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CREB Signaling in Neural Stem/Progenitor Cells: Implications for a Role in Brain Tumors

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1. Introduction

Since its discovery in the PC12 rat pheochromocytoma cell line (Montminy & Bilezikjian 1987) the cAMP Response Element Binding (CREB) protein has been implicated in a variety of neuronal responses such as excitation, long-term memory formation, neural cell proliferation and opiate tolerance. Its importance is underscored by the attention this factor has attracted in the neuroscience community, as evidenced by the thousands of citations in the academic bibliographic databases. CREB is a transcription factor which potentially regulates the transcription of hundreds or even thousands of genes in neurons. A variety of protein kinases possess the capability of driving CREB phosphorylation and activation, placing CREB at a hub of multiple intraneuronal signalling cascades. The array of neuronal functions attributed to CREB has expanded recently, with studies showing that CREB has role in neural stem/progenitor cell growth, differentiation and survival. This data, together with complementary studies in tissues outside the CNS showing that CREB activation has oncogenic effects has led to the hypothesis that CREB has an important role in brain tumour biology. Therefore, CREB is a factor which sits within a molecular network potentially integrating signalling events regulating neural stem cells and neurogenesis, neural cancer cells and other cells within brain tumors.

To gain an the understanding of the link between stem cells normally residing in the adult brain and the stem cells which can give rise to a brain tumour, it is important to introduce the concepts relating to the so-called 'cancer stem cell hypothesis'. Indeed, one of the most important advances in brain tumour biology has been the discovery that tumors can develop from cells with stem cell-like characteristics. The reason for the excitement is better understood when one considers the nature of treatments of typical cancers/tumors in a patient. The most relevant example to consider in the context of this chapter is the most common and deadly brain tumour, glioblastoma multiforme (high-grade glioma). Gliomas are difficult to treat and patients usually succumb within months to 1-2 years, even with multiple treatment approaches. Standard treatments rely on 'debulking' of the tumour(s), achieved by surgical excision and/or cytotoxic therapies, usually radiation and chemotherapy. Almost inevitably, this first treatment is followed by relatively rapid relapse

and aggressive tumour recurrence. Considering the existence of glioma cancer stem cells, which give rise to the original tumour mass, it has become clear that these cells, which although few in number, probably lie at the periphery or even outside the main tumour mass and are also resistant to current cytotoxic therapies. Thus, surgery only removes the large tumour mass and cancer stem cells within, sparing other cancer stem cells outside the main tumour mass. These surviving cancer stem cells are able to give rise to the recurring/secondary tumors, which have also evolved to become more resistant to further treatments. Thus, much research has focussed on stem cell biology in the context of cancer and the processes which give rise to cancer stem cells or tumour initiating cells. Research on the mechanisms that play a role in neural birth and brain development are gaining traction in the understanding of brain tumour biology, since there must be common molecular genetic mechanisms operating in both normal/non-tumor neural stem cells and neural cancer stem cells. Indeed, once the parallel mechanisms are understood, then the differences will also become apparent. These differences will also provide the rational basis for therapeutic targeting of neural cancer stem cells.

Aside from contributing to furthering the understanding of the ongoing cellular plasticity of the brain, the knowledge that adult organs, including the brain, harbour stem and progenitor cells throughout the life of the organism has helped develop new concepts on what happens when these cells accumulate mutations in the context of diseases such as cancer. Indeed, the understanding of cancer stem cells has provided a new optimism in the development of novel strategies for cancer therapy (Schatton *et al.* 2009). The signalling networks operating in normal neural and brain tumour initiating cells involve complex molecular networks. At the hub of these networks are the transcription factors, which determine which genes are expressed, when they are expressed and how much of each corresponding mRNA is expressed. There are many transcription factors which have been identified as being important for neural stem cell function but research linking transcription factors regulating normal stem cells and cancer stem cells is still at an early stage. In fact, little is known about what distinguishes a cancer stem cell from a physiologically normal stem cell.

