pathways. Each MAPK pathway is composed of three signaling modules that are activated sequentially: a MAP3K, a MAP2K and a MAPK. Some substrates of the MAPKs are shown. Abbreviations: **ATF**, Activating transcription factor; **ASK**, Apoptosis Signal-regulating kinase; **DLK**, Dual leucine zipper-bearing kinase; **ER**, Estrogen receptor; **ERK**, Extracellular signal-regulated kinase; **Hsp27**, Small heat shock protein 27; **JNK**, c-Jun amino-terminal kinase; **MAPKAP**, MAP kinase-activated protein kinase; **MEF**, MAP/ERK kinase; **MEK**, MAP/ERK kinase; **MEKK**, MEK kinase; **MLK**, Mixed-lineage protein kinase; **MNK**, MAP kinase-interacting kinase; **MSK**, mitogen- and stress-activate protein kinase; **NFAT**, Nuclear factor of activated T-cells; **PLA2**, Phospholipase A2; **PPAR**, Peroxisome proliferative-activated receptor; **RSK**, Ribosomal S6 kinase; **STAT**, Signal transducer and activator of transcription; **TAK**, Transforming growth factor activated-β-protein kinase; **TAO**, One thousand and one amino acids. *Adapted from Aouadi et al., 2006*

All MAPKs share a common mechanism of activation: together with upstream MAPK kinases (MAP2Ks) and MAPK kinase kinases (MAP3Ks), they form highly conserved kinase cascades. A specific subset of MAP2Ks is responsible for phosphorylating each MAPK on a tyrosine and a threonine residue in their activation loop. MAP3Ks, in turn, activate different combinations of MAP2Ks. The initiation of this kinase cascade consists in the activation of MAP3Ks either by an upstream MAPK kinase kinase kinases (MAP4Ks) or by auto-phosphorylation, when brought into proximity by adaptors or scaffolds. This organization of MAPKs into signaling modules is highly conserved from yeast to man, and serves to ensure specificity and amplification of the signal.

Most of the evidence implicating MAPKs in neuronal survival comes from *in vitro* studies, and relies on the use of chemical inhibitors, dominant negative proteins, cell lines and *in vitro* cultured neurons. Although these techniques are essential in elucidating the precise molecular mechanisms regulating neuronal survival and death, they also carry with them some serious limitations. For example, drugs and gene constructs expressing dominant negative proteins can have non-specific effects by affecting unrelated pathways, and this can give us false indications of physiological effects. Now, careful examination of the knockouts for each of the MAPKs should indicate to us which *in vitro* results corresponds to an *in vivo* reality.

I. Genetic Evidence Implicating JNK Signaling in Neuronal Survival

Knockout studies have implicated JNK in neuronal progenitor apoptosis *in vivo*. Deleting any single one of the three *Jnk* genes did not lead to any obvious developmental defect. However, when both *Jnk1* and *Jnk2* were deleted, developmental apoptosis was reduced in the hindbrain and increased in the forebrain neural epithelium of E9.5 embryos. At E10.5, apoptosis was increased in both the forebrain and hindbrain regions. This suggests that JNK1 and JNK2 play redundant roles in region-specific apoptosis of progenitor cells in the developing neural tube. On the other hand, $Jnk1^{-/-}$; $Jnk3^{-/-}$ and $Jnk2^{-/-}$; $Jnk3^{-/-}$ double knockouts revealed no obvious developmental defects (Kuan et al., 1999; Sabapathy et al., 1999). Therefore, JNK3 seems to play a very limited role during development. Instead, JNK3 contributes to apoptosis in adult neurons in response to

stress and injuries such as kainate, ischemia and axotomy (Keramaris et al., 2005; Kuan et al., 2003; Yang et al., 1997).

Genetic evidence on the role of JNK in post-mitotic neurons during development is still lacking. This is due to the fact that the *Jnk1*$^{-/-}$*; Jnk2*$^{-/-}$ knockout embryos die around E11.5 (Kuan et al., 1999), when post-mitotic neurons normally begin to be generated. Therefore, studies on the role of JNK in post-mitotic neurons are conducted using *in vitro* models (Bhakar et al., 2003; Bruckner et al., 2001; Yeiser et al., 2004; Friedman, 2000). Another strategy consists of disrupting the JNK pathway specifically in the nervous system by using mice with a conditional mutation in c-Jun, an important effector of JNK *in vitro* (Palmada et al., 2002). This has a limited effect on the final number of neurons (Raivich et al., 2004).

II. Lack of Genetic Evidence Implicating P38 Signaling in Neuronal Survival

Similarly to JNK, P38 was suggested to play a role in neuron cell fate. *In vitro* studies suggested that P38 contributes to NGF-induced neural differentiation of PC12 cells (Morooka and Nishida, 1998; Iwasaki et al., 1999) and to their apoptosis in response to neurotrophin-deprivation (Le-Niculescu et al., 1999). Also, P38 was shown to mediate survival of cultured CGNs in response to calcium influx (Mao et al., 1999). However, knockouts for each of the four *P38* genes, *P38*α,β,γ and δ has failed to confirm any role for P38 in the nervous system so far (Adams et al., 2000; Allen et al., 2000; Beardmore et al., 2005; Mudgett et al., 2000; Sabio et al., 2005; Tamura et al., 2000). While the *P38*β,γ and δ knockouts were viable and fertile, the *P38*α, knockout died around E11.5 due to severe defects in placenta formation (Adams et al., 2000; Mudgett et al., 2000). Restoring the expression of P38α in the extra-embryonic tissue was sufficient for the *P38*α knockout embryo to develop normally (Adams et al., 2000).

Mice in which the map kinase kinases MKK3 and MKK6, the two main P38 activating kinases, were delete, died around E11.5, as a result of placental and vasculature defects. No NTDs or another obvious neural phenotype was observed in these embryos (Brancho et al., 2003).

III. Genetic Evidence Implicating ERK Signaling in Neuronal Functions

ERK1, 2 and 5 were suggested to promote survival of PC12 cells in response to neurotrophins (Liu et al., 2003; Wiese et al., 2001), but this has not been confirmed *in vivo*. Instead, studies in knockouts established that ERKs are necessary for early embryonic development, axon outgrowth and activity-dependent plasticity. *Erk2* and *Erk5* knockouts died between E6.5-11.5 and E9.5-11.5, respectively, due to severe defects in placenta formation (Fischer et al., 2005; Hatano et al., 2003; Hayashi, 2004; Regan et al., 2002 ; Saba-El-Leik et al., 2003; Sohn et al., 2002; Yan et al., 2003; Yao et al., 2003). Therefore, these studies could not address the role of ERK2 and ERK5 later in development. *ERK1* knockouts developed normally, but showed behavioral changes and reduced long-term potentiation (LTP) (Pagès et al., 1999; Selcher et al., 2001). To circumvent the problem of early embryonic lethality, Zhong and colleagues deleted B-Raf and Raf1, two MAP3Ks responsible for activating ERK1 and ERK2, specifically in the nervous system. Although DRGs had significantly reduced levels of ERK activation, their survival was not affected. Instead, neuronal outgrowth was abnormal (Zhong et al., 2007).

The NF-κB Pathway Plays a Role in Neuronal Survival *in vivo*

The transcription factor nuclear factor-κB (NF-κB) may play a role in neuronal survival during development. Its ability to promote survival was first established in the immune system, along with its role in immune responses. In non-stimulated cells, NF-κB is retained in the cytosol by the inhibitor of κB (IκB) protein. Upon stimulation by pro-inflammatory cytokines such as IL-1β and TNFα, the IκB kinases IKK1 and IKK2 are activated by MAP3Ks and phosphorylate IκB, thereby targeting it for proteasomal degradation. NF-κB is now free to translocate to the nucleus where it induces expression of pro-inflammatory genes. In the nervous system, NF-κB can be activated by p75NTR, TNFR, electrical activity, soluble amyloid β-precursor protein, oxidative stress and glutamate, and can upregulate the expression of genes coding for pro-survival proteins, such as inhibitors of apoptosis proteins (IAPs), Bcl-2, and manganese superoxide dismutase (MnSOD) (Mattson and Camandola, 2001).

Most genetic evidence points to a pro-survival role for NF-κB in neurons. For example, mice lacking the p50 NF-κB subunit showed increased sensitivity to glutamate excitotoxicity in the hippocampus (Yu et al., 1999) and premature neural degeneration (Lu et al., 2006). Surprisingly, the same mice also showed reduced neuronal damage after ischemia, illustrating that in some pathological circumstances NF-κB could be pro-apoptotic (Schneider et al., 1999).

The role NF-κB plays in developing neurons is still unclear. Li and colleagues reported that *Ikk1*$^{-/-}$*; Ikk2*$^{-/-}$ double knockout mice, which completely lacked NF-κB activity, had NTDs and an increase in apoptosis at the level of the hindbrain neuroepithelium. This phenotype closely resembled that of E10.5 *Jnk1*$^{-/-}$; *Jnk2*$^{-/-}$ knockout embryos. In addition, a two-fold increase in the number of apoptotic cells was observed in the spinal cord and DRGs. However, the identity of the apoptotic cells was not established (Li et al., 2000). Therefore, it remains unclear whether NF-κB normally protects post-mitotic neurons from developmental apoptosis. To address this issue, our lab generated a transgenic mouse that expresses β-galactosidase under the control of NF-κB response elements. Surprisingly, high constitutive transcription from the NF-κB promoter was observed in neuronal tissue such as the developing neocortex and olfactory bulb. Blocking NF-κB in cortical neurons *in vitro* led to a reduction in cell viability. This constitutive NF-κB transcriptional activity is likely to be the result of autocrine signals or electric activity (Bhakar et al., 2002). It could play a role in protecting neurons from undergoing apoptosis outside of specific developmental windows.

Other Signaling Pathways Implicated in Neuronal Survival During Development

Other signaling pathways have been implicated in neuronal survival. For example, p53, a tumor suppressor protein that causes growth arrest or apoptosis in response to DNA damage, could play a role in apoptosis of neural epithelial cells, neural progenitors and post-mitotic neurons. Female *p53*$^{-/-}$ embryos showed high frequency NTDs and exencephaly (Armstrong et al., 1995). Also, sympathetic neurons from *p53*$^{-/-}$ mice cultured *in vitro* were more resistant to neurotrophin deprivation-induced apoptosis (Aloyz et al., 1998). JNK and other kinases can phosphorylate p53 and thereby lead to its

stabilization and apoptotic potential. An example of a survival pathway that should also be considered is the PI3-K pathway (Dudek et al., 1997).

Crosstalk Between the Different Signaling Pathways

Life and death decisions depend on the balance between multiple inputs that converge on a cell at a given time. For example, JNK and NF-κB are often activated by the same ligands or stress, although they can mediate similar or opposite cell fate decisions. In some circumstances, NF-κB can inhibit the JNK pathway, and this can favor survival over apoptosis (De Smaele et al., 2001; Tang et al., 2001). This complexity explains why JNK or NF-κB activation are associated with different cell fates depending on cellular context.

Prelude to Chapter 2

Testing the *in vivo* relevance of the different signaling pathways is difficult for several reasons. First, there is a significant level of redundancy among MAPKs. Generation of double or even triple knockouts could be needed to elucidate their role. Alternatively, deletion of a common upstream activator, such as a MAP3K, or important downstream effectors could allow shutting down a pathway, and thereby solve the issue of redundancy. Second, several of these MAPKs have important developmental functions, and their deletion cause early embryonic lethality. Generating embryos with nervous system-specific deletions could circumvent this problem. Third, some of these pathways could by themselves play a limited role in neuronal apoptosis, and a much more meticulous phenotypic analysis would be needed to uncover defects. Finally, the neural tube phenotypes are difficult to analyze, and determining which cell populations are affected (neural progenitors, neuroblasts or projecting neurons) can be difficult and time-consuming. In sight of this, a neuron-specific deletion would allow addressing the role of MAPKs specifically in post-mitotic neurons.

Deletion of MAP3Ks *in vivo* is efficient in shutting down MAPK pathways and provides important insights into their function. An overview of the knockouts for some of the 21 MAP3Ks identified to date, suggests that MAP3Ks may have tissue and ligand-

specific functions. Mitogen ERK kinase kinase (MEKK) 1 for example, is important in eyelid closure (Yujiri et al., 2000), while MEKK2 and Transforming growth factor-β (TGF-β)-activated kinase (TAK1) contribute to T cell receptor (TCR) signaling in T cells (Guo et al., 2002; Liu et al., 2006; Wan et al., 2006). NTDs or neuronal migration defects were found in mice deficient in MEKK4, dual leucine zipper-bearing kinase (DLK), TAK1 and mammalian Ste20-like kinase (Mst3B) (Abell et al., 2005; Chi et al., 2005; Hirai et al., 2006; Irwin et al., 2006; Sarkisan et al., 2006; Shim et al., 2005). However, the MAP3Ks responsible for JNK, P38 and NF-κB signaling in post-mitotic neurons during development have not been identified yet. We highly suspect TAK1 to play an important role, for reasons discussed in the next chapter.

