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Transcriptional Regulation by cAMP and Ca^{2+} Links the Na^+/Ca^{2+} Exchanger 3 to Memory and Sensory Pathways

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Abstract

The signaling cascades triggered by neurotrophins such as BDNF and by several neurotransmitters and hormones lead to the rapid induction of gene transcription by increasing the intracellular concentration of cAMP and Ca^{2+}. This review examines the mechanisms by which these second messengers control transcriptional initiation at CRE promoters via transcription factor CREB, as well as at DRE sites via transcriptional repressor DREAM. The regulation of the *SLC8A3* gene encoding the Na^+/Ca^{2+} exchanger 3 (NCX3) is taken as an example to illustrate both mechanisms since it includes a CRE site in the promoter and several DRE sites in the exon 1 sequence. The upregulation of the NCX3 by Ca^{2+} signals may be specifically required to establish the Ca^{2+} balance that regulates several physiological and pathological processes in neurons. The regulatory features and the expression pattern of *SLC8A3* gene suggest that NCX3 activity could be crucial in neuronal functions such as memory formation and sensory processing.

Index Entries: Transcription factors; ATF/CREB; DREAM; Na^+/Ca^{2+} exchanger 3; BDNF; cAMP; Ca^{2+}; gene expression; memory.

Introduction

The second messengers cyclic adenosine monophosphate (cAMP) and Ca^{2+} control a large array of biological functions. In neurons, the elevation of Ca^{2+} at presynaptic terminals is involved in the release of neurotransmitters and of the neurotrophin Brain-Derived Neurotrophic Factor (BDNF) *(1–4).* Their binding at postsynaptic receptors triggers fast synaptic transmission and initiates a cascade of events, mostly mediated by the rise in the cytosolic cAMP and Ca^{2+}, which causes long-term changes in the activity of post-synaptic cells by regulating gene expression *(5–9).* Neuronal activity-dependent gene expression is crucial for survival and establishment of neuronal

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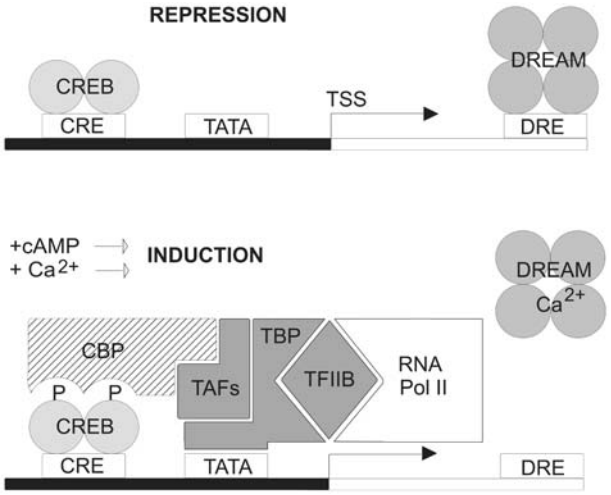

Fig. 1. Example of the transcriptional regulation occurring at CRE and DRE promoter sites. **(Repression)** Transcription factors of the ATF/CREB family are constitutively expressed and bind the CRE sequence without activating transcription. The tetrameric transcription factor DREAM binds the DRE and represses transcriptional activity. **(Induction)** The rise in second messengers cAMP activates protein kinase A that phosphorylates Ser residues in the KID domains of ATF/CREB. The phosphorylation favors the interaction with the coactivator CBP which, in turn, recruits the basal transcription activator factors (TAFs), TATA binding protein (TBP) and transcription factor TFIIB. The resulting complex stabilizes RNA polymerase II and favors the assembly of the transcriptional initiation complex. Ca^{2+} binding to the EF-hand domain of DREAM produces a conformational change in the protein with consequent detachment from DRE sites and relief of transcriptional repression.

connections during development as well as for neurotransmitter release, synaptic plasticity, maintenance of long-term potentiation (LTP), induction of chronic pain, and the dependence on abusive drugs. The transcription factors of the cAMP responsive element binding protein (CREB) family are essential in these functions *(10–16).* The rise in intracellular cAMP activates the cAMP-dependent protein kinase A (PKA) that phosphorylates Ser-133 of transcription factor CREB (Fig. 1) *(5,17–19).* The cytosolic Ca^{2+} rise activates Ca^{2+}-dependent protein kinases, such as Ca^{2+}-calmodulin dependent kinase II and IV (CaMKII and CaMKIV) that also phosphorylates CREB *(20–23).* The activation of CREB induces the rapid expression of immediate early genes (IEGs) without the need of new protein synthesis *(6).* Some members of the IEGs encode transcription factors that are classified in three subgroups: Fos, Jun, and Krox *(24);* these factors, in turn, modulate transcription of several late genes involved in specific neuronal functions. The Fos and Jun proteins have been widely characterized and their transcriptional activity depends on their dimerization, through their basic leucine zipper domain (bZIP), to form transcription factor AP1 (activator protein 1) *(24,25).* Activation of transcription factor of the ATF/CREB family also directly modulates transcription of several other genes, including the CRE (cAMP response element) in their promoter sequences. The transcriptional repressor downstream responsive element antagonistic modulator (DREAM) contributes an additional mechanism of transcriptional regulation by the cAMP and Ca^{2+} signals in neurons *(26).* It binds to downstream regulatory element (DRE) located in the first exon of genes such as c-*fos* and prodynorphin. It includes EF-hands motifs capable of binding Ca^{2+} and leads to a conformational change in the protein. The consequence is the transcriptional derepression by the detachment of DREAM from the DRE sites (Fig. 1).

To function as second messengers, cAMP and Ca^{2+} levels must be strictly controlled. Their concentration, rising as a consequence of receptor stimulation, must return to basal levels to allow subsequent responses. The concentration of cAMP is controlled by the activity of multiple forms of phosphodiesterases, some of which are regulated by Ca^{2+}-calmodulin (CaM) *(27–29).* The cytosolic Ca^{2+} is maintained at low concentration by two main systems: the Ca^{2+} ATPase enzyme and the Na^{+}/Ca^{2+} exchanger (NCX). The activity of the ATP-driven Ca^{2+} pumps of the plasma membrane (PMCA) and of the endoplasmic reticulum (SERCA) is present in most cell types and their function is to extrude Ca^{2+} from the cells

and to sequester Ca^{2+} from the cytosol, respectively. They operate with high affinity but low turnover rates. The Na^+/Ca^{2+} exchange activity is an important system in Ca^{2+} homeostasis especially in excitable tissues. NCX is a reversible transporter since it can operate in both directions; the movement of Ca^{2+} in or out of the cell depends on the net electrochemical driving force. This transporter exchanges three Na^+ against one Ca^{2+} across the plasma membrane, and hence, its activity is electrogenic. The NCX exhibits 10-fold lower affinity for Ca^{2+} but 10- to 50-fold higher turnover rate than the ATP-driven Ca^{2+} pumps *(30).* The NCX protein is abundantly expressed in neurons. It is prevalently localized in regions of the plasma membrane where a large traffic of Ca^{2+} ions occur, especially at synapses and growth cones *(31,32).* Although its precise role is not yet completely clear, the dominant expression of NCX and its localization suggests that this molecule is critical in the maintenance of the Ca^{2+} homeostasis in neurons.