2. Neural stem cells and neurogenesis

The origins of the mammalian central nervous system lie within the neuroepithelium, a thin layer of developing nerve cells. Much of this early developmental period in vertebrates is dedicated to organising the structure of the brain. This organisation precedes a period of rapid cellular expansion, the peak of neurogenesis.

The discovery that neurogenesis persists in the adult vertebrate brain was contrary to the long-held dogma, oft quoted as Santiago Ramon y Cajal's statement referring to the central nervous system that "...nothing may be regenerated". Of course, the available methods over a century ago made it almost impossible to observe or measure the minute fraction of nerve cells undergoing cell division amongst the billions of postmitotic cells in an adult mammalian brain. Since Cajal's time there were sporadic but important reports on the existence of mitotic cells in mature adult mammalian brains (Allen 1912; Altman & Das 1965). The prevailing understanding of neurogenesis is that neural stem cells arise during embryogenesis, and a fraction of these persist into adulthood within discrete regions of adult brain ("neurogenic regions") (reviewed in (Abrous *et al.* 2005)). These cells are distinct

from other, non-neural cell types in the brain (most notably microglia – the "immune cells" of the brain) which retain the ability to proliferate, but cannot generate cells of other neural lineages. Cells fulfilling the criteria of "stemness" (self-renewal, multipotentiality) have been identified in the brains of higher vertebrates, including humans (Eriksson *et al*. 1998). The best characterised neurogenic regions in higher vertebrates lie in the sub-ventricular zone of the lateral ventricles and the sub-granular zone of the hippocampus. The number of proliferating cells and newborn neurons in the dentate gyrus, olfactory bulb and sub-ventricular zone decreases with age (Altman & Das 1965; Kuhn *et al*. 1996), consistent with an age-dependent decline in neurogenic potential. As mentioned previously, there are many factors which regulate neurogenesis, including transcription factors. The CREB transcription factor has only recently been recognised to play an important role in this process. This factor is at the hub of multiple signalling cascades, which are active in neural stem cells and regulates the expression of a series of downstream target genes important for stem cell survival and growth (see Figure 1).