Chapter 2

Development and Validation of Tools to Analyze the Role of the TAK1 in Neurons *in vitro* and *in vivo*

An Introduction to TAK1

TAK1 Emerges as a Key MAP3K in JNK, P38, and NF-κB Signaling Downstream of Multiple Receptors

The MAP3K TAK1 has received much attention lately, as accumulating *in vivo* evidence reveals its central role in activating NF-κB, P38 and JNK downstream of multiple receptors. Although TAK1 was first identified as a kinase that could be activated by transforming growth factor-β (TGF-β) (Yamaguchi et al., 1995), most genetic studies now link it to the TNF receptors, IL-1/Toll-like receptors, and B and T cell receptors. Three different *Tak1* deletion mutants were generated, and all died between E9.5-11.5 (Jadrich et al., 2006; Sato et al., 2005; Shim et al., 2005). This is likely to be the result of a proliferation defect, as mouse embryonic fibroblasts (MEFs) derived from E9.5 $Tak1^{-/-}$ embryos failed to grow *in vitro* (Sato et al., 2005; Shim et al., 2005). In addition, *Tak1* mutants showed severe vascular defects, both in the embryo proper and in the yolk sac. The reduced access to nutrients could explain the early embryonic lethality. The vascular phenotype closely resembled that of the TGF-β type I and type III receptor mutants, and could be the result of defective TGF-β signaling (Jadrich et al., 2006, Larsson et al., 2001, Li et al., 1999a). Finally, one group also reported NTDs in the head and tail regions of $Tak1^{-/-}$ embryos (Shim et al., 2005). The vascular and neural tube phenotypes could also simply be the consequences of a generalized defect in proliferation.

Since TAK1 plays such a crucial role in early development, studies on the role of TAK1 in later stages rely on conditional knockouts. Two separate groups generated conditional knockouts, and revealed essential roles of TAK1 in the adaptive immune system. Sato and colleagues generated a transgenic mouse in which two LoxP sites flank exon II of *Tak1*. When $Tak1^{flox/flox}$ mice were crossed to a mouse expressing Cre-recombinase in B cells, Cre-mediated recombination between the LoxP sites resulted in the deletion of the floxed region. A smaller protein (ΔTAK1), lacking the adenosine triphosphate (ATP)-binding site was produced. This gene product was unable to activate NF-κB and activating protein-1 (AP-1)-dependent transcription *in vitro*. A B cell-specific deletion of exon II of *Tak1* resulted in severely deficient immune responses. *In vitro*, B cells derived from these mice failed to activate NF-κB, P38 and JNK downstream of

Toll-like receptors, and JNK activation by B cell receptor cross-linking was impaired (Sato et al., 2005).

In parallel, another conditional *Tak1* mutant, with the first exon being floxed, was generated. The deletion of exon I, which contains the transcriptional start site, prevented the expression of TAK1. Two studies on mice with a T cell-specific deletion of exon I of *Tak1* implicate TAK1 in T cell development, survival and function. *In vitro*, TAK1-deficient T cells failed to activate NF-κB and JNK downstream of TCR stimulation and TNFα (Liu et al., 2006; Wan et al., 2006). Finally, studies on immortalized MEFs deficient in TAK1 revealed that TAK1 also activates NF-κB, P38 and JNK downstream of IL-1β, a molecule involved in coordinating immune responses (Sato et al., 2005; Shim et al., 2005). The role of TAK1 in the immune system is highly conserved during evolution, as drosophila TAK1 (dTAK1) is essential for drosophila innate immunity against gram-negative bacteria. The dTAK1 kinase mediates NF-κB activation by the immune deficiency (Imd) pathway, and this is critical to the synthesis of antibacterial peptides in response to infection (Vidal et al., 2001).

TAK1 Mediates Survival Signaling in Various Physiological Contexts

In addition to its role in the survival of MEFs (Sato et al., 2005; Shim et al., 2005) and T cells (Liu et al., 2006; Wan et al., 2006), TAK1 also plays a role in keratinocyte survival. Mice with an epidermis-specific deletion of exon II of *Tak1* had a severe skin inflammatory condition, which was most likely the consequence of the massive increase in keratinocyte apoptosis in the epidermis (Omori et al., 2006; Sayama et al., 2006). Omori and colleagues observed that TAK1-deficient keratinocytes failed to activate both NF-κB and JNK in response to TNFα *in vitro*. In addition, TNFα caused apoptosis in TAK1-deficient keratinocytes, whereas TNFα did not affect the survival of wild type keratinocytes. The same was observed for TAK1-deficient MEFs compared to wild type MEFs (Sato et al., 2005; Shim et al., 2005). These results illustrate the ability of TNFα to simultaneously activate a pro-survival pathway, mediated by NF-κB and JNK in this context, and a pro-apoptotic pathway. Under normal circumstances, NF-κB overrides the apoptotic signal. However, when the survival pathway is disrupted, by deleting an

upstream activator such as TAK1 or IKK2, the apoptotic pathway is free to take over the control of cell fate (Li et al., 1999b). *Tak1* deletion in a *Tnfr*$^{-/-}$ genetic background almost completely rescued keratinocytes from apoptosis, since in this context, the apoptotic signal normally emanating from the TNF-receptor was also lost. The role for TAK1 in TNF receptor-mediated signaling is also evolutionary conserved. The dTAK1 kinase mediates Basket/JNK activation in response to the stress-inducible TNF ligand Eiger. However, in this context, TAK1 activation leads to apoptosis (Kauppila et al., 2003).

The Expression of TAK1 During Development Suggests a Role for TAK1 in the Maturation of the Nervous System

TAK1 is highly expressed in the developing mouse nervous system. Immunohistochemisty (IHC) on E10.5, E12.5 and E14.5 embryos revealed that the expression of TAK1 is ubiquitous but highest in the nervous system. At E12.5, expression was very high in the spinal cord, DRG, nasal epithelium and non-proliferating cells of the hindbrain and forebrain. At E14.5, TAK1 was expressed in most developing organs, and its expression remained high in the DRGs, spinal cord and trigeminal ganglia. TAK1 was found mostly in the cell body but also to a lesser extent in the axons of neurons (Jadrich et al., 2003). This expression profile suggests that TAK1 plays a role in the development of the nervous system. In fact, a role for TAK1 in mediating BMP-dependent apoptosis of neural progenitors has been suggested. In this context, the MAPK P38, rather than JNK, was activated by BMP and led to apoptosis (Kendall et al., 2005). However, this data was not corroborated by *in vivo* studies. Also, TAK1 was expressed in the differentiating layer of the developing brain, and its expression was very low in the ventricular zone, the region of neural progenitor proliferation (Jadrich et al., 2003). This pattern of expression is more consistent with a role for TAK1 in differentiating or mature neurons. Whether TAK1 plays any role in post-mitotic neurons has not been addressed yet.

Rationale and Statement of Hypothesis

The JNK and NF-κB pathways are strongly suspected to be important in neuronal survival during development. However, the upstream activating MAP3Ks responsible for their activation in the developing nervous system remain unknown. Accumulating evidence suggests that TAK1 is key to activating JNK, P38 and NF-κB signals downstream of multiple receptors. Because TAK1 is expressed in the developing nervous system and given the role of TAK1 in the survival of different cell types, we hypothesized that TAK1 plays a role in neuronal survival during development.

In order to study our hypothesis, we focused on the development of experimental tools. We developed a genetic strategy to delete *Tak1* specifically in neurons during development. To prepare for the analysis of mutant embryos, we tested several antibodies on paraffin-embedded tissues. In parallel, we tested lentiviral vectors as a means for delivering ribonucleic acid (RNA) interference reagents or Cre recombinase into neurons *in vitro*.

Materials and Methods

Materials, siRNAs and miRNA Primers. Cell culture medium, antibiotics and supplements were purchased from Hyclone, unless indicated otherwise. NGF was purchased from Cedarlane (Cat# CLMCNET-001). SiRNAs and miRNA primers directed against *Tak1* were developed using Invitrogen's BLOCK-iT^{TM} RNAi designer program. Stealth siRNA duplex oligoribonucleotides 393 and 1311 were designed against the regions 551-575 (5'-CCG TGT GAA CCA TCC TAA TAT TGT A-3') and 1470-1494 (5'-GAG TGC TGA CAT GTC TGA AAT AGA A-3') of the human transcript A of *Tak1* (NM_003188), respectively. The miRNAs 1020 and 1473 target the regions 1020-1041 (5'-ATC ATG TGG GCT GTT CAT AAT-3') and 1473-1496 (5'-GCT GAC ATG TCT GAA ATA GAA-3') of the *Tak1* transcript, respectively. The miRNA primers were first hybridized, and then ligated into the pcDNA^{TM}6.2-GW/EmGFP-miR plasmid, according to the instructions provided in the Invitrogen user manual for the BLOCK-iT Pol II miR RNAi Expression Vector Kits.

Cell culture, Transfection, and Infection. Human embryonic kidney (HEK) 293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% bovine calf serum (BCS), 2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin in 5% CO_2 at 37^{o}C. The rat PC12 cell line was maintained in DMEM supplemented with 6% BCS, 6% horse serum, 2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin in 10% CO_2 at 37^{o}C. The human breast cancer cell line MDA-MB-231 was maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin in 5% CO_2 at 37^{o}C. Cells were seeded 18-24 h prior to transfection. 293T-HEK were transfected using the standard calcium phosphate transfection method. PC12 and MDA-MD 231 cells were transfected using Lipofectamine 2000 or Lipofectin (both from Invitrogen). Briefly, PC12 and MDA-MB-231 cells were plated in 12 well plates at densities of 0.6 x 10^5 and 1.0 x 10^5 cells/well, respectively. Prior to transfection, the medium was changed to antibiotic free medium. To each well, 4 μl of Stealth siRNA duplex oligoribonucleotides (20 μM, Invitrogen) and 2 μl of Lipofectamine 2000 or Lipofectin were added according to the manufacturer's instructions. The medium was not changed until cells were harvested 48 h later.

Infections of 293T-HEK cells, PC12 cells, and cerebellar granule neurons (CGNs) consisted in adding the virus directly to the culture medium. When indicated, polybrene (Sigma-Aldrich) was added directly to the medium at the time of infection to a final concentration of 6 μg/ml. Pictures of EGFP fluorescence of cells were taken on a Nikon Eclipse TE200 microscope.

Western Analysis. Cytoplasmic cell extracts were obtained by lysing cells in a buffer containing 1% Nonidet-P40, 10 mM Tris (pH 8), 150 mM NaCl, 10% glycerol and a protease inhibitor 'cocktail' (Roche). Lysates were normalized for protein content using the bicinchoninic acid assay (BCA, Pierce) and by diluting in Laemmli sample buffer (120 mM Tris (pH 6.8), 4% SDS, 10% glycerol, 100 mM DTT). Whole cell extracts were obtained by lysing cells directly in Laemmli sample buffer. Samples were boiled for 5 min, separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Immunoblots were blocked in TBS-T (10 mM Tris (pH 7.4), 150 mM NaCl, 0.2% Tween 20) containing 5% (w/v) dry skim milk and then incubated with primary and secondary antibodies diluted in blocking solution. The anti-TAK1 antibody was purchased from UBI (Cat# 07263), the anti-actin antibody from ICN Biomedicals (Cat# 69100), the anti-GFP antibody from Molecular Probes (Cat# A-6455) and the anti-Cre antibody (Abcam, Cat# ab24580) was a kind gift from Dr. Stefano Stifani (McGill University, Montreal, Quebec, Canada). Immunoreactive bands were detected using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (PerkinElmer, Life Sciences).

Primary Cultures of Mouse Cerebellar Granule Neurons and Hippocampal Neurons. CGNs were prepared from P6-P8 mouse pups. Cerebella were freed of meninges and dissociated, first using a razor blade, and then by incubation for 15 min at 37^{o}C in 3 ml of HHGN (1X Hanks Balanced Salt Solution (HBSS), 2.5 mM Hepes (pH 7-7.6), 35 mM glucose, 100 μg/ml penicillin/streptomycin) containing 0.125% trypsin (Gibco, #15090-046). The trypsin was inhibited by addition of 5 ml of DMEM containing 10% FBS. After 2 min of centrifugation at 1000 rpm, the tissue was further dissociated by gently triturating 8 times with a 1 ml syringe and 20 G needle, and then 2 times with a 22 G needle. The suspension was then spun for 10 min at 1500 rpm. The pellet was

resuspended in 5 ml of DMEM containing 10% FBS, and filtered through a 40 μm cell strainer. Live cells were counted using the Trypan blue exclusion method. Cells were plated on Poly-L-Lysine (Sigma, Cat# P4832)-coated plates at a density of 2.6 x 10^5 cells/cm^2 in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/ml penicillin/streptomycin, and 25 mM KCl. After 2 days, cytosine-ß-D-arabinofuranoside (AraC, Sigma) was added directly to the medium to a final concentration of 10 μM. Neurons were maintained in 5% CO_2 at 37°C.