Three isoforms of the murine Na^+/Ca^{2+} exchanger NCX1-2-3 have been cloned and identified as products of distinct genes *(33–35).* The NCX1 protein is mostly expressed in heart but is also found abundantly in the brain and at variable levels in many other cell types. The NCX2 and NCX3 isoforms are mainly expressed in brain and skeletal muscle. All three human *SLC8A1-2-3* genes, encoding NCX1-2-3, respectively, have been identified *(36–38).* The *SLC8A* genes, although highly similar in the coding regions, differ markedly in the regulatory regions. The regulation of *SLC8A1* gene transcription was first investigated: three promoter regions which control tissue-specific expression of the gene in heart, kidney, and brain have been identified so far *(39).* The human *SLC8A3* promoter, recently identified, directs transcription in neurons and is potentially active in muscle cells *(40),* whereas the NCX2 promoter region is still unknown. The *SLC8A3* promoter includes CRE and AP1 sites that mediate transcriptional regulation by cAMP and Ca^{2+}. Furthermore, the first exon of the *SLC8A3* gene includes several DRE sites potentially capable of binding the transcriptional repressor DREAM. Thus the second messengers cAMP and Ca^{2+} have the potential to regulate transcription of this transporter at least by two distinct mechanisms: via the activation of transcription factors of the CREB family and consequent induction of IEG to form AP1 complexes, and via transcriptional derepression by the detachment of DREAM from DRE sites (Fig. 1).

This article examines the mechanisms that control transcriptional activity via CRE and DRE promoters in neurons. The regulation of the *SLC8A3* promoter by BDNF and by second messengers cAMP and Ca^{2+} is exemplified in this review. This, together with the expression pattern of the *SLC8A3* gene, suggests that the NCX3 activity may be crucial in controlling intracellular Ca^{2+} balance involved in the regulation of neuronal functions, such as memory formation and sensory processing.

Tissue-Specific Expression and Different Regulation of SLC8A1–3 Genes

Although many studies have focused on the posttranscriptional regulation of NCX protein synthesis and activity, there are few studies addressing the problem at the level of transcription initiation. In fact, although the *SLC8A* genes are very similar in their coding regions, they nevertheless differ markedly in the organization and structure of their promoter regions, suggesting different transcriptional control. Immunohistochemistry and *in situ* hybridization studies on rat brain support this possibility. In addition, the three NCX1 isoforms are differentially expressed in several brain areas, suggesting distinct functional roles *(41).*

SLC8A1 Promoters

The existence of alternative promoters in the NCX1 gene was suggested by the observation that NCX1 transcripts are expressed in a

tissue-specific manner *(42).* A complex multipartite structure of the human and rat *SLC8A1* promoters has been reported. The proximal promoter activated transcription of the reporter gene in cultured aortic smooth muscle cells, and showed features of housekeeping guanine and cytosine (GC)-rich promoters with constitutive activity. However, transcription could be increased by two additional upstream promoters *(43).* Three distinct tissue-specific transcriptional start sites of *SLC8A1* specify transcripts including alternative exon 1 sequences (5'UTR). The cardiac promoter is upstream of the kidney and brain promoter, respectively. The cardiac promoter is TATA-less, but includes several enhancer elements: the serum responsive element (SRE) and CArG elements that enhance promoter activity in cardiomyocytes. The upregulation of NCX1 transcription by the serum responsive factor and GATA-4 is essential for the development of neonatal cardiomyocytes, suggesting that NCX1 proteins as α-MHC, cardiac troponin C, and skeletal α-actin may be upregulated during heart development, as well as in pathological conditions such as cardiac hypertrophy and heart failure *(44).* The upregulation of the NCX1 transcript by α-adrenergic stimulation was found to be mediated by the E-boxes and by an additional sequence *(45).* The cardiac promoter also indicates AP1 sites that could enhance transcription following the induction of IEGs by several stimuli. At present, the functional analysis of the alternative *SLC8A1* promoters has been limited to the cardiac promoter: NCX1 protein is mostly expressed in heart. Unlike the cardiac and the kidney promoter that drive expression only in the specific tissues, the brain NCX1 transcript indicates ubiquitous expression, although it is more copious in brain. The ubiquitous expression could be determined by the several elements potentially binding specific protein 1 (SP1).

SLC8A3 Promoter

The human *SLC8A3* minimal promoter was identified in the 250 bp region immediately upstream of the exon 1 (Fig. 2). It shows features similar to the *SLC8A1* brain promoter in that it includes SP1 and AP2 elements that confer constitutive transcription and tissue specific expression, respectively. The sequence of human *SLC8A3* promoter and of the exon 1, as that of the rat brain *SLC8A1* promoter, is very rich in GC bases. These form a typical cytosine and guanine dinucleotide (CpG) island as it occurs at the 5' end of several housekeeping genes and oncogenes. While these GC rich sequences usually stay unmethylated, abnormal methylation occurs in some pathological conditions as during oncogenic transformation, e.g., the α2 (VI) collagene gene undergoes extensive methylation in SV-40 transformed fibroblasts that inhibit transcription from the AP2 sites *(46–49).*

The sequence of the *SLC8A3* promoter and that of *SLC8A1* brain promoter include multiple AP2 sites (GCCNNNGGC) potentially important for regulation in neurons. Members of the AP2 transcription factor family have been shown to be important regulatory molecules for the vertebrate development of neuronal tube, neuronal crest derivatives, skin, and urogenital tissue. These members also show differences in spatial and temporal expression during mouse development *(50).* The tissue-specific expression of AP2 is highest in ectodermal cell lineage, including neuroepithelium and neural crest *(51).* Several repeated consensus elements for transcription factors AP2 are found in neuron specific gene promoters (e.g., choline acetyltransferase, proenkephalin; human fragile mental retardation promoter [FMR1], *[52,53]*) as well as in other cellular genes, (i.e., keratin, sodium-phosphate cotransporter [Npt2] of kidney proximal tubules and viral genomes as MMTV *[54–56]*).

In contrast to the *SLC8A1* gene, whose transcription initiates from multiple tissue-specific promoters, a single promoter region may determine the tissue-specific expression of the *SLC8A3* gene. The *SLC8A3* promoter indicates neuronal expression. Furthermore, it also includes elements for the muscle-specific transcription factors MyoD and GATA factors 2/3; in agreement is the fact that the ortholog

Fig. 2. The structure of the *SLC8A3* gene promoter/exon 1. The minimal promoter region of the human *SLC8A3* gene is comprised of a short nucleotide stretch starting from nucleotide position (–263). Position +1 defines the site of transcriptional initiation. Consensus elements for binding transcription factors are shaded, as well as DRE sites in the exon 1 sequence (underlined).

rodent gene is predominantly expressed in brain and skeletal muscles *(35).*

The *SLC8A3* promoter also includes elements potentially binding inducible transcription factors AP1, and Egr-1 (also known as NGF1-A, Krox24, Zif/268, or Tis 8). These are a subgroup of IEGs that are activated rapidly and transiently in neuronal cells in response to environmental stimuli, such as neurotransmitters and neurotrophin receptor stimulation. They regulate the expression of subsets of genes termed delayed-response genes. The AP1 complex is formed by the dimerization of the Jun proteins (c-Jun; JunB and Jun D) and Fos proteins (c-Fos, FosB, Fra-1, etc.) through their leucine zipper motifs. The AP1 complex binds to the consensus sequence TGACTCA. This element, also termed TPA response element (TRE), was first indicated as the element responsive to phorbol ester activation of protein kinase C (PKC). The Jun proteins can also form homodimers or heterodimers with Fos; dimerization with transcription factors of the ATF/CREB family is also possible. The composition of the AP1 complex can affect the affinity for consensus sequences and influence transactivation potential. The regulation of Jun and Fos proteins by phosphorylation has been deeply investigated and a considerable amount of literature is available *(24,25,57).*