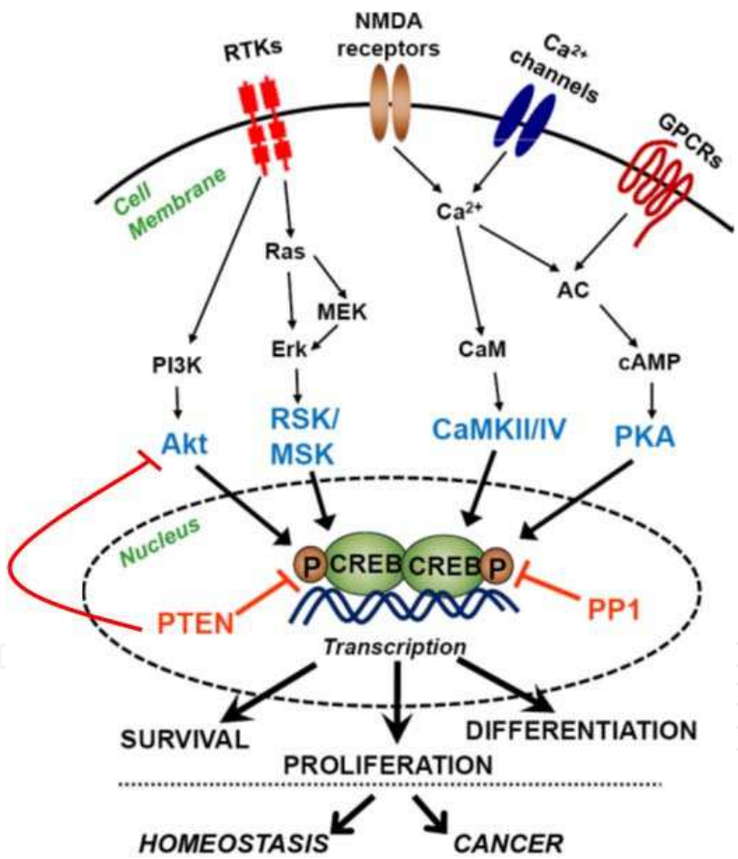

Fig. 1. Several pathways lead to CREB phosphorylation/activation to promote cell survival, proliferation and differentiation. In the context of neural stem cells and cancer, Receptor Tyrosine Kinases (RTKs) are important, since their ligands such as EGF and PDGF are growth factors necessary for cell survival and proliferation. However, the role of the other pathways shown, remain to be investigated in this context. Note that dephosphorylation of CREB via phosphatases occurs via the activity of PTEN and PP1. PTEN may be critical in the context of brain tumors and CREB signalling, as it is often mutated in gliomas.

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3. The CREB transcription factor family

Transcription factors are the terminal convergence points of many signalling pathways, these genes function as effector molecules to activate downstream target genes which in turn regulate NSPC proliferation, cell-cycle exit, induction of differentiation and survival (for a concise review see (Ahmed *et al.* 2009)). The precise cell stage at which a particular transcription factor is active determines its contribution to the cell's progression from immaturity to maturity.

CREB is a nuclear-localised basic leucine zipper superfamily transcription factor, acting as a conduit between upstream signalling kinases and downstream target-gene transcription. Three major isoforms of CREB are known (α, Δ and γ), all transcribed from the same gene, CREB1. Although the best characterised member of the CREB family is CREB itself (Montminy & Bilezikjian 1987), the family also includes CREM (Foulkes et al. 1991) and ATF1 (Hai *et al.* 1988), products of distinct genes. These transcription factors are able to homodimerize or heterodimerize with each other, bind to cyclic-AMP Response Element (CREs) sequences present in target gene promoters and are activated by serine-threonine kinases targeting the phosphorylation of their Kinase-Inducible Domain (KID). Thus, there is an inherent functional redundancy in the CREB transcription factor family which has been shown in mouse knockout studies, where CREB deletion results in an upregulation of CREM expression in an attempt to compensate for many of the cellular functions normally attributed to CREB (Blendy *et al.* 1996; Mantamadiotis *et al.* 2002). Phosphorylation of the KID then causes increased affinity to various transcriptional coactivators such as CREB-Binding Protein (CBP), p300 and the Transducers Of Regulated CREB activity (TORCs), which then leads to the assembly of the transcriptional machinery and transcription initiation. The CREB transcription factor family are potent transcriptional activators although there is some evidence that in certain contexts these factors are capable of repressing transcription (Rutberg *et al.* 1999).

3.1 CREB in NSPCs and neurogenesis

CREB's role in embryonic brain development and neurogenesis is conserved across at least two vertebrate species separated by over 300 million years of evolution, as studies in zebrafish embryos show that CREB has a role in developmental neurogenesis and in midbrain-hindbrain patterning (Dworkin *et al.* 2007). There is also evidence that CREB has a role in the regeneration of the simple nerve net in *Hydra* species and the more complex nervous system of the roundworm *Caenorhabditis elegans* (Chera *et al.* 2007; Ghosh-Roy *et al.* 2010).

In the developing mouse brain, the active phosphorylated form of CREB is seen in cells clustered in the neurogenic regions at E14.5, a time when the brain takes on recognisable neuro-anatomical features and neurogenesis peaks and becomes regionally localised. These

regions include the ventricular zones of both the lateral and third ventricles and the olfactory bulb (Dworkin *et al.* 2009). Consistent with a role in neurogenesis, the activated, phosphorylated form of CREB, pCREB is enriched and restricted to the neurogenic zones of the adult mouse brain, whereas total (unphosphorylated and phosphorylated) CREB protein is present in almost all cells of the brain (Figure 2).