Primary cultures of hippocampal neurons were prepared by Peng Huashan and the protocol has been previously described (Banker and Goslin, 1998). Briefly, hippocampi from E18.5 rat embryos were dissected on ice in HBSS, and dissociated by gentle trituration with a fire-polished Pasteur pipette. The neurons were then resuspended in DMEM containing 10% fetal calf serum and the supplement N2 (Invitrogen). Neurons were plated on 100 μg/ml poly-L-lysine and 20 μg/ml laminin (Sigma)-coated 12 mm glass coverslips (Fisher) at a density of 1.5 x10^4 cells/cm^2, placed on a feeder layer of astrocytes. After 12 h, the medium was changed to Neurobasal medium supplemented with B27 (Invitrogen). The feeder layer was prepared from the cortex of E18.5 embryos, by shaking off microglias. Cultures were kept at 37°C in a 5% CO_2 humidified incubator for two weeks before infection by lentivirus.

Immunohistochemistry (IHC). IHC was performed on cerebella of P9 Sprague Dawley rat pups and E12.5 mouse embryos, fixed for 2 days in 10% Neutral Buffered Formalin (Surgipath, Cat#00600) at room temperature, dehydrated through a graded ethanol series and embedded in paraffin. The 5 μm sections were dried for 24 h at 60°C, and then deparaffinated and rehydrated. Slides were rinsed in TBS-T and cooked at 100°C for 10 min in citrate buffer (pH 6-6.2)(Lab Vision, Cat# AP-9003-500). Slides were rinsed twice in TBS-T, before being processed in a Lab Vision 360 Autostainer. First, endogenous peroxidase activity was blocked by a 5 min incubation in 3% peroxide. Then the slides were blocked by incubating for 5 min in Large Volume UltraV block (Lab Vision, Cat# TA-125-UB). Next, the slides were incubated for 1 h with primary antibodies diluted in Ultra Antibody Diluent (Lab Vision, Cat# TA-125-UD). The anti-p75NTR antibody was incubated for 90 min. The Value Primary Antibody Enhancer detection system (Lab

Vision, Cat#TL-125-PBS) was used according to the manufacturer's instructions for all antibodies, except the TAK1 antibody, which was detected using a biotinylated goat anti-rabbit secondary antibody (Lab Vision, Cat#TR-125-BN). Slides were then incubated for 10 min with AEC (Lab Vision, Cat#TA-125-SA) for colorimetric development. In the case on the anti-p75NTR antibody, rat immunoglobulin was added to the Enhancer solution to a final concentration of 20 μg/ml. The nestin antibody developed by Susan Hockfield was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. The nestin antibody was used in a 1:10 dilution. The anti-NeuN and anti-neurofilament M (NF-M) antibodies were purchased from Chemicon (Cat# MAB377 and AB1987) and used in 1:3000 and 1:1000 dilutions, respectively. The p75NTR antibody was directed against the p75NTR intracellular domain (Roux et al., 1999) and used in a 1:250 dilution. Mounted slides were analyzed under the bright field of a Nikon Eclipse 55i microscope.

β-Galactosidase Assay. Embryos were fixed for 20 min in 2% PLP (2% paraformaldehyde (PFA), 74 mM L-lysine monohydrochloride, 10 mM sodium periodate in 0.1 M phosphate buffer (pH 7.4)) at 4^{o}C and then assayed for β-galactosidase activity by incubation in X-gal solution (80 mM dibasic sodium phosphate, 20 mM monobasic sodium phosphate, 2 mM MgCl2, 0.2% IGEPAL, 1 mg/ml sodium deoxycholate, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 800 mg/ml X-gal (4-chloro-5-bromo-3-indolyl-β-galactoside, (Sigma)) for 4 h at 37^{o}C, in the dark. Samples were then washed with Phosphate Buffered Saline (PBS) and post-fixed in 4% PFA in PBS. Pictures of whole embryos were taken on a Zeiss SteREO Dicovery.V12 microscope using the Northern Eclipse V.5 program.

Production of Lentivirus. Production of HIV-derived lentivirus pseudotyped with the VSV-G envelope protein were obtained by transient transfections of a transfer vector, the viral core packaging plasmids pRSV-Rev and pMDLg/pRRE and the VSV-G envelope protein vector pMD2.G in 293T-HEK cells. Both the envelope protein vector and the viral core packaging plasmids were obtained from Addgene and Invitrogen. The miRNA expression cassettes 1020 and 1473 were transferred from the pcDNA6.2 plasmid to the

transfer vector pLenti6/V5-DEST™ by performing a rapid BP/LR recombination reaction, according to the instructions provided in the Invitrogen user manual for the BLOCK-iT Pol II miR RNAi Expression Vector Kits. The pCMV-Cre-EGFP and pCMV-EGFP transfer plasmids (Ahmed et al., 2004) were obtained form Joost Verhaagen (Netherlands Institute for Brain Research, Amsterdam, Netherlands). On the day prior to transfection, 1.0 x 10^6 cells were plated in 150 cm^2 cell culture flasks. For each flask, 20 μg of transfer vector, 10 μg of pMDLg/pRRE, 5 μg of pRSV-Rev and 6 μg of pMD2.G were cotransfected using the calcium phosphate method. The medium was replaced after 6 h with 15 ml of 293T-HEK medium supplemented with non-essential amino acids (NEAA, GIBCO, Cat#11140-076) and sodium pyruvate (GIBCO, Cat#11360-070). The conditioned medium was harvested 24, 26 and 48 h after the transfection, and replaced by fresh 293T-HEK medium supplemented with NEAA and sodium pyruvate. The medium was cleared by low-centrifugation (1000 rpm for 5 min) and filtered through 0.45 μm filters. The virus was concentrated by centrifugation at 23,000 rpm, for 2 h at 4°C.The pellet was resuspended in 80 μl sterile PBS and stored at –80°C as 20 μl aliquots. The titer was determined by infecting 293T-HEK cells with serial dilutions of the LV-EGFP and LV-Cre-EGFP virus, and estimating the ratio of GFP-positive cells under a Nikon Eclipse TE200 microscope.

Mice, Genotyping and Breeding. The nervous system specific *Ta1::Cre* transgenic mouse line was a kind gift from Lino Tessarelo (National Cancer Institute, Frederick, MD, USA) and its generation has been described previously (Coppola et al., 2004). Screening for the *Cre* transgene was done by conducting polymerase chain reaction (PCR) for 35 cycles (94°C for 60 s, 55°C for 60 s, 72°C for 120 s) using the following oligonucleotides: 5'-GTG GCA GAT GGC GCG GCA ACA CCA TT-3' and 5'-GCC TGC ATT ACC GGT CGA TGC AAC GA-3'. A 750 base pairs (bp) product indicated the presence of the transgene. The *Tak1*$^{flox/flox}$ mice were generously provided by Shintaro Sato and Shizuo Akira (Japan Science and Technology Agency) and previously described (Sato et al., 2005). The *Tak1* floxed and wild type alleles were distinguished by conducting PCR for 35 cycles (94°C for 30 s, 67°C for 60 s, 74°C for 60 s) using primers FW1: 5'-GGC TTT CAT TGT GGA GGT AAG CTG AGA-3' and FW2: 5'-GGA ACC CGT GGA TAA

GTG CAC TTG AAT-3'. PCR on the wild type and floxed allele yielded a 280 bp and a 320 bp band, respectively. The *Rosa26* mice were obtained from William J. Muller (McGill University, Montreal, Quebec, Canada) and previously described (Soriano, 1999). The *Rosa26* wild type and mutant alleles were distinguished by conducting PCR for 30 cycles (94^{o}C for 30 s, 58^{o}C for 45 s, 72^{o}C for 60 s) using primers W1: 5′-AAA GTC GCT CTG AGT TGT TAT-3′, W2: 5′-GGA GCG GGA GAA ATG GAT ATG-3′ and T: 5′-GCG AAG AGT TTG TCC TCA ACC-3′. PCR on the mutant and wild type allele yielded a 400 bp and a 650 bp band, respectively. All mice were backcrossed to the C57BL/6 strain.

To generate embryos with a neuron-specific deletion in *Tak1*, *Tak1*$^{flox/wt}$*; Ta1::Cre* mice were bred to *Tak1*$^{flox/flox}$ mice. To confirm that *Ta1::Cre* is located on the same chromosome as *Tak1*, *Tak1*$^{flox/wt}$*; Ta1::Cre*$^{+/-}$ were bred to C57BL/6 mice. The day on which a vaginal plug was detected was considered day 0 of gestation. All animal experiments were done with the approval of the McGill University Animal Care Committee.

Results

In order to investigate the role of TAK1 in a neuronal setting, we developed and validated a genetic strategy to delete exon II of *Tak1* specifically in neurons of the developing nervous system. We also developed molecular tools aimed at knocking down TAK1 in neurons, *in vitro* and *in vivo*. IHC on paraffin-embedded cerebelli from postnatal day (P) 9 pups revealed that TAK1 is expressed in all neuronal layers of the cerebellum. Finally, we tested and optimized IHC protocols on paraffin-embedded embryos for different antibodies that could be useful for the future analysis of the nervous system of mutant embryos.

Genetic Strategy for Deleting *Tak1* Specifically in Neurons During Development

To investigate the role of TAK1 in neuronal survival during development, we decided to generate embryos with a neuron-specific deletion of exon II of *Tak1*. To carry out this strategy, we took advantage of a mouse line with a conditional mutation in *Tak1*. In this line, the exon coding for the ATP-binding domain of TAK1 (exon 2) has been flanked by two LoxP sites, hence it is "floxed". Expression of the enzyme Cre recombinase in cells with floxed *Tak1* alleles results in the deletion of the floxed exon (**Figure 5.A**). The TAK1 protein produced from the mutated locus lacks its ATP-binding domain and is therefore unable to activate downstream signaling pathways (Sato et al., 2005). We chose the *Ta1::Cre* mouse to express Cre specifically in newly generated neurons. The transcriptional activity of the Ta1 alpha-tubulin promoter is almost entirely restricted to post-mitotic neurons and is first detected at E9.5, when neurogenesis begins (Gloster et al., 1999). Therefore, we expected to delete exon II of *Tak1* specifically in post-mitotic neurons, shortly after they exit the cell cycle.

To verify that the expression of Cre in the *Ta1::Cre* mouse is restricted to the neural tube and sufficient to mediate the deletion of floxed sequences, we bred the *Ta1::Cre* line to the Cre-reporter line *Rosa26*. In these mice, LacZ expression is blocked by a floxed sequence in the promoter region. Cre-mediated deletion of that inhibitory sequence allows LacZ to be expressed. Regions with LacZ enzymatic activity were detected by the appearance of a dark blue product after addition of X-gal to whole embryos. Blue staining was observed in the developing spinal cord and brain of E13.5

and E12.5 *Ta1::Cre*$^{+/-}$*; Rosa26*$^{+/-}$ embryos (**Figure 1.A-D**). Some light blue staining was detectable in the DRGs of E13.5 embryos (**Figure 1.A**). No LacZ staining was detected outside the nervous system of any of the *Ta1::Cre*$^{+/-}$*; Rosa26*$^{+/-}$ mice (**Figure 1.A-D**), except one (**Figure 1.I,J)**, nor was any staining detected in *Ta1::Cre*$^{-/-}$*; Rosa26*$^{+/-}$ mice (**Figure 1.E-H**). This confirms that Cre is specifically active in the developing nervous system. Surprisingly, one out of the 11 *Ta1::Cre*$^{+/-}$*; Rosa26*$^{+/-}$ embryos, showed generalized LacZ staining, with highest expression in the neural tube, in the developing limbs and in what appeared to be the endothelial cells of the umbilical cord (**Figure 1.I,J**).