The transcription factors of the Egr or Krox family are rapidly induced in mammalian brain by a variety of stimuli; in contrast to Fos and Jun they lack the bZip domain and bind DNA by zinc finger motifs. The activation of the *N*-Methyl D-Aspartate (NMDA) receptor

by glutamate transiently induces Egr-1 within minutes, similarly to the induction of c-*fos* *(58).* The induction of Egr-1-mRNA has been reported in a variety of primary cultures of cerebellar, corticostriatal, cortical, and hippocampal neurons. The in vivo expression of *Egr-1* is strongest in the hippocampus and cerebellum of adult rats and considerably high in neocortex, striatum, and hippocampal CA1 neurons. Following stimulation by intraperitoneal administration of NMDA, the Egr-1 mRNA increases in the cortex, cerebellum, and hippocampus *(59).* The blockade of NMDA receptor by MK-801 selectively downregulates the expression of *Egr-1* but does not alter c-*fos* and AP1 expression *(60).* It has been suggested that the activation of NMDA receptor by physiological synaptic activity specifically increases the expression of *Egr-1*, whereas kainate-evoked seizures induced all four members of the Egr family *(61,62).* Thus, although the role of Egr-1 element in the regulation of *SLC8A3* gene expression has not yet been documented experimentally, it seems likely that NMDA-receptor activation may cause the upregulation of SLC8A3 mRNA. The consequent increase in NCX3 protein activity may be specifically required to extrude Ca^{2+} following its entry through the activated NMDA-receptor channel. A functional link between NMDA receptor and NCX3 is also suggested by their high expression in the hippocampus, especially in the CA1 region *(59,63).*

The *SLC8A3* promoter also indicates a CRE element–218 (TGACGTGC) originally identified in the promoter of the somatostatin gene as the 8-bp sequence TGACGTCA *(64,65).* The CRE element is included in several other cAMP-responsive gene promoters, such as c-*fos*, *Zif/268*, or *Egr-1* and proenkefalin *(66–68).* In addition, it was shown that the CRE element mediated the transactivation of the c-*fos* gene in response to Ca^{2+} influx as well as cAMP thus also named Ca^{2+} response element (CaRE), *(5).* The presence of the CRE element has attracted much attention since it could confer rapid inducibility to the *SLC8A3* gene, similar to that of IEGs. The *SLC8A3* gene also includes several DRE elements (with central core nucleotide sequence GTCA) in the region downstream of the TATA box and in the exon 1 (Fig. 2), potentially able to bind the cAMP and Ca^{2+}-regulated transcriptional repressor DREAM *(26,69,70).* The transcriptional regulation in response to BDNF, cAMP, and Ca^{2+} signals via the CRE and DRE sites will be examined in the following subheading.

Primary Signals Converging on CREB

A summary of the signaling cascades activated by the BDNF stimulation of TrkB receptor as well as by the activation of several neurotransmitter and hormone receptors through the second messengers cAMP and Ca^{2+} is shown in Fig. 3. Transcription factors of the CREB family binding the CRE element are the target of the specific signal transduction pathways, each of which is controlled by the activation of specific protein kinase-coupled receptor systems. The molecular studies on memory formation point out the central role of the signaling cascades that regulate CREB-dependent gene transcription in different organisms such as *Aplysia*, *Drosophila*, and mice; in line with this, mutations in the human gene for CREB coactivator binding protein (CBP) result in a syndrome characterized by mental retardation *(15).*

BDNF

The neurotrophin BDNF is one of the major regulators of CREB function *(12).* BDNF and its affinity receptor TrkB are produced in about one-third of sensory neurons *(71)* localized, prevalently, in the dorsal horn of the rat spinal cord *(72).* Embryonic chick sensory neurons depend on BDNF or Neurotrophin 3 (NT3) earlier than nerve growth factor (NGF) for their survival *(73).* The deletion of the respective genes in mice causes the loss of about 30% of neurons in dorsal root ganglia (DRG), suggesting that BDNF is essential for survival and development of a subpopulation of sensory neurons. It also modulates cortical

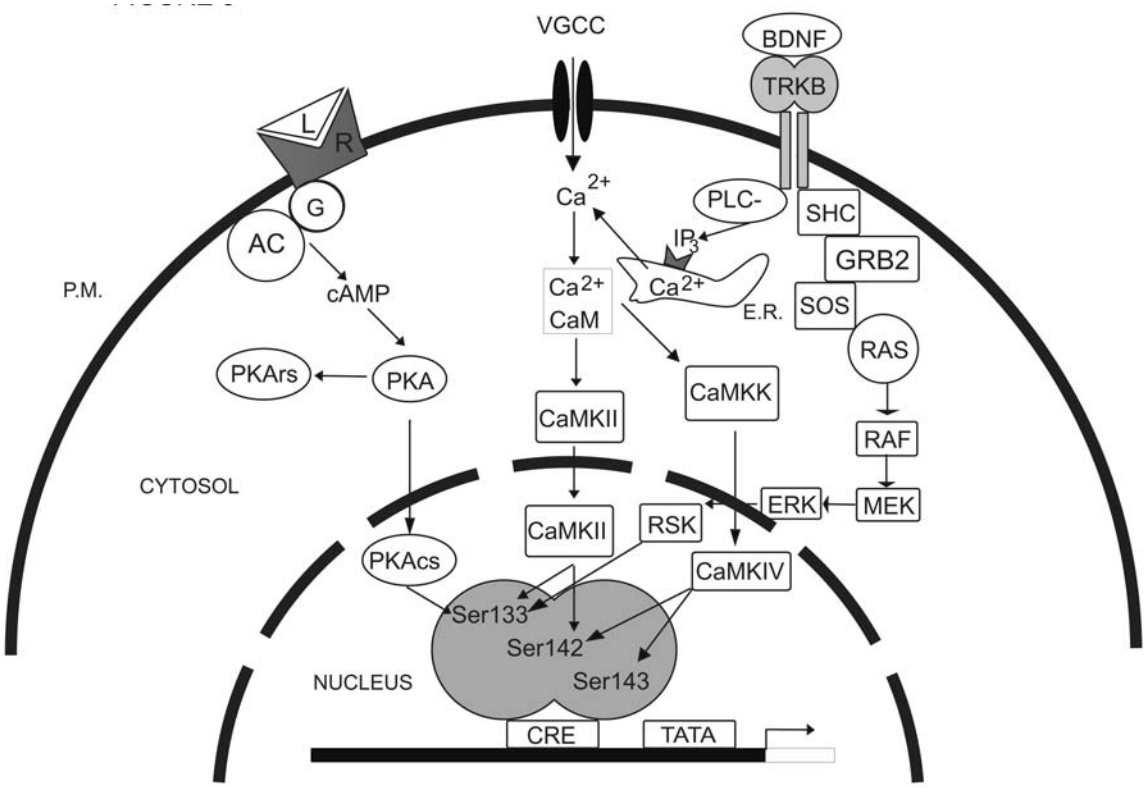

Fig. 3. Signaling pathways regulating transcription at CRE sites. Activation of neurotransmitter receptors and plasma membrane (PM) depolarization causes the opening of voltage-gated Ca^{2+} channels (VGCC) and consequent Ca^{2+} influx. The Ca^{2+} rise regulates transcription by activating nuclear CaMKII and CaMKIV that phosphorylate transcription factors of the ATF and CREB family at three specific serine residues (Ser-133, Ser-142, and Ser-144 in CREB). Ligands (L) binding to G protein-coupled receptors (R) stimulate the production of cAMP which binds to protein kinase A (PKA) and favors the dissociation of the regulatory subunit (PKArs) from the catalytic subunit (PKAcs). The latter translocates to the nucleus where it regulates transcription by phosphorylation of CREB (Ser-133). Binding of the neurotrophin BDNF to its high affinity receptor TrkB leads to auto-phosphorylation of the intracellular receptor segment and consequent activation of two signaling cascades converging on transcription factor CREB. One pathway involves the activation of the phospholipase C-γ (PLCγ) which breaks phosphatidyl-inositol into diacylglycerol and inositol trisphosphate (IP_3). While the latter binds to its receptor in the membranes of the endoplasmic reticulum (ER) and promotes Ca^{2+} release, increased cytosolic Ca^{2+} levels activate CaMKs which phopshorylate CREB. A second pathway activated by phosphorylated TrkB receptor involves the interaction with SHC, GRB2, and SOS to activate the small GTP binding protein RAS. This causes activation of the first protein kinase RAF which, in turn, activates the kinase cascade including MEK, ERK, and RSK, which leads to the phosphorylation of transcription factors CREB (Ser-133).