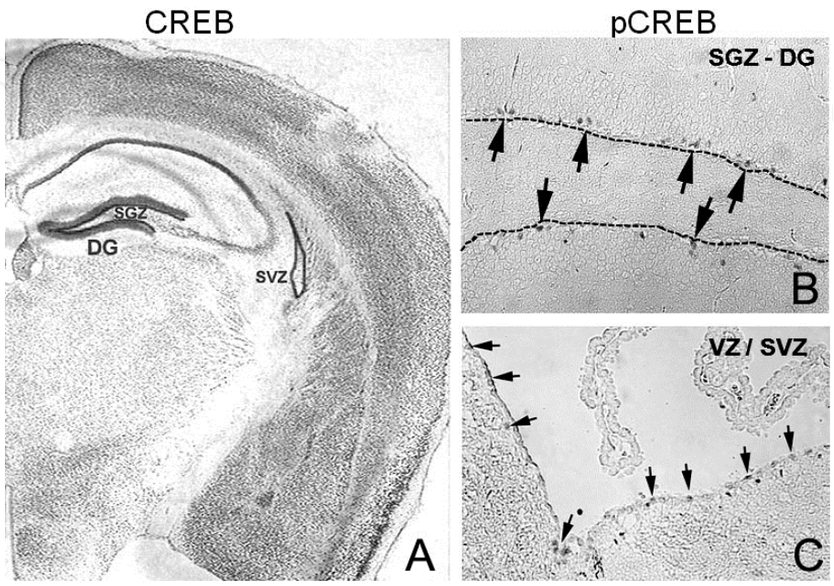

Fig. 2. Immunohistochemical analysis of CREB protein expression in mouse brain.
A) A coronal section of mouse brain showing the global expression of CREB protein (phosphorylated and unphosphorylated). The positive signals are evident as the dark nuclear staining in each cell/neuron. The neurogenic zones, SGZ (sub-granular zone) of the dentate gyrus (DG) located in the hippocampus and SVZ (sub-ventricular zone) are indicated by the dark lines and labels (x4 power). B) In contrast to total CREB protein expression, phospho-CREB expression is evident and restricted to the neurogenic sub-granular zone (SGZ) of the hippocampal dentate gyrus (DG), where some positive cells are indicated by the arrow heads. C) phospho-CREB expression is evident in the SVZ, where some positive cells are indicated by the arrow heads. (B & C x100).

Regulated transient CREB phosphorylation and de-phosphorylation is a well described mechanism by which neuronal activity is regulated in many regions of adult mouse brain (Lonze & Ginty 2002). Moreover, CREB is required for the survival of post-mitotic neurons in mouse brain (Ao *et al.* 2006; Dworkin *et al.* 2009; Giachino *et al.* 2005; Herold *et al.* 2010; Mantamadiotis *et al.* 2002; Riccio *et al.* 1999), while the role of CREB signalling in the proliferation and migration stages of immature neurons is less well defined. In a number of studies, the use of phospho-specific CREB antibodies demonstrate that constitutive CREB activation is restricted to cells in neurogenic regions (Bender *et al.* 2001; Dworkin *et al.* 2007; Dworkin *et al.* 2009; Fujioka *et al.* 2004; Gampe *et al.* 2011; Giachino *et al.* 2005; Herold *et al.* 2010; Nakagawa *et al.* 2002). In zebrafish, phosphorylated CREB is expressed throughout the highly proliferative embryonic brain but in the adult expression is restricted to cells in the proliferative zones (Dworkin *et al.* 2007), in patterns identical to those previously reported for proliferating cells (Grandel *et al.* 2006). Taken together, these data suggest a role for

CREB in proliferating cells in the post-natal adult vertebrate brain. Furthermore, pCREB is also expressed in zones of NSPC migration (Giachino *et al.* 2005), indicating it may also function in maintaining survival of migratory neuroblasts.