To generate embryos in which both *Tak1* alleles are deleted in neurons, we bred *Tak1*$^{flox/flox}$; *Ta1::Cre*$^{-/-}$ mice to *Tak1*$^{flox/wt}$*; Ta1::Cre*$^{+/-}$ mice. Assuming that *Tak1* and *Ta1::Cre* are located on separate chromosomes, we expected that this cross would yield 25% *Tak1*$^{flox/flox}$*; Ta1::Cre*$^{+/-}$ embryos, as well as 3 other genotypes, *Tak1*$^{flox/flox}$*; Ta1::Cre*$^{-/-}$*, Tak1*$^{flox/wt}$*; Ta1::Cre*$^{+/-}$, and *Tak1*$^{flox/wt}$*; Ta1::Cre*$^{-/-}$ also at frequencies of 25% each (**Figure 2.B**). We collected the embryos between E12.5 and E17.5. Surprisingly, out of a total of 34 embryos, none had the expected *Tak1*$^{flox/flox}$*; Ta1::Cre*$^{+/-}$ genotype, only one had the *Tak1*$^{flox/wt}$; *Ta1::Cre*$^{-/-}$ genotype, and all other embryos had either one of the parental genotypes (**Figure 2.A**). Since *Tak1* is an essential gene for mouse development, we first thought that the *Tak1*$^{flox/flox}$*; Ta1::Cre*$^{+/-}$ genotype might be embryonic lethal due to a nervous system malformation. However, this could not explain why *Tak1*$^{flox/wt}$; *Ta1::Cre*$^{-/-}$ embryos were also obtained in significantly lower ratios than the expected 25%. This genotypic distribution could best be explained if *Tak1* and *Ta1::Cre* on the same chromosome (**Figure 2.C**). In this scenario, generating *Tak1*$^{flox/flox}$*; Ta1::Cre*$^{+/-}$ embryos would depend on the *Tak1*$^{flox/wt}$*; Ta1::Cre*$^{+/-}$ parent transmitting a chromosome bearing both the floxed *Tak1* allele and the *Ta1::Cre* transgene. Such a chromosome would be the result of a recombination event between the chromosome bearing the floxed *Tak1* allele and the chromosome bearing the *Ta1::Cre* transgene. Recombination events are usually rare. This explains why we obtained *Tak1*$^{flox/flox}$*; Ta1::Cre*$^{+/-}$ and *Tak1*$^{flox/wt}$; *Ta1::Cre*$^{-/-}$ at ratios significantly lower than those expected.

To confirm our suspicion that the floxed *Tak1* allele and the *Ta1::Cre* transgene are located on the same chromosome, we crossed *Tak1*$^{flox/wt}$*; Ta1::Cre*$^{+/-}$ mice to wild

type C57Bl/6 mice (*Tak1*$^{wt/wt}$*; Ta1::Cre*$^{-/-}$*)*. We predicted that the offspring would inherit either the floxed *Tak1* allele or the *Ta1::Cre* transgene. In rare cases, we expected that recombination events between both chromosomes would lead to *Tak1*$^{wt/wt}$*; Ta1::Cre*$^{-/-}$ or *Tak1*$^{floxt/wt}$*; Ta1::Cre*$^{+/-}$ genotypes (**Figure 3.A**). As predicted, a majority (98%) of the total 100 mice inherited only one of the genes, while 1% inherited both, and 1% none (**Figure 3.B**). A large number of *Tak1*$^{flox/flox}$*; Ta1::Cre*$^{+/-}$ embryos can now be obtained by breeding the *Tak1*$^{floxt/wt}$*; Ta1::Cre*$^{+/-}$ male (**Figure 3.B**) to *Tak1*$^{flox/flox}$*; Ta1::Cre*$^{-/-}$ females. This male was only obtained on month before the thesis submission deadline, after several months of intensive breeding. Therefore, we could not generate *Tak1*$^{flox/flox}$*; Ta1::Cre*$^{+/-}$ embryos.

Attempts at Knocking Down TAK1 Using Small Interfering RNAs

To examine the role of TAK1 in survival signaling, we attempted to knockdown TAK1 in the PC12 rat cell line using RNA interference (RNAi). PC12 cells are a classic model for studying the molecular signaling events leading to neuronal differentiation, survival and apoptosis. Addition of NGF to PC12 cells causes them to differentiate into neuron-like cells that, like sympathetic neurons, are dependent on NGF for their survival. TAK1 is expressed at similar levels in non-differentiated (naïve) and differentiated PC12 cells as a 70kDa protein (**Figure 4.B**). We tried knocking down TAK1 using two different small interfering RNAs (siRNAs), 393 and 1311, directed against regions that are 100% conserved between the four human and the rat *Tak1* transcripts (**Figure 4.A**). The mouse sequence only differs by one nucleotide. Both siRNAs knocked down TAK1 expression in the human breast cancer cell line MDA-MB-231 by approximately 80% and 50%, respectively. However, the same siRNAs, transfected using the same Lipofectamine 2000 transfection protocol, had no effect on TAK1 levels in naïve PC12 cells (**Figure 4.C**). This failure of knocking down TAK1 was most likely due to the limited transfectabilty of PC12 cells. The maximal transfection efficiency obtained using a plasmid expressing the traceable marker green fluorescent protein (GFP), was about 15%. To increase the transfection efficiency, we tried increasing the amount of Lipofectamine used. We also tried Lipofectin, which according to its manufacturer can yield higher transfection efficiencies than Lipofectamine when used on certain cell types.

Finally, we tried transfecting the PC12s in suspension. None of these strategies were successful in affecting TAK1 levels (data not shown).

Attempts at Knocking Down TAK1 Using Lentivirus-Delivered MicroRNAs

To address the issue of limited delivery of RNA interfering reagents into PC12 cells and neurons in general, we turned to viral vectors. Lentivirus infects both dividing and non-dividing cells, such as neurons (Naldini et al., 1996). Thus we were tempted to try a new kit by Invitrogen, that provided the plasmids necessary for producing human immunodeficiency virus (HIV)-derived lentivirus, pseudotyped with the envelope glycoprotein G of the vesicular stomatitis virus (VSV-G), in 293T-HEK cells. In this system, RNA interference is achieved by the lentiviral delivery of a microRNA (miRNA) cassette that is reverse transcribed and stably integrated into the host's genome. The miRNA cassette then continuously produces pre-miRNA transcripts, which are processed to miRNAs that target complementary messager RNA (mRNA) sequences in a similar manner than siRNAs, and thereby lead to a stable and long-term knock down of the targeted protein. We designed two miRNA primers, 1020 and 1473, with the BLOCK-iT^{TM} Invitrogen program, that are 100% complementary to regions conserved in the mouse, rat and human *Tak1* transcripts (**Figure 4.A**). The primers were ligated into the pcDNA^{TM}6.2-GW/EmGFP-miR plasmid, which contains the necessary flanking regions for the proper processing of the miRNAs. In addition, emerald GFP (EmGFP) is transcribed co-cistronically with the pre-miRNA, thereby providing a means of tracking the expression of the pre-miRNA. In order to confirm that the sequences 1020 and 1473 could target the *Tak1* mRNA, we co-transfected plasmids p1020 and p1473 with a plasmid coding for a TAK1-GFP fusion protein in 293T-HEK cells. The expression of TAK1-GFP (100kDa) was significantly impaired by p1473, and to a lesser extent by p1020 (**Figure 4.D**).

We produced lentivirus carrying the 1473 miRNA cassette (LV-miRNA-1473-EmGFP), and infected PC12 cells to determine optimal conditions for the knockdown of endogenous TAK1. In order to increase the infection efficiency, we added the cationic polymer polybrene (Manning et al., 1971). After 6 days, levels of TAK1 were not affected although EmGFP was detected in the lysate of PC12 cells infected with the

highest concentration of lentivirus, confirming the expression of the pre-miRNA (**Figure 4.E**). From the examination of the infected cells under a fluorescence microscope, we estimated that less than 30% of the PC12 cells expressed EmGFP at the highest concentration of virus used. We also tried infecting mouse CGNs *in vitro* with this lentivirus, without any success (data not shown).

Generation and Validation of a Cre Lentivirus to Delete a Floxed *Tak1* Allele in Neurons

As part of an alternative strategy to suppress TAK1 *in vitro*, and possibly *in vivo*, we produced a lentivirus coding for Cre (**Figure 5.C**). This virus could later be used to delete exon II of *Tak1* in neuron derived from $Tak1^{flox/flox}$ embryos or pups. Another application of this virus would be to inject it directly into the brain of $Tak1^{flox/flox}$ pups to delete *Tak1 in vivo*. To produce the lentivirus, we used a pCMV-Cre-EGFP plasmid, which expressed a fusion protein of Cre and enhanced GFP (EGFP) from the constitutive cytomegalovirus (CMV) promoter (Ahmed et al., 2004). It also contains the necessary sequences for packaging into viral particles and stable integration of the Cre transgene into the genome of the infected cell (**Figure 5.B**). Because we had no success in infecting neurons with lentiviral particles produced using the Invitrogen kit, we tried using the envelope and the viral core packaging plasmids available from Addgene. This time, we were able to infect rat hippocampal neurons (**Figure 5.D**). However, the virus was eventually toxic to neurons, and caused extensive neuronal death.

TAK1 Is Expressed in All Neuronal Populations of the P9 Rat Cerebellum

TAK1 is ubiquitously expressed throughout the embryo between E10.5 and E14.5 (Jadrich et al., 2003). To gain further insight into the expression of TAK1 during development, we performed IHC on paraffin-embedded cerebelli of P9 rat pups. TAK1 immunoreactivity was detected in all neuronal regions of the P9 cerebellar cortex. Cells in the external granule layer (EGL) that is recognizable by its intense p75NTR staining (**Figure 6.B**) were intensely stained. Some cells were stained in the molecular layer (ML). Cells in the Purkinje layer (PL) and in the internal granule layer (IGL) also exhibited TAK1 immunoreactivity (**Figure 6.A**). Intense and more heterogeneous

staining was detected in the deep nuclei of the cerebellum (**Figure 6.F**). Omitting the primary antibody did not produce any staining (**Figure 6.C,D**) thereby confirming that the staining was specific to the TAK1 and p75NTR antibodies. The staining appeared to be mostly cytoplasmic in Purkinje neurons and deep nuclei neurons (**Figure 6.E,F**).

Immunohistochemical Tools for Analyzing the Spinal Cord of Mutant Embryos

For the future analysis of the spinal cord of mutant embryos, we tested and optimized IHC protocols with three different antibodies. We tested neuron-specific antibodies directed against a nuclear protein, neuronal nuclei N (NeuN), and an axonal protein, neurofilament M (NF-M). Other groups have used these antibodies to analyze the effect of gene deletion on neuronal populations and on axonal outgrowth (Ransome et al., 2004; Watanabe et al., 2006). We also tested an anti-nestin antibody that specifically stains neuronal precursors. This antibody is commonly used to evaluate the effect of gene deletion on neuronal progenitor populations (Golestaneh et al., 2006). Since *Tak1* should only be deleted in post-mitotic neurons, nestin-expressing cells should not be affected, and should therefore serve as an internal control for the future analysis of the nervous tissue of mutant embryos.

In transverse sections of the spinal cord of paraffin-embedded E12.5 embryos, NeuN staining was restricted to the ventral and dorsal horns, whereas no staining was detected in the ventricular zone, the floor plate, roof plate or marginal zone (**Figure 7.A**). Staining of the motorneurons in the ventral region was more intense than staining in the dorsal region. Staining was also detected in the DRGs. The staining seemed to be restricted to the cell bodies of the neurons. The anti-nestin antibody stained the neural progenitors lining the spinal canal and radial glia, which extend processes from the pial surface to the central canal (**Figure 7.B**). Some nestin immunoreactivity was also present in the floor plate. The neuronal processes in the sensory and motor nerves, and in the developing spinal white matter were stained by the anti- NF-M antibody (**Figure 7.C**).

To look at the effect of the deletion of exon II of *Tak1* on the JNK, P38 and NF-κB pathways and cleavage of caspase 3 *in vivo*, we tested antibodies directed against p-JNK, p-P38, p-Jun, the NF-κB family member p65 and cleaved caspase 3. These

antibodies are routinely used in our lab for Western blot analysis of neuronal cultures exposed to various stress. Despite trying a diversity of fixatives, antigen-retrieval protocols and detection reagents, none of these antibodies showed any specific staining in the spinal cord of paraffin-embedded embryos. We also tried these antibodies on paraffin-embedded thymus of P9 rat pups, without any success. At this developmental stage, the intense selection of thymocytes results in a significant number of apoptotic cells in this organ. The JNK and P38 signaling pathways were suggested to play a role in the regulation of thymocyte development and apoptosis (Ricón et al., 2003).

Chapter 3

Discussion and Closing Remarks

Discussion

To investigate the role of TAK1 in neuronal survival, we developed several experimental tools. We validated a genetic strategy to delete exon II of *Tak1* specifically in neurons in the developing nervous system. We also tested lentiviral vectors as a means for delivering miRNAs directed against *Tak1* into PC12 cells and CGNs. We were unsuccessful in knocking down TAK1 in PC12 cells and could not infect neurons using lentivirus produced from the packaging plasmids provided by Invitrogen. When we used the lentiviral packaging plasmids from Addgene, the lentivirus could infect neurons, but also caused extensive neuronal death. We suspect that the traceable marker EGFP of the lentivirus was toxic to neurons.