dendritic growth in response to neuronal activity *(74)* and enhances neurotransmitter release in neuromuscular junctions of *Xenopus* embryos *(75).*

In addition to its trophic effects, BDNF regulates synaptic plasticity in various brain areas. BDNF induces a rapid depolarization of hippocampal neurons as does glutamate; it also enhances glutamatergic synaptic transmission and increases the phosphorylation of NMDA-receptor subunits *(76–80).* Transfection of primary rat cortical neurons with the BDNF-green

fluorescent protein (GFP) fusion construct localized the BDNF-GFP in secretory granules, pertaining to the regulated pathway of secretion. Its localization in the vicinity of synaptic junctions has suggested its involvement in synaptic transmission *(81).* A high-frequency stimulation of glutamatergic synapses of hippocampal neurons triggered the release of BDNF-GFP from secretory granules, indicating a role of BDNF in synaptic plasticity *(82).* Furthermore, BDNF enhances LTP in the hippocampus in vivo *(83)* and BDNF-deficient mice display markedly impaired LTP rescued by treatment with BDNF *(84).* Genetic and pharmacological interference with BDNF or TrkB in rats impairs learning and memory *(85).*

The binding of the neurotrophin BDNF to tyrosine kinase receptors (Trk) leads to receptor dimerization and autophosphorylation of specific tyrosine residues in its intracellular domain. This allows for the interaction with the protein SHC, phospholipase C-γ (PLC-γ), and phosphatidylinositol-3 kinase (PI3K) that are consequently activated *(86–88).* Tyrosine phosphorylation of SHC by TrkB receptor triggers the SHC/Grb2/Sos interaction that leads to Ras activation. Furthermore it phosphorylates Raf that, in turn, activates mitogen activated protein kinase (MEK), extracellular regulated protein kinases Erk1-2, and ribosomal kinase Rsk *(89).*

The active PLC-γ cleaves phosphatidylinositol-4,5-bisphosphate to generate inositoltrisphosphate (IP3) and diacylglycerol (DAG), which in turn promotes the release of Ca^{2+} from endoplasmic reticulum and the activation of PKC, respectively. In hippocampal neurons, the Ca^{2+} released from internal stores also activates CaMKII which in turn stimulates the mitogen-activated protein kinases (MAPK) and a second downstream kinase MAPK-activated protein kinase II (KAPK-2) both of which can phosphorylate directly CREB at Ser-133. The neutrophic activity exerted by BDNF on synaptic plasticity appears to be mediated by CaMKII phosphorylation of CREB in the rat hippocampus *(90,91).* Intra-hippocampal microinfusion of BDNF triggering LTP in the dentate gyrus leads to the rapid phosphorylation of MAP and ERK kinases coupled to CREB activation. The selective inhibition of the two kinases indicates that they are required for induction but not for the maintenance of LTP *(92).* In addition, activated PI3K activates other kinases e.g., Akt *(93,94),* a serine-treonine protein kinase shown to phosphorylate the mammalian target of rapamycin (mTOR). The activation of this signaling pathway is important for the upregulation of mRNA translation *(95,96).* In addition, this pathway has been shown to be associated with consolidation of spatial memory *(85).* Thus BDNF is critical for synaptic plasticity, including short- and long-term memory formation.

Ca^{2+}

The depolarization of the plasma membrane during synaptic activity largely determines the entry of external Ca^{2+} through VGCC. The Ca^{2+} increase triggers neurotransmitter release from synaptic vesicles. The central excitatory neurotransmitter in mammalian brain (L-Glutamate) binds to ionotropic receptors (i.e., NMDA) that are ligand gated Ca^{2+} and Na^{+} channels. The Ca^{2+} rise in the cytosol produces short- and long-term changes in neuronal physiology that are determined by the control on gene expression exerted by this second messenger in the nucleus. For example, the elevation of synaptic Ca^{2+} within 1–2 μm from the cell surface during synaptic activity is sufficient to activate CaMKs in the nucleus of hippocampal neurons *(97).* CaMKs inhibitors block synaptically activated phosphorylation of CREB, and antisense oligonucleotides against CaMKIV abolish the phosphorylation of CREB in hippocampal neurons *(97,98).* The dual phosphorylation of CREB at Ser-133 and Ser-142 by CaMKII produces a negative effect on transcription *(99,100).* The Ca^{2+}-induced gene transcription via CREB requires the triple phosphorylation at Ser-133, Ser-142, and Ser-143 by the combined activity of CaMKIV and CaMKII *(23).* The phosphorylation of Ser-133 is a critical step in CREB activation because it favors the recruitment of the

CBP, which can interact with basal transcription factors, and the RNA polymerase II to stimulate transcription (Fig. 1). However, when CREB is phosphorylated at three sites by CaMKs, its interaction with CPB is impaired, although transcription is activated, suggesting that specific transcriptional complexes at CRE promoter could be selectively assembled following a rise in cytosolic Ca^{2+} *(23).*

L-Glutamate also stimulates metabotropic glutamate receptors (mGluRs) that are G-protein coupled receptors. Of the eight subtypes of mGluRs characterized, thus far mGluR1 and mGluR5 are coupled with the activation of PLC-γ *(101,102).* The selective activation of mGluR5 receptor subtype was shown to promote the phosphorylation of CREB and the consequent induction of c-*fos* in striatal neurons *(103).* The other members of the mGluR family are coupled via specific G proteins to the adenylyl cyclase and regulate cAMP levels, as is the case of serotonin receptors.

cAMP

The binding of several neurotransmitters and hormones to their specific receptors can either stimulate or inhibit the production of cAMP. For example, some of the serotonin (5-HT) receptor subtypes (5-HT_4, 5-HT_{5a}, 5-HT_6, 5-HT_7) stimulate cAMP production in postsynaptic cells, while others (5-HT_{1B}, 5-HT_{1C}, 5-HT_{1D}, 5-HT_{1E}) inhibit its production *(104).* These receptors belong to the class of seven transmembrane receptors binding to trimeric GTP-binding proteins (G proteins). These receptors are coupled to the adenyl cyclase that catalyzes the synthesis of cAMP from adenosine triphosphate via stimulatory or inhibitory G proteins (Gs or Gi/o, respectively). The differential expression of the adenylyl cyclase isoforms in brain and their specific regulatory features accurately control the level of cAMP in response to extracellular stimuli. The increased cAMP concentration activates protein kinase A (PKA), which phosphorylates transcription factors of the ATF/CREB family *(105,106).*

Transcription Factors of the ATF/CREB Family

CREB is a member of a large protein family (ATF/CREB) binding to CRE promoter elements of genes regulated by cAMP and Ca^{2+}, the best characterized examples of which are the IEGs *(c-fos)* and those encoding for neuropeptides (VIP, somatostatin, proenkefalin etc.) *(64–68).* The multigene family of cAMP-responsive transcription factors includes at least 10 structurally related proteins that are expressed in a wide variety of cell types where they regulate several physiological processes including circadian rhythms, pituitary gland function, memory, and LTP *(15,18,107,108).* The cAMP-responsive transcription factors CREB, cAMP responsive element modulator (CREM), and activating transcription factor 1 (ATF1) are encoded by three genes in mammals that share a high degree of structural conservation *(18,109,110).* These are proteins of bZip class that dimerize through their leucine zipper domain and contact the DNA directly with the adjacent basic region. The bZip proteins can form homo- and heterodimers that bind to the consensus sequence (CRE) in the promoters of several genes *(111).* The ATF/CREB factors can dimerize with Fos and Jun proteins determining AP1 complexes with distinct DNA binding properties and transactivation potential *(25,57,68).* Homodimers of Jun proteins are also possible as well as heterodimers of Jun and ATF/CREB polypeptides. In addition the dimerization of CREB/CREM polypeptides with DREAM is also possible *(70,112),* (*see also* "Ca^{2+} and cAMP regulation via transcriptional repressor DREAM").