A number of CREB mouse mutants have been critical to the investigation of CREB function in vivo. Transgenic mice expressing a dominant-negative mutant CREB shows that CREB has a role in cell expansion and survival in the pituitary gland (Struthers *et al.* 1991) and seminiferous tubules of the testis (Scobey *et al.* 2001). CREB over-expression on the other hand results in increased cellular proliferation (Shankar & Sakamoto 2004; Zhu *et al.* 2004). Mice with germline deletion of all CREB isoforms show a decrease in the size of the corpus callosum and an increase in lateral ventricle area (Rudolph *et al.* 1998), consistent with a decrease in cellularity and displayed significant defects in brain development which were attributed to neurogenic defects (Dworkin *et al.* 2009).

Since loss of CREB leads to an upregulation of the related factor CREM as a compensatory mechanism for CREB loss, a more sophisticated approach was needed to assess the role of CREB signalling loss. Therefore, mice were generated with a germline deletion of CREM and lacking CREB specifically in neural cells. These brain-specific compound CREB-CREM mutant mice displayed severe neuronal death (Mantamadiotis *et al.* 2002), stressing the importance of CREB signalling in neuronal survival. Further studies on on NSPCs derived from CREB-null mice displayed severe defects in survival, cellular expansion and neurosphere forming potential (Dworkin *et al.* 2009). An important question on whether CREB is also important for neural expansion comes from studies in mice where a transcriptionally constitutive active fusion of the CREB DNA-binding domain with the transactivation domain of Herpes Simplex Virus, VP-16-CREB has demonstrated that CREB-dependent genes contribute to neurogenesis (Zhu *et al.* 2004). Similarly, a constitutively active CREB mutant leads to an overproduction of neural cells in zebrafish embryos while a dominant-negative CREB mutant which is able to silence kinase-induced CREB activation, has the opposite effect and inhibits neurogenesis (Dworkin *et al.* 2007).

The upstream or downstream factors associated with the CREB-dependent mechanisms promoting proliferation are not well understood. However, activation of the PI3K/Akt pathway by FGF-2 in cultured adult hippocampal NSPCs resulted in increased CREB phosphorylation and increased progenitor proliferation and decreased differentiation, as did over-expression of wild-type CREB (Peltier *et al.* 2007). Furthermore, increasing cGMP, Akt and GSK3β activity, upstream signals, which phosphorylate CREB, in adult SVZ-derived neurospheres increased NSPC proliferation, whereas down-regulating these signals resulted in decreased proliferation (Peltier *et al.* 2007). Recent work also shows that CREB-dependent NSPC proliferation and neurogenesis is mediated via EGF-induced activation of both PKA (Iguchi *et al.* 2011) and ERK (Gampe *et al.* 2011). All the above mentioned studies were performed in animal model organisms or NSPCs derived from these. So far there are no reports on the role of the CREB pathway in human NSPCs but recent work shows that CREB is activated and functional in neurogenic cells in the adult primate (Japanese macacque) brain (Boneva & Yamashima 2011).

3.2 CREB's oncogenic properties

There are numerous reports in cell, animal and human tissue studies showing a positive correlation between the level of CREB expression and activation and malignancy. A role for

CREB-mediated transcription in cancer was first reported through the identification of a chromosomal translocation t(12;22)(q13;q12) in clear cell sarcomas of soft tissue to give a fusion protein EWS-ATF1 (Zucman *et al.* 1993). This chimaeric protein, consisting of the N-terminal region of EWS (Ewing's Sarcoma) fused with the C-terminal DNA-binding domain of the CREB-related protein ATF1, generates a constitutively active transcription activator capable of binding to the promoters of CREB/ATF1 target genes, which in turn promote tumour development and growth. More recently, a EWS-CREB1 fusion was discovered in a clear cell sarcoma variant (Antonescu *et al.* 2006) and angiomatoid fibrous histiocytomas (Rossi *et al.* 2007).