IHC on P9 rat cerebellum revealed TAK1 immunoreactivity in all neuronal populations of the developing cerebellar cortex and in the deep nuclei. Finally, we tested several antibodies on paraffin-embedded embryos. A combination of antibodies directed against NeuN, NF-M and nestin seemed well-suited for future analysis of the spinal cord of mutant embryos.

Strategies for Achieving a Neuron-specific Deletion of exon II of *Tak1 in vivo*

To study the role of TAK1 *in vivo*, we took advantage of a mouse line with a conditional mutation in *Tak1*. The second exon of *Tak1* is floxed, and can be deleted by Cre. The mutant allele yields a protein missing its ATP-binding site (K63) and that cannot activate JNK, P38 and NF-κB dependent transcription (Sato et al., 2005). Previously, another catalytically inactive form of TAK1, in which lysine 63 (K63) was mutated, was shown to act as a dominant suppressor of JNK activation in the liver (Bradham et al., 2001). Therefore, the phenotype observed in *Tak1* mutants could be the result of TAK1 acting as a dominant negative suppressor of other MAP3Ks, in addition to a loss of TAK1 function. The observed phenotype would be more dramatic than that of a knockout. For example, embryos expressing a catalytically inactive form of MEKK4 show a more severe phenotype than the complete MEKK4 knockout (Abell et al., 2005; Chi et al., 2005). However, one advantage of working with a mutant rather than with a complete knockout is that the presence of a protein could avoid compensation by other

MAP3Ks. Also, if TAK1 has functions independent of its kinase activity, these would be preserved.

Multiple Cre-expressing lines are now available to achieve time- and region-specific deletions with the Cre/LoxP system (reviewed in Gavériaux-Ruff and Kieffer, 2007). The *Ta1::Cre* transgenic line seemed to be ideal for our study, since it expresses Cre specifically in post-mitotic neurons throughout the developing PNS and CNS as early as E9.5, when neurogenesis begins. Some exceptions are sympathetic neuroblasts and a small population of neuronal progenitors in the ventricular zone of the developing cortex (Gloster et al., 1999). Other lines that we considered are the *NSE-BAC::Cre, mNF-H::Cre, nestin::Cre, Synapsin::Cre* and *MLC2v::Cre3* transgenic lines, which all broadly express Cre in the nervous system (Morozov et al., 2003; Gavériaux-Ruff and Kieffer, 2007). The *NSE-BAC::Cre* line is the result of a "knockin" of a bacterial artificial chromosome, which expresses Cre under the control of the neuron-specific enolase (NSE) promoter. The expression of Cre in the nervous system of *NSE-BAC::Cre* embryos has not been characterized yet (Lee et al., 2001). Other *NSE::Cre* lines are available that express Cre during development, however the expression does not cover all regions of the nervous system (Cinato et al., 2001; Kwon et al., 2006; Frugier et al., 2000). The *mNF::H-Cre* line starts expressing Cre only at E18.5, when most of the neuronal apoptosis of sensory, sympathetic and motor neurons has already occurred (Hirasawa et al., 2001). On the other hand, expression of Cre starts early in the *nestin::Cre* line (Zimmerman et al., 1994). The nestin promoter initiates the expression of Cre in neuronal progenitors, and this should insure that the deletion of exon II of *Tak1* would be transmitted to all neurons. However, we wished to avoid affecting TAK1 levels in progenitors, because we suspect that TAK1 could be necessary for controlling their survival (Kendall et al., 2005), proliferation or differentiation. The *Synapsin::Cre* line starts expressing Cre in differentiating neurons at E12.5 and Cre is broadly expressed throughout the developing CNS and DRGs (Zhu et al., 2001). This transgenic line would therefore be appropriate for our study. One possible complication when breeding this line to mice with a floxed allele is that the floxed allele can be deleted in the germ-line of male progeny, although synapsin is not normally expressed in the testis (Rempe et al., 2006). Finally, the *MLC2v::Cre3* line expresses Cre specifically in neurons of the

developing and adult CNS and PNS. However, the expression of Cre is not as homogeneous throughout the neural tube as the one we observed in the *Ta1::Cre* line (**Figure 1**), and some regions exhibit very low levels of Cre activity. Also, it is not clear whether expression of Cre is present in neuronal progenitors (Banares et al., 2005).

The degree of Cre-mediated deletion and the expression pattern of Cre can change with the age of the transgenic line. Successive meiotic recombinations could be responsible for altering the environment of the transgene and thereby its expression. A broadening of the Cre expression pattern was observed in the *CaMKIIα:::iCre* line (Fukaya et al., 2003; Tsien et al., 1996). To confirm that the expression of Cre was still restricted to the nervous system of *Ta1::Cre* embryos and was sufficient to mediate the deletion of floxed sequences, we bred *Ta1::Cre* mice to the Cre reporter line *Rosa26*. As expected, robust Cre activity was detected throughout the developing nervous system of E12.5 and E13.5 double heterozygous embryos. Surprisingly, one embryo showed generalized Cre activity, with the highest activity being in the nervous system. This could be the result of a recombination, which could have altered the transgene's genetic environment.

Surprisingly, we were unable to obtain embryos with a $Tak1^{flox/flox}$; $Ta1::Cre^{+/-}$ genotype by crossing $Tak1^{flox/wt}$; $Ta1::Cre^{+/-}$ mice to $Tak1^{flox/flox}$ mice. At first, we suspected that the deletion of exon II of *Tak1* in developing neurons could cause NTDs that might be embryonic lethal. This would be surprising, as in many knockouts with NTDs, such as the *TRAF6*, *MEKK4*, *caspase 9* and *caspase 3* knockouts, some embryos survive until later stages of embryonic development or even until birth (Abell et al., 2005, Chi et al., 2005; Hakem et al., 1998; Kuida et al., 1996; Lomaga et al., 2000). We also suspected that "leaky" Cre expression from the T*a1* promoter could have resulted in the deletion of exon II of *Tak1* in regions where TAK1 function is vital, such as in the embryonic vasculature (Jadrich et al., 2006). In particular, we observed high levels of Cre activity in the endothelial cells of the umbilical cord of a single $Ta1::Cre^{+/-}$; $Rosa26^{+/-}$ embryo, out of 11 embryos with the identical genotype. However, it seemed much more likely that our inability to generate $Tak1^{flox/flox}$; $Ta1::Cre^{+/-}$ mice was the result of *Ta1::Cre* and *Tak1* being located on the same chromosome. In fact, the genotypic

distribution of the embryos corresponded to that expected for genes located on the same chromosome (**Figure 2.A,C**). Because the *Ta1::Cre* line was generated by the random insertion of the transgene into the genome, the chromosomal location of the transgene was so far unknown. To confirm our suspicion, we bred double heterozygous mice to wild type C57Bl/6 mice. Again, the genotypic distribution of the offspring was consistent with that expected for two linked alleles (**Figure 3.A,B**).

Delivery of RNA Interference Reagents Into Neurons and PC12 Cells

To address the role of TAK1 in survival signaling, we attempted to knockdown TAK1 in PC12 cells and CGNs *in vitro.* We faced several difficulties, and these were representative of some of the common obstacles that researchers encounter when trying to knock down gene expression in neurons. Transfection of siRNAs directed against *Tak1* using Lipofectamine had no effect on TAK1 levels in the rat PC12 cell line, although the sequences were efficient in knocking down TAK1 in the human MDA-MB-231 cell line. PC12 cells, and even more so neurons, are notoriously resistant to transfection, and this could explain our failure to knock down TAK1. Transfection efficiencies using calcium phosphate or lipid reagents rarely exceed 20% in PC12 cells, and range around 1% in neurons (Banker and Goslin, 1998). A possible explanation why neurons are so difficult to transfect is the fact that they are post-mitotic. In dividing cells, break down of the nuclear envelope during mitosis facilitates the entry of DNA and RNA interference (RNAi) reagents into the nucleus (Banker and Goslin, 1998; Mortimer et al., 1999). siRNA was shown to knockdown gene expression by assembling RNA-induced silencing complexes (RISC) both in the cytoplasm and nucleus (Robb et al., 2005). Furthermore, neurons and PC12 cells are particularly sensitive to the toxic effects of transfection reagents (Banker and Goslin, 1998). Why neurons are more sensitive to transfection reagents compared to other post-mitotic cells is unknown. Alternative transfection methods include the use of newer lipid reagents, gene guns, micro-injection or electroporation. Only electroporation yields high enough transfection efficiencies in neurons for performing loss-of-function studies using siRNAs. Jia and colleagues achieved 85% transfection efficiency in rat CGNs and almost 90% knockdown of their protein target (Jia et al., 2007). The transfection efficiency obtained in our lab and

promised by the manufacturer are closer to 60%. Limitations of this method are its toxicity and transient nature. Neurons must be transfected the day of the dissection, and cannot be transfected again, thereby limiting the time for analysis to the first few days *in vitro*, when CGNs or cortical neurons are still considered immature (Contestabile, 2001; Kriegstein and Dichter, 1983). Performing a TAK1 knockdown with this method could affect both progenitor differentiation and post-mitotic CGN survival *in vitro*, and the results would be difficult to interpret.

To circumvent the problems of delivery and obtain stable expression of RNAi reagents in PC12s and neurons, we decided to use lentiviral vectors. Lentivirus derived form HIV and pseudotyped with the envelope glycoprotein-G of the vesicular stomatitis virus (VSV-G) appeared as the ideal vector, since numerous reports showed infection of neurons *in vivo* and some *in vitro* using this vector (Ahmed et al., 2004; Dull et al., 1998; Fleming et al., 2001; Naldini et al., 1996; Miyoshi et al., 1997). Also, lentivirus allows for stable and long-term expression of trangenes or RNAi sequences. Once the lentivirus's RNA content and proteins are released into the cytosol, the RNA is reverse transcribed to double stranded DNA, transported to the nucleus and integrated into the host genome by the viral proteins (Blömer et al., 1997; Miyoshi et al., 1997). Adeno-associated virus (AAV) or recombinant adenovirus, which are also used to infect neurons, do not integrate into the genome. Also, they will preferentially infect glial cells in the brain or in mixed cultures, whereas the opposite seems to true for lentivirus (Banker and Goslin, 1998; Duale et al., 2005; Fleming et al., 2001).

To insure maximal bio-safety, the HIV-derived lentivirus was rendered defective in replication by successive deletions of the sequences that were non-essential to the insertion of a transgene. The third generation lentivirus system now only utilizes 3 HIV genes: the *gag*, *pol* and *rev* genes (Dull et al.,1998). Also, the segregation of the different viral genes onto different plasmids that do not contain regions of homology greatly limits the chance of recombination between the different plasmids or with wild-type virus and generation of replication-competent virus. Finally, to further decrease the risks to the user, we will no longer use miRNAs directed against human sequences.

Recombinant lentivirus can be used to insert either short hairpin RNA (shRNAs) or miRNA expression cassettes into the host's genome. Both shRNA and miRNAs direct

gene silencing through components shared with the RNAi pathway. Only miRNAs are endogenously expressed. They are small single-stranded RNA sequences, on average 22 nucleotides in length, which have various physiological functions (Kloosterman and Plasterk, 2006). We preferred using the miRNAs system, because miRNAs were reported to achieve higher levels of knockdown than shRNAs (Chang et al., 2006). In addition, miRNAs can be transcribed from cell-specific promoters, a potentially useful feature for achieving a neuron-specific knockdown *in vitro* or *in vivo*. This is not possible with the shRNA system that uses RNA polymerase III promoters rather than RNA polymerase II promoters (Rubinson et al., 2003). Another advantageous feature of the miRNA system, is the possibility to express several miRNAs co-cistronically, and thereby knock down several targets simultaneously or increase the efficiency of knockdown of a single target. In our experiment, EmGFP and the pre-miRNA 1473 were transcribed co-cistronically. Therefore, the detection of GFP by Western analysis on lysates from PC12 cells infected with LV-GFP (1ul/ml) was an indirect confirmation that the pre-miRNA 1473 was expressed (**Figure 4.E**). However, no knockdown of TAK1 was observed after 6 days. EmGFP fluorescence was detected in no more than 30% of cells. In this situation, EmGFP is not a perfect indicator of infection efficiency, because the EmGFP transcript is partially processed and degraded to produce the miRNA. Therefore, only a portion of the EmGFP transcript actually produces EmGFP protein. Hence the number of infected cells could be much higher. Because miRNA 1473 did not affect levels of endogenous TAK1 in 293T-HEK cells, we suspect that the miRNA 1473 is not efficient enough in targeting the *Tak1* mRNA transcribed from an endogenous promoter. A slow turnover rate of the TAK1 protein or the death of cells in which TAK1 was deleted could also explain this result.

