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CK2 Contributes to the Synergistic Effects of BMP7 and BDNF on Smad 1/5/8 Phosphorylation in Septal Neurons

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UNIVERSITY OF MIAMI

CK2 CONTRIBUTES TO THE SYNERGISTIC EFFECTS OF BMP7 AND BDNF ON SMAD 1/5/8 PHOSPHORYLATION IN SEPTAL NEURONS

By
Florence Chaverneff

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Florence Chaverneff

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The combination of bone morphogenetic protein 7 (BMP7) and neurotrophins (e.g. brain-derived neurotrophic factor, BDNF) protects septal neurons during hypoglycemic stress. I investigated the signaling mechanisms underlying this synergistic protection. BMP7 (5 nM) increased phosphorylation and nuclear translocation of BMP-responsive Smads 1/5/8 within 30 min in cultures of rat embryonic septal neurons. BDNF (100 ng/ml) enhanced the BMP7-induced increase in phospho-Smad levels in both nucleus and cytoplasm; this effect was more pronounced after a hypoglycemic stress. BDNF increased both Akt and Erk phosphorylation, but pharmacological blockade of these kinase pathways (with wortmannin and U0126, respectively) did not reduce the Smad phosphorylation produced by the BMP7+BDNF combination. Inhibitors of casein kinase II (CK2) activity reduced the (BMP7 + BDNF)-induced Smad phosphorylation, and this trophic factor combination increased CK2 activity in hypoglycemic cultures. These findings suggest that BDNF can increase BMP-dependent Smad phosphorylation via a mechanism requiring CK2. Preliminary results indicate that a cytoplasmic component robustly inhibits CK2. Protection of septal cholinergic neurons during a hypoglycemic stress is inhibited by a CK2 inhibitor and by a Phosphatidylinositol 3-kinase inhibitor, indicating that increases in CK2 activity and in Smad phosphorylation are only part on the protective mechanisms.
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Abbreviations

ACh  Acetyl choline
ActR  Activin receptor
Akt  Protein kinase B
Alk  Activin receptor-like kinase
Az  Ornithine decarboxylase antizyme
BAMBI  BMP and activin membrane-bound inhibitor
BMP  Bone morphogenetic protein
BMPR  Bone morphogenetic protein receptor
BRE  BMP-responsive elements
CamKII  Calmodulin kinase II
CBP  CREB binding protein
ChAT  Choline acetyl transporter
CK2  Casein kinase 2
CNS  Central nervous system
CREB  cAMP-regulated enhancer binding protein
DAG  Diacylglycerol
Dpp  Decapentaplegic
E15  Embryonic day 15
EGF  Epidermal growth factor
Erk  Extracellular signal-regulated kinase
FGF  Fibroblast growth factor
GDNF  Glial cell line-derived neurotrophic factor
Grb-2  Growth factor receptor-bound protein 2
GSK-3β  Glycogen synthase kinase-3beta
HES  Hairy/Enhancer of split
Mad  Mother against decapentaplegic
MAPK  Mitogen-activated protein kinases
MEK  MAPK extracellular signal-regulated kinase
MH  Mad homology domain
MW  molecular weight
NIC  Notch receptor intracellular domain
OAZ  Olf-1/EBF-associated Zinc Finger
ODC  Ornithine decarboxylase
Op  Osteogenic protein
p75NTR  pan neurotrophic receptor
PARP  Poly (ADP-ribose) polymerase
PI3K  Phosphatidylinositol 3-kinase
PKC  Protein kinase C
PLCγ1  Phospholipase C
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue deleted on chromosome 10</td>
</tr>
<tr>
<td>RSK</td>
<td>pp90 Ribosomal S6 kinase</td>
</tr>
<tr>
<td>SCP</td>
<td>Small C-terminal domain phosphatases</td>
</tr>
<tr>
<td>Shc</td>
<td>Src homology 2 domain-containing transforming protein C</td>
</tr>
<tr>
<td>Smad</td>
<td>Sma- and Mad-related protein</td>
</tr>
<tr>
<td>Smurf</td>
<td>Smad ubiquitination regulatory factor</td>
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<td>TAB</td>
<td>TAK binding proteins</td>
</tr>
<tr>
<td>TAK</td>
<td>TGF-β-activated kinase</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA box binding protein</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>Tob</td>
<td>Transducer of ErbB-2</td>
</tr>
<tr>
<td>Trk</td>
<td>Tropomyosin-related kinase</td>
</tr>
<tr>
<td>TRPM7</td>
<td>Transient receptor potential melastatin 7</td>
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Evidence for survival-promoting effects of neurotrophins in the central nervous system (CNS) is abundant in the literature, starting with the discovery of nerve growth factor (NGF) by Levi-Montalcini & Angeletti, 1963. Work from our laboratory has shown that combinations of bone morphogenetic proteins (BMPs 6 or 7) and neurotrophins preserve Choline acetyl transferase activity (ChAT) both during a hypoglycemic stress and low-density stress (Fig. 1.1 and Nonner et al., 2001). This combination of trophic factors is much more effective than either factor separately in preserving cholinergic function. The aim of the work reported in this document was to determine the mechanisms underlying this synergistic protective effect by BMPs and neurotrophins in cultures of rat embryonic septal neurons.

**Fig. 1.1 Combinations of BMPs and neurotrophins preserve Choline acetyl transferase (ChAT) activity during hypoglycemia.** In control medium, during hypoglycemia, ChAT activity is reduced to 13% of that from normoglycemic sister cultures. BMPs 6 or 7 (2 nM) in combination with any of the four neurotrophins (100 ng/ml, hatched bars) significantly increases ChAT activity when the trophic factor combination is applied during hypoglycemia, but not when applied alone (hatched bars in C group for control). The neurotrophins also failed to have a significant effect when applied alone (open bars). Nonner et al., 2001.
I will first introduce neurotrophins, emphasizing the pathways they activate to mediate their survival-promoting effects. I will then describe BMP trophic factors, discussing their role, expression pattern and signaling pathways in the central nervous system. Reported evidence for synergistic effects of BMPs and neurotrophins will be presented. Finally, since our studies determined that CK2 may be involved in the synergy between BMP- and neurotrophin pathways for the survival of septal neurons, I will introduce this kinase and describe its complicated and still largely unresolved regulation.

1. Neurotrophins, neurotrophic receptors and effects they mediate in the basal forebrain

Four neurotrophins are present in the mammalian brain, brain-derived neurotrophic factor (BDNF), NGF, neurotrophin 3 (NT3) and neurotrophin 4-5 (NT4-5). Proteolytic cleavage of pro-neurotrophins (30-35 kDa) produces the mature neurotrophins (12-13 kDa). Pro-neurotrophins have recently been shown to exert a role in promoting cell death by binding with high affinity to the pan neurotrophic receptor, p75NTR (Lee et al., 2001), whereas mature neurotrophins preferentially bind tropomyosin-related kinase receptors (Trk) to promote cell survival. Beyond their neuroprotective role during development, neurotrophins are also implicated in synapse formation and function, dendritic and axonal outgrowth as well as cell proliferation and migration. This diversity of biological functions by these trophic factors involves several levels of specification. First, neurotrophins bind specifically to different Trk receptors: NGF and NT-3 bind TrkA, BDNF, NT-3 and NT-4 activate TrkB and NT-3 is a ligand for TrkC, whereas all neurotrophins can bind p75NTR (Fig. 1.2). Second, the particular ligand binding the neurotrophic receptor determines the nature of the biological effect. For
example, BDNF and NT-4 both bind TrkB but only NT-4 promotes survival of D-hair mechanoreceptors (Minichiello et al., 1998). Third, timing of neurotrophin binding (constitutive release vs. regulated secretion) affects their role (Kafitz et al., 1999). Finally, the location of neurotrophin receptors (on the cell soma, pre-synaptically on axons or post-synaptically on dendrites) influences the nature of the response.

a. Neurotrophin signaling mediated through Trk receptors

All three Trk receptors are expressed by basal forebrain neurons and both BDNF and NGF elicit responses in vivo and in vitro. Survival of the basal forebrain cholinergic population in culture is enhanced by BDNF, NGF and NT4-5 (Nonomura et al., 1995). NGF increases ChAT activity and increases protein and mRNA levels of both p75NTR and TrkA (Li et al., 1995). TrkA expression alone or co-expressed with p75NTR is required for NGF’s trophic effects (Hempstead et al., 1991; Jing et al., 1992). Similar effects are mediated by BDNF through TrkB receptors on basal forebrain cholinergic neurons (Alderson et al., 1990). NT-3 and NT-4 both increase ChAT activity in vitro through TrkC and TrkB respectively (Friedman et al., 1993). Divergent findings concerning the effect of neurotrophins on the survival of basal forebrain cholinergic neurons have been

Fig. 1.2 Receptor selectivity for neurotrophins. All four neurotrophins and pro-neurotrophins can bind the pan neurotrophic receptor, p75NTR, whereas the Trk receptors are more specific (Adapted from Segal, 2003).
reported: some studies found an increase in the number of cells with detectable cholinergic markers such as p75NTR (Sobreviela et al., 1994) in response to NGF (Alderson et al., 1990; Svendsen et al., 1994), while others could not detect survival-promoting effects of the neurotrophins (Nonner et al., 1992, 1993 & 1994). These discrepancies may be due to differences in basal stress levels. Stress increases survival-promoting effects by the neurotrophins on basal forebrain cholinergic neurons (Nonner et al., 1996). This work focuses on BDNF/TrkB, because both are widely distributed in the adult brain and TrkB is present on both cholinergic and non-cholinergic septal neurons.

**Fig. 1.3 Survival signals downstream of Trk receptors are mediated through activation of the PI3 kinase/Akt pathway or the Mek/Erk pathway.** PI3 kinase-activated Akt promotes survival either by enhancing transcription of pro-survival genes (through phosphorylation of IκB and CREB) or by inhibiting apoptotic processes (through phosphorylation of Forkhead or Bad). Activation by Ras and Raf of the MEK/Erk pathway downstream of Trk receptor through adapter proteins (Shc, Grb-2, SOS) leads to activation of Rsk. Activated Rsk promotes survival, either by enhancing expression of the pro-survival gene Bcl-2 (through CREB) or by inhibiting pro-apoptotic Bad (Modified from Yuan & Yankner, 2000).
Signaling of neurotrophins via Trk receptors causes phosphorylation of tyrosine residues on their cytoplasmic domain, which allows binding of adaptor proteins through their src-homology-2 or phosphotyrosine-binding motifs (reviewed in Pawson & Nash, 2000). Binding of these adaptor proteins permits activation of the mitogen-activated protein kinase extracellular signal-regulated kinase (MEK)/ Extracellular signal-regulated kinase (Erk), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) and phospholipase C-γ1 (PLC-γ1) signaling pathways (Kaplan & Miller, 2000 and Fig. 1.3).

i. PI3K/Akt signaling

Once activated downstream of Trk receptors, PI3K generates phosphatidyl inositol bis- and trisphosphates that regulate signal transduction cascades. Akt is the major target downstream of PI3K that mediates survival signals through Trk receptors. Akt exerts these effects by inhibitory phosphorylation of pro-apoptotic proteins (reviewed in Yuan & Yankner, 2000 and Fig. 1.3). For example, binding of the Bcl-2 family member, Bad to Bcl-xl results in increased activation of the proapoptotic Bax. Phosphorylation of Bad by Akt allows Bad to bind 14-3-3 proteins, preventing Bad from promoting apoptosis by binding to Bcl-xl (Datta et al., 1997). Akt also phosphorylates IκB (thereby targeting it for degradation), preventing it from sequestering NFκB in the cytoplasm. NFκB is then free to translocate to the nucleus and activate the transcription of neuronal survival-promoting genes (Middleton et al., 2000). Forkhead transcription factor FKHRL1, upon phosphorylation by Akt, binds 14-3-3 proteins and is sequestered in the cytoplasm, preventing it from activating genes involved in cell death, such as the Fas ligand (Brunet et al., 1999). Another example of a target of Akt is cAMP-regulated enhancer binding protein, CREB. Phosphorylation of CREB downstream of PI3K/Akt
stimulates the recruitment of CREB binding protein, CBP and activates genes coding for prosurvival factors (Du & Montminy, 1998; Riccio et al., 1999).

ii. Mek/Erk signaling

Trk activation also leads to activation of Ras via adapter proteins that include She, Grb-2 and the Ras exchange factor, SOS. Neuronal survival is promoted downstream of Ras, through activation of the PI3K/Akt or the Mek/Erk (mitogen-activated protein kinase, MAPK, extracellular signal-regulated kinase/extracellular signal-regulated kinase) pathway (Fig. 1.3). In the MEK/Erk pathway, Ras, activated by SOS can in turn activate Raf. This leads to the sequential activation of MEK1/2, Erk1/2 (=MAPK) and pp90 ribosomal S6 kinase, Rsk (Xing et al., 1996). CREB phosphorylation can result from activation of both the MEK/Erk and the p38 MAPK pathways (Xing et al., 1998). Several survival-promoting effects of neurotrophins are mediated downstream of Trk receptors through the Mek/Erk pathway. Thus, NGF-mediated phosphorylation of CREB promotes expression of the pro-survival gene Bcl-2, allowing survival of sympathetic neurons (Riccio et al., 1999). BDNF enhances survival of cerebellar neurons via activation of the MEK/Erk pathway, both by activating pro-survival genes through CREB phosphorylation and by inhibitory phosphorylation of the pro-apoptotic Bad through activation of Rsk (Bonni et al., 1999).

iii. PLCγ1 signaling

Another pathway through which neurotrophins exert their survival-promoting effects via the Trk receptors is by activation of phospholipase C (PLCγ1). Hydrolysis of phosphatidylinositides by PLCγ1 generates diacylglycerol (DAG) and inositol triphosphates, which leads to increased intracellular Ca^{2+} (Vetter et al., 1991). Calcium can
in turn activate Ca\textsuperscript{2+}-calmodulin kinases. Intracellular calcium and its receptor protein, calmodulin (CaM) were shown to transduce neurotrophin signals responsible for survival in PC12 cells (Egea \textit{et al.}, 2001) and in neocortical neurons (Silva \textit{et al.}, 2000).

\textbf{b. Neurotrophin signaling mediated through p75\textsuperscript{NTR}}

p75\textsuperscript{NTR} is expressed by basal forebrain cholinergic neurons throughout life and its activation by pro-NGF may lead to the death of this neuronal population during development (Naumann \textit{et al.}, 2002). All four neurotrophins bind p75\textsuperscript{NTR} with similar affinity and were shown to exert some of their survival-promoting effects via this receptor (Barrett & Bartlett, 1994). Activation of the p75\textsuperscript{NTR} by neurotrophins recruits the adaptor protein TRAF-6 (Khursigara \textit{et al.}, 1999). Association of p75\textsuperscript{NTR} with other neurotrophic receptors determines the nature of downstream effects. For example, binding of proneurotrophins to p75\textsuperscript{NTR} and the receptor sortilin with high affinity results in cell death (Fig. 1.4 and Nykjaer \textit{et al.}, 2004). But, when binding to p75\textsuperscript{NTR}, NGF promotes neuronal survival through the NF\kappa B pathway (in sympathetic neurons, Maggirwar \textit{et al.}, 1998 and in sensory neurons, Hamanoue \textit{et al.}, 1999). Neurotrophins can also induce neuronal cell death by binding to p75\textsuperscript{NTR} in the absence of Trk receptor activation (Friedman, 2000). Other effects of NGF’s chronic action on p75\textsuperscript{NTR} include increased cholinergic and glutamatergic neurotransmission in medial septal cholinergic neurons (Huh \textit{et al.}, 2008).
2. Bone Morphogenetic Proteins and their receptors

a. BMPs and their expression pattern

BMP trophic factors comprise a group of over 20 members that belong to the transforming growth factor-β (TGF-β) superfamily of secreted molecules, which also includes activins (Fig. 1.5). Based both on their structure and evolutionary conservation, they can be subdivided into several categories. 60A, BMP5, BMP6 (or Vgr1), BMP7 (or Osteogenic protein 1, OP1), BMP8a (OP2) and BMP8b (OP3) belong to one group. Dpp (Drosophila’s decapentaplegic gene product), BMP2 and BMP4 constitute another group. BMP9, BMP10 and Dorsalin form a third family (reviewed in Ebendal et al., 1998).