The transcriptional activators ATF1/CREB are ubiquitous in neurons and other cell types; their gene promoter regions are rich in GC, indicating features of constitutive expression. Their activity induces transcription of IEGs within minutes, without requiring *de novo* protein synthesis. They are linked to the various signal transduction pathways by several kinases that rapidly activate them by phosphorylation. The N-terminal portion of the

ATF/CREB polypeptides includes a phosphorylation box (P-box) or kinase-inducible domain (KID) which can be phosphorylated by PKA as well as by several other kinases (Fig. 3). Adjacent regions rich in glutamine (Q1 and Q2 regions in CREB) contact cofactors of the basal transcription complex. Alternative splicing generates isoforms of the CREM gene (CREM α, β, and γ) that lack the Q-region and act as transcriptional repressors *(113,114).* The isoform of CREM, termed cAMP early repressor (ICER), is the unique inducible transcription factor in this family. The ICER mRNA is synthesized from an alternative promoter (P2) located in an intron close to the 3′ end of the CREM gene; it includes CRE elements that mediate induction by cAMP. The ICER is the smallest transcription factor described thus far: it is composed of the C-terminal segment of CREM and contains only the DNA-binding domain. Thus, it competes with the CREB/ATF polypeptides for binding to CRE elements, but, lacking the P-box domain, is unable to activate transcription and functions as a transcriptional repressor *(115).* This negative regulatory circuit accounts for the transient nature of transcriptional activation by ATF/CREB and predicts that transcriptional activation must be followed by a refractory period. The ICER promoter is also a target of ICER negative modulation.

A second feature that characterizes the transient nature of CREB induction is the rapid dephosphorylation of the activating phospho-Ser residues. The Ca^{2+} increase responsible for kinase activation can also activate phosphatase calcineurin (PP-2B) and thus the phosphatase PP-1 *(98,116,117).* Calcineurin is a Ca^{2+}-dependent serine threonine phosphatase that can dephosphorylate proteins directly or indirectly by inactivating the phosphatase inhibitor-1-phosphate cascade. This, in turn, activates PP1 which dephosphorylates several target proteins, including NMDA and α-amino-3-hydroxy-5-methyl-4 isoxazole propionic acid (AMPA) ionotropic receptors as well as CREB *(118–120).* Calcineurin is involved in the regulation of important biological processes such as cell cycle control, T-cell activation, and memory formation. Calcineurin is particularly enriched in synapses, and is important for plasticity of hippocampal neurons *(121–123).* LTP and long-term depression (LTD) are linked with postsynaptic cytosolic Ca^{2+} rise. However, the phosphatase activity prevails in LTD whereas the kinase activity predominates in LTP. For example, in the establishment of LTP, the activation of AMPA receptor is achieved by phosphorylation through CaMKII *(124,125).* In contrast, a parallel decrease in AMPA receptor phosphorylation occurs during LTD that depends on the activity of phosphatase, particularly calcineurin *(126,127).* The selective calcinuerin inhibitors FK506 and cyclosporin, and inhibitors of protein phosphatase 1 (PP1) and PP2A (okadaic acid or caliculin A, respectively), prevent LTD.

SLC8A3 Promoter Induction by BDNF Requires the CRE Site

BDNF is one of the main regulators of CREB-induced gene transcription whose pivotal functions in the development of sensory neurons and in the induction of LTP in hippocampal neurons have been examined previously. The BDNF-induced gene transcription is activated by at least two signaling pathways coupled to the TrkB receptor that lead to the phosphorylation of serine residues in the kinase-inducible domain of CREB: the Ca^{2+}-mediated pathway which activates CaMKs and that involving the activation of MAPK triggered by the GTP-binding protein Ras (Fig. 3) *(12,128).* The regulation of the *SLC8A3* promoter by BDNF has been studied during differentiation of the human neuroblastoma SH-SY5Y. This cell line can be fully differentiated into a homogenous neuronal-like population of sympathetic ganglion neurons by the sequential treatment with retinoic acid (RA) and BDNF *(129,130).* RA slowly increases the transcriptional activity, which reaches five-fold over basal level after four days. The kinetics of *SLC8A3* promoter activation by RA indicates

that the effect of RA is indirect. This suggestion is supported by the lack of RA response elements or of sites for binding NF-kB/Rel which is activated at early stages in differentiating SH-SY5Y cells *(131).* The stimulation with RA of a human neuronal teratocarcinoma cell line increases the levels of AP-2 that became maximal after 48–72 h, suggesting that the numerous AP2 sites in the *SLC8A3* promoter could enhance the basal expression of the *SLC8A3* gene in neurons.

The differentiation of SH-SY5Y cells by RA induces a variety of biochemical and morphological changes; in particular it induces the expression of the BDNF receptor TrkB in the plasma membrane *(129).* The stimulation with BDNF rapidly induces *SLC8A3* promoter activity in stably transfected cells *(40).* In transiently transfected cells the kinetics of induction of the reporter activity is similar (Fig. 4), reaching maximal levels 4–5 hours following exposure to BDNF. The induction of the *SLC8A3* promoter by BDNF in differenting SH-SY5Y cells is similar to the induction of the *c-fos* promoter by NGF in cultured embryonic chicken DRG neurons *(132).* The fast promoter induction is consistent with the regulation by CREB. The deletion of the CRE element in the *SLC8A3* promoter causes the loss of stimulation by BDNF (Fig. 4). The slow upregulation of the *SLC8A3* promoter observed after 4 h of exposure to BDNF could be determined by transcription factors of the IEG family, since consensus elements for the binding of AP1 and Egr-1 are present in the *SLC8A3* promoter.

The phopshorylation event in Ser-133 of CREB is a crucial step for the activation of transcriptional activity and for the consequent induction of IEGs. The phosphorylation of CREB by BDNF has been shown to occur in cultured neurons from rat visual cortex *(133).* In differentiating SH-SY5Y cells, BDNF stimulates the rapid and transient phosphorylation of CREB at Ser-133, with P-CREB being maximal after 1 h and returning to basal levels after 4 h *(40).* A similar time course of CREB phosphorylation was monitored in PC12 cells exposed to NGF. P-CREB peaked 30 min after

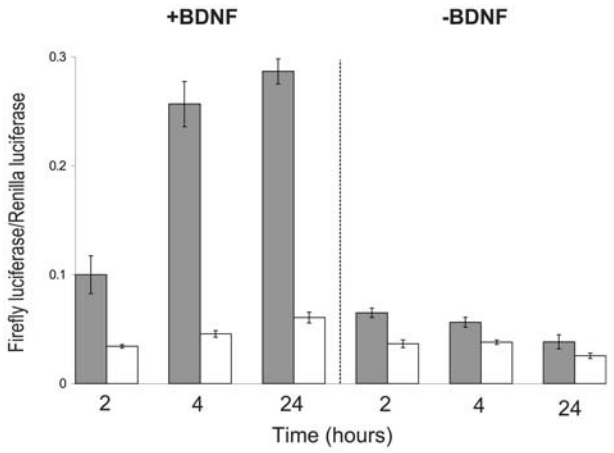

Fig. 4. The fast induction of the *SLC8A3* promoter by BDNF requires the CRE. Proliferating SH-SY5Y cells were transiently transfected with the *SLC8A3* promoter construct, including the 250 bp of the promoter region and 362 bp of the exon 1 sequence (Fig. 2) linked to the firefly luciferase gene (gray bars) or, alternatively with the promoter construct lacking the CRE site (open bars). Transfected cells were cultured for 4 d in the presence of retinoic acid (10 μ*M*), then washed and incubated without serum in the presence of BDNF (+BDNF; 50 ng/mL), or in the absence of BDNF (–BDNF). The luciferase activity was determined following the addition of BDNF at the times indicated. The activity of the firefly luciferase was normalized to that of *Renilla* luciferase expressed from pRLTK to correct transfection efficiency. Values are the mean (±SD) of two experiments carried out with triplicate samples.

the stimulation with NGF and correlated with the induction of ICER repressor produced from the CREM gene, which was maximal after 2 h *(134).* The quantification of NCX3 protein in SH-SY5Y cells by a specific antibody (made available by Dr. K.D. Philipson) indicated an increase after RA treatment and a further increase by the addition of BDNF that was maximal (about threefold) after 4 h, *(40).* The profile of NCX3 protein upregulation was consistent with that of the reporter assay, indicating that BDNF triggers the fast increase of NCX3 mRNA and protein, which is preceded by the transient phosphorylation of CREB.