Despite Invitrogen's promises, the viral particles we generated did not infect neurons *in vitro.* Several of our collaborators experienced the same problem. However, our collaborator Philippe Seguela observed that the same virus infected hippocampal neurons *in vivo*, which means that a specific permissive condition necessary for the infection of neurons was not met *in vitro.* We tried to generate lentivirus with plasmids obtained from Addgene instead, and were happily surprised that the lentivirus generated was able to infect CGNs and hippocampal neurons *in vitro* (**Figure 5.D**), since both sets

of plasmids from Addgene and Invitrogen were originally generated in the Trono lab (Dull et al., 1998).

Another surprising finding was that lentivirus generated using the Addgene plasmids was selectively toxic to neurons, and the rapidity of the progression to neuronal death was dose-dependent. Our collaborator Brigitte Ritter observed a similar neurotoxic effect when she infected hippocampal neurons with a lentivirus produced using the Addgene plasmids and the miRNA transfer vector from Invitrogen (pLenti6/V5-DEST™). When she swapped the sequence for EmGFP in the miRNA transfer vector with the sequence for the red fluorescent protein (RFP), infected hippocampal neurons survived for several weeks *in vitro*. Therefore, the EmGFP, and most likely also the EGFP, were responsible for the neurotoxicity of the lentivirus. Forms of GFP optimized for high levels of expression and brighter fluorescence, such as EGFP, were previously reported to be toxic to some neurons (Krestel et al., 2004). A suggested mechanism for this toxicity is the inhibition of polyubiquitination by EGFP (Baens et al., 2006).

Expression of TAK1 in the Developing Nervous System

A previous report revealed that TAK1 levels were ubiquitous during development and highest in the neural tube of E12.5 embryos (Jadrich et al., 2003). To further characterize the expression of TAK1 in the developing nervous system, we performed IHC on paraffin-embedded cerebelli from P9 rat pups, using the same antibody as Jadrich and colleagues did. At that stage of development, neuronal progenitors are actively dividing and generating immature granule neurons in the external granule layer (EGL). These immature neurons then migrate through the molecular layer (ML) and the Purkinje cell layer (PL), to the internal granule layer (IGL) where they become mature CGNs. Apoptosis is observed both in the IGL and EGL at that stage of development. We observed that TAK1 was expressed in all layers of the developing cerebellar cortex, including the EGL and IGL. This is consistent with a potential role for TAK1 in the regulation of the survival of CGN progenitors and post-mitotic CGNs (Contestabile, 2002). Some staining was also detected in the deep nuclei, a region where the levels of *Tak1* mRNA are elevated in the adult, according to the Allen Brain Atlas (Lein et al., 2007).

Immunohistochemical Tools to Examine the Nervous System of Mutant Embryos

In anticipation of mutant embryos, we tested IHC protocols for markers of neurons and neuronal progenitors. Neurons were stained with an antibody directed against NeuN, a neuron-specific DNA-binding protein of unknown function. NeuN is expressed as early as E9.5, when neurons first exit the cell cycle, and continues to be expressed throughout development (Mullen et al., 1992). It is mostly restricted to the cell bodies, and its homogenous distribution allows for easy quantification using the ImageJ software. A limitation of this antibody is that it does not stain sympathetic neurons, Purkinje neurons, most neurons of the internal nuclear layer of the retina, as well as several other neuronal populations in the adult (Mullen et al., 1992; Wolf et al., 1996). An antibody that can be used to examine whether *Tak1* deletion affects axonal migration is the NF-M antibody (Watanabe et al., 2006). Finally, an antibody directed against nestin was used to stain neural progenitors. Predominantly, the antibody stained radial glia, which project across the spinal cord and were recently shown to be neuronal progenitors (Anthony et al., 2004; Malatesta et al., 2003). Since *Tak1* should only be deleted in post-mitotic neurons, nestin-expressing cells should not be affected, and should therefore serve as an internal control for the future analysis of the nervous tissue of mutant embryos.

We preferred to perform IHC on paraffin-embedded tissue rather than on frozen tissue for several reasons. First, the tissue architecture is much better preserved. Also, the possibility of automating the staining procedure by using an Autostainer in collaboration with Dr. Barry Bedell was very appealing, as it allows for the rapid analysis of a large number of sections, while limiting the variability between sections. One of the major limitations of working with paraffin-embedded tissues is that stronger fixation is needed, and this can prevent certain antibodies from binding to their antigens. This is less of a problem for antigens that are exposed at the cell surface, or are abundantly expressed, such as the cytoskeletal protein NF-M. However, we were unable to detect the phosphorylated forms of JNK, P38, and c-Jun, or the NF-κB family member, p65.

The most commonly used fixatives contain formaldehyde, which cross-links proteins and thereby preserves protein structure but can hinder antibody diffusion. On the other

hand, fixatives containing the coagulants trichloroacetic acid (TCA) or methanol can expose some antigenic sites that were buried in the native protein by denaturating them, rather then cross-linking them (Kiernan, 1999). TCA has also been used in immunofluorescence with antibodies directed against phosphorylated sites, as it is could inhibit endogenous phosphates (Hayashi et al., 1999). We tried different fixatives containing formaldehyde, methanol, or TCA on thymus tissue from P9 rat pups. Thymus tissue should have high levels of endogenous P38 and JNK activity (Ricón et al., 2003). However, in none of these fixation conditions did the phospho-specific antibodies produce any staining.

The access of antibodies to antigens can also be improved by subjecting the tissue to antigen retrieval. Most antigens can be revealed by heating for 2 min in 0.1 M citrate buffer (pH 6) at 100^{o}C in a pressure cooker (Kiernan, 1999). For some antigens the pH needs to be lower. We tried antigen retrieval protocols in citrate buffer, hydrochloric acid and formic acid, without any success.

Future Directions and Closing Remarks

To investigate the role of TAK1 in neuronal survival, we developed several experimental tools. We validated a genetic strategy to delete exon II of *Tak1* specifically in neurons in the developing nervous system. We also tested lentiviral vectors as a means for delivering miRNAs directed against *Tak1* into PC12 cells and CGNs. By immunohistochemistry (IHC), we determined that TAK1 is expressed in all neuronal layers of the P9 cerebellum. We also tested and optimized IHC protocols on paraffin-embedded embryos for different antibodies that could be useful for the analysis of the nervous system of mutant embryos.

Our genetic strategy was complicated by the fact that both *Tak1* and *Ta1::Cre* are located on the same chromosome. However, we have now generated a mouse in which *Ta1::Cre* and *Tak1* co-segregate during meiosis. Breeding this male to $Tak1^{flox/flox}$ females should yield 50% $Tak1^{flox/flox}$; $Ta1::Cre^{+/-}$ embryos. The nervous system of these embryos could then be examined by IHC using NeuN, NF-M and nestin antibodies.

Now that we established lentivirus as an effective way to infect neurons *in vitro*, loss-of-function experiments will be greatly facilitated. We expect that the knockdown of TAK1 in CGNs cultured for 6 days *in vitro* will negatively affect their ability to survive under depolarizing conditions. Alternatively, a LV-Cre-RFP virus could be used to delete exon II of *Tak1* in neurons derived from $Tak1^{flox/flox}$ mice. A LV-Cre-RFP lentivirus could also be used to examine the role of TAK1 in neuronal survival in the adult. The virus could be injected into the hippocampus of $Tak1^{flox/flox}$ mice. This experiment will need to be carefully controlled for the toxicity of the virus itself by injecting a control virus (LV-EGFP) into the hippocampus on the contralateral side. The ability of lentiviruses to stably transfer genes or RNAi reagents into neurons *in vivo* opens many experimental and therapeutic opportunities.

Figures

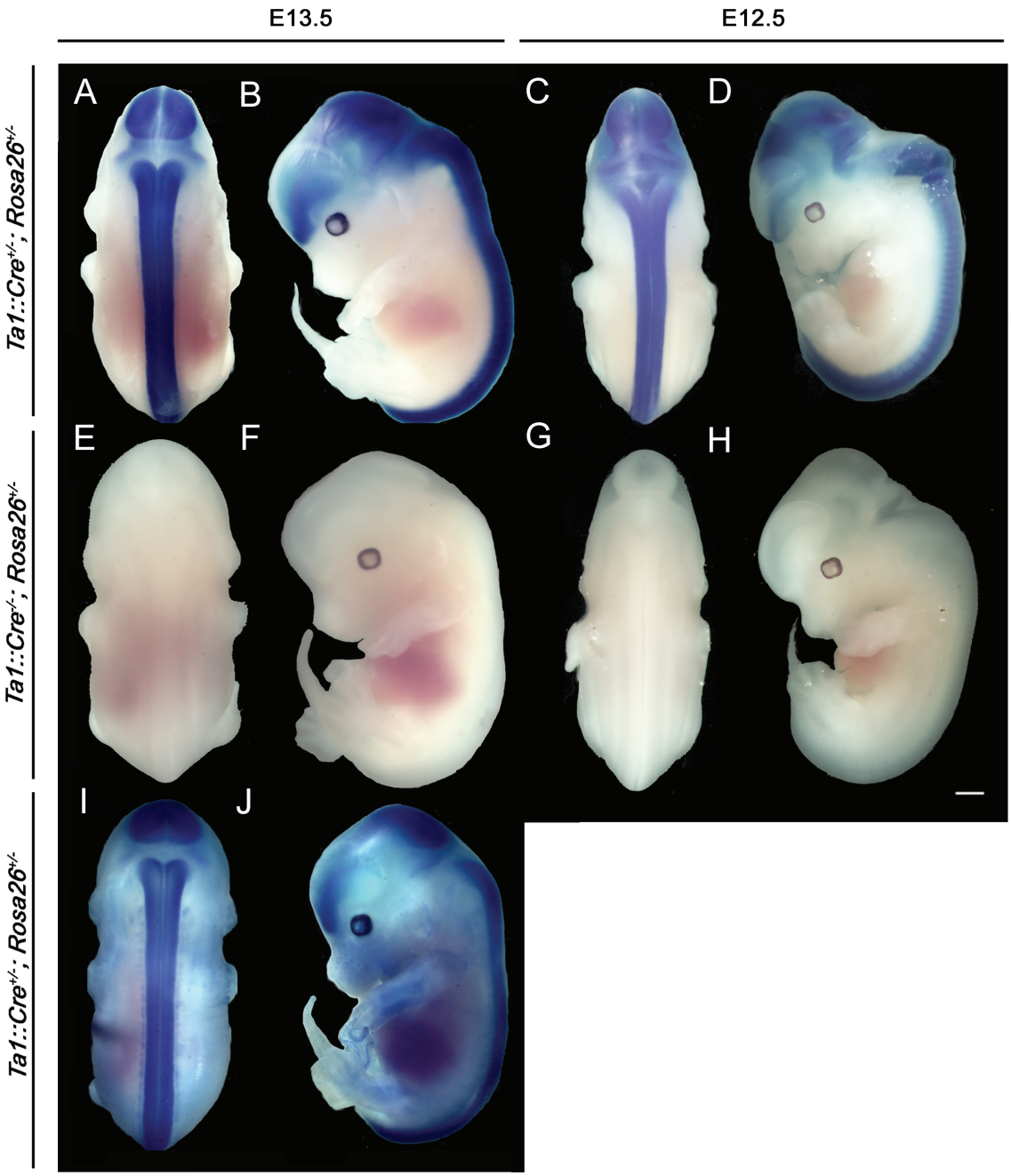


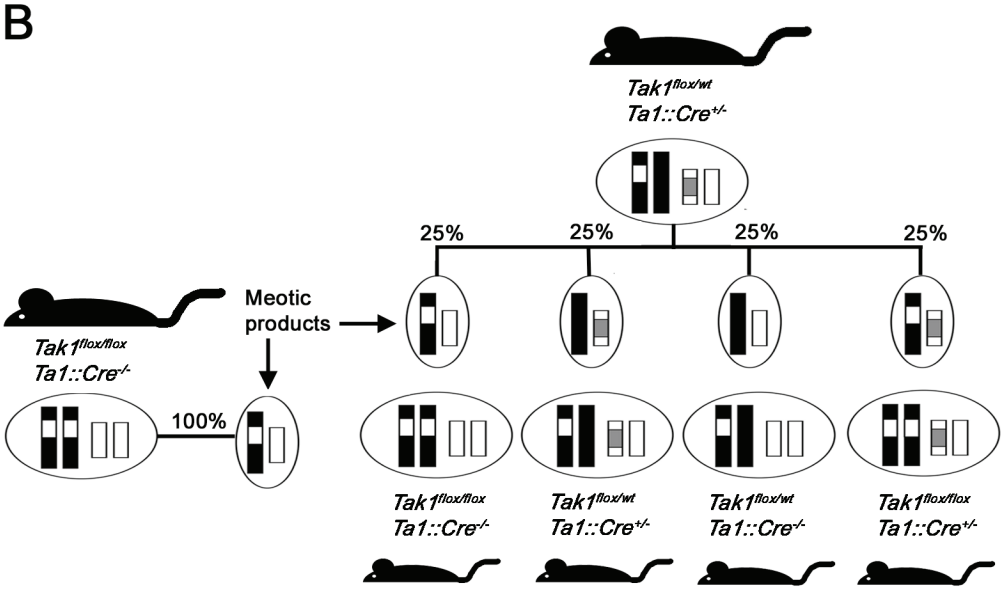
Figure 1. Cre activity in E12.5 and E13.5 Ta1::Cre+/-; Rosa26+/- embryos is restricted to the nervous system, except for one (legend continued on p.44).