Both neurons and astrocytes were found to express BMPs 6 & 7 (in neurons: Tomizawa et al., 1995 and in glia: Schluesener & Meyermann, 1994). BMP6 mRNA is present in the basal forebrain of rats at all stages of development with a peak in expression perinatally, returning to E15/E18 levels at adulthood (Tomizawa et al., 1995). At these stages, BMP6 transcript is also present in the hippocampus and the neocortex.
target areas for basal forebrain’s cholinergic neurons (Tomizawa et al., 1995). Transcripts for BMPs 2-7 are detected in adult mouse and rat brains (Charytoniuk et al., 2000). BMP6 & 7 transcripts are expressed in adult rat brain’s meninges and choroid plexus. BMP7 is also present in septal regions, whereas BMP4 transcripts are expressed in myelinated (probably oligodendrocytes) structures. Levels of BMP4 transcript were found to gradually increase during development, peaking 3 weeks postnatally and decreasing only slightly thereafter (Fan et al., 2003). Our work focused on BMPs 6 and 7.

b. BMP receptors and their expression pattern

BMP signaling is mediated through a complex of transmembrane serine/threonine kinase receptors of two types (I and II). The BMP family includes three type I: IA or activin receptor-like kinase (Alk-3), IB or Alk-6 and Alk-2 and three type II receptors: BMP-RII and activin type II receptors (Act), ActR and ActRIIB. BMP7 binds to Alk2, Alk3 and Alk6 and to BMPR-II, ActRII and ActRIIB, (Macias-Silva et al., 1998).
In a study of expression of BMP-R transcripts in the adult rat brain, only low expression of the type I receptor was detected in regions including the striatal neuroepithelium and the thalamus for BMPR-IA and in the olfactory epithelium for BMPR-IB, but transcripts for the type II receptor were found in several areas including olfactory bulbs, hippocampus, hypothalamus, cortex and striatum (Zhang et al., 1998; Charytoniuk et al., 2000). In the mouse basal forebrain, both mRNA and protein for BMPR-IB and BMPR-II as well as mRNA for BMP-RIA were detected at early developmental stages (E14, E17). Others found messenger for BMPR-IA to display widespread expression throughout the brain, whereas that for BMPR-IB showed more restricted distribution (e.g. in the anterior olfactory nuclei, Zhang et al., 1998). In the adult brain, mRNA for BMPR-II was found to be expressed in several regions including the cortex and the hippocampus, but mRNA for BMPR-IA and -IB were not detected (Söderström et al., 1996). Messengers for ActRIIB and for BMPR-IA, -IB and -II were detected in basal forebrain of rats at embryonic day (E) E14, E17 and in the adult basal forebrain of rats, whereas ActRII was present at E14 and E17, but not at the adult stage (Lopez-Coviella et al., 2006). Thus, the receptors required for activation of the BMP signaling pathway by BMP6/7 are expressed at the stage (E15) studied here. Transcripts for BMP signaling molecules, Smads 1, 4 and 5 were also present at those same early stages but no Smad8 messenger was detected until adulthood (Lopez-Coviella et al., 2006).
c. The BMP signaling pathway

Signaling in the BMP pathway is mediated from the cell membrane to the nucleus by Sma- and Mad-related proteins, Smads. Smads are the vertebrate homologues of drosophila’s mothers against decapentaplegic (Mad) and C. Elegans’ Sma proteins. There are three categories of Smads: receptor-activated Smads (Smads 1, 5 and 8 for BMPR; Smads 2 and 3 downstream of TGF-β receptors), co-Smad (Smad 4) and inhibitory Smads (Smads 6 & 7; reviewed in Shi et al., 2003).

i. Canonical BMP signaling

Transduction in the BMP signaling pathway is initiated by binding of BMPs as homo- or heterodimers to transmembrane serine/threonine kinase type II BMP receptors (BMP-R II, see Fig. 1.6). The type II receptors bind BMPs with low affinity and are constitutively active serine/threonine kinase receptors. Type I receptors, on the other hand, bind BMPs with high affinity. Association of BMPs to their receptors allows recruitment of type I BMPR (BMPR-I), which gets phosphorylated by the type II receptor on serine and threonine residues in its intracellular domain (Wrana et al., 1994). Activated BMPR-I recruits and phosphorylates BMP-responsive Smads (Smads 1, 5 or 8) on their C-terminal SSXS motif, thereby activating them. Once phosphorylated, R-Smads dissociate from the receptor complex and bind the common Smad, Smad 4. This Smad complex translocates to the nucleus where, along with transcription co-factors (including CBP and p300), it binds to promoter regions of target genes (reviewed in Zwijsen et al., 2003).
Fig. 1.6 Nucleocytoplasmic shuttling in the Smad signaling pathways. In the absence of stimulation (a), activation of BMP-responsive Smads (Smads 1/5/8) and TGF-β-responsive Smads 2 & 3 by their receptors is prevented by binding to the cytoplasmic retention molecules, Endofin and SARA (Smad anchor for receptor activation) respectively. Upon binding of ligands (b), receptor-activated Smads detach from the cytoplasmic retention molecules and get phosphorylated by the receptors on their C-terminal SSXS motif, allowing them to bind the co-Smad (Smad4) and translocate to the nucleus where the complex activates gene transcription. Nuclear import and export are facilitated by the presence of nuclear localization signal (NLS) and nuclear export sequence (NES), respectively, which also allow nucleocytoplasmic shuttling of Smad 4 and Smad1 (not shown). After gene activation, receptor-responsive Smads are dephosphorylated and exported to the cytoplasm for recycling (adapted from Reguly & Wrana, 2003).

ii. Non-canonical BMP signaling

Several non-canonical BMP-induced pathways have been identified to date. For example, the p38 MAPK pathway can be activated downstream of BMPs in a Smad-independent manner. Binding of BMP2 to the isolated type I receptor triggers recruitment
of the type II receptor and thus can activate the p38 MAPK pathway (Hassel et al., 2003). BMPs 2 & 4 enhance activity of the Map kinase kinase TAK1 and its binding proteins TAB1 & 2, thereby activating the p38 MAPK pathway (Kimura et al., 2000). Treatment of kidney proximal tube epithelial cells with low concentrations of BMP7 results in phosphorylation of p38 MAPK and its nuclear accumulation (Motazed et al., 2008). Another example of BMP activation of Smad-independent pathways relies on the finding that BMP2 activates both p38 MAPK and c-Jun N-terminal kinase, JNK, in osteoblasts (Guicheux et al., 2003). BMP2 was also shown to promote apoptosis in osteoblasts by activating a PKC-dependent pathway (Haÿ et al., 2001). Finally, identification of an interaction between the C-terminal tail of BMPR-II and the cytoskeletal regulator LIM kinase 1 revealed another alternative pathway whereby BMPs inhibit actin depolymerization (Foletta et al., 2003).

iii. Smad structure

Of the eight vertebrate Smads, Smads 2 & 3 are activated downstream of TGF-β and activin receptors whereas Smads 1, 5 & 8 are activated by the BMPR. Smad proteins are composed of three domains, the Mad homology domain (MH1) at the N-terminus, a proline-rich linker and a MH2 domain at the C-terminus (Fig. 1.7 and reviewed in Derynck & Zhang, 2003). Both MH1 and MH2 domains are highly conserved across species and among R- and co-Smads, whereas the linker region is divergent. The MH1 domain is involved in binding with cytoplasmic partners and contains a nuclear localization signal involved in nuclear import. It is also through the MH1 domain that DNA binding and interaction with nuclear proteins occurs. Inhibitory Smads (Smads 6 & 7) lack the MH1 domain and inhibit Smad signaling by competing for binding with co-
Smad. Recognition and phosphorylation by the BMPR type I takes place in the MH2 domain, more specifically, on the C-terminal SSXS motif. Interaction of R-Smads with Smad 4, inhibitory Smads, adapters in the cytoplasm and transcription factors in the nucleus takes place on the MH2 domain.

iv. BMP-induced genes

Two BMP-responsive regions (BMP-responsive elements, BRE) were identified in the promoter of the basic helix-loop-helix inhibitor Id1, a direct target of BMP (but not of Transforming growth factor-β, TGF-β) trophic factors (Korchynskyi & ten Dijke, 2002). Identification of these BRE sequences led to the identification of BMP-induced Smad signaling target genes. Smads display weak DNA binding on this BRE sequence.
Fig. 1.8 Selection of BMP/BMP-responsive Smads target genes. Modified from Zwijsen et al., 2003.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Big-3</td>
<td>seven WD-40 repeat protein</td>
</tr>
<tr>
<td>Btk</td>
<td>Ser/Thr protein kinase</td>
</tr>
<tr>
<td>Runx2/ebfa1/OSF2</td>
<td>DNA binding subunit of core binding transcription factor CBP</td>
</tr>
<tr>
<td>Dlx 2, Dlx 3, Dlx 5</td>
<td>homeobox proteins</td>
</tr>
<tr>
<td>Gata2, Gata4</td>
<td>zinc finger transcription factors</td>
</tr>
<tr>
<td>Hex</td>
<td>homeobox protein</td>
</tr>
<tr>
<td>Id1, Id2, Id3</td>
<td>inhibitors of basic helix-loop-helix proteins</td>
</tr>
<tr>
<td>JunB</td>
<td>proto-oncogene</td>
</tr>
<tr>
<td>Msx 1</td>
<td>homeobox protein</td>
</tr>
<tr>
<td>Msx 2</td>
<td>homeobox protein</td>
</tr>
<tr>
<td>Nkx2.5</td>
<td>homeobox protein</td>
</tr>
<tr>
<td>Noggin</td>
<td>BMP antagonist</td>
</tr>
<tr>
<td>Osteopontin (OPN)</td>
<td>bone matrix protein</td>
</tr>
<tr>
<td>Osteoprotegrin (OPG)</td>
<td>soluble decoy TGFβ receptor</td>
</tr>
<tr>
<td>Smad6, Smad7</td>
<td>inhibitory Smad</td>
</tr>
<tr>
<td>Tbx2</td>
<td>T-box transcription factor</td>
</tr>
<tr>
<td>Tlx2</td>
<td>homeobox protein</td>
</tr>
<tr>
<td>Vent2</td>
<td>homeodomain transcription factor</td>
</tr>
</tbody>
</table>

(Shi et al., 1998) and efficient Smad-induced gene activation requires the presence of transcription co-activators.

The Olf-1/EBF-associated Zinc Finger, OAZ, allows specific BMP-responsive Smad binding to DNA three different ways: it cooperates with BMP-responsive Smads (but not with TGF-β-responsive Smads, Smads 2 and 3), it recognizes BRE in target genes and it is expressed only in particular cell types (Hata et al., 2000). BMP-induced genes include zinc finger transcription factors (GATA2, GATA4), the proto-oncogene JunB and the homeodomain transcription factor, Vent2 (Fig. 1.8 and reviewed in Zwijsen et al., 2003). Other BMP target genes code for BMP-responsive Smad interacting proteins and are involved in negative feedback loops of BMP signaling (e.g. Smads 6 & 7, reviewed in Zwijsen et al., 2003).

The pattern of gene expression in E14 mouse basal forebrain purified cholinergic neurons induced by BMP9 was examined by microarray analysis (Lopez-Coviella et al., 2006). Treatment of basal forebrain cholinergic neurons with BMP9 results in upregulation of genes coding for: transcription factors such as the helix-loop-helix Id1 & 3, also highly expressed in control cultures and Mdfi, Gata2, Msx1, Msx2 with little expression in control cultures. BMP9 treatment also upregulates expression of signal
transduction molecules (e.g. noggin, ciliary neurotrophic factor, NGF receptor, BMPR-IA), extracellular matrix and adhesion molecules and of enzymes, transporters and chaperonins.

v. Intracellular regulation of BMP signaling

The BMP-mediated Smad signaling pathway, like most other signaling pathways, is regulated by negative feedback loops that restrict the duration of its activation. Several molecules have been identified in vertebrates as negative regulators of the BMP pathway (summarized in Fig. 1.9). BMP and activin membrane-bound inhibitor (BAMBI) acts as a dominant-negative receptor in cells. Binding of BAMBI to BMP type I receptors inhibits association with type II receptors, thereby preventing BMP signaling (Onichtchouk et al., 1999). Inhibitory Smads (I-Smads 6 & 7) constitute another type of BMP-signaling inhibitory molecule. Phosphorylation of BMP-responsive Smads by BMPR type I is inhibited by binding of I-Smads to this receptor (Imamura et al., 1997). Smad 6 can also inhibit Smad signaling by binding Smad 4, thus preventing its binding to Smad1 (Hata et al., 1998). Expression of I-Smads is induced by BMPs and therefore constitutes a negative feedback loop (Afrakhte et al., 1998). Tob was shown to block Smad signaling in a BMP-dependent manner by binding to Smads 1, 4, 5 & 8, thus creating another negative feedback loop (Yoshida et al., 2000).
Fig. 1.9 Inhibition and feedback regulation of Smad signaling. Inhibition in the Smad signaling pathway is achieved by Smurf1 (which targets R- and co-Smads to the proteasome), by the transcriptional co-repressors, Ski and SnoN and by dephosphorylation of the C-terminal SSXS motif (by small C-terminal domain phosphatases, SCP 1, 2 & 3 and by Ser/Thr protein phosphatases, PPM1A). Feedback negative regulation in response to BMPs is achieved by BAMBI (BMP and activin membrane-bound receptor, which, by binding BMPR-1, acts as a dominant-negative receptor) and by I-Smads (Smads 6 & 7, which both prevent binding of R-Smads to the type I receptor or to Smad4, for Smad6, modified from Itoh & ten Dijke, 2007).

Other inhibitory molecules do not contribute to feedback regulation of BMP-responsive Smad signaling, since their expression is independent of BMP activation. For example, ubiquitin ligase Smurf 1 (Smad ubiquitination regulatory factor) targets Smads 1 & 5 to proteasomal degradation by ubiquitination (Zhu et al., 1999). The putative function of these Smurfs is to regulate basal levels of Smads that are available for BMP signaling (Zhu et al., 1999). Other regulators of BMP-responsive Smad signaling act at the nuclear level. Ski and the related SnoN act as transcriptional co-repressors of BMP signaling by binding the MH2 domain of Smads 1 and 5 (Wang et al., 2000).
Dephosphorylation of Smads at the C-terminal site for phosphorylation by BMPR-I by serine/threonine protein phosphatase, PPM1A (Duan et al., 2006) and by small C-terminal domain phosphatases 1, 2, and 3 (SCP1-3, Knockaert et al., 2006) also terminates BMP signaling.

vi. Cross-talk with other signaling pathways

The identification of several phosphorylation sites for MAPK, protein kinase C (PKC) and calmodulin kinase II on Smads (see Fig. 1.7) suggests cross-talk with BMP-induced Smad signaling. For example, interaction of BMP/Smad and TGF-β1-mediated pathways, counteracts epithelial-to-mesenchymal transition induced by TGF-β1 (Zeisberg et al., 2003). Interaction of BMP2-induced Smad signaling with the Wnt-β catenin pathway leads to increased levels of the Wnt transducer, β-catenin (Fischer et al., 2002; Nakashima et al., 2005). Interaction between Smad1 and the cleaved intracellular domain of the Notch receptor, NIC leads to formation of a complex that also includes the co-activators p300 and P/CAF. Thus, Smad and Notch signaling cooperate to activate transcription of a Notch target gene, Hairy/Enhancer of split (Takizawa et al., 2003). Other examples include calmodulin’s binding to Smad1, which prevents Smad1 phosphorylation by Erk2 (Scherer & Graff, 2000), which inhibits nuclear accumulation of Smad1 (Kretzschmar et al., 1997). Finally, the Smad pathway was found to interact with the JAK/STAT pathway. Smads inhibit association between STAT5 and its co-activator CREB. This blocks activation of STAT5’s target genes (Cocolakis et al., 2008).

d. Biological effects of BMPs in the CNS

The BMP trophic factors act locally. The presence of extracellular inhibitors of BMPs and their high affinity for extracellular matrix proteins allows them to establish
discrete patterns in the CNS. Originally identified in bones for their role in osteoblast differentiation (Urist, 1965), BMPs emerged as essential regulators of the developing nervous system. For example, during gastrulation, inhibition of BMP signaling by follistatin allows the formation of neuroectoderm from mesoderm (Hemmati-Brivanlou et al., 1994). During neurogenesis, a gradient of BMP4 in the neural tube permits the establishment of dorso-ventral patterning (Piccolo et al., 1996). BMPs are selectively expressed in rhombomeres, which carry positional information and are constituted from neural crest cells that have migrated from the hindbrain. BMP4 expression also leads to induction of segmental apoptosis (Graham et al., 1994).

However, these trophic factors are not just expressed during development; their expression is maintained in the adult nervous system (Zhang et al., 1998) and rapidly upregulated following CNS injury. BMP6 is upregulated after induced ischemia and mRNA for BMP7 is increased after injury to the spinal cord (Martinez et al., 2001; Setoguchi et al., 2001). BMPs promote survival of neuronal progenitor cells from the subventricular zone (Mehler et al., 1997) and gliogenesis of neural progenitors (Mehler et al., 2000). Consistent with this, is evidence for neuroprotective effects of BMPs administered prior to (BMPs 6 & 7, Wang et al., 2001) or following ischemia (BMP7, Liu et al., 2001).