Since the transcriptional activation was abolished by the elimination of the CRE site, it can be concluded that the induction of the *SLC8A3* promoter by BDNF is mediated by CREB. At present, NCX3 is the sole Ca^{2+} transporter known to be coordinately transcribed with IEGs and neuropeptides by CREB.

Control of cAMP and Ca^{2+} Signals on the SLC8A3 Promoter Activity

The regulation of transcription of the *SLC8A3* promoter by the elevation of cAMP and the effect of the partial depolarization of the plasma membrane with KCl to increase intracellular Ca^{2+} was investigated in proliferating and differentiated SH-SY5Y cells (Fig. 5). The *SLC8A3*-reporter activity was induced about twofold in proliferating and differentiated cells by the treatment with the membrane permeable cAMP derivative N^6, 2′-*O*-Dibutyryladenosine-3′:5′-cyclic monophosphate (Bt_2cAMP). In contrast, the induction by cAMP of the reporter construct lacking the CRE was completely abolished in both proliferating and differentiated SH-SY5Y cells (Fig. 5).

In proliferating cells, the Ca^{2+} increase induced by the partial depolarization of the plasma membrane with 30 m*M* KCl, downregulated transcription from the *SLC8A3* promoter by approx 40% in comparison with untreated control cells. The activity of the *SLC8A3* promoter lacking the CRE site was decreased by the Ca^{2+} elevation as the wild-type promoter, indicating that the CRE was not involved in the downregulation. Thus, the possibility that the downregulation of the basal transcription activity could be produced by CaMKII phosphorylation of CREB at Ser-142 may be ruled out. This conclusion is also supported by the failure of the CaMKII inhibitor KN-93 to reverse the inhibition. The negative effect of Ca^{2+} on basal *SLC8A3* transcription could depend on the downregulation of AP2 consequent to the activation of PLC and PKC by depolarization with KCl, shown to occur in SH-SY5Y cells *(135,136).*

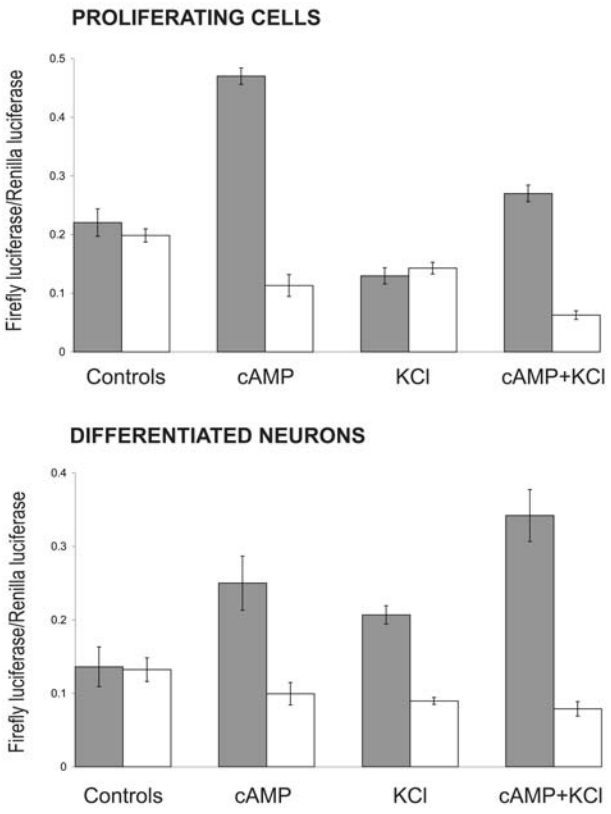

Fig. 5. Regulation of the *SLC8A3* promoter by cAMP and and Ca^{2+}. **(Proliferating cells.)** SH-SY5Y cells were transiently co-transfected with the wild-type *SLC8A3* promoter construct (gray bars) or with the *SLC8A3* promoter lacking the CRE site (open bars) in combination with pRLTK. The elevation of intracellular cAMP was induced by the membrane permeant derivative Bt_2cAMP (2 m*M*), whereas the partial depolarization of the plasma membrane was induced by increasing the KCl concentration in the medium to 30 m*M*. Both treatments were performed during the last 16 h of the second day after transfection. Samples of untreated cells were used as controls. **(Differentiated neurons)** SH-SY5Y cells, transiently transfected as described previously, were cultured for 4 d in the presence of retinoic acid (10 µ*M*) and thereafter with BDNF (50 ng/mL) for four additional days. Aliquots of cells were then treated for 16 h with Bt_2cAMP (2 m*M*), KCl (30 m*M*), or a combination of both. The values are the mean (±SD) of triplicate samples from 2–3 experiments.

The inhibitory effect of the Ca^{2+} elevation was also evident when cAMP and Ca^{2+} levels were raised simultaneously. At variance with the basal activity, the CaMKII inhibitor KN-93 reversed the downregulation of the cAMP-stimulated activity by Ca^{2+} *(40).* The possibility that CaMKII activity could be involved in the downregulation was supported by the evaluation of anti-phospho Ser-133 immunoreactivity following the cAMP and Ca^{2+} elevation. CREB was strongly phosphorylated following the cAMP increase, whereas the depolarizing treatment was ineffective. However, when cAMP and Ca^{2+} were elevated simultaneously, phospho-CREB was reduced, raising the possibility that phosphorylation of the Ser-142 by the CaMKII could decrease the affinity of the antibody to anti-phospho Ser-133. However, data were not conclusive because the possibility that KN-93 inhibitor could instead block Ca^{2+} entry through VGCC was not ruled out *(137,138).* Furthermore the cAMP-stimulated activity of the promoter lacking the CRE site was strongly downregulated by Ca^{2+} in proliferating cells (Fig. 5). In cells partially differentiated by RA, the negative response to the Ca^{2+} elevation was similar to that in proliferating cells, whereas the stimulation with BDNF for 1 d abolished the negative effect *(40).* In fully differentiated neurons exposed to BDNF for 4 d the *SLC8A3* promoter was clearly induced by Ca^{2+} (Fig. 5). The promoter induction by Ca^{2+} is mediated by transcription factors of the ATF/CREB family since it is completely abolished in the absence of the CRE/CaRE sequence (Fig. 5). The stimulation of the promoter activity may be mediated by CaMKII and CaMKIV phophorylation of CREB, since the CaMK activity could be upregulated during the differentiation promoted by BDNF of the neuroblastoma cell line.