CREB has been implicated in contributing to the progression of several other tumour types (Conkright & Montminy 2005; Rosenberg *et al.* 2002). Analysis of prostate tumors from patients demonstrated that pCREB expression was restricted to poorly-differentiated prostate cancers and bone metastatic tissue but not to non-tumour benign prostate glands (Wu *et al.* 2007). Increased mRNA levels of CREB are also a feature of breast cancer tissue compared to non-tumour mammary tissue and the level of CREB expression correlated with disease progression and survival (Chhabra *et al.* 2007). In non-small-cell lung cancer the expression levels of CREB and pCREB were elevated in tumour compared to adjacent normal tissues and increased CREB expression correlated with poor patient survival (Seo *et al.* 2008). Human ovarian tumors also exhibit increased CREB expression and ovarian tumour cell lines in which CREB expression is silenced display significantly reduced proliferation (Linnerth *et al.* 2008). Some of the best studies implicating CREB in cancer development come from evidence showing that CREB has a role in the development of bone marrow malignancies. The oncogenic virus human T-cell leukemia virus type 1 (HTLV-1) is strongly associated with T-cell leukemia (ATL) [29, 30]. T-cell oncogenic transformation mediated by the HTLV-1 Tax oncoprotein requires intact CREB signalling (Smith & Greene 1991). Moreover, increased CREB and pCREB expression is seen in bone marrow from patients with ALL (acute lymphoid leukemia) and AML (acute myeloid leukemia) compared to that from healthy patients (Crans-Vargas *et al.* 2002). In addition, CREB expression and in some cases increased CREB gene copy number correlates with disease stage in leukemia patients where CREB overexpression is associated with accelerated relapse and event-free survival (Crans-Vargas *et al.* 2002; Pigazzi *et al.* 2007; Shankar *et al.* 2005). Finally, CREB also appears to regulate malignant melanoma biology by promoting tumour cell survival and metastasis (Jean & Bar-Eli 2000; Melnikova *et al.* 2010).

How CREB regulates tumour growth is still a question that remains unanswered. An obvious approach to unravel the underlying CREB-mediated oncogenic mechanisms is to determine the array of "cancer-associated" genes which CREB directly regulates at the level of transcription. Several genes known to be directly regulated by CREB are implicated in tumourigenesis and uncontrolled proliferation. CREB directly regulates several cell-cycle control genes known to be aberrantly expressed in hyper-proliferative disorders, including *cyclin D1* (Pradeep *et al.* 2004), *cyclin A1* and *A2* (Desdouets *et al.* 1995)(Shankar and Sakamoto, 2004), *bcl-2* (Wilson *et al.* 1996), *HEC1* (a cell-cycle regulatory protein which localizes to the kinetochore in mitosis and is implicated in cancer progression (H. Y. Cheng *et al.* 2007) and *cyclin D2*. Increased *cyclin D2* transcription following CREB transactivation has been implicated in regulating the proliferation of lymphocytes, putatively through phosphorylation of CREB by PI3K and PKA (Assanah *et al.* 2006). In cultured mouse

embryonic fibroblasts (MEFs), phosphorylation of CREB by LiCl increases cyclin D2 expression, whereas inhibition of the CREB-cyclin D2 pathway by the tumour-suppressor phosphatase PTEN decreases the abundance of cyclin D2 mRNA and protein (Huang *et al.* 2007), indicating that CREB-mediated regulation of cyclin D2 may be a conserved partnership regulating proliferation. VEGF was also increased in tandem with increased CREB signalling in metastatic prostate cancer derived from human bone (He *et al.* 2007), strongly supporting a direct role for CREB in mediating cellular proliferation and possibly metastasis. In human brain tumour derived cell lines there is evidence that CREB can be activated by prostaglandin E_2 via the PKA pathway to stimulate cell proliferation (Bidwell *et al.* 2010). Thus, data from cell lines, animal models and importantly patient tumour samples, indicate that CREB not only serves as a diagnostic marker but also has a role in promoting and supporting the development tumors in a variety of cell and tissue types. In the next section we discuss the evidence that suggests CREB may also be an important factor in brain tumour development and growth.