In cell culture, BMPs elicit numerous effects ranging from enhanced survival to stimulation of differentiation. For example, BMPs 4 & 7 induce an adrenergic phenotype in neural crest cultures (Reissmann et al., 1996). BMP7 promotes survival of cerebellar granule cells (Yabe et al., 2002). BMP4 promotes neurogenesis in the olfactory epithelium (Shou et al., 2000). Indirect effects of BMPs through glial cells include the
promotion of survival of midbrain dopaminergic neurons and differentiation of
serotonergic neurons (Jordan et al., 1997; Galter et al., 1999). BMPs also affect neurite
outgrowth. BMPs 2, 6 & 7 induce dendritic growth in sympathetic neurons (Guo et al.,
1998), BMP6 promotes neurite outgrowth of cerebellar granule cells (Yabe et al., 2002)
and BMP7 enhances dendritic outgrowth of cerebral cortical neurons and hippocampal
neurons (Le Roux et al., 1999; Withers et al., 2000). Some of these effects on the
morphological development of neurons are Smad-independent, such as BMP2’s
promotion of neurite outgrowth of PC12 cells (via p38 MAPK, Iwasaki et al., 1999) and
BMP7’s induction of dendritic arborization in cortical neurons (through LIM kinase 1,
Lee-Hoeflich et al., 2004). Others have found an inhibitory effect of BMP2 on neurite
outgrowth of postnatal cerebellar neurons, in a LIMK1-dependent manner (Matsuura et
al., 2007). Opposite effects of BMPs in vitro have also been identified, that are
reminiscent of their role in segmental apoptosis during embryogenesis. For example,
BMP4 induces programmed cell death during eye development in chicks (Trousse et al.,
2001).

More closely related to our study, mRNA for BMP9 was most abundant in the
basal forebrain at E14, and BMP9 was found to induce the cholinergic phenotype in this
neuronal population, as indicated by its effect on increasing levels of ACh, ChAT and
vesicular ACh transporter (Lopez-Coviella et al., 2000). BMPs 6 & 7 also robustly
upregulated ACh levels in this neuronal population (Lopez-Coviella et al., 2000).
3. BMPs are complementary to neurotrophins in promoting neuronal survival

   a. Effects of the synergy between BMPs and neurotrophins

Neurotrophins and BMPs have cooperative effects on several neuronal populations, often resulting in increased survival. For example, BMP7 potentiates neuronal survival by NT-3 or GDNF in peripheral ganglia (Bengtsson et al., 1998); BMPs 2, 4, 7 & 12 potentiate the survival-promoting effects of NT-3 and NGF on dorsal root ganglion neurons (Farkas et al., 1999); BMPs 6 & 7 promote the protection of septal neurons by all 4 neurotrophins against a hypoglycemic stress (Nonner et al., 2001). Synergy between BMPs and neurotrophins can also result in other types of effects: BMPs 4 & 6 potentiate the effects of NT-3 both on survival and neurite outgrowth of peripheral neurons (Althini et al., 2004); BMP2 and BDNF/NT-3 cooperate to increase the number of calbindin-positive striatal neurons (Gratacòs et al., 2001).

Synergy between trophic factors can be achieved by interactions at the level of their transmembrane receptors. Such interactions have been identified between BMPs, their receptors and neurotrophins and their receptors. Treatment of superior cervical ganglia with BMP2 resulted in increased mRNA levels of BDNF’s Tyrosine kinase receptor, TrkB (Zhang et al., 1998). BMP2 induced TrkB phosphorylation and increased protein levels of BDNF in striatal neurons (Gratacòs et al., 2001). In a colon cancer cell line, TrkC was reported to interact with BMP receptor type II (BMPR-II), thereby preventing signaling through the BMP-responsive Smad pathway (Jin et al., 2007). This interaction inhibited BMP tumor suppressor activity and so, might contribute to TrkC’s role in tumor growth (Jin et al., 2007).
b. Investigating the mechanisms underlying synergy between BMPs and neurotrophins for protection of septal neurons

The combination of BMPs and neurotrophins gave synergistic effects for protection of septal neurons (Fig. 1.1). These two types of trophic factors classically activate different signaling pathways (see sections 1.a, 1.b and 2.c of this Chapter). However, there is evidence for common signaling pathways activated by neurotrophins and BMPs. For example, NGF directly activates Smad3 (but not Smad1) in PC12 cells, without requiring TGF-β receptor-mediated phosphorylation of Smad3 (Lutz et al., 2004). NGF was also found to activate p38 MAPK (which is a non-canonical pathway for BMPs) in primary sensory neurons of dorsal root ganglia (Ji et al., 2002). I tested whether BMPs enhance neurotrophin-mediated signaling (in the PI3K/Akt and MEK/Erk pathways) and whether neurotrophins enhance BMP signaling (in the Smad signaling pathway). It is also possible that the BMP+neurotrophin combination activates yet another signaling pathway. We found that Casein kinase 2 (CK2) is activated both by BMP7 and BDNF during hypoglycemia and might underlie the synergy between these trophic factors for protection of septal neurons.

4. CK2: a pleiotropic kinase

Studies presented in this document show an involvement of protein kinase CK2 (formerly known as casein kinase 2) in mediating the synergy between BMPs and neurotrophins in increasing Smad1/5/8 phosphorylation. This synergy may also have a role in survival of septal neurons following cellular stress. Mechanisms that might underlie this synergy are detailed below.
CK2 is a Serine/Threonine protein kinase with two catalytic (any combination of α and α’) and two regulatory (β) subunits, forming a heterotetramer. This kinase is highly conserved from yeast to human and is essential for cell survival. A knockout of both catalytic subunits is lethal in yeast (Hanna et al., 1995). In mammals, disruption of either the CK2α or the CK2β gene leads to embryonic lethality (Lou et al., 2008; Buchou et al., 2003). Despite the fact that CK2 was one of the first kinases to be identified in the 1950s, very little is known about the mechanisms that lead to its activation. Two of the characteristics that render CK2 challenging to study are its numerous substrates (>300) and its mode of regulation. The wide diversity of CK2 substrates contributes to the uncommon pleiotropy of the kinase. These substrates can be grouped into different functional categories (listed in Meggio & Pinna, 2003; reviewed in Pinna, 2002): signaling molecules (e.g. calmodulin, PTEN, disheveled), transcription factors (e.g. CREB, Hoxb-6 & 7, Iκβ, p53, TFIIA), proteins involved in DNA and RNA function or protein synthesis (e.g. DNA topoisomerasers I and IIα, DNA ligase, RNA polymerases I and III, EIF2). Many of these substrates are crucial in cell signaling, whether at the level of gene expression, gene transcription, translation or other stages of signaling. Other substrates of CK2 include viral proteins, cytoskeletal proteins and metabolic enzymes.

5. Regulation of CK2 activity

The mode of CK2 regulation has been controversial for many years. It is thought that it is constitutively active (and unregulated) because its activity has been detected in unstimulated tissue extracts and cell culture and because it is active when expressed in bacteria as a recombinant enzyme. However, several studies have found CK2 activity to
be affected by various stimuli. Sayed et al. (2000) reported that the stress factors anisomycin, arsenite, and tumor necrosis factor-alpha, stimulate CK2 enzymatic activity in HeLa cells. CK2 is also activated and translocates from the cytoplasmic compartment to the nuclear matrix in response to heat shock (Davis et al., 2002). More closely related to the present study, neurotrophins can activate CK2. In rat hippocampal slices, both BDNF and NT-4 treatments lead to robust activation of CK2 (Blanquet, 1998). Also, in cultured hippocampal neurons, NGF, by binding to p75NTR, inactivates glycogen synthase kinase-3beta (GSK-3β), leading to CK2 activation and axon elongation (Arevalo & Rodriguez-Tébar, 2006).

Olsten & Litchfield (2004) suggest that different subpopulations of CK2, defined by both their subcellular localization and subunit composition, are present in cells. They hypothesize that these subpopulations are subject to discrete mechanisms of regulation, in an attempt to reconcile the ideas that CK2 can both be constitutively active and regulated.

a. Regulation by small molecules

Although CK2 is thought to be devoid of classical second messenger regulation, its activity can be regulated by small molecules. CK2 is activated by positively-charged compounds (e.g. polyamines in vitro, Meggio et al., 1992 and in vivo Shore et al., 1997, Fig. 1.10) and inhibited by negatively-charged compounds (e.g. heparin, Lin et al., 1991, Fig. 1.10). More recently, CK2 activity was found to be upregulated by inositol tetra-, penta- and hexakisphosphates (IP4, IP5 and IP6 respectively) but only in the presence of a yet unidentified negative regulator, allowing for low basal activity (Solyakov et al., 2004).
b. Regulation by interaction with cellular proteins

The protein-protein interactions involving CK2 serve different functions. Some proteins, by binding CK2, directly regulate the kinase’s catalytic activity (e.g. FGF-1, Skjerpen et al., 2002). Other proteins that bind CK2, target the kinase to specific subcellular localizations (e.g. CKIP-1, Bosc et al., 2000). Binding of CK2 to some proteins was shown to affect CK2’s ability to phosphorylate its substrates. For example, binding of CK2 to Pin1 inhibits phosphorylation of topoisomerase II by CK2 (Messenger et al., 2002, Fig. 1.10). But binding of CK2 to the FACT complex increases CK2’s selectivity for phosphorylation of p53 (Keller et al., 2001).

c. Phosphorylation of CK2

Both regulatory (β) and catalytic (α and α’) subunits of CK2 can be subject to phosphorylation including auto-phosphorylation. Phosphorylation of CK2 occurs on a serine residue for the β subunit (Litchfield et al., 1991) and on serine and threonine residues (Litchfield et al., 1992) and tyrosine residues for the α subunit (by Src tyrosine kinases, Heriche & Chambaz, 1998). Tetramer assembly triggers auto-phosphorylation of CK2β on serine residues (Litchfield et al., 1991), which increases stability of the subunit and therefore may inhibit its proteasome-dependent degradation (Zhang et al., 2002). None of the phosphorylation events previously mentioned seem to affect CK2’s catalytic activity directly. However, auto-phosphorylation of the catalytic subunit on tyrosine residues is required for full activation of the kinase in the absence of CK2β (Donella-Deana et al., 2001).
d. Activation by CK2

CK2 is known to activate protein substrates by phosphorylation through its serine-threonine kinase activity (by phosphorylating Akt: Di Maira et al., 2005) and by protein–protein interaction (e.g. by binding to Akt, Guerra, 2006 or to MKP3, Castelli et al., 2004, summarized in Fig. 1.10). In some cases, both phosphorylation and binding of CK2 to its substrate may be required for activation, as with the molecular chaperone Cdc37 (Miyata & Nishida, 2005). The CK2 tetramer phosphorylates some substrates better than CK2’s free catalytic subunits (e.g. Rev: Marin et al., 2000), whereas the free catalytic subunits phosphorylate some substrates better than the holenzyme (e.g. calmodulin:...
Sacks et al., 1992). Other substrates can be activated by CK2 either as free catalytic subunits or as a tetramer (e.g. I-2: Marin et al., 1994).

It is thought that constitutive activation of the CK2 holoenzyme (the tetrameric form) is permitted by association of the catalytic and the regulatory subunits. This association may be dispensable for activation of the kinase, since interaction between the activation loop and C-terminal segment of the isolated catalytic subunit is sufficient for the protein kinase to be active (Sarno et al., 2002). However, I present two different lines of evidence that CK2 is not constitutively active in cultured septal neurons. First, as others have also found CK2 activity changes in response to trophic factor stimulation (see Chapter II). Second, preliminary studies (described in Chapter III) indicate that CK2 may actually be subjected to robust inhibition by a cytoplasmic component.

6. Rationale

Signaling pathways have long been studied in isolation, however, the complexity and importance of interactions that exist between them have led to more integrative studies. Cells are exposed to many factors, each of which triggers intracellular cascades of events. Interactions between pathways allow integration of different signals and so increase the range of cellular responses. The aim of this study was to understand the mechanisms whereby a combination of BMP7 and BDNF protects septal neurons against cellular stress.

Embryonic day 15 septal cells grown for 5 to 7 days are used in this study. At this age and in the culture conditions used, 95% of cells are neurons (Tedeschi et al., 1986). Neurons in the septal region are cholinergic or GABAergic. Cholinergic phenotype in the
basal forebrain can be determined using markers such as \( p75^{\text{NTR}} \). GABAergic neurons are of three different phenotypes that can be discriminated using calcium-binding proteins (calbindin, calreticulin and parvalbumin). Cholinergic septal neurons express TrkA and \( p75^{\text{NTR}} \). TrkB is expressed by non-cholinergic basal forebrain neurons. Septal neurons express mRNA for BMPR types Ia, Ib and II that can mediate BMP6/7 signaling (Lopez-Coviella et al., 2006 and Fig. 4.2).

We tested the hypothesis that BMP7 and BDNF exhibit synergy for protection of septal neurons due to interactions at the level of their signaling pathways. Several pathways that are activated downstream of neurotrophins mediate their survival-promoting effects (summarized in section 1 of this Chapter). However, the Smad signaling pathway is the most commonly activated pathway downstream of BMPs. Therefore, interactions between BMPs and neurotrophins may affect Smad signaling. In particular, such interactions may upregulate activity in the Smad signaling pathway. Upon BMP binding to its receptor, Smads 1/5/8 get phosphorylated by type I BMP receptors. The phosphorylated Smads then translocate to the nucleus and act as transcription factors. Increased levels of Smads phosphorylated by the BMP receptors are therefore an indication of activation of the BMP signaling pathway. Some of the interactions between BMP- and neurotrophin-mediated pathways may be reflected by changes in levels of phospho-Smads.

Despite growing evidence in the literature of interactions between BMPs and neurotrophins, this is the first mechanistic report of their intracellular interactions. This study will therefore bring fundamental knowledge to our understanding of how trophic factors affect neuronal populations. Indeed, neurotrophins are necessary for the growth
and maintenance of neurons. However, neurotrophins may not be sufficient to protect these same neurons against severe insults and may require other types of trophic factors (e.g. BMPs).
Chapter II

CK2 contributes to the synergistic effects of BMP7 and BDNF on Smad 1/5/8 phosphorylation in septal neurons

1. Background

A combination of BMPs (6 and/or 7) and neurotrophins (nerve growth factor (NGF) and BDNF) protect septal neurons from both a hypoglycemic stress and a stress produced by inhibition of phosphatases 1A and 2A by okadaic acid (Nonner et al., 2001 & 2004). This combination was much more effective than either BMPs or neurotrophins alone. BMPs and neurotrophins also exert cooperative trophic effects in other neuronal populations. For example, BMPs 4, 6 and 7 potentiate the survival-promoting effects of neurotrophin 3 (NT-3) in sympathetic ganglia (Bengtsson et al., 1998; Althini et al., 2004). BMPs 2, 4, 7 and 12 potentiate the survival-promoting effects of NT-3 and NGF on dorsal root ganglion neurons (Farkas et al., 1999). BMP2 and BDNF/NT-3 cooperate to increase the number of calbindin-positive striatal neurons and neurite outgrowth in sympathetic ganglion neurons (Gratacòs et al., 2001; Zhang et al., 1998). The goal of this study was to determine the mechanisms underlying the complementary effects of BMPs and neurotrophins in the protection of cultured embryonic septal neurons during hypoglycemia.

Neurotrophins signal via TrkA, TrkB, TrkC and p75NTR receptors, and all of these receptors are expressed in cultures of basal forebrain neurons (Springer et al., 1987; Vazquez and Ebendal, 1991; Merlio et al., 1992). In these cultures, the neurons expressing TrkA and p75NTR are almost all cholinergic (Sobreviela et al., 1994); TrkB and TrkC receptors are more widespread (Merlio et al., 1992; Cheng & Mattson, 1994).
TrkA, alone or co-expressed with p75\textsuperscript{NTR}, mediates NGF’s trophic effects, which include increased survival of cholinergic neurons, increased choline acetyl transferase (ChAT) activity and enhanced protein and mRNA levels of both TrkA and p75\textsuperscript{NTR}, both in vitro and in vivo (Fischer et al., 1987; Hempstead et al., 1991; Jing et al., 1992; Li et al., 1995; Nonomura et al., 1995). The intracellular pathways that mediate the survival-promoting effects of neurotrophins include phosphatidylinositol 3-kinase (PI3K) in NGF-dependent PC12 cells, sympathetic neurons and neocortical neurons (Yao et al., 1995; Crowder et al., 1998; Mazzoni et al., 1999; Cheng et al., 2003; reviewed in Kaplan & Miller, 2000), mitogen-activated protein kinase (MAPK) in sympathetic ganglion neurons (Mazzoni et al., 1999; Atwal et al., 2000) and calcium-calmodulin in PC12 cells and neocortical neurons (Silva et al., 2000; Egea et al., 2001; Cheng et al., 2003).