A different phosphorylation pattern of ATF/CREB polypeptides in response to the cAMP elevation in differentiating neurons was also observed. The cAMP elevation induced during the first day of exposure to BDNF apparently causes the phosphorylation of transcription factor ATF1 instead of CREB *(40).* The phosphorylation of transcription factor ATF-1 following the cAMP elevation may be the consequence of the developmental change induced by BDNF. It is possible that specific isoforms of CaMKs could be upregulated in differentiated neurons. In vitro studies have indicated that CaMKII can phosphorylate Ser-63 of ATF-1, corresponding to Ser-133 of CREB, but is unable to phosphorylate Ser-72 of ATF-1, corresponding to the inhibitory Ser-142 of CREB. Therefore transcription factor ATF-1 can be only positively regulated by CaMKII, suggesting that transcriptional activation of cAMP-responsive genes by CaMKII may be mediated by ATF-1 *(139).* The Ca^{2+} elevation does not change the extent of anti-phospho Ser-133 immunoreactivity; however, it is possible that the activation of CaMKII and CaMKIV by BDNF induces the phosphorylation of CREB at Ser-142 and Ser-143 as well. This explanation arises from the findings that Ca^{2+} influx through L-VSCCs or glutamate treatment to activate the NMDA receptor induced the coordinate phosphorylation of Ser-133, Ser-142, and Ser-143 *(23).* The triple phosphorylation disrupts the interaction of the KIX domain of CREB with CBP, indicating that a transcription complex, different from that induced by cAMP, may trigger the specific program of gene expression modulated by the Ca^{2+} pathway. The use of selective inhibitors suggests that CaMKs may phosphorylate Ser-142, whereas the Ras-MAPK/Rsk pathway is probably not involved *(23).* The triple phosphorylation of CREB that is required for the activation of gene transcription by Ca^{2+} is developmentally regulated, since it is operative in embryonic cortical neurons (E18) cultured for 6–8 d but absent at earlier stages. In line with this findings, the upregulation of *SLC8A3* transcription by Ca^{2+} influx is greater in fully differentiated SH-SY5Y exposed to BDNF for 4 d than in cells exposed to BDNF for 1 d.

Ca^{2+} and cAMP Regulation via Transcriptional Repressor DREAM

DREAM was first identified as the transcriptional repressor of the human gene for the opioid peptide dynorphin, which is involved in memory formation and chronic pain adaptation. These processes cause profound changes in *c-fos* and prodynorphin gene expression in specific neurons. Dynorphin peptide controls the release of neurotransmitters, and in the hippocampus blocks LTP by inhibiting excitatory neurotransmission from synaptic terminals *(140,141).* The DNA sequence required for the transactivation of prodynorphin transcription by cAMP- and Ca^{2+} has been identified in the first exon of the gene (at position +40), and termed dynorphin downstream regulatory element (DRE) *(69).* The transcription factor binding to the DRE was identified subsequently and includes four Ca^{2+} binding domains of the EF-hand type; to date, it is the sole transcription factor known to be directly regulated by Ca^{2+}. When loaded with Ca^{2+}, DREAM undergoes a conformational change that causes its detachment from DRE sites (Fig. 2), thus relieving the transcriptional repression *(26,142).* The ability to bind DNA of some members of the basic helix-loop-helix (bHLH) transcription factor family is also regulated by Ca^{2+}, through the interaction with Ca^{2+}-loaded calmodulin *(143–145).* Recently it has been shown that the deduced amino acid sequence of DREAM is identical to that of calsenilin and KChIP3 proteins, although they function in different contexts. Calsenilin was identified by the yeast two-hybrid system to interact with the C-terminal portion of presenilin-2. Calsenilin is an essential component of the γ-secretase activity, and regulates the cleavage of C-terminal peptides of presenilin-1 and-2. Mutations in the presenilin genes have been linked to autosomal dominant familial Alzeimer's disease *(146–150).* KChIP3 is a member of a protein family that interacts with voltage-dependent K^+ channels (Kv4) also known to modulate the kinetic properties of A-type current in a Ca^{2+}-dependent manner *(151).* DREAM, calsenilin, and KChIP3 are the products of a unique gene *(152,153),* although the proteins are localized in different cellular compartments: nucleus, endoplasmic reticulum, or lysosomes and plasma membrane, respectively. Regulation of mRNA translation at alternative start codons may generate proteins with variable N-terminal peptides, which could be important for their localization and function. Furthermore, isoforms lacking the EF-hand domains are generated by alternative splicing in mouse *(153).* No alternatively spliced transcripts of the human calsenilin/DREAM/KChIP3 have been identified so far. The regional distribution of the protein and mRNA in rat brain indicates elevated expression in the hippocampus, especially in the molecular layer of the dentate gyrus, as well as in various thalamic nuclei and cerebellum, particularly in the granule layer of the cerebellar cortex. Calsenilin/DREAM/KChIP3 is strongly represented in axons, dendrites, and nerve terminals and its localization matches that of Kv4.2. Conspicuous expression of the protein was also observed in the key relay centers for sensory processing in brain and spinal cord associated to the pain pathways involving prodynophink receptor system, as well as to visual, auditory, and olfactory pathways, suggesting that Calsenilin/DREAM/KChIP3 could play a role in all kinds of sensory processing *(153–155).* The physiological role of calsenilin/DREAM/KChIP3 is not yet well-established and it is likely that much work will be required to clarify the multiple Ca^{2+}-dependent regulatory activity of the protein in the different cell compartments.

The involvement of DREAM in pain modulation has been shown in *dream* −/− mice which display reduced sensitivity to acute and chronic pain but behave normally in motor function, learning, and memory. The lack of DREAM elevates prodynorphin expression in the spinal cord, resulting in the activation of dynorphin selective (k)-opiate receptor. This indicates that DREAM constitutively suppresses prodynor-

phin expression in the spinal cord neurons, thus functioning as a modulator of pain gating *(156).* The mechanism of DREAM activation was studied in the neuroblastoma cell line (NB69). The release of Ca^{2+} from internal stores by caffeine treatment activated prodynorphin gene transcription. The transcriptional derepression was insensitive to the broad kinase inhibitor staurosporin but sensitive to agents that alter Ca^{2+} homeostasis, indicating that transcriptional derepression is promoted directly by the Ca^{2+} increase *(157).* However, PIP3 kinase was shown to mediate the accumulation of Hrk protein by causing detachment of DREAM from the 3′ untranslated region of hrk *gene* in hematopoietic progenitor cells. This mechanism leads to cellular apoptosis through the induction of death-related genes. The transcriptional silencing of the apoptotic *hrk* gene via DREAM is promoted by interleukin-3 (IL-3), *(158).* In mouse brain, DREAM mRNA is increased by pentylenetetrazol (PTZ) and kainic acid-induced seizures and is maximal 7 to 8 h postinjection. The upregulation is sensitive to MK801 inhibitor, suggesting that Ca^{2+} influx though the NMDA receptor is necessary for the induction of DREAM following seizures *(159).* By contrast, induction of seizures with kainic acid for 24 h reduced the expression of DREAM in mouse brain and in cultured hippocampal neurons. In addition, DREAM is significantly decreased in the hippocampus of epileptic patients *(160),* suggesting differences in the regulation of DREAM expression that depends on the duration of the epileptogenic stimulus.

The mechanism of derepression of the prodynorphin gene expression by the activation of PKA induced by forskolin has been studied in NB69 and SK-N-MC human neuroblastoma cells *(70).* The stimulation of the PKA pathway causes the detachment of DREAM from DRE sites independently of Ca^{2+} binding to the EF-hands domains. The dimerization of DREAM and αCREM through the interaction between their leucine-charged residue-rich domains (LCD) prevents the binding of DREAM to DRE sites. It occurs by interaction between two LCD domains of αCREM, located in the KID and bZIP domains, respectively, with two LCDs of DREAM. The phosphorylation of αCREM by PKA increases affinity for DREAM. It is possible that the dimerization of αCREM with DREAM may compete with the dimerization of αCREM with transcription factors of the CREB family. The specific dimerization of DREAM with the transcriptional repressor αCREM could relieve the repression on the CRE-dependent transcription by αCREM or εCREM isoforms. The LCD is also present in the KID of CREB; KID is essential for the interaction of CREB with CBP that activates transcription. It has been shown that CRE-dependent transcription may be affected by the dimerization of Ca^{2+}-DREAM with CREB. The dimerization impairs the recruitment of CBP thus blocking transcriptional activation by CREB *(112).* The ability of DREAM to bind CREB is Ca^{2+}-dependent but does not require phosphorylation of CREB. Although these findings suggest that increased Ca^{2+} signaling or PKA activation could result in transcriptional regulation at DRE and CRE sites, further investigation will help to clarify how cAMP and Ca^{2+} signals influence the choice of dimerization partner between the polypeptides of the CREB/ATF family and DREAM.