4. Converging evidence for the involvement of CREB in brain cancer

Various studies using brain tumour cell lines suggest that signalling pathways involving CREB activation are important for tumour cell growth and differentiation (Bidwell *et al.* 2010; Golan *et al.* 2011; Kim *et al.* 2010; Morioka *et al.* 2010). To date there has been no evidence linking CREB to brain cancer development or progression in vivo, although a number of recent findings linking CREB activity to PTEN and growth factors, together with the knowledge of CREB's role in NSPC biology, lend support to the view that CREB is an important factor in brain tumour signalling pathways. Of note, recent data shows that CREB is a protein target of PTEN phosphatase activity and that PTEN loss induces CREB-dependent gene expression and cell growth (Boneva & Yamashima 2011). PTEN is a tumour suppressor gene frequently mutated in many cancers including the most aggressive forms of brain cancer, glioblastoma multiforme and related astrocytomas. Indeed PTEN expression appears to directly affect glioblastoma growth as well as glioma-initiating cell proliferation and self-renewal (R. B. Cheng *et al.* 2011). Thus, PTEN loss-of-function mutations would lead to loss of CREB deactivation, allowing the over activation of CREB-dependent cell survival and growth signals in brain cancer stem cells or brain tumour initiating cells (BTICs). Other important signalling pathways in patient brain tumour cells are the epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor (PDGFR) pathways (Brennan *et al.* 2009). EGFR activation is important for glioma stem/progenitor cell growth and resistance to anti-cancer treatments (Murat *et al.* 2008). EGF is able to induce CREB in NSPCs in vivo (Gampe *et al.* 2011); most likely acting through EGFR induced CREB activation via the Ras-MAPK dependent kinase, RSK-2 (Xing *et al.* 1996). Furthermore, there is evidence that CREB is activated in human glioma cells lines and that inhibition of CREB leads to reduced survival of glioma cells (Malla *et al.* 2010). This study also shows that PDGFR-dependent PI3K/Akt signals which converge upon CREB are important for tumour invasiveness, a process which BTICs use to migrate and generate metastatic tumors.

Data from the Human Protein Atlas (www.proteinatlas.org) shows that CREB is highly expressed in all glioma patient samples tested (24 cases) and consistent with the mouse data, human brain also shows robust CREB expression in neurogenic zones (Figure 3). Data from

our own laboratory shows that human glioma tumour tissue (40 cases) exhibits robust pCREB expression compared to only weak staining in non-tumour tissue controls (unpublished data). This implies that the CREB pathway is overactive in human glioma cells, thereby driving the survival and growth of these cells. More interest is the potential role that CREB may be playing in the glioma stem cells, which are the cellular source of the tumour and which may also be responsible for the relapse of tumour growth following therapy. Data from primary mouse NSPCs shows that CREB is required for the expression of various growth and survival factors including BDNF, NGF, PACAP and Bcl-2 (Dworkin *et al.* 2009). It is likely that the expression of growth and survival factors will be dependent upon CREB-dependent transcription.