BMPs signal via type I and type II BMP receptors (BMPR); type I BMPRs include activin receptor-like kinases (Alks), and type II receptors, BMPR-II and activin type II receptors (ActR). BMP7 can bind to several type I receptors (Alk2, BMPR-IA = Alk3 and BMPR-IB = Alk6) and to several type II receptors (BMPR-II, ActRII and ActRIIB, Macias-Silva et al., 1998). Mouse basal forebrain expresses mRNA for several type I (BMPR-IA, IB, Alk1) and type II (ActR-II, IIB) BMPRs and protein for BMPR-IB and II at early developmental stages; some BMPRs are also expressed in the adult (Lopez-Coviella et al., 2006). In the classical BMP signaling pathway, BMPR-II binds the BMP ligand and then recruits and phosphorylates BMPR-I, which in turn phosphorylates intracellular Smads 1, 5 and 8. These Smads then translocate to the nucleus where they regulate transcription of target genes (Lein et al., 2002, Angley et al., 2003, Chalazonitis et al., 2004, Lopez-Coviella et al., 2006). Transcripts for Smads1 and
5 and the co-Smad, Smad4, are also present in basal forebrain at early developmental stages as well as in the adult (Lopez-Coviella et al., 2006)

BMP+neurotrophin-induced protection of septal neurons during hypoglycemia is reduced by inhibitors of the PI3K and the Mek/Erk pathways as well as by inhibitors of CK2 (Nonner et al., in preparation). CK2 is a highly conserved serine/threonine kinase with two catalytic (α and/or α’) and two regulatory β subunits. Knockout of the β regulatory subunit is embryonic lethal (Buchou et al., 2003) and knockout of both catalytic subunits is lethal in yeast (Hanna et al., 1995).

Work presented here demonstrates that BMP7 increases Smad phosphorylation in septal neurons, and that BDNF enhances BMP7-induced Smad phosphorylation and nuclear translocation, especially after a hypoglycemic stress. These effects are blocked by inhibitors of CK2 activity, but not by inhibitors of PI3K, Erk or Ca\(^{2+}\)-dependent signaling. These findings suggest that CK2 activation contributes to the synergistic stress-protective effects of BMP7 and BDNF in septal neurons.

2. Materials and Methods

a. Cell culture

The septal region of the basal forebrain was dissected from embryonic day 15 rat embryos following euthanasia of pregnant rats with 100% CO\(_2\). Dissociated cells were plated at 600 cells/mm\(^2\) (as described in Nonner et al., 2004) using a defined medium (N5, Kawamoto & Barrett, 1986) supplemented with 1 mg/ml of a 55 kDa serum fraction (Kaufman & Barrett, 1983). This serum fraction contains selenoprotein-P and, when added to growth media, allows for longer survival of embryonic central neurons and
slower proliferation of non-neuronal cells than whole serum (Yan & Barrett, 1998). In these cultures, >95% of the cells were neurons (Tedeschi et al., 1986). Cells were maintained at 37 °C in 95% O₂/5% CO₂. Immunocytochemical experiments used glass-bottom cell culture dishes; Western blot and CK2 assays used 60-mm cell culture dishes, all coated with poly-L-lysine.

b. Hypoglycemic stress

Hypoglycemia was produced by washing 5 to 7 day-old cultures three times in medium containing normal salts (in mM: 125 NaCl, 2.7 KCl, 1.5 MgCl₂, 0.05 MgSO₄, 2 CaCl₂, 0.83 NaH₂PO₄, 24 NaHCO₃, 2 HEPES), 1 mg/ml bovine serum albumin (BSA) and 50 µM glucose (1% of the normal glucose concentration). Unless otherwise noted, cells were maintained in this hypoglycemic medium for 6 h with trophic factors added during the last hour. Inhibitors of signaling pathways were added 1 h prior to trophic factor addition. Unstressed control cultures were washed an equal number of times, but maintained throughout in the original N5 growth medium, except that the serum fraction was replaced with 1 mg/ml BSA to avoid possible effects of serum components on Smad signaling pathways (David et al., 2008). BMP7 (combined with 5 nM BMP6 in Fig. 2.1) was applied at the optimal concentration for increasing ChAT activity (5 nM, Nonner et al., 2001). BMP7 was as effective in inducing Smad phosphorylation, whether alone or combined with BMP6. Neurotrophins were applied at 100 ng/ml (monomer concentration of ≈7.4 nM; active dimer concentration≈3.7 nM), which produces maximal stimulation of ChAT activity. Some experiments used both NGF and BDNF, but in most cases, the neurotrophin was BDNF alone, since the majority of septal neurons express TrkB. Also,
BDNF alone was as effective as when combined with NGF in upregulating Smad phosphorylation in the presence of BMP7.

c. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde and blocked in phosphate-buffered saline (PBS) with 0.1% Triton containing 10% donkey serum. Primary antibodies (incubated at 4 °C overnight) were rabbit anti-phospho Smad1/5/8 (1:200, gift from Dr. C.H. Heldin, Uppsala University, Uppsala, Sweden; or 1:50, Cell Signaling Technology, Danvers, MA, USA; these antibodies recognize Smad sites that become phosphorylated in response to activation of BMP receptors); mouse anti-casein kinase IIα and goat anti-casein kinase IIα’ (both at 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA); and mouse anti-BMP receptor II (1:75, R&D Systems, Minneapolis, MN, USA). Secondary antibodies, all from Molecular Probes (Eugene, OR, USA), were 488 donkey anti-mouse, 1:2000; 488 donkey anti-rabbit, 1:300; and 555 donkey anti-rabbit, 1:1000. Mounting medium with 4’,6-diamidino-2-phenylindole (DAPI) was used for nuclear counterstain (Vector Labs, Burlingame, CA, USA).

Cells were imaged using a Hamamatsu-ER CCD camera (Bridgewater, NJ, USA) and a 60X oil-immersion objective, numerical aperture, 1.45 (Olympus, Center Valley, PA, USA). All images of control and experimental treatment groups were collected using identical exposure time, gain and magnification. Custom macros written in an image analysis program (V++, Digital Optics, Browns Bay, Auckland, New Zealand) were used to measure fluorescence of nuclear and cytoplasmic phospho-Smads 1/5/8 in cells counter-stained with DAPI (details in Fig. 4.1). In all experiments, controls without primary antibodies gave negligible staining.
d. Western blots and cell fractionation

Lysis for Western blots was performed using modified RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 1% NP-40, 0.25% Na deoxycholate, pH 7.4). For fractionation, cells were scraped in a low-salt lysis buffer (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.05% NP-40, pH 7.9) and centrifuged (3000 rpm, 4 °C, 10 min). The supernatant was taken as the cytoplasmic fraction. The pellet was resuspended in the same lysis buffer and sonicated for 30 s at 4 °C to lyse membranes and help solubilize DNA. Membranes were removed by a 5000 g centrifugation for 10 min; the supernatant was taken as the nuclear fraction. All lysis buffers contained a protease inhibitor cocktail with a broad specificity for inhibition of serine, cysteine, aspartic and aminopeptidases (P8540, Sigma-Aldrich, St. Louis, MO, USA); phosphatase inhibitor cocktail for serine/threonine protein phosphatases and L-isozymes of alkaline phosphatase (Sigma-Aldrich 2850); and phenylmethanesulfonyl fluoride (1 mM). Protein levels were determined using either a Bradford assay (Bio-Rad, Hercules, CA, USA) or a detergent-compatible assay (Pierce, Rockford, IL, USA). Before loading lysates onto gels, samples were heated at 95 °C for 10 min in loading buffer (60 mM Tris-HCl, 10% glycerol, 5% β-mercaptoethanol, 2% SDS, 0.05% bromophenol blue, pH 6.8).

Samples normalized to equivalent protein levels were loaded onto 10% Tris-HCl pre-cast minigels (Bio-Rad). Gels were electroblotted onto nitrocellulose membranes (Bio-Rad) blocked in Odyssey blocking buffer (927-40000, Li-Cor Biosciences, Lincoln, NE, USA). Primary antibodies, diluted in Odyssey blocking buffer with 0.1% Tween 20, were rabbit anti-phospho-Smad 1/5/8 (1:1000); mouse anti-phospho Akt (1:100); rabbit anti-poly (ADP-ribose) polymerase (PARP), 1:1000; mouse anti-p42/44 MAPK, 1:100.
(all from Cell Signaling Technology); mouse anti-class III β tubulin (1:1000, TuJ1, Covance, Berkeley, CA, USA); rabbit anti-transcription factor IID (TFIID, 1:200) and rabbit anti-TATA box binding protein (TBP, 1:200, both from Santa Cruz Biotechnology); and rabbit anti-neurofilament 200 (1:1000, Sigma-Aldrich). Secondary antibodies were IRDyes 700 and 800 anti-rabbit and IRDyes 700 and 800 anti-mouse (all at 1:5000, Rockland, Gilbertsville, PA, USA) and Alexa-Fluor 680 donkey anti-goat IgG (Molecular Probes). Membranes were scanned on the Odyssey infrared imaging system (Li-Cor Biosciences), with band intensities determined using Odyssey software and normalized to those of the loading control. TBP was detected in the nuclear but not the cytoplasmic fraction, validating the procedure for subcellular fractionation.

e. Assay for CK2 activity

Cells were washed twice in PBS and homogenized in a lysis buffer (in mM: 150 NaCl, 50 Tris-HCl, 25 β-glycerol phosphate, 10 NaF, 5 Na pyrophosphate, 2 EGTA, 1 thioglycolate, 0.1% Triton, pH 7.45). Inhibitor cocktails were as described above for Western blots; an anti-tyrosine phosphatase cocktail (Sigma-Aldrich) was also added. Protein concentrations were measured in the cleared lysates (Bradford assay) and equivalent amounts of protein (2.5 - 5 µg) were used for the kinase assay. Lysates (5 - 10 µl) were incubated for 12 min at 30 °C with a CK2 substrate peptide (RRRDDSDDDD, 200 µM, Upstate/Millipore, Temecula, CA, USA) and 1 - 10 µCi γ32P-ATP (Perkin-Elmer, Waltham, MA, USA). The assay was performed following the manufacturer’s protocol except that following trichloroacetic acid addition, samples were centrifuged (8000 rpm, 15 min) to remove phosphorylated cellular proteins. 25 µl of the supernatant was pipetted onto filter paper squares (P81 phosphocellulose, Upstate), which were
washed, placed in vials with scintillation fluid (ScintiSafe, Fisher Scientific, Pittsburgh, PA, USA) and counted with a scintillation counter. The assay was calibrated using recombinant CK2 (CKII, New England Biolabs, Ipswich, MA, USA). With this assay, 1 unit (or 2 ng) of CKII corresponds to $10^{-3}$ pmol phosphate transferred to the substrate peptide (200 µM) in 1 min at 30 °C in a reaction volume of 50 µl.

f. Reagents

Trophic factors: recombinant human BMP6 and BMP7 (Calbiochem, LaJolla, CA, USA); recombinant human NGF and BDNF (Alomone Labs, Jerusalem, Israel). Pharmacological inhibitors: PI3K/Akt pathway, wortmannin (100 nM, Calbiochem), 1L-6-Hydroxymethyl-\textit{chiro}-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (=Akt inhibitor, 5 µM, Alexis, San Diego, CA); MAPK pathway: U0126 (10 µM, Sigma-Aldrich); calmodulin kinase II pathway, intracellular Ca$^{2+}$ buffer 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM, 25 µM, Sigma-Aldrich); inhibitor of CK2 as a tetramer or as free catalytic subunits, 4,5,6,7-tetrabromobenzotriazole (TBB or TBBt, 20 µM) and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB, 20 µM); inhibitor of CK2 as a tetramer, but not as free catalytic subunits, 4,5,6,7-tetrabromobenzimidazole (TBBz, 20 µM, all CK2 inhibitors from Sigma-Aldrich).

g. Statistical Analyses

Averages (from at least 3 experiments performed on different biological samples) are expressed as means ± SEM. Data were analyzed using GraphPad Prism software (LaJolla, CA, USA) with a one-way analysis of variance followed by Dunnett’s test for multiple comparisons to control or by Newman-Keuls test for pairwise comparison.
Regression analysis with non-parametric correlation (Spearman test, GraphPad InStat, La Jolla, CA, USA) was used to test for changes in phospho-Smad levels and CK2 activity over time.

3. Results

   a. Neurotrophin enhancement of BMP7 signaling is greater during hypoglycemia

   Phosphorylation of Smads 1/5/8 by BMPR type I indicates activation of the BMP signaling pathway. Fig. 2.1 shows results of immunocytochemical experiments in which phosphorylated Smads 1/5/8 (P-Smad) were fluorescently labeled as described in Materials and Methods. Fig. 2.1A shows a scatter plot of a representative experiment comparing average P-Smad fluorescence in the cytoplasmic and nuclear compartments of individual neurons. Neurons exposed to BMP6/7 for 1 hr (open circles) showed more intense P-Smad staining than controls (open squares) in both compartments. P-Smad staining was further increased when neurotrophins (BDNF and NGF, filled circles) were also present. In most cells treated with BMPs ± neurotrophins, the average intensity of nuclear staining exceeded that of cytoplasmic staining. Results of these experiments are summarized in Fig. 2.1B, which plots the average ratio of nuclear/cytoplasmic P-Smad staining, a measure of translocation. BMP6/7 increased this ratio, and the ratio was further increased (by 14%) when neurotrophins were also present. Additional immunocytochemical experiments confirmed the presence of BMPR type II in both cholinergic and non-cholinergic neurons (Fig. 4.2).
Fig. 2.1 BMP6/7 and a BMP-neurotrophin combination treatment increase nuclear and cytoplasmic P-Smad levels. Septal cells at 5 days in vitro were treated for 1 h with BMP6/7 alone or in combination with BDNF+NGF, then stained with an anti-P-Smad 1/5/8 antibody and counterstained with DAPI. Average values of nuclear and cytoplasmic fluorescence were measured as detailed in Fig. 1 of Supporting Information. A) Log scale scatter plot of P-Smad fluorescence in the nucleus (y axis) and cytoplasm (x axis). Each point represents one neuron. Points situated above the 45° identity line represent cells in which nuclear fluorescence exceeded cytoplasmic fluorescence. B) Ratio of nuclear to cytoplasmic P-Smad fluorescence, a measure of nuclear translocation. * indicates significant difference from control or between the indicated trophic factor groups, assessed with one-way ANOVA followed by Newman-Keuls test (p<0.05, n≥22 cells per group, from two different experiments).
The effects of BMP7 ± BDNF on P-Smad levels were also tested using Western blots of nuclear (Fig. 2.2A) and cytoplasmic (Fig. 2.2B) subcellular fractions. Both nuclear and cytoplasmic P-Smad levels increased within 30 min following BMP7 addition, and this increase was sustained for at least 2 h (Fig. 2.2C, D). Sister cultures treated with both BMP7 and BDNF showed a greater increase in nuclear P-Smad at 30 and 60 min than cultures treated with BMP7 alone (Fig. 2.2C). Possible reasons for the apparent decrease in nuclear P-Smads after 90-120 min in BMP7 + BDNF are considered in the Discussion of this Chapter.

Because the combination of BMP7 and BDNF protects septal neurons during a hypoglycemic stress (Nonner et al., 2001 and in preparation), we compared the effects of these trophic factors on nuclear and cytoplasmic P-Smads under control and hypoglycemic conditions. Fig. 2.3 shows that a 6 h exposure to hypoglycemia alone increased P-Smad fluorescence in both the nucleus (73% increase, compare Fig. 2.3A & B) and cytoplasm (70% increase, Fig. 2.3C & D), compared to non-stressed controls. BMP7 increased both nuclear and cytoplasmic P-Smad fluorescence by a greater percentage in hypoglycemic cultures than in non-stressed cultures. The enhancement of P-Smad fluorescence produced by the combination of BMP7 and neurotrophin was also greater under hypoglycemic conditions (40% greater in nucleus, 37% greater in cytoplasm, compared to BMP7 alone; see also Fig. 2.6). BDNF alone had no significant effect on P-Smad fluorescence in either control or hypoglycemic conditions (Fig. 2.3, see also Figs. 2.5, 2.6).
**Fig. 2.2** Time course of Smad 1/5/8 phosphorylation following addition of BMP7 ± BDNF. Western blots of nuclear (A) and cytoplasmic (B) fractions from cell lysates after the indicated incubation periods with BMP7 or BMP7 plus BDNF. Loading controls for the cytoplasmic and nuclear compartments were poly (ADP-ribose) polymerase and TATA box binding protein, respectively. C, D) Quantification of nuclear (C) and cytoplasmic (D) blots like those shown in A and B. Averaged band intensities for P-Smad were normalized to their respective loading controls and this ratio was normalized to that of controls not exposed to trophic factors (plotted at 0 min). Regression analysis with Spearman non-parametric correlation showed that BMP7+BDNF increased nuclear P-Smad more than BMP7 alone during the first 60 min of incubation (data from ≥ 4 different experiments, p<0.01).