Figure 1. Cre activity in E12.5 and E13.5 *Ta1::Cre*$^{+/-}$*; Rosa26*$^{+/-}$ embryos is restricted to the nervous system, except for one. E13.5 *Ta1::Cre*$^{+/-}$*; Rosa26*$^{+/-}$ embryos **(A, B, I, J)**, E13.5 *Ta1::Cre*$^{-/-}$*; Rosa26*$^{+/-}$ embryo **(E, F)**, E12.5 *Ta1::Cre*$^{+/-}$*; Rosa26*$^{+/-}$ embryo **(C, D)**, and E12.5 *Ta1::Cre*$^{-/-}$*; Rosa26*$^{+/-}$ embryo **(G, H)** incubated with LacZ staining solution to reveal Cre activity. Strong LacZ staining was detected in the developing spinal cord and brain of *Ta1::Cre*$^{+/-}$*; Rosa26*$^{+/-}$ embryos **(A, B, C, D, I, J)**. Light staining was detected in the developing DRGs **(A)**. *Ta1::Cre*$^{-/-}$*; Rosa26*$^{+/-}$ embryos were not stained **(E, F, G, H).** One *Ta1::Cre*$^{+/-}$*; Rosa26*$^{+/-}$ embryo showed ubiquitous LacZ staining **(I, J)**. Scale bar = 1mm in **(H),** applies to **(A-J)**.

A

Genotype	*Tak1*$^{flox/flox}$ *Ta1::Cre*$^{-/-}$	*Tak1*$^{flox/wt}$ *Ta1::Cre*$^{+/-}$	*Tak1*$^{flox/wt}$ *Ta1::Cre*$^{-/-}$	*Tak1*$^{flox/flox}$ *Ta1::Cre*$^{+/-}$	Total
Number of embryos	16	17	1	0	34
Percentage	47%	50%	3%	0%	100%

B



C

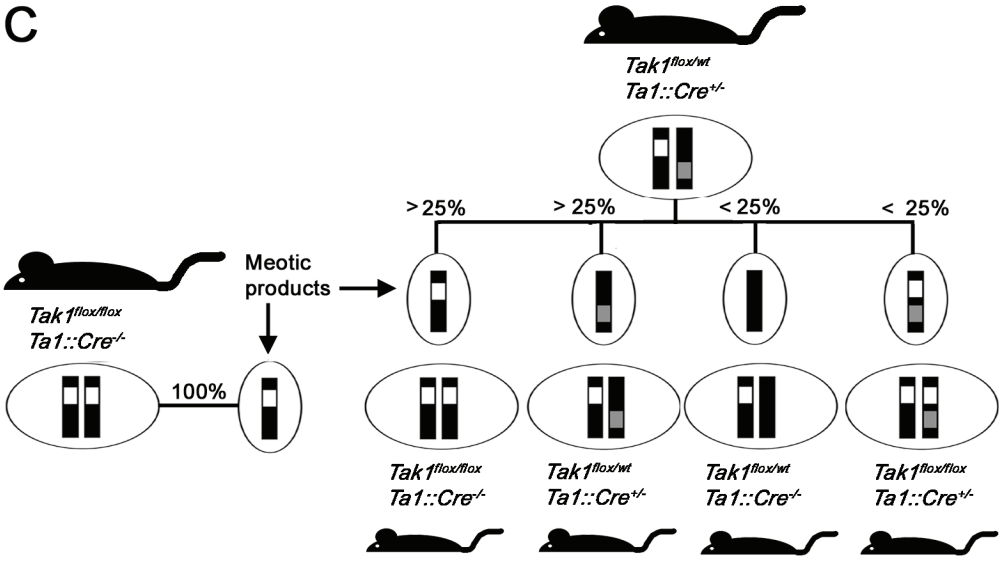
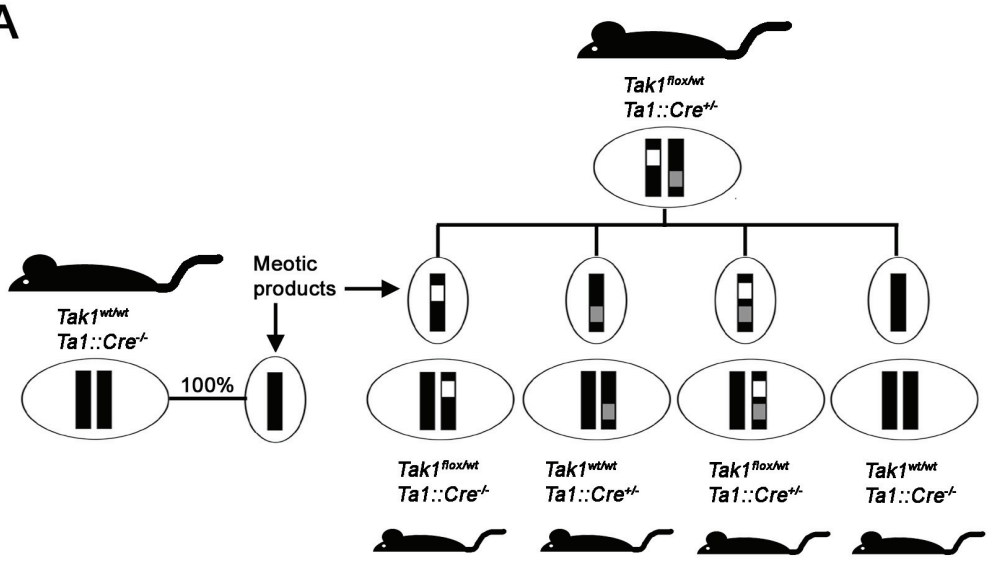


Figure 2. **Breeding strategy for generating embryos with a neuron-specific deletion in *Tak1*** (legend continued on p.46)**.**

Figure 2. **Breeding strategy for generating embryos with a neuron-specific deletion in *Tak1*.** *Tak1*$^{flox/wt}$*; Ta1::Cre*$^{+/-}$ mice were bred to *Tak1*$^{flox/flox}$*; Ta1::Cre*$^{-/-}$ mice. **(A)** Number and percentage of embryos obtained for each of the expected genotypes. Note that none of the 34 embryos had the *Tak1*$^{flox/flox}$*; Ta1::Cre*$^{+/-}$ phenotype. **(B)** Prediction of the genotypic distribution assuming that *Ta1::Cre* (gray box) is located on a separate chromosome (white bar) than *Tak1* (white box on a black bar) **(C)**. Prediction of the genotypic distribution assuming that *Tak1* and *Ta1::Cre* are located on the same chromosome. **(B,C)** The possible meotic products for each parent are shown, along with the expected frequencies. Fusion of the gametes can result in embryos with 4 different combinations of *Tak1* and *Ta1::Cre* alleles, and their expected frequencies depend on whether *Tak1* and *Ta1::Cre* are located on separate chromosomes or not.

A



B

Genotype	*Tak1*$^{flox/wt}$ *Ta1::Cre*$^{-/-}$	*Tak1*$^{wt/wt}$ *Ta1::Cre*$^{+/-}$	*Tak1*$^{flox/wt}$ *Ta1::Cre*$^{+/-}$	*Tak1*$^{wt/wt}$ *Ta1::Cre*$^{-/-}$	Total
Number of mice	52	46	1	1	100
Percentage	52%	46%	1%	1%	100%

Figure 3. *Tak1* and *Ta1::Cre* are located on the same chromosome. *Tak1*$^{flox/wt}$; *Ta1::Cre*$^{+/-}$ mice were bred to C57BL/6 mice. **(A)** The genotypic distribution of the offspring is predicted, based on the assumption that *Tak1* and *Ta1::Cre* are both located on chromosome 4. **(B)** Number and percentage of mice obtained for each of the 4 genotypes predicted in **(A)**.

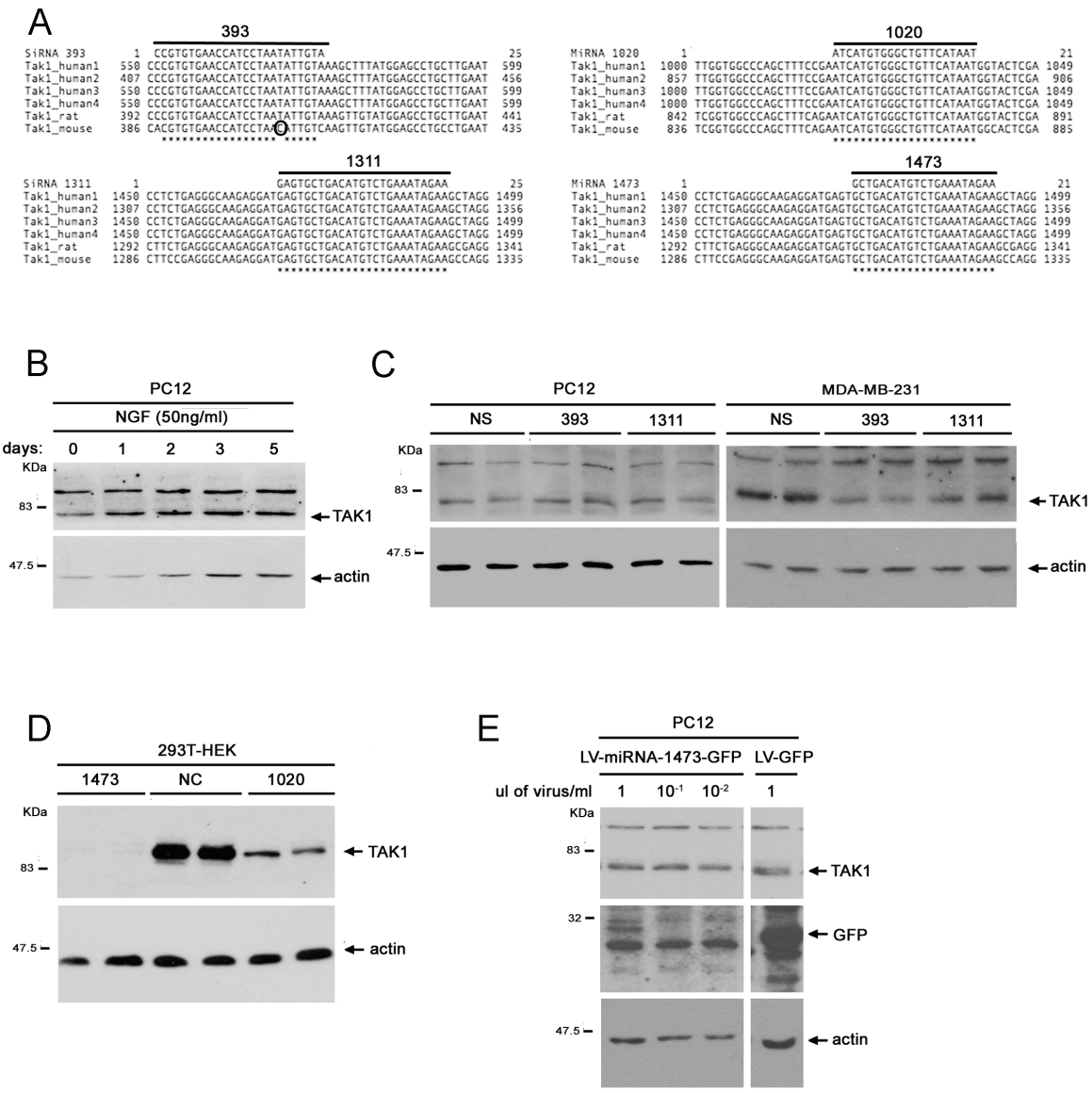


Figure 4. Attempts at knocking down TAK1 in PC12 cells, using siRNAs or lentivirus-delivered miRNAs directed against *Tak1*. (A) ClustalW alignment of the 4 human (NM_003188, NM_145331, NM_145332, NM_145333), the single mouse (NM_172688) and the single rat (XM_232855) *Tak1* transcripts. Only the regions containing the sequences targeted by siRNAs 393 and 1311 and miRNAs 1020 and 1473 are shown. A star indicates a perfect alignment between the nucleotides of the *Tak1* transcripts and the miRNAs or siRNAs at a given position. **(B)** PC12 cells were differentiated for 1-5 days by addition of NGF (50ng/ml) to serum-free medium, or were left untreated (0 days of differentiation). **(C)** PC12 and MDA-MB-231 cells were transfected with siRNAs 393 and 1311 or a non-specific (NS) siRNA. **(D)** 293T-HEK cells were transfected with a plasmid coding for TAK1-GFP, together with plasmids coding for miRNAs 1473 and 1020, or TRPC5, as a negative control (NC). **(E)** PC12 cells were infected with different concentrations of LV-miRNA-1473-GFP. LV-GFP lentivirus was used as a control. Cells were lysed 6 days post-infection. **(B-E)** All lysates were analyzed by immunoblot for levels of TAK1 and actin. Lysates of PC12s infected with lentivirus were also analyzed for levels of GFP **(E)**.