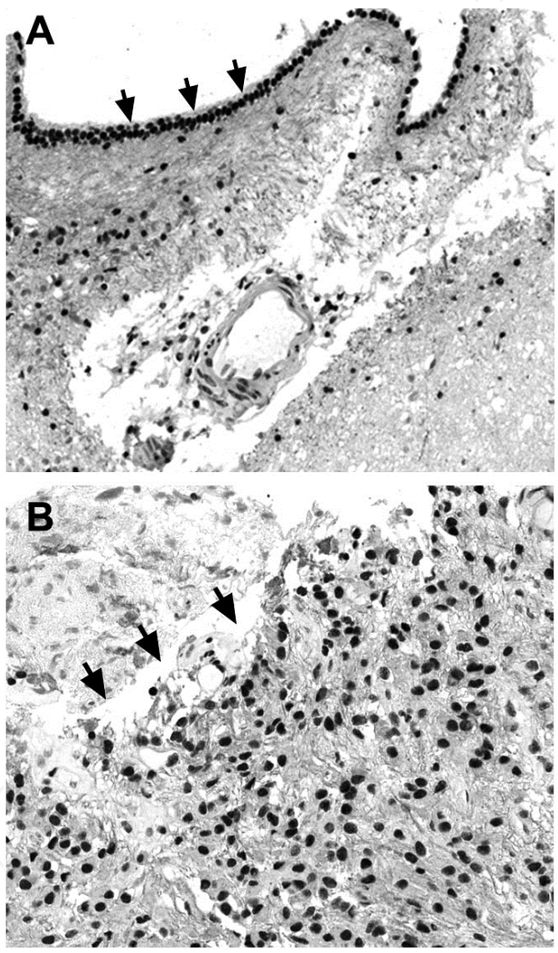

Fig. 3. CREB expression is human brain.
A) CREB expression is enriched in the human brain SVZ, as seen by the intense nuclear staining of cells lining the ventricular space (indicated by arrow heads). B) Intense CREB expression is clearly evident in human high grade glioma. The non-tumour cells show weak staining (behind the arrow heads). According to the Human Protein Atlas data, 100% (24 cases) of brain tumour samples tested exhibited strong CREB expression (Uhlen et al., *Nat Biotechnol.* 2010 28(12):1248-50 and http://www.proteinatlas.org). The images were from the Human Protein Atlas database.

5. Conclusion

In conclusion, there is significant emerging experimental data implicating the CREB signalling pathway in the development and maintenance of brain tumors. Investigation of the CREB signalling pathway and transcriptome in glioma cell lines, BTICs and new animal models will shed light on the importance of this pathway in glioma biology. This knowledge will provide an opportunity to investigate novel drug targeting approaches in glioma treatment, targeting CREB itself or an upstream or downstream component of the CREB-pathway. Opinions on whether widely expressed factors which are critical to cell function are good targets vary widely and have evolved over the last decades. CREB may well prove to be a good anti-tumour target in the brain, as tumors seem to express high levels of the activated phosphorylated form. This is in contrast with the physiologically normal adult brain which only transiently exhibits pCREB expression only in discreet nuclei responsible for a specific neuronal response (eg. the suprachiasmatic nucleus in response to visual light stimulation). This observation together with the ever advancing drug delivery technologies may allow targeting of CREB in brain tumors with minimal toxicity to neurons outside the tumour.

6. Acknowledgements

This work has been supported by an FP7 Marie Curie IRG (IRG231032/Neurogencreb), Karatheodori Grant (2010-4735 Uni Patras) and the Department of Pathology, The University of Melbourne.

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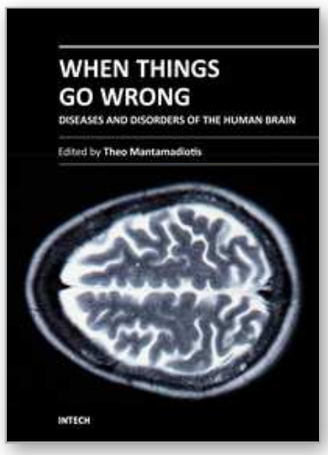

When Things Go Wrong - Diseases and Disorders of the Human Brain

Edited by Dr. Theo Mantamadiotis

ISBN 978-953-51-0111-6
Hard cover, 238 pages
Publisher InTech
Published online 29, February, 2012
Published in print edition February, 2012

In this book we have experts writing on various neuroscience topics ranging from mental illness, syndromes, compulsive disorders, brain cancer and advances in therapies and imaging techniques. Although diverse, the topics provide an overview of an array of diseases and their underlying causes, as well as advances in the treatment of these ailments. This book includes three chapters dedicated to neurodegenerative diseases, undoubtedly a group of diseases of huge socio-economic importance due to the number of people currently suffering from this type of disease but also the prediction of a huge increase in the number of people becoming afflicted. The book also includes a chapter on the molecular and cellular aspects of brain cancer, a disease which is still amongst the least treatable of cancers.

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