These results suggest that hypoglycemia enhances P-Smad signaling even in the absence of added trophic factors and enhances the stimulatory effects of both the BMP7 and BMP7+ BDNF combination on P-Smad signaling.
Fig. 2.3 Hypoglycemia increases the enhancement of nuclear and cytoplasmic P-Smad levels produced by both BMP7 alone and a BMP7 + BDNF combination. Cultures grown for 5 days in vitro were subjected to hypoglycemic stress (B, D) or to serum-free medium with normal glucose (A, C). During the last hour, BMP7 alone, BDNF alone, or BMP7 + BDNF were added, and nuclear (A, B) and cytoplasmic (C, D) P-Smad fluorescence were measured, as in Fig. 1. * indicates significant increase from control or indicated group with p<0.01, using one-way ANOVA followed by Newman-Keuls test; each group represents measurements of P-Smad fluorescence in at least 25 cells.

b. BDNF enhancement of BMP7-induced Smad phosphorylation is independent of PI3K/Akt and Mek/Erk pathways, but requires CK2

Intracellular signaling pathways activated by BDNF include the PI3K/Akt and Mek/Erk pathways (reviewed in Kaplan & Miller, 2000). We confirmed this activation in septal cultures by demonstrating increased levels of phosphorylated Akt (P-Akt) and p42-44 Erk (P-Erk) on Western blots within 15 min after addition of BDNF and also
confirmed that the PI3K inhibitor wortmannin (100 nM) selectively blocked the increase in P-Akt and the Mek1/2 inhibitor U0126 (10 µM) selectively blocked the increase in P-Erk (Fig. 4.3). Fig. 2.4A-C shows Western blot evidence that BMP7 did not increase Akt or Erk phosphorylation, and that the combination of BMP7 with BDNF did not increase Akt or Erk phosphorylation above the levels achieved by BDNF alone. Fig. 2.4 also shows that the BDNF-induced increase in Akt and Erk phosphorylation was not blocked by TBB (20 µM), an inhibitor of CK2 (Sarno et al., 2005).

We also assessed the effect of these inhibitors on BMP7-mediated signaling, measured as increases in nuclear P-Smad levels. Fig. 2.5A summarizes immunocytochemical evidence that the increase in nuclear P-Smad produced by (BMP7 + BDNF) under hypoglycemic conditions was not reduced by inhibitors of the PI3K/Akt pathway (wortmannin, PI3K inhibitor) or by the Mek/Erk inhibitor, U0126. This increase in nuclear P-Smad was also unaffected by BAPTA-AM (25 µM), indicating that it is unlikely that Ca^{2+}-dependent activation of calmodulin kinase (Blanquet & Lamour, 1997) is required. Fig. 2.5B shows that the (BMP7 + BDNF)-induced increase in nuclear P-Smad was reduced by the CK2 inhibitor, TBB. These results were confirmed using Western blots under both normoglycemic (Fig. 2.6A) and hypoglycemic conditions (Fig. 2.6B). Similar results (not shown) were obtained with another CK2 inhibitor, DRB (20 µM) and with Ly 294002 (20 µM), which inhibits both the PI3K/Akt pathway and CK2 (Davies et al., 2000).
Fig. 2.4 BDNF-induced increases in phosphorylated Akt and Erk are not modified by BMP7 or a CK2 inhibitor, TBB. A) Western blot of cell lysates exposed for 15 min to BMP7, BDNF, or their combination. One experimental group was pretreated for 1 h with 20 µM TBB prior to trophic factor addition. B and C) Quantification of blots for P-Akt (B) and P-Erk (C), normalized to the loading control (TATA box binding protein, TBP). * indicates significant difference from control (p<0.001), one-way ANOVA followed by Dunnett’s test.
Fig. 2.5  The increase in nuclear Smad fluorescence produced by (BMP7 + BDNF) is not reduced by inhibitors of PI3K/Akt, Mek/Erk, or Ca$^{2+}$-dependent signaling (A), but is reduced by a CK2 inhibitor (B). Cells under hypoglycemic conditions were pre-treated with pharmacological inhibitors for 1 h prior to and during a 1 h incubation with the indicated trophic factors. Nuclear P-Smad fluorescence was measured immunocytochemically, as described for Fig. 1. Wortmannin (Wort, 100 nM), Akt inhibitor (5 µM), U0126 (10 µM), BAPTA-AM (25 µM). * indicates significant difference from control or indicated group, one-way ANOVA followed by Newman-Keuls test (p<0.001, n=6 to 54 cells).

Taken together, the results of Figs. 2.4-6 indicate that classical BDNF-activated PI3K/Akt, Mek/Erk, and calcium-calmodulin pathways are not required for BDNF’s enhancement of BMP7-induced Smad phosphorylation and suggest that CK2 activity is required for this synergistic trophic factor effect.

c. The combination of BDNF and BMP7 increases CK2 activity under hypoglycemic conditions
Fig. 2.7A presents both immunocytochemical and Western blot evidence that both catalytic subunits of CK2 (α and α’) are present in septal neurons. For both subunits, most of the CK2 immunostaining was cytoplasmic. No changes in the distribution of either CK2 subunit were observed following a 1 h exposure to trophic factors (not shown).

CK2 has been reported to be activated by neurotrophins in hippocampal slices (BDNF and NT-4, Blanquet, 1998) and hippocampal cultures (NGF, Arevalo & Rodriguez-Tébar, 2006). Fig. 2.7B plots the summed results of 5 experiments in which septal cell lysates were assayed for their ability to phosphorylate a peptide substrate specific for CK2 following 5-60 min exposures to BDNF. CK2 activity increased within
1 h of BDNF exposure, compared to non-treated controls. This upregulation of CK2 activity was maintained after 2 h in BDNF (not shown).

**Fig. 2.7** Localization of catalytic subunits of CK2 in septal neurons and activation of CK2 by BDNF and BMP7. **A)** Immunocytochemical staining (top) and immunoblotting (bottom) for CK2α and CK2α’. Loading markers were neurofilament 200 for CK2α and TBP for CK2α’. **B)** CK2 activity assayed after different durations of exposure to BDNF. Linear regression analysis of values averaged from 5 experiments (normalized to untreated controls) showed a significant increase over 60 min of BDNF incubation (r=0.53, p<0.01). **C, D)** Effects of a 1 h exposure to BMP7, BDNF or a BMP7+BDNF combination on CK2 activity, measured under normoglycemic (C) and hypoglycemic (D, 3 h) conditions. Experimental values were corrected by subtracting values measured in the absence of cell lysates, then normalized to values measured in the absence of trophic factors. The activity of control lysates was ~5.4x10^-6 pmol/µg protein/min. CK2 inhibitors (TBBt and TBBz) inhibited this control activity by 78% and 89%, respectively (not shown). *significant difference from control, one-way ANOVA followed by Dunnett’s test (p<0.05, n=10 experiments). Calibration bars in A: 2 µm
Since stress can also activate CK2 (Sayed et al., 2000; Davis et al., 2002) and since BMP7 + BDNF combinations produce a greater increase in P-Smad levels during hypoglycemia (Figs. 2.3, 2.6), we compared CK2 activities measured following a 1 h exposure to BMP7, BDNF or (BMP7 + BDNF) under normoglycemic (Fig. 2.7C) and hypoglycemic (Fig. 2.7D) conditions. The only significant change was a modest (24%) increase in CK2 activity with the BMP7 + BDNF combination under hypoglycemic conditions. These results, combined with the effects of CK2 inhibitors described above, are consistent with the hypothesis that CK2 plays an active and/or permissive role in the synergistic trophic effects of BMP7 and BDNF.

4. Discussion

The results of this study confirm and extend evidence for BMP signaling in the basal forebrain. Previous work demonstrated the presence of BMP9 and BMP receptors in the basal forebrain and Smad phosphorylation in response to BMP9 and presented evidence that BMP9 upregulates and maintains the cholinergic phenotype in the basal forebrain (Lopez-Coviella et al., 2000, 2006). BMP7 studied in the present paper interacts with a different subset of receptors than BMP9 (Macias-Silva et al., 1998; Brown et al., 2005; David et al., 2007). BMPs 6 and 7 (which belong to the same BMP subfamily) dramatically increase the stress-protective effects of neurotrophins (Nonner et al. 2001, 2004). Synergistic trophic effects of BMPs and neurotrophins have also been reported in other neuronal populations (see Introduction). The present work investigated the mechanisms underlying the synergistic effects of BMP7 and BDNF in cultured embryonic septum.
a. Pathways mediating synergy between BMP7 and BDNF signaling

Both immunocytochemical and Western blot evidence demonstrates that BMP7 (like BMP 9) increases both nuclear and cytoplasmic levels of P-Smads 1/5/8 in septal cultures (Figs. 2.3 & 2.6). The rapid increase in P-Smad levels (within 30-60 min of exposure to BMP7) suggests that this response was mediated by BMP activation of neuronal receptors rather than an indirect effect mediated via other cells (e.g. glia). This idea is consistent with the presence of BMP receptors and BMP-responsive Smads in septal neurons (Lopez-Coviella et al., 2006; and Fig. 4.2).

BMP7-induced increases in nuclear and cytoplasmic Smad phosphorylation were enhanced by BDNF, especially during hypoglycemia (Fig. 2.3). The decline in nuclear P-Smad measured 90-120 min after exposure to BMP7 + BDNF under hypoglycemic conditions (Fig. 2.2C) may have been due to Smad dephosphorylation, as reported for TGF-β-responsive Smads 2 and 3 (Inman et al. 2002). BDNF-initiated signaling pathway(s) may have sped the disappearance of nuclear P-Smad. For example, phosphorylation of Smad1 in its central linker region by Erk2 may decrease P-Smad by targeting it to the proteasome (Sapkota et al., 2007). This might explain why BMP7 effects on neurite outgrowth from sympathetic ganglion neurons were enhanced by the Mek1/2 inhibitors PD98059 and U0126 (Althini et al., 2004; Kim et al., 2004a).

Although BDNF enhanced BMP7-induced intracellular signaling, the converse was not true. The BDNF-induced increase in phosphorylation of Akt and Erk1/2 was not increased by BMP 7. Thus, it is unlikely that BMP7 induced a rapid general up-regulation of TrkB-mediated signaling.
The PI3K/Akt and Mek/Erk pathways are important for stress protection, because inhibitors of these pathways block BMP7+BDNF-mediated protection of septal neurons during hypoglycemic and phosphatase inhibitor stresses, and Mek1/2 inhibitors block BMP7-induced protection of cortical neurons stressed by hydrogen peroxide (Cox et al., 2004; Nonner et al., in preparation). However, neither inhibitors of PI3K/Akt nor inhibitors of Mek1/2/Erk reduced Smad phosphorylation by BMP7+BDNF (Fig. 2.5A).

b. CK2 activity contributes to the synergistic effects of BMP7 and BDNF on Smad phosphorylation

The CK2 inhibitor, TBB, reduced stress-protective effects of BMP+neurotrophin combinations during both hypoglycemic and phosphatase inhibitor stresses (Nonner et al., in preparation), as well as the BDNF-induced increase in BMP7-stimulated Smad phosphorylation (Figs. 2.5B & 2.6). BMP7 + BDNF also increased CK2 activity during hypoglycemia (Fig. 2.7). These findings suggest a role for CK2 in the synergistic stress protection mediated by this trophic factor combination.

Blanquet (1998) and Arevalo & Rodriguez-Tébar (2006) recorded marked upregulation of CK2 activity in hippocampal tissue in response to neurotrophins (5-fold and 2-fold respectively). However, after phosphorylation events mediated by other kinases were eliminated from the assay, the growth factor-induced increase in CK2 activity we measured was relatively small, only 24% with a combination of BMP7+BDNF during hypoglycemia (Fig. 2.7D). But even small changes in total CK2 activity could have important functional consequences, especially if they increase recruitment of transcription factors to the Smad signaling complex, since Smads by themselves bind DNA with only low affinity (Shi et al., 1998). It is also possible that
different subpopulations of CK2 coexist within cells and are subjected to discrete modes of regulation (Olsten & Lichtfield 2004).

There are additional reports of up-regulation of CK2 activity during cellular stress. For example, CK2 activity is upregulated in response to heat shock (Davis et al., 2002), anisomycin and arsenite (Sayed et al. 2000) and expression of the huntingtin protein containing poly-glutamine repeats (Fan et al., 2008). The ability of Smads to induce transcription of ATF3, a stress-activated gene (reviewed in Hai et al., 1999), also suggests a possible role for Smads in cellular responses to stress (Kang et al., 2003).

In summary, BMP+neurotrophin combinations such as BMP7+BDNF exert synergistic stress-protective effects in cultures of embryonic septal neurons. Evidence presented here suggests that this synergy is due more to BDNF-mediated enhancement of BMP7-initiated signaling pathways than to BMP7-mediated enhancement of classical BDNF-initiated signaling pathways. BMP7-initiated phosphorylation of Smad1/5/8 in both nuclear and cytoplasmic compartments was enhanced by BDNF, especially under hypoglycemic conditions. Studies using inhibitors of CK2 and an assay for CK2 activity suggest an important role for this enzyme in the synergistic trophic effects of BMP7 and BDNF.
Chapter III

General Discussion

My work provided evidence for synergistic activation of a Smad signaling pathway by BMP7 and BDNF (Chapter II) that is especially prominent during hypoglycemia and requires CK2 activity. While CK2 is known to promote the survival of many non-neuronal cells, this work is the first direct evidence that CK2 may be critical for trophic factor-induced protection of neurons. I will first discuss mechanisms that might underlie the synergy between BMP7 and BDNF and propose a model integrating these different possibilities. My study shows a correlation between an increase in CK2 activity and enhanced P-Smad levels in response to a BMP7+BDNF combination during hypoglycemia. I will discuss the nature of the interactions between Smads and CK2, since it is likely that Smads 1/5/8 are direct substrates of CK2, as indicated by the presence of putative phosphorylation sites for CK2 on Smads. I will also present work from our laboratory investigating the nature of the pathways responsible for synergy between BMPs and neurotrophins for protection of septal neurons during hypoglycemia. Mechanisms of protection by the BMPs + neurotrophin combination during hypoglycemia are discussed, integrating results from the presented studies.

The results presented in Chapter II and Section 2 of this chapter indicate that CK2 is required for both the synergistic effects of BMP7 and BDNF on Smad1/5/8 phosphorylation and for the neuroprotective effects of these trophic factors on septal cultures. Yet, activation of CK2 by trophic factors is controversial since it is thought to be a constitutively active kinase. This paradox is discussed both in terms of my results
and results from the literature. A model is proposed that integrates changes in CK2 activity reported in Fig. 2.7 C & D in response to BMP7 alone or in combination with BDNF or in response to BDNF alone. We encountered limitations of the standard kinase assays used in this study to measure changes in CK2 activity in response to the trophic factor treatments. These limitations are discussed, focusing on the errors they could introduce when studying a kinase that is subject to reversible regulation, as CK2 might be. Evidence for a negative regulator of CK2 is presented. The presence of a regulator of CK2 will require further validation, utilizing an improved kinase assay.

Our work shows involvement of CK2 in modulating Smad signaling as well as protection from stress. Although this is not the first study showing regulation of CK2 in response to stimuli, this is the first study showing a role of CK2 in neuroprotection. We will discuss the implication of these results, integrating them with the literature related to activation of CK2 by stimuli. Lastly, possible future directions are presented.

1. Possible mechanisms underlying cooperativity between BMP7 and BDNF for upregulation of P-Smad levels

BDNF increases P-Smad levels in the presence of BMP7 (Fig. 2.3.B & D). This increase occurs at the same sites (C-terminal serines) as those phosphorylated by BMPR-I in response to BMP7. This upregulation by BDNF of BMP7-induced Smad1/5/8 phosphorylation by BMPR might occur in several ways. Different hypothesis are described below and summarized in Fig. 3.1 (#1 to 4).