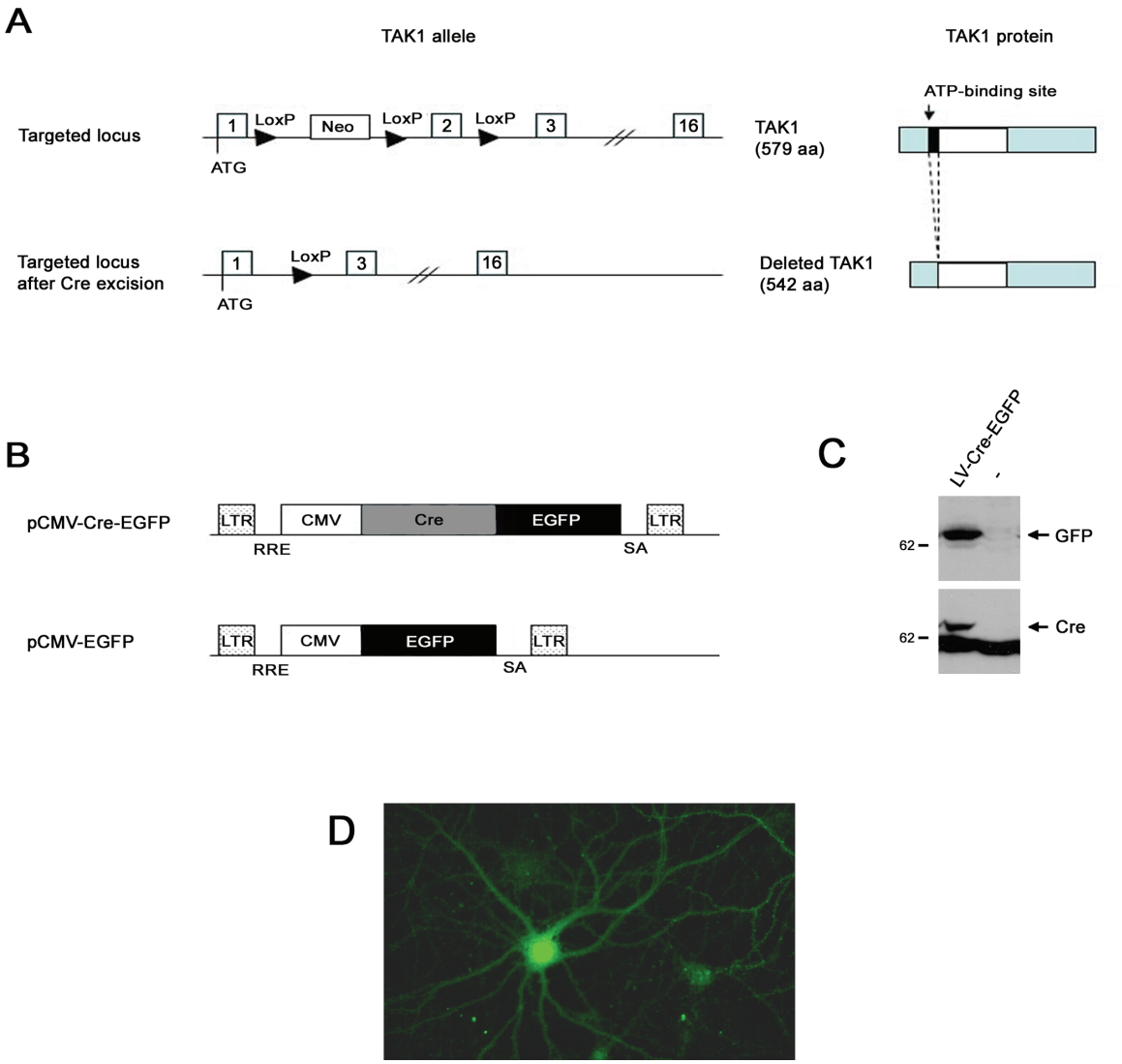


Figure 5. Generation and validation of a Cre-EGFP lentivirus, in order to delete a floxed *Tak1* allele in neurons. (A) Structure of the conditional *Tak1* allele and TAK1 protein before and after Cre excision. The LoxP sites are indicated as black triangles. Numbered boxes represent the different exons. The ATP-binding domain of TAK1 is shown as a black box and the kinase domain as a white rectangle. **(B)** Schematic representation of the transfer vectors used to produce LV-EGFP and LV-Cre-EGFP lentivirus. Only the region containing the CMV promoter, the sequence coding for Cre or the Cre-EGFP fusion protein, the Rev responsive element (RRE), splice acceptor site (SA) and Long Terminal Repeats (LTRs) are indicated. **(C)** Western blot analysis of the expression of EGFP, Cre and actin, in 293T-HEK cells 2 days after infection with a lentivirus carrying the pCMV-Cre-EGFP plasmid. **(D)** EGFP fluorescence in a rat DIV14 hippocampal neuron, 2 days after infection with LV-Cre-EGFP.

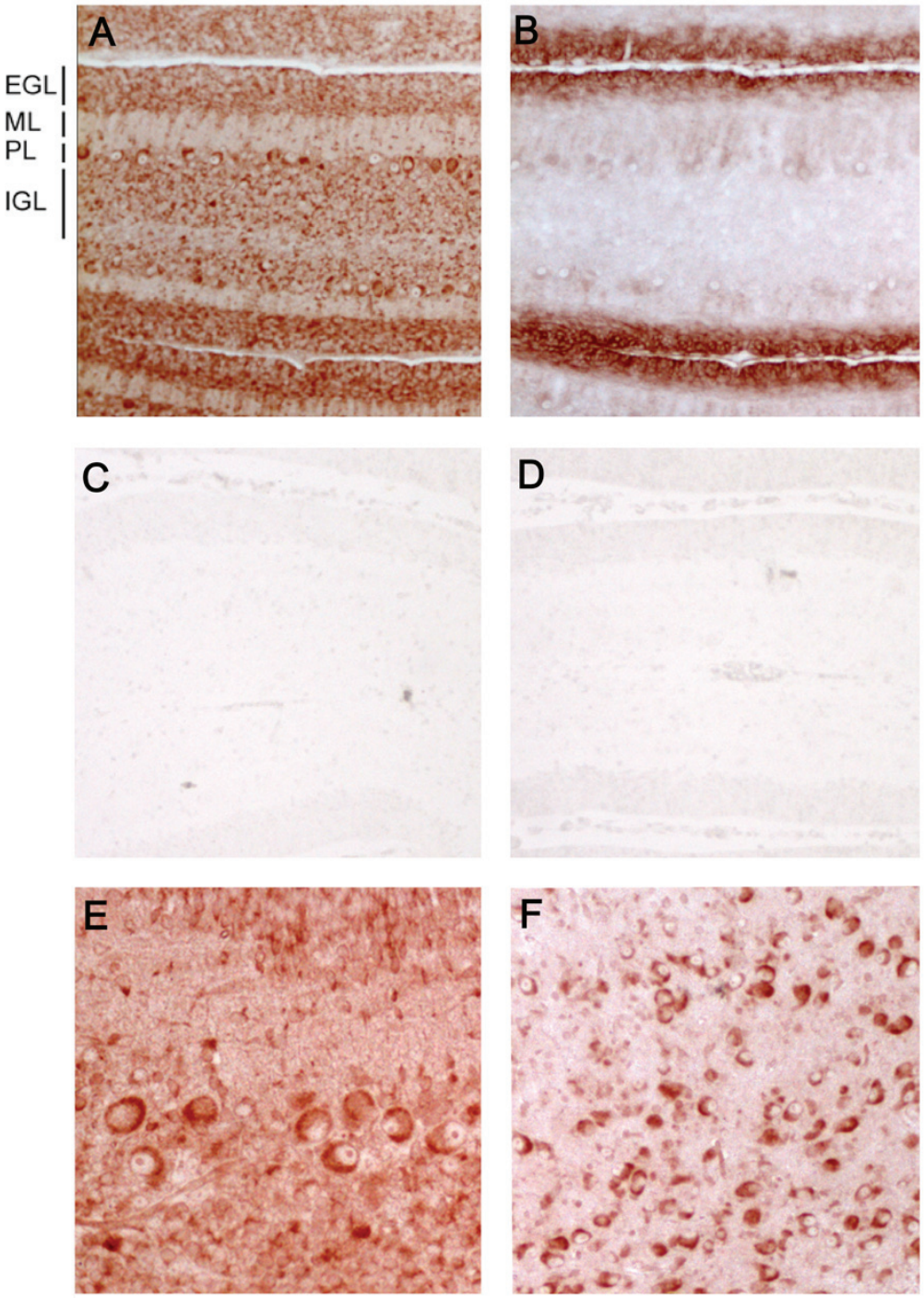


Figure 6. TAK1 is expressed in the different neuronal populations of the P9 cerebellum. Paraffin-embedded cerebelli from P9 rat pups were stained for TAK1 and p75NTR. **(A)** TAK1 immunoreactivity in the external granule layer (EGL), molecular layer (ML), Purkinje layer (PJ) and internal granule layer (IGL) of the cerebellar cortex. **(B)** p75NTR immunoreactivity in the EGL and PL. **(C,D)** Absence of immunoreactivity when the primary antibody is omitted. **(E)** Higher magnification of the same area of the cerebellar cortex shown in **(A)**. **(F)** TAK1 immunoreactivity in the deep nuclei region.

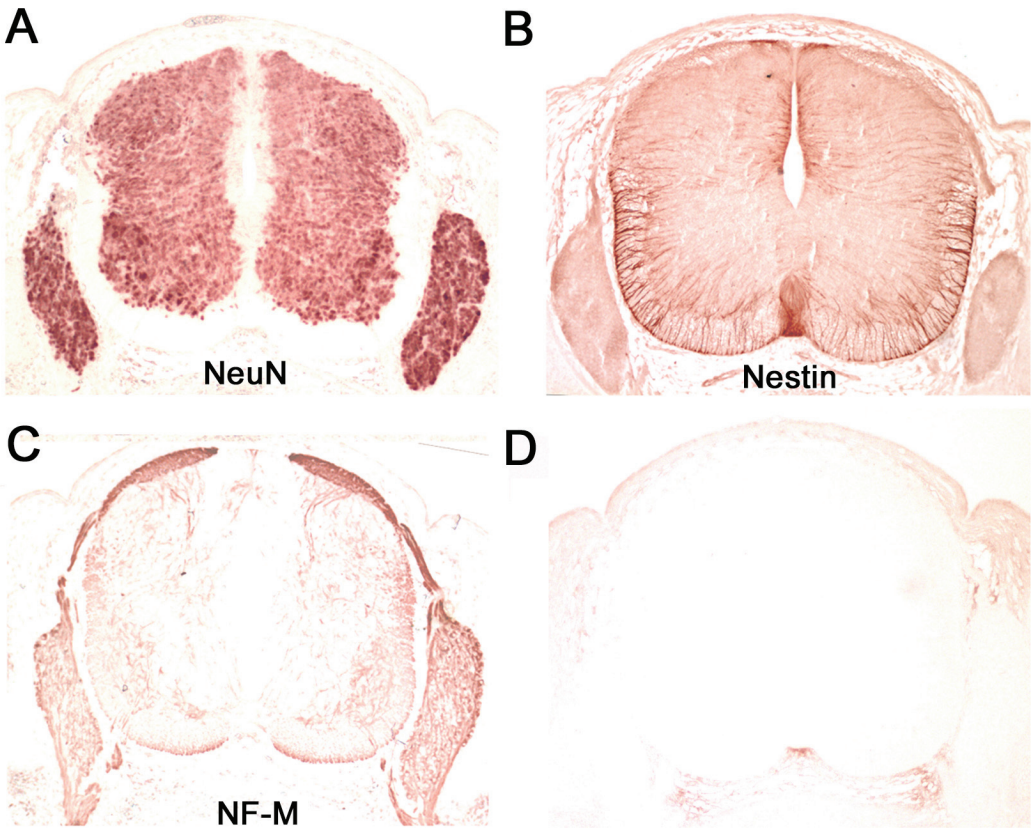


Figure 7. Nestin, NeuN and NF-M staining in the E12.5 spinal cord. IHC on transverse sections of paraffin-embedded E12.5 embryos, using antibodies directed against NeuN **(A)**, nestin **(B)**, and NF-M **(C)**, or no primary antibody **(D).**

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