Increased Smad availability at the plasma membrane could produce this increase in Smad phosphorylation by BMP receptors. This increased availability could be achieved through modulation of endofin’s activity (#1 in Fig. 3.1). Endofin was recently
shown to act as an anchor for Smad1, bringing it to the membrane to be activated by BMPR (Shi et al., 2007). Endofin increases both Smad1 phosphorylation and nuclear localization upon binding of BMP to its receptor (Shi et al., 2007). Thus, co-activation by BDNF and BMP7 may increase P-Smad levels compared to BMP7 alone by positively regulating endofin (#1 in Fig. 3.1). This idea could be tested by co-immunoprecipitation of P-Smad and endofin following BMP7+BDNF treatment.

However, endofin also negatively regulates BMP-induced Smad signaling. Binding of protein phosphatase 1c (PP1c) to endofin on its protein phosphatase binding motif leads to PP1c-mediated BMPR-I dephosphorylation, thus terminating BMP signaling (Shi et al., 2007 and #2 in Fig. 3.1). A protein activated downstream of BDNF might interfere with binding of PP1c to endofin and so result in greater P-Smad levels in cells treated with a BMP7+BDNF combination, compared to BMP7 alone (#2 in Fig. 3.1). Decreased levels of endofin bound to PP1c upon treatment with the combination of trophic factors would confirm this hypothesis.
Fig. 3.1 Possible mechanisms downstream of TrkB for upregulation of P-Smad levels by a BMP7+BDNF combination treatment. 1: Activation of endofin would result in recruitment of more Smads to be phosphorylated by the BMPR. 2: Inhibition of PP1c’s binding to endofin, would prevent the subsequent dephosphorylation of BMPR-I by PP1c. 3: Activation of importin would enhance its binding to Smads’ Nuclear localization signal (NLS) and their recruitment to the BMPR. 4: Inhibition of C-terminal phosphatases SCP1-3, that would otherwise terminate BMP-induced Smad signaling. Red and green arrows indicate inhibition and activation respectively. Blue and black arrows indicate recruitment and phosphorylation of a molecule respectively. Dashed lines indicate hypothetical relationships.

Fig. 2.2 shows that co-incubation of septal cells with BMP7 and BDNF leads to faster Smad phosphorylation than treatment with BMP7 alone. This induction of the Smad signaling pathway may also lead to a higher turnover of Smads and increased Smad availability for phosphorylation by the BMP receptors. Higher rates of nuclear import of Smads in response to a BMP7+BDNF combination would allow for increased turnover. Nuclear import requires the presence of a nuclear localization signal (NLS, reviewed in Mattaj et al., 1998), located in the MH1 domain for receptor-responsive Smads (Fig. 1.7). Association of Smads’ NLS with importins 7 & 8 leads to docking of Smads to the cytoplasmic side of the nuclear pore and translocation of this Smad complex to the nucleus (Xu et al., 2007). BDNF activation may facilitate binding between importins 7/8
and BMP-responsive Smads, which could result in enhanced nuclear translocation of P-
Smads activated by BMP7 (#3 in Fig. 3.1). Pull-down assays of importins 7/8 and P-
Smad that would show increased association of these proteins when cells are treated with
BMP7+BDNF than with BMP7 alone would verify this possibility.

Events occurring downstream of BDNF that would interfere with feedback
regulation (summarized in section 2.c.v of Chapter I) of the BMP signaling pathway
might also explain increased P-Smad levels by the BMP7+BDNF combination, compared
to BMP7 only treatment. Small polymerase II C-terminal phosphatases (SCP1-3)
dephosphorylate Smads 1/5/8 at the C-terminal target site for phosphorylation by BMPR-
I (Duan et al., 2006; Knockaert et al., 2006 and #4 in Fig. 3.1). Inhibition of either of
these phosphatases by a mechanism downstream of BDNF activation might result in
prolongation and/or upregulation of Smad signaling (#4 in Fig. 3.1).

Nature of the interactions between Smads and CK2

The study reported in Chapter II does not resolve the exact nature of interactions
between the Smad and BDNF-induced signaling. Blocking CK2 activity with TBB
inhibits the increase in P-Smad levels by the BMP7+BDNF combination (Fig. 2.5.B &
2.6). Therefore, the increase in CK2 activity by the BMP7+BDNF combination during
hypoglycemia (Fig. 2.7) might lead to the observed increase in BMPR-mediated Smad
phosphorylation in response to the same trophic factor combination (e.g. Fig. 2.3).
Scansite software (http://scansite.mit.edu/, Obenauer et al., 2003) was used to search for
sites on Smads that have consensus sequences matching those for phosphorylation by
acids, where X is any non-basic residue, the letter size is roughly proportional to the
residue’s frequency at that position and residues in bold letters are phosphoacceptors; from Pinna, 2002). Using low stringency (high sensitivity but low specificity), Smad 1 (GI:3192871/AAC19116, SwissProt: P97588) is predicted to get phosphorylated by CK2 on Thr405 in the MH2 domain, whereas Smad8 (GI:2689629/AAC53515/O54835) is predicted to get phosphorylated by CK2 at Ser214 in the linker region. The surrounding sequences being: SVNHGFETEYELTKM for Smad1 and PQSPGSPSESDSPYQ for Smad8 match requirements for phosphorylation by CK2 (phosphorylated residues in bold). However, different prediction softwares produce different hits. For example, PredPhospho (http://www.ngri.re.kr/proteo/PredPhospho.htm., Kim et al., 2004b) predicts phosphorylation of the following residues: on Smad1: Ser 99, Thr 171, Thr 208, Ser 324, Thr 405; on Smad5 (GI: 5706366/BAA83093/ Q9R1V3): Ser 100, Ser 161, Thr 172, Ser 256, Ser 321; on Smad8: Ser 103, Thr 176, Ser 212, Ser 214, Ser 286. Another software, NetPhosK (http://www.cbs.dtu.dk/services/NetPhos/, Blom et al., 1999) predicts phosphorylation by CK2 on: Ser 99 and Thr 405 for Smad1, on Ser 100 for Smad5 and on Ser 103 & 216 for Smad8. None of these prospective CK2 phosphorylation sites are sites recognized by the antibody used to recognize P-Smad (which is specific to the two C-terminal serine residues) in results reported in Chapter II. Mass spectroscopy analysis of the phosphorylated sites on Smads in response to the BMP+BDNF combination would determine which sites on Smads are phosphorylated in response to BMP7+BDNF.

As more extensively described in the following section, increased survival by the BMP+neurotrophin combination may be explained by cooperativity between Smads and other transcription factors. CK2, activated downstream of the trophic factor combination
during stress (Fig. 2.7) might phosphorylate, thereby activate other transcription factors besides the Smads and so enable them to bind the Smad transcriptional complex. As reviewed in Meggio & Pinna (2003), a number of CK2 substrates are transcription factors.

2. Possible mechanisms underlying cooperativity between BMPs and neurotrophins in protecting septal neurons

Neurotrophins have a long-established role in promoting neuronal survival. In particular, neurotrophins mediate survival by activation of the PI3K/Akt pathway, the Mek/Erk pathway or the calmodulin kinase pathway (reviewed in section 1 of Chapter I). BMPs can also enhance survival of neurons. BMP6 is protective both *in vitro* against hydrogen peroxide and *in vivo* against induced ischemic injury (Du *et al*., 2007; Wang *et al*., 2001). Pre-treatment with BMP7 reduces ischemia-induced brain infarction (Cox *et al*., 2004). BMPR-II and activin receptor type I are upregulated following mild brain injury (Lewén *et al*., 1997) and release of BMP6 is increased following mild ischemia (Martinez *et al*., 2001).

Synergy between BMPs and neurotrophins can result in increased neuronal survival (as reviewed in section 3.a of Chapter I); in particular, combinations of neurotrophins and BMP trophic factors protect septal cholinergic and septal non-cholinergic neurons from a hypoglycemic stress (Nonner *et al*., 2001).
Fig. 3.2 Inhibitors of the PI3K/Akt pathway and of CK2 block BMP+neurotrophin-induced protection of septal choline acetyl transferase (ChAT) activity during a hypoglycemic stress. Sister cultures were stressed by a 24 h exposure to hypoglycemia (0 glucose) with (hatched bars) or without (open bars) trophic factors (BMP6 and BMP7 both at 5 nM; NGF and BDNF, both at 100 ng/ml). Some of these cultures were also exposed to inhibitors of the Mek/Erk pathway (U0126, 10 µM) of the PI3 kinase/Akt pathway (Wortmannin, 200 nM), or of CK2 (TBB, 40 µM) during the trophic factor incubation. At the end of the 24 h stress, cultures were returned to normal medium for 24 h, then assayed for ChAT activity. Each bar indicates the mean ± SEM of 13-19 culture wells from 2-3 experiments. * indicates significant difference from hypoglycemia+trophic factors control, using ANOVA followed by Dunnett’s test, p<0.01. Data collected by Doris Nonner.

Pathways underlying the protective effects of a neurotrophin (NGF and BDNF) plus BMP6/7 combination were studied using pharmacological inhibitors. In the experiments presented in Fig. 3.2 and Table 1 (performed by Doris Nonner), ChAT activity measurements indirectly assess for protection of septal cholinergic neurons, whereas Alamar Blue reducing activity gives an indication of protection of all septal cells (including glia).
Table 1

<table>
<thead>
<tr>
<th>Drug (D)</th>
<th>(Hypo+D)/Hypo</th>
<th>Significance</th>
<th>(Hypo+TFs+D)/(Hypo+TFs)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wortmannin</td>
<td>0.99±0.19</td>
<td>ns</td>
<td>0.77±0.06</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>U0126</td>
<td>0.95±0.04</td>
<td>ns</td>
<td>0.87±0.04</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>TBB</td>
<td>0.7±0.07</td>
<td>p&lt;0.05</td>
<td>0.82±0.04</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>0.93±0.16</td>
<td>ns</td>
<td>0.18±0.07</td>
<td>p&lt;0.01</td>
</tr>
</tbody>
</table>

Table 1 Inhibitors of PI3 Kinase, Mek1/2 and CK2 reduce trophic factor (TF)-induced protection of septal neurons during hypoglycemia (Hypo). Sister cultures treated as described in Fig. 3.2 were assayed for Alamar Blue reducing activity. TBB by itself significantly increased the severity of the stress (Hypo+D/Hypo value significantly less than 1). All tested drugs significantly decreased the TF-mediated protection of Alamar blue reducing activity during the stress ((Hypo+TFs+D)/(Hypo+TFs)) values significantly less than 1). Each value represents the mean ± SEM for 14-29 culture wells in each of 2-5 separate experiments. Data were analyzed as in Fig. 3.2 and collected by Doris Nonner.

a. Involvement of the PI3K/Akt and MEK/Erk pathways in protection from hypoglycemia

Both the PI3K/Akt and the MEK/Erk pathways are activated by the trophic factor combination (Fig. 2.4). The 24 h hypoglycemic stress reduced ChAT activity to 13% of non-stressed controls (Nonner et al., 1996). Neither of the inhibitors tested (U0126, wortmannin or TBB) had by itself any effect on ChAT activity (open bars in Fig. 3.2). A combination of BDNF/NGF+BMP6/7 rescues ChAT activity (Fig. 3.2). Treating septal cultures with the Mek1/2 inhibitor, U0126, during a 24 h hypoglycemic stress did not
reduce protection by BMPs+neurotrophins, as indicated by ChAT measurements (hatched bars in Fig. 3.2). However, the PI3K inhibitor, wortmannin, blocked the protective effects on septal cholinergic neurons by the trophic factor combination during hypoglycemia (hatched bars in Fig. 3.2). This same concentration of wortmannin blocked the BDNF-induced increase in P-Akt (Fig. 4.3). Both U0126 and wortmannin reduced the BMP+neurotrophin-induced preservation of Alamar-reducing activity (Table 1).

Several mechanisms could underly the need for activation of the PI3K/Akt pathway during hypoglycemia. Hypoglycemia can involve oxidative stress and mitochondrial damage (Singh et al., 2004), and so upregulation of anti-apoptotic proteins such as Bcl-2, also known to occur following Erk activation (observed in Fig. 4.3 in response to BDNF & Wang et al., 2006) might protect from this stress.

Another mechanism that could help the cells survive during hypoglycemia is translocation of glucose transporters to the cell surface as is known to occur in response to insulin via the PI3K pathway (reviewed in Kandror, 2003). The PI3K pathway is important for neuronal survival during ischemia (Xu et al., 2008) and NGF protection of hippocampal neurons from oxygen and glucose deprivation is mediated in part by the PI3K/Akt pathway through downregulation of transient receptor potential melastatin 7 (Jiang et al., 2008). Similar saving mechanisms could protect from hypoglycemia.

b. Involvement of CK2 in trophic factor-induced protection

The CK2 inhibitor, TBB increased the severity of hypoglycemia in the absence of trophic factors (see Table 1, left columns). TBB also attenuated the trophic factor-induced protection of ChAT activity (Fig. 3.2) and the Alamar Blue signal (Table 1, right columns). Thus, even without the added trophic factors, CK2 contributes to cell survival
and may act in a permissive manner to aid protection given by the trophic factors. CK2 activity increased 20% after 60 min of exposure to BDNF and BMP7 during hypoglycemia (Fig. 2.7). This additional activation of CK2 probably contributes to protection in the presence of the trophic factors. The mechanisms underlying a role for CK2 in the protection from hypoglycemia are not known, but some possibilities are discussed in terms of studies implicating CK2 in protection from other cells. Protective mechanisms involving the nucleus will be discussed first, followed by mechanisms protecting from excitotoxic damage.

CK2 activity is enhanced in response to several types of stress, possibly allowing it to activate saving mechanisms. CK2 activity is upregulated in response to anisomycin and arsenite in HeLa cells (Sayed et al., 2000) and in response to heat shock in several cell lines including LNCaP cells (Davis et al., 2002). Enhanced association of CK2 with the nuclear matrix allows CK2 to exert its protective effects in response to heat shock (Davis et al., 2002). The nuclear matrix is implicated in several processes including DNA repair. Matrix association with CK2 is thought to reflect a role of CK2 in nuclear integrity, allowing for its stress-protective effects (Guo et al., 2001; Davis et al., 2002). In all cancer cell lines that have been studied, CK2 was found to be greatly upregulated and to display elevated levels in the nuclear compartment, whereas its distribution is more uniform between the nuclear and cytoplasmic compartments in non-tumor cells (Faust et al., 1999; Laramas et al., 2007). We did not observe any relocalization of CK2 to the nuclear compartment following 1 h of trophic factor treatment during hypoglycemia (not shown). However, translocation of CK2 to the nucleus might occur several hours after the onset of stress.
Mechanisms of cell damage from hypoglycemia are not completely understood, but probably involve excitotoxic damage from ATP depletion (Mattson et al., 1993), increased reactive oxygen species (Liu et al., 2003), activation of PARP (Suh et al., 2003), depletion of NAD and both nuclear (Kalimo et al., 1985) and mitochondrial damage (Mattson et al., 1993; Liu et al., 2003). NMDA receptor-mediated currents and toxicity are exacerbated in striatal neurons from a transgenic mouse model of Huntington’s disease, leading to excitotoxic damage from dysregulated glutamatergic signaling (Shehadeh et al., 2006). In these cells, CK2 activity is enhanced and inhibition of CK2 activity potentiates toxicity, pointing to its protective role through modulation of NMDA receptor function (Fan et al., 2008). Thus, it is possible that during the hypoglycemic stress, enhanced CK2 activity (as observed in Fig. 2.7), by inhibiting NMDA receptor function, results in reduced intracellular Ca\(^{2+}\), thus allowing for increased ChAT activity (Fig. 3.2; see also Nonner et al., 2000).

c. Effect of mRNA synthesis inhibitors on trophic factor-induced protection

Protective effects of trophic factors often require expression of pro-survival proteins (e.g. Bcl-xl, Bcl-2). Actinomycin D, a blocker of mRNA synthesis, reduced the protective effects by the trophic factor combination on Alamar Blue reducing activity during hypoglycemia (Table 1). This result indicates that new gene transcription is required for the saving effects by the trophic factors.

Phosphorylation of Smads in the linker region by kinases activated downstream of BDNF may allow for recruitment of additional transcription factors to the Smad signaling complex. Such a signaling complex may bind regulatory regions of different genes than those activated by Smads alone. In neuroepithelial cells, formation of a complex
comprised of signal transducers for leukemia inhibitory factor and BMP2 (STAT3 and Smad1 respectively) allows induction of astrocytic differentiation (reviewed by Fukuda & Taga, 2005). Co-occurrence of binding sites for Smad1, STAT3 and other transcription factors in the regulatory regions of numerous genes favors cooperation between these transcription factors (Chen et al., 2008). Also, Milk and Mixer of the paired-like homeodomain transcription factors interact with TGF-β-induced Smad2 on a “Smad interaction motif” and recruit the activated Smad2/Smad4 complex to their binding site on goosecoid’s promoter (Germain et al., 2000). Transcription of goosecoid by the Mixer/Smad complex compared to Mixer alone is enhanced at least 10 times. This “Smad interaction motif” is also required for Fast-1/Smad2 interaction (Germain et al., 2000). It is thus conceivable that binding of BDNF to its receptor would lead to activation of (a) transcription factor(s) that would associate with the BMP7-activated Smad1/Smad4 complex. This association would allow for transcription of neuroprotective genes not activated downstream of either trophic factor alone. These cooperative interactions between transcription factors could be very important since Smads by themselves bind DNA with low affinity (Shi et al., 1998).

3. Regulation of CK2

Activation of CK2 by stimuli

CK2 is often considered to be a constitutively active kinase because it is active when recombinantly expressed in bacteria. However, mounting evidence indicates that CK2 can be activated in response to various stimuli (Sayed et al., 2000; Davis et al., 2002; Solyakov et al., 2004; Arevalo & Rodriguez-Tebar, 2006). A constitutive kinase whose activity can be regulated by stimuli poses a contradiction. Thus, the hypothesis
that various subpopulations of CK2 may coexist in cells has been advanced (Olsten & Litchfield, 2004). These subpopulations, defined both by their subcellular localization and by their subunit composition, might be subject to different modes of regulation. Modest changes in total cellular CK2 activity (Fig. 2.7) could result in neuroprotection if only a fraction of the total CK2 population were affected downstream of the trophic factors.

Co-activation of BMP7 and BDNF could lead to enhanced CK2 activity through regulation of levels of ornithine decarboxylase (ODC, summarized in Fig. 3.3). ODC is a key enzyme in the biosynthesis of polyamines (e.g. spermidine, spermine; reviewed in Tabor & Tabor, 1976). Increased polyamine levels lead to upregulation of CK2 activity (Shore et al., 1997). Upon BMP activation, the interaction between Smad1, a beta subunit of the proteasome (HsN3) and ODC’s inhibitory enzyme, ornithine decarboxylase antizyme (Az) is enhanced (Lin et al., 2002). The downward trend in CK2 activity that we observed in response to BMP7 (Fig. 2.7.C) may be due to Az bound to the Smad complex; this interaction with Az would inhibit ODC by targeting it to the proteasome for degradation (Hayashi et al., 1996). Reduced ODC levels would result in decreased polyamine levels and attenuated CK2 activity (left panel in Fig. 3.3). Trophic factors can activate ODC (e.g. NGF) (Volonte & Greene, 1990). This activation of ODC could result from activation of Az’s inhibitor, Oazin, which traps Az, thus preventing Az from degrading ODC (Hayashi et al., 1996). Upon co-activation of BMP7 and BDNF, Oazin would bind to Az on the activated Smad complex. This binding of Oazin would inhibit degradation of ODC by Az and result in enhanced polyamine levels and thus increased CK2 activity (as measured in Fig. 2.7.D and right panel in Fig. 3.3). Oxidative stress (e.g.
from hypoglycemia) also upregulates polyamines in rat embryonic hippocampal neurons (Yatin et al., 1999). Therefore, the increase in CK2 activity by co-treatment of BMP7 and BDNF during hypoglycemia (Fig. 2.7.D) might be explained by regulation of ODC levels. These hypotheses can be tested by measuring levels of ODC. Treatment of septal cells in normoglycemic conditions would decrease ODC levels compared to non-treated controls. Co-incubation of BMP7 and BDNF during hypoglycemia would raise ODC levels.

**Fig. 3.3 Possible mechanism for the increase in CK2 activity in response to a BMP7+BDNF combination.** Upon BMP7 activation (left panel), a complex forms between P-Smad, the proteasome subunit (HsN3) and ornithine decarboxylase antizyme (Az). Az might bind and target ornithine decarboxylase (ODC) to the proteasome for degradation, leading to reduced levels of polyamines. Reduced polyamines levels would result in decreased CK2 activity. When both BMP7 and BDNF are activated (right panel), BDNF might activate the Az inhibitor (Oazin), which would be recruited to the Smad/Az/HsN3 complex. This activation of Oazin would lead to inhibition of Az and subsequent activation of ODC, resulting in increased levels of polyamines. Upregulation of polyamine levels would enhance CK2 activity. Oxidative stress (e.g. hypoglycemia) also leads to increased polyamine levels. Red, green and blue arrows indicate inhibition, activation and recruitment respectively. Dashed lines indicate hypothetical relationships.
Limitations/problems with the CK2 assay

The lysate assay used in this study (see section 2.d of Chapter II for methods) is subject to artifacts. Background counts can stem from kinase activity other than CK2 phosphorylating the peptide substrate. Two different negative controls were used in the assay, one in which specific CK2 inhibitors (TBBt and TBBz) was added to the kinase reaction and another in which no cell lysate was present. Activity recorded in the presence of the inhibitors was twice as large as that in the absence of lysate (not shown), indicating that these inhibitors do not completely block CK2, or that other kinases (not targeted by the TBB inhibitors) phosphorylate the peptide substrate as well.

In this in vitro kinase assay, lysates are greatly diluted in the buffer used to lyse cells. If CK2 was subject to reversible regulation by a cell component activated by the trophic factors (as addressed in the following section), such a regulator would be diluted more than 100 times and its regulatory effect would be underestimated.

Another important source of error lies in the method used to capture the phosphorylated peptide substrate in this assay. This peptide substrate is positively charged and binds the negatively-charged phosphocellulose membrane through ionic interactions. We excluded a major source of background by including a centrifugation step following protein precipitation with trichloroacetic acid. Trophic factor treatment leads to phosphorylation of numerous proteins in cells. These proteins are phosphorylated during the kinase reaction, by CK2 and/or other kinases, and will also incorporate $^{32}$P-ATP. Most proteins have positively-charged amino acids and thus bind the phosphocellulose filter paper. If one does not precipitate these proteins, they will also contribute to the signal for CK2 activity. Also, the ionic interactions between the peptide
substrate and the filter paper are labile and can be disrupted by washes needed after capturing the peptide. We therefore utilized a different strategy in some experiments. A similar peptide, but carrying a biotin label was used as the substrate (see Appendix 2.a.). This modified peptide was captured on a streptavidin-coated membrane. This method overcomes some of the issues with unspecific binding that arise from binding relying on ionic interactions.

4. Evidence for a possible negative regulator of CK2

It is thought that CK2 is constitutively active (and unregulated) because its activity is often high in unstimulated tissue extracts and because CK2 is fully active when expressed in bacteria as a recombinant enzyme. However, we found that cell lysates produced a robust inhibition (approximately 80%) of recombinant CK2 activity (Fig.

![Fig. 3.4 Inhibition of recombinant CK2 by neuronal lysates.](image)

CK2 assays, in which recombinant CK2 (rec) was incubated alone or in combination with total cell lysates, high molecular weight “high MW” or “low MW” fractions of those same lysates were performed. Total lysates as well as both fractions strongly inhibited CK2 activity. The “high MW” fraction tended to inhibit recombinant CK2 less than corresponding total lysates, although not reaching significance. The strongest inhibition of CK2 tended to be obtained by the “low MW” fraction. Similar amounts of total protein were used for total lysates and “high MW” fractions and equivalent volumes of “Low MW” fractions were used in the assay. * indicates significant difference with \( p<0.05 \), \( n=3 \) (ANOVA followed by Newman-Keuls).
3.4. The first step to characterize this negative regulator was to determine its size. We used ultrafiltration membranes to separate cell lysates by molecular weight (MW, see Fig. 3.5 for MW fractionation of cell lysates and Appendix 2.b). Filtration cells with a molecular weight cut-off of 50 kDa were used to fractionate cell lysates into “high MW”...
and “low MW” fractions (see Fig. 3.5 and Appendix 2.b). CK2 activity was retained entirely in the “high MW” fraction, whereas the negative regulator activity was found in the “low MW” fraction (Fig. 3.6). CK2 activity in the “high MW” fraction tended to be higher than that of the corresponding cleared lysate (with protein normalization, Fig. 3.6), consistent with removal of a reversible lower molecular weight inhibitor.

The fact that some negative regulation was still present in the “high MW” fraction after removal of the “low MW” fraction is probably due to the nature of the method that we used to separate cell components by their MW. In this method, proteins tend to clog the pores of the ultrafiltration membrane, thus preventing passage of a fraction of small molecules.

We also used size-exclusion chromatography to better separate lysate components according to their MW (see Appendix 2.c). In this approach, cleared lysates were first spun on ultrafiltration membranes. The “low MW” fraction devoid of CK2 activity (see Fig. 3.6), was further fractionated using size-exclusion chromatography (Sephadex G-50, see Fig. 3.5). Fractions collected from the chromatography column were concentrated on 10 kDa cut-
off ultrafiltration membranes (Fig. 3.5). Several of these fractions did almost completely inhibit recombinant CK2 activity (Fig. 3.7). This inhibition was present in several consecutive fractions. Additional experiments in which the “high MW” fraction would be processed through a Sephadex G-50 column might be worthwhile in further separating CK2 from the negative regulator.

In other experiments, a different cell extraction method was used to extract only small cytoplasmic molecules. In these experiments, cells still attached to the culture dish were permeabilized using saponin (0.04 mg/ml) or low levels of Triton X-100 (0.001%), thus creating small perforations in the plasma membrane (see Appendix 2.d). This step

![Graph showing CK2 activity](image)

**Fig. 3.7 Recombinant CK2 is robustly inhibited by the “low MW” fraction further purified by size-exclusion chromatography.** “Low MW” fractions from ultrafiltration with 50 kDa cut-off membranes were further processed onto Sephadex G-50 columns and further concentrated on 10 kDa cut-off filters. CK2 activity of recombinant CK2 alone or in combination with total lysates, “high” or “low MW” fractions processed through a Sephadex column (fractions 2 to 4) was measured. Fractions 2 to 4 almost completely inhibited recombinant CK2’s activity. The “high MW” fraction inhibited recombinant CK2 activity less than corresponding total lysates, indicating removal of some of the negative regulator from this fraction. Numbers below the graph represent CK2 activity in counts per minute (cpm). The activity measurements are from 2 experiments. Samples were normalized as in Fig. 3.5. Equal volumes of fractions 2, 3 and 4 were used in the assay.
frees cytoplasmic components of small size, while proteins of larger size (including CK2) remain in the cells. These “cell extracts” inhibited recombinant CK2 (not shown), indicating that the regulator is probably located in the cytoplasm. However, we could only obtain a small portion of the total negative regulator activity with this method.

Another argument in favor of the presence of a negative regulator of CK2 in lysates comes from a “titration” of the effect of this negative regulator-containing fraction on recombinant CK2 (Fig. 3.8). Inhibition of recombinant CK2 increased in an approximately linear manner with increasing amounts of the “low MW” fraction in the kinase reaction (Fig. 3.8). The shape of this titration curve is consistent with that expected from a reversible negative regulator.

**Fig. 3.8 Dose-response curve for inhibition of recombinant CK2 by the “low MW” fraction.** Increasing volumes of the “low MW” fraction used in the kinase reaction were added to 1 unit of recombinant CK2. Total volumes of “low MW” fraction + recombinant CK2 were kept constant. CK2 activity was measured as in previous experiments.
Experimental problems and caviats

In some experiments, the “kinase reaction” was performed in the absence of the “low MW” fraction, which was added only at the end of the “kinase reaction” (referred to as “late addition”). This “late addition” of the “low MW” fraction also resulted in partial inhibition of recombinant CK2 (not shown). Inhibition of recombinant CK2 by this “late addition” is possible only if a component of the “low MW” fraction dephosphorylates the substrate peptide or impairs binding between the peptide substrate and the phosphocellulose filter paper. Dephosphorylation of the CK2 substrate peptide with the “late addition” of the “low MW” fraction might occur if phosphatases are present in this fraction. Inhibition was obtained with a “low MW” fraction which does not contain detectable levels of protein (using the Bradford method of detection), but trace levels of proteins (including phosphatases) might still be present. “Low MW” fractions kept at 4°C for 2 days, inhibited CK2 as well as fractions collected immediately prior to the “kinase reaction” (not shown). However, phosphatases could be stable in those conditions. Serine, threonine and tyrosine phosphatase inhibitors were re-added (they are already present in the lysis buffer) to the “low MW” fraction before the “kinase reaction” with recombinant CK2. Yet, inhibition by the “low MW” fraction of recombinant CK2 was still present (not shown), indicating that inhibition is unlikely to be due to a phosphatase. Further experiments in which proteases would be added to the “low MW” fraction prior to incubation with recombinant CK2, would help further test whether the negative regulator is a phosphatase or other protein.

Other controls were also performed to test the possibility that artifacts (introduced by the assay) are responsible for the observed inhibition of recombinant CK2 by the “low
Substrate depletion during the kinase reaction would lead to lower levels of detected phosphorylation. However, performing the “kinase reaction” in the presence of an excess of substrate produced similar levels of inhibition (not shown), indicating that inhibition of recombinant CK2 is not due to depletion of the peptide substrate.

The inhibition of recombinant CK2 by the “low MW” fraction could also be due to interference with the binding of the substrate peptide to the filter paper. To test for binding interference, an aliquot of the “low MW” fraction was spotted on the filter paper prior to addition of the reaction mix, in which recombinant CK2 alone had been incubated with the substrate peptide. This “spotting” of the “low MW” fraction did not reduce the activity measured (not shown). This result argues against a component of the “low MW” fraction interfering with binding of the substrate peptide to the filter paper by blocking binding sites on the paper.

Small molecules (e.g. ATP, Mg\(^{2+}\)), present in the “low MW” fraction could be responsible for inhibition by the “low MW” fraction of recombinant CK2. To test for this, fractions that came through the 50 kDa filter were further processed using a 10 kDa filter (Fig. 3.5). The fraction retained on the 10 kDa filter, in which the concentration of small molecules from the lysate is greatly reduced, still retained the inhibitory effect.

As mentioned in the previous section, the lysate-based radioactive assay is plagued by both non-specific binding and to precarious binding of the peptide substrate. Using a biotinylated CK2 substrate peptide that specifically binds a streptavidin-coated membrane could minimize these issues. We still recorded high levels of inhibition of recombinant CK2 by the “low MW” fraction with this modified version of the radioactive assay (not shown). However, the “late addition” of the “low MW” fraction using the
The streptavidin-biotin system still displayed inhibition (not shown). A possible problem with this strategy is that cells contain biotin. Cellular biotin could potentially bind the streptavidin membrane and compete for binding with the biotinylated peptide substrate.

Despite all the control experiments performed, I still cannot explain the inhibitory effect of the “late addition” of the “low MW” fraction on recombinant CK2 activity. Several commercially available fluorescent kinase assays are designed to screen inhibitors of kinases using recombinant proteins. An inhibitory effect of the “low MW” fraction on recombinant CK2 using such an assay (in which capture of a phosphorylated substrate is not an issue) might allow us to test for the presence of a negative regulator in the “low MW” fraction.

Further experiments could be performed to determine the nature of this negative regulator. For example, it might be isolated by using recombinant CK2 in an affinity column to purify the active inhibitory component from the “low MW” fraction. A molecule, thus isolated could be tested directly for its capacity to inhibit recombinant and endogenous CK2. Protein negative regulators of CK2 have been identified (Kim et al., 2006; Meggio et al., 2000). However, it is unlikely that the regulator responsible for inhibition of recombinant CK2 in the study presented here is a protein, due to its size. Consistent with our results, Solyakov et al., also recorded strong inhibition of CK2 in rat liver by an unidentified heat-stable regulator. These authors were however unable to separate out this negative regulator from CK2. Our results provide a way to isolate such a molecule.
5. CK2, trophic factors, cell survival and protection from stress

There is substantial evidence for the involvement of CK2 in survival signaling, in the mediation of protection from stress and in trophic factor signaling (summarized in Fig. 3.9). The studies described in Chapter II and section 2 of this Chapter place CK2 at the crossroads of signaling pathways involved in these three phenomena. Indeed, inhibition of CK2 robustly reduces protection of septal neurons from hypoglycemic stress by the trophic factor combination (Fig. 3.2 and Table 1) and CK2 activity is upregulated in response to a trophic factor combination during hypoglycemic stress (Fig. 2.7).

The requirement of CK2 for cell viability is supported by evidence of embryonic lethality of CK2 subunit knockouts (detailed in section 4 of Chapter I). The role of CK2 in survival is strengthened by the observation that its inhibitor, TBB, leads to apoptosis in Jurkat cells (Ruzzene et al., 2002). Relocation of CK2 to the nuclear compartment was observed in response to chemical agents (etoposide and diethylstilbestrol) that mediate apoptosis. This cell death was prevented by prior overexpression of CK2 (Guo et al., 2001). Phosphorylation by CK2 of some of its substrates counteracts apoptosis by preventing their cleavage by caspases (e.g. with the transcription factor Max of the Myc/Max/Mad network that is targeted by caspases in Fas-mediated apoptosis, Fig. 3.9.b & Krippner-Heidenreich et al., 2001). CK2 phosphorylates the apoptotic proteins haematopoietic lineage cell-specific protein 1 (HS1, Ruzzene et al., 2002), Max (Krippner-Heidenreich et al., 2001 and Fig. 3.9) and Bid (Desagher et al., 2001) in Jurkat cells, preventing their cleavage by caspases 3, 5 and 8, respectively. Similarities in the consensus site for phosphorylation by CK2 and for degradation by caspases have been
Fig. 3.9 Summary of the involvement of CK2 in the response to stress signals and in the mediation of survival and growth factor signaling. (a) Formation of a stress-induced complex between CK2 and p38MAPK leads to phosphorylation of p53 by activated CK2. Tumor necrosis factor α (TNF-α) activates CK2, which phosphorylates NF-κB, thereby increasing its transcriptional activity. CK2-phosphorylated IκB is degraded, leading to activation of NF-κB. (b) Activation of Wnt signaling activates Dishevelled (Dvl), which, along with β-catenin is phosphorylated by CK2. Phosphorylated β-catenin translocates to the nucleus, thus increasing cell survival. CK2 forms a complex with the RNA polymerase III (Pol III) subunit, TATA box binding protein (TBP). Phosphorylation by CK2 of Bid and Max prevents their cleavage by caspases and the resulting apoptosis occurring in Fas ligand signaling. (c) Activation by NGF of CK2 leads to inhibition of PTEN and increased levels of phosphatidyl inositol-3 phosphates (IP3). This results in inhibition of Glycogen synthase kinase-3beta (GSK3β) and increased axonal outgrowth. Adapted from Ahmed et al., 2002.

Upon DNA damage, transcription is repressed in a CK2-dependent manner, underlying a role for CK2 in stress signals (Ghavidel & Schultz, 2001). RNA polymerase III (PolIII) is kept active by binding to the β subunit of CK2 and phosphorylation by CK2.
of TATA box binding protein (TBP), a subunit of PolIII (Fig. 3.9). When DNA damage occurs, the catalytic subunits of CK2 dissociate from the CK2/TBP complex, leading to interruption of activity of PolIII and transcriptional repression (Ghavidel & Schultz, 2001). This CK2-mediated repression of RNA polymerase III is thought to help maintain cell viability following stress by using energy sources to repair DNA rather than to continue transcription (Ghavidel & Schultz, 2001). Repression of RNA polymerase III also allows for DNA repair mechanisms to occur, since DNA repair would otherwise be obstructed by elongating mRNA (Aboussekhra and Thoma, 1998). Our studies (Chapter II, sections 3.c & 4.b) bring additional support for the hypothesis that stress can enhance CK2 activation (e.g. in mediating responses to: UV irradiation, Keller et al., 2001 and heat shock, Gerber et al., 2000, see section a) of Fig. 3.9).

CK2 is emerging as a mediator of trophic factor survival signals, along with the well-established protein kinases (Akt, Ras, Erk and GSK-3β, see Chapter I, section 1). For example, upregulation of CK2 activity in the substantia nigra contributes to the neuroprotective effect of Glial cell line-derived neurotrophic factor (GDNF) on dopaminergic neurons (Chao et al., 2006). Also, activation of CK2 by NGF leads to inactivation of phosphatase and tensin homologue deleted on chromosome 10 (PTEN). Inactivation of PTEN allows levels of phosphatidylinositol 3-phosphate to rise, leading to inactivation of Glycogen synthase kinase-3beta (GSK-3β) and increased axonal outgrowth (Arevalo & Rodriguez-Tébar, 2006). Finally, treatment of hippocampal slices with BDNF or NT-4 robustly upregulates CK2 activity, which might contribute to synaptic plasticity (Blanquet, 1998).
6. Future directions

The study presented in Chapter II shows that one of the effects of the BMP7+BDNF combination is to upregulate BMP receptor-mediated Smad phosphorylation (e.g. Fig. 2.1). However, phosphorylation of Smads 1/5/8 at other sites (e.g. in the central linker region) are not detected with the antibody used in this study. Such phosphorylation events may occur in response to the BMP7+BDNF combination. Identification of these sites by phosphopeptide analysis (e.g. by mass spectrometry) may implicate additional signaling pathways in the upregulation of Smad signaling.

Studies presented in Chapter II and section 2 of this Chapter do not determine whether BDNF mediates its effects on Smad signaling and protection of septal cells via the p75NTR or through TrkB. This question might be answered using in similar experiments pro-NGF, which is a specific ligand for p75NTR. Upregulation by pro-NGF of BMP7-induced Smad phosphorylation and ChAT activity would implicate p75NTR. However, in the basal forebrain, p75NTR is only expressed by the cholinergic population, which represents approximately 5% of total cells. It is thus unlikely that effects of neurotrophins on P-Smad would be mediated via this receptor.

The BMP+neurotrophin combination requires new gene transcription to mediate neuroprotection in the septum during hypoglycemia (Table 1). Section 2.c of this Chapter discusses the possibility that neuroprotection by the trophic factor combination is mediated through formation of a molecular complex integrating transcription factors activated downstream of BMPs and neurotrophins. Such a transcriptional complex could display different DNA binding properties than its components separately. In order to test this hypothesis, microarray studies might be performed, comparing genes activated by a
BMP7+BDNF combination to genes activated by BMP7 alone and BDNF alone. Regulatory regions of genes displaying greater upregulation in response to the combination of trophic factors would be analyzed. In particular, transcription factor binding sites in the vicinity of “Smad binding elements” may give an indication of Smad binding partners. Potential partners (e.g. transcription factors) could be confirmed by P-Smad pull-down assays. Such studies would make it possible to better define the nature of interactions between BMP-induced Smad signaling and pathways activated downstream of neurotrophins and CK2. It may also give insight into genes that promote survival of septal neurons during cellular stress.

Neuropathological changes that occur in Alzheimer’s disease (AD) mainly consist of neurofibrillary tangles of hyperphosphorylated Tau protein and of senile plaques composed of amyloid-β (Aβ) peptide. Accumulation of Aβ is thought to result from dysregulated proteolytic cleavage of amyloid precursor protein, APP. The cascade of events that follows includes generation of free radicals, oxidative damage to mitochondria and inflammatory processes, resulting in neuronal loss or dysfunction. The basal forebrain cholinergic neuronal population projects to the cerebral cortex and the hippocampus and is among the first to be affected in AD. Both AD (reviewed in Fukui & Moraes, 2008) and hypoglycemia (Liu et al., 2003) are thought to involve oxidative stress and mitochondrial damage. A combination of BMP+neurotrophins protects these basal forebrain cholinergic neurons from hypoglycemia (Fig. 3.2 and Table 1) and so might also protect in AD models. There is evidence for protective roles of BMPs and neurotrophins in Aβ-related damage due to toxicity. Aβ peptide produces deposits in blood vessels, which cause ischemia (Zhang et al., 1997), and overexpression of APP
increases infarct size produced by middle cerebral artery occlusion (Zhang et al., 1997). BMP7 injected into cisterna magna leads to functional recovery after induced focal cerebral infarction in rats and to enhanced glucose utilization and local cerebral blood flow in regions ipsilateral to ischemic injury (Liu et al., 2001). Because of its dendritic growth-promoting properties (Guo et al., 1998; Le Roux et al., 1999), BMP7 might help reverse the damaging effects of excess glutamate after focal cerebral ischemia. Both transcript and protein levels of BDNF, as well as levels of its TrkB receptor are decreased in the hippocampus of AD brains (Allen et al., 1999; Murer et al., 2001). Perhaps the most convincing evidence that impairment of the neurotrophin system can produce brain damage similar to that seen in AD, is that mice expressing antibodies against NGF develop Alzheimer-like pathology in the normal targets of the basal forebrain neurons (Capsoni et al., 2000). NGF protects hippocampal and cortical neurons from hypoglycemic stress (Cheng et al., 1991), and clinical trials in which neurotrophins were administered intracerebrally have shown promising results (reviewed in Schulte-Herbrüggen et al., 2008). Therefore, experiments in which combinations of BMP7 and BDNF would be chronically administered to the basal forebrain (e.g. via implanted cannulae) of mouse models of AD (e.g. anti-NGF mice) would allow to test the neuroprotective effects of this trophic factor combination in vivo. Indeed BMPs have not been tested for their protective effects in AD, whether alone or in combination with neurotrophins. Spatial memory tests that measure hippocampal function, which is affected in AD would be performed to assess the effects of such a treatment.

Finally, CK2 is greatly upregulated in many cancers and this upregulation leads to dysregulated cell cycle and tumorigenesis (e.g. Münstermann et al., 1990; Gapany et al.,
1995; Landesman-Bollag et al., 2001). Transfection of cancer cells with antisense CK2 or siRNA for CK2 induces apoptosis (Faust et al., 2000; Wang et al., 2001; Ahmad et al., 2006). Thus, identifying a molecule that has potent inhibitory CK2 activity has tremendous potential in establishing strategies to stop tumor growth.
Fig. 4.1 Objective measurement of average nuclear and cytoplasmic intensities of P-Smad 1/5/8 immunofluorescence. A region of interest (ROI) encompassing a neuron was placed around a cell in the phase image (A) using a macro in V++ (phase program in Supporting Material 1). The nucleus within this ROI was determined by thresholding the DAPI image (B, excitation 360 nm, emission 450 nm) using a macro in V++ (mask program in Supporting Material 2). The mask created by this thresholding (D) was applied to the P-Smad fluorescence image (C, excitation 488 nm, emission 520 nm) to measure P-Smad fluorescence within the mask (E) and outside of the nuclear mask but within the cytoplasm (panels F, G). To avoid errors at the edge of the nucleus, the nuclear mask was “eroded” by one pixel to yield a mask that fell entirely within the nucleus. This mask (values of 1 within the nucleus and zero outside the nucleus, panel D) was multiplied by the P-Smad image to yield a measure of nuclear P-Smad fluorescence (E). The original nuclear mask was then dilated by 2 pixels (≈1 µm) to yield a mask slightly larger than the nucleus. A ring-shaped mask extending 2 pixels outward from this dilated mask defined a region near the nucleus but entirely within the cytoplasm (F). The mask of this cytoplasmic ring multiplied by the P-Smad image gave a measure of cytoplasmic P-Smad fluorescence (G). These measures were converted to area-independent average values for a given cell by dividing the summed P-Smad pixel values within each mask by the number of pixels within the mask. These measurements were made automatically; the only input from the operator was to define a region of interest containing the cell in the phase contrast image. Calibration bars: in A, 5 µm, B - G, 2 µm.
Fig. 4.2 Both cholinergic and non-cholinergic septal neurons express BMP receptor type II and P-Smad.

Staining of three representative septal neurons for DAPI, BMP receptor type II (BMPR-II), P-Smad and \( p75^{NTR} \). The middle cell is positive for \( p75^{NTR} \), a marker for cholinergic neurons in the septum. All three cells shown stain for P-Smad1/5/8 (even in the absence of BMP treatment) and BMPR-II. All neurons stained for BMPR-II (≥ 40 neurons in each of 5 different experiments).
Fig. 4.3 Inhibitors of the PI3K/Akt and Mek/Erk pathways block BDNF-induced phosphorylation of Akt and Erk. Sister cultures were pre-treated for 1 h with specific kinase inhibitors before exposure to BDNF for 15 min. Wortmannin (100 nM) was used to block PI3K and hence prevent Akt phosphorylation. An inhibitor of Mek1/2, U0126 (10 µM), prevented the increase in phosphorylated Erk1/2.
2. Methods used to isolate CK2’s regulator

   a. Modified radioactive CK2 assay with biotinylated substrate peptide

   In this modified version of the CK2 assay, the protocol is similar to that described in the Methods section of Chapter II (2.d.), until the end of the incubation with the peptide substrate. Here, we used a similar peptide substrate, but containing a biotin label at its N-terminus (synthesized by GenScript Corporation, Scotch Plains, NJ, USA). The membrane used to capture the substrate was coated with streptavidin at high density (V2861, Promega, Madison, WI, USA). Use of the biotinylated peptide substrate in conjunction with a streptavidin-coated membrane minimizes non-specific binding. After incubation with γ\(^{32}\)P-ATP, the reaction was stopped with 25 µl 7.5 M guanidine HCl. The centrifugation following the end of the reaction became unnecessary, since proteins do not contain biotin and would not bind the membrane. 15µl of the reactions were spotted onto the filter membrane. The ultrafiltration membranes were washed according to the manufacturer’s (Promega) protocol and counted in a scintillation counter.

   b. Ultrafiltration of lysates

   Ultrafiltration was always performed at 4°C after clearing of the lysates. The ultrafiltration cells that were used had different molecular weight cut-offs (all from Millipore, Bedford, MA, USA). To isolate “high MW” fractions, cleared lysates were added to 50 kDa or 30 kDa filters and centrifuged at 14000 g for 15 to 30 min (depending on volume, until >95% of the lysate flowed-through). Membranes were washed several times with lysis buffer. Usually, 50 kDa membranes gave better separation of the negative regulator than the 30 kDa membranes (i.e. less inhibition by the “high MW” fraction of recombinant CK2 than the cleared lysates and higher CK2 activity, see Fig.
3.5 & 3.6). We therefore used the 50 kDa membranes. Proteins retained on the membrane were resuspended in lysis buffer (30-60 µl) and this was considered the “high MW” fraction. The flow-through from those membranes (including that from the different washes) was added to another ultrafiltration unit with a MW cut-off of 10 kDa which separated small molecules (e.g. Mg\(^{2+}\), ATP). The negative regulator retained on the membrane (there was no inhibition from the flow-through) was considered the “low MW” fraction. Both “high” and “low MW” fractions were used directly in the assay, unless the “low MW” fraction was further separated onto Sephadex columns (see next section). Proteins were quantified (with the Bradford assay) in the “high MW” fraction and the cleared lysates, and similar amounts (usually 5 µg of total protein) were used in the assay. Using this detection method, no proteins were present in the “low MW” fraction.

c. Size-exclusion chromatography with Sephadex G-50

0.5 g of superfine Sephadex G-50 (fractionation range: 1.5 to 30 kDa, Pharmacia Fine Chemicals, Uppsala, Sweden), diluted in 10 ml dH\(_2\)O was poured into a column (1 cm in diameter) and allowed to settle. PBS was used as the chromatography buffer. Calibration was performed using 125 µl Blue Dextran added to the column to determine the void volume (2 ml; pre-void volume, 1 ml). After all of the Blue dextran was loaded, PBS was added. Calibration of MW for fractions was performed using a mix of 100 µl 3 mg/ml carbonic anhydrase (MW=30 kDa) and 100 µl phenol red (MW=400 Da), diluted in dH\(_2\)O.

325 µl of “low MW” fractions were added to the column, mixed with 75 µl phenol red for a total volume of 400 µl. The first 3 ml of the eluate were considered void
volume. Fractions of 0.5 ml were collected. Phenol red started appearing in fraction #5. Fractions from the column were concentrated on 10 kDa ultrafiltration cells as described above and a volume of 9 µl of each of those fractions was used in CK2 assay reactions.

d. Permeabilization of cultured cells

Attached cultures were washed twice with cold PBS. CK2 lysis buffer containing either 0.01% Triton X-100 or saponin (0.04 mg/ml) was added to the dish (150 µl per 35-mm diameter well), incubated on ice for 10 min. The buffer/”cell extract” was then collected by tilting the dish and kept on ice until used in the assay. Cells still attached to the dish were scraped on ice in a similar buffer but with 0.1% Triton X-100. Lysates but not “cell extracts” were then cleared. Proteins were quantified in both lysates and “cell extracts” which contained some protein, indicating that permeabilization freed some cytoplasmic proteins along with small cytoplasmic molecules. Efficient cell permeabilization was confirmed by labeling with propidium iodide (PI, Invitrogen). PI is a membrane impermeant dye which, when bound to nucleic acids can be detected by fluorescence microscopy (excitation, 535 nm; emission, 620 nm). Cell permeabilized with 0.01 % Triton or saponin showed clear nuclear labeling with PI, indicating successful permeabilization.
References