The regulation of *SLC8A3* gene transcription by DREAM has been investigated recently because the exon 1 sequence indicated several potential binding sites for DREAM (Fig. 2). In the neuroblastoma SH-SY5Y cells, the induction of transcription by cAMP and Ca^{2+} of the *SLC8A3* promoter is predominantly mediated by the CRE sequence (Fig. 5). In line with these results is the low amount of DREAM immunostaining in the nucleus of SH-SY5Y cells. Calsenilin/DREAM/KChIP3 immunoreactivity is much stronger in the internal stores and in the plasma membrane. However, the overexpression of recombinant DREAM downregulates the *SLC8A3* reporter activity, indicating that the DRE sites in the SLC8A3 exon 1 sequence could be targets of DREAM binding (Gabellini N., unpublished results).

Role of the NCX Proteins in Neurons

The primary structure of the NCX proteins shows a high degree of sequence conservation suggesting similar membrane topology. All of them contain ten transmembrane segments implicated in ion transport and a large hydrophilic loop with cytosolic location *(161).* The cytosolic region includes important domains for the catalytic modulation of the ion exchange activity, such as the site for inhibition by intracellular Na^+ and that for activation by intracellular Ca^{2+} *(162–164).* Tissue-specific alternative splicing modifies the C-terminal portions of the hydrophilic loop of the NCX1 and NCX3 *(165–169),* whereas no splice variant of the NCX2 has been reported. The spliced NCX1 isoforms expressed in oocytes exhibit significant variability in the kinetic features of ion transport and in their regulatory properties *(170).* The regions modified by splicing could mediate the modulation of the exchange activity by phosphorylation. The first evidences that ATP and analogs with a hydrolizable terminal phosphate enhance the Ca^{2+} efflux activity were obtained in squid axons *(171,172).* Subsequent studies revealed that PKC activation by growth factors in vascular smooth muscle cells increased the activity of the cardiac NCX1 isoform by phosphorylation of Ser residues in the intracellular loop of the protein *(173).* Recent evidence suggests that NCX1 may also be phosphorylated by PKA, and that phosphorylation can be accomplished by the association of NCX1 with the catalytic and regulatory subunits of PKA in a macromolecular complex that also includes PKC and the serine/threonine protein phosphatases PP1 and PP2A *(174,175).* The functional comparison of the three NCX proteins stably expressed in BHK cells show similar ion transport affinity. However, while the depletion of cellular ATP inhibits NCX1 and NCX2, it fails to inhibit NCX3. The three proteins have different putative phosphorylation sites and are possibly substrates of distinct protein kinases, although their phosphorylation pattern has yet not been clarified. The activity of NCX1 and NCX3 can be stimulated by PKA and PKC *(176).*

All three NCX isoforms are expressed in the brain and spinal cord, however, the use of specific antibodies has revealed marked cell-specific expression and different localization. In rat hippocampal cultures, NCX1 and NCX3 are both expressed in neurons. However, while NCX1 is markedly expressed in the majority of the cells, NCX3 immunoreactivity is restricted to a small population of neurons and the NCX2 isoform is almost exclusively found in glial cells *(177).* The use of specific antibodies against peptides of the NCX1, NCX2 *(178),* and of NCX3, indicates that the latter isoform is the most represented member of the NCX family in SH-SY5Y cells (Gabellini N., unpublished results). The differential expression of the *SLC8A1–3* genes has been documented in rat brain by *in situ* hybridization and immunohystochemistry. All three *SLC8A1–3* transcripts are highly expressed in the cerebral cortex and in several other brain areas, including the hippocampus, thalamus, particular layers of the motor cortex, several hypothalamic nuclei, and cerebellum. The *SLC8A2* mRNA is present in all hippocampal subregions, striatum, and paraventricular thalamic nucleus, whereas *SLC8A3* mRNA is found predominantly in the hippocampus, thalamus, amygdala, and cerebellum. All three gene transcripts are found in the spinal cord, however, *SLC8A2* and *SLC8A3* are the most abundant.

In the hippocampus, NCX3 immunoreactivity is particularly evident in the CA1 and CA3 subregions whereas NCX1 and 2 appear to be expressed predominantly in the mossy fiber projections to the CA3 region *(41,63).* These pathways are essential for the occurrence of LTP and LTD *(179).* The physiological function of NCX2 in vivo has been explored in NCX2 knockout mice. In hippocampal neurons lacking NCX2, the decrease of the Ca^{2+} elevation following depolarization was shown to be significantly delayed. The induction of LTP in the CA1 subregion of mutant mice was favored by a lowered frequency threshold; in accordance, mice lacking NCX2 displayed enhanced per-

formance in several memory and learning tests *(180).* This study shows that the Ca^{2+}-efflux activity of NCX2 is important in the regulation of Ca^{2+} homeostasis in hippocampal neurons involved in cognitive function. Although the regulation of transcription of the *SLC8A2* gene promoter remains to be investigated, it may be suggested that both NCX3 and NCX2 activity are critical in controlling the duration and the amplitude of the Ca^{2+} elevation that determines the differential induction of LTP or LTD. Thus, the induction of *SLC8A3* gene transcription by CREB-mediated signals and the subsequent upregulation of NCX3 activity may constitute a physiological means to regulate the balance of LTP versus LTD in hippocampal neurons.

In several thalamic nuclei, all three NCX isoforms are represented. Strong NCX2 immunoreacivity is found in neurons of the hypothalamic arcuate and mammillary nuclei *(41)* suggesting that the current activated by orexin and serotonin could be generated by NCX2 *(181,182).* In rat ventromedial hypothalamic nucleus where NCX2 is abundantly expressed, NCX activity produces an inward current that depolarizes neurons and increases their firing frequency following mGluR1 stimulation *(183).* In basal ganglia and the amygdala, the level of the NCX3 protein is lower than that of the two other isoforms; however, NCX3 is abundantly expressed in nucleus accumbens indicating its involvement in the control of motor coordination. The immunoreaction of the NCX proteins in the cerebellum is very intense; Purkinje cell layer showed dominant expression of NCX1 and NCX3.

The NCX proteins appear to be expressed at pre- and postsynaptic sites. NCX1 and NCX3 are clustered in the plasma membrane of hippocampal neurons at specific sites of contact with the endoplasmic reticulum and are separated from the sites of neurotransmiter release *(41,63).* The NCX proteins, revealed with a wide-ranging anti-NCX antibody, are localized in the neuronal somata and in presynaptic nerve terminals of chick ciliary ganglia and are confined to specific micro-domains of the plasma membrane corresponding to sites of junction with the endoplasmic reticulum *(184).* In agreement with these results, the immunostaining of differentiated SH-SY5Y cells with a specific antibody to NCX3 displayed strong immunoreaction in particular regions of the plasma membrane, probably corresponding to the junctions with endoplasmic reticulum, and in neurites especially in their terminals and in growth cones (Fig. 6). By contrast, the immunoreaction of PMCA stained the plasma membrane and presynaptic terminals uniformly, it was markedly clustered very close to the site of neurotransmitter release, or active zones *(30,184).* Following an action potential, the nerve terminal depolarizes through the opening of VGCC which is located in the active zone, and the increased local Ca^{2+} concentration triggers the vesicular release of neurotransmitters. The different subcellular localization of the two main systems for Ca^{2+} extrusion suggests that PMCA, which displays high affinity for Ca^{2+}, may lower Ca^{2+} concentration in the active zone, whereas NCX, with lower affinity for Ca^{2+}, may be involved in the extrusion of Ca^{2+} which has been temporarily sequestered in the endoplasmic reticulum by the SERCA pumps. This explanation is in line with recent findings showing that NCX is activated by the stimulation of receptors coupled to IP3 production and Ca^{2+} release from internal stores *(181–183).*

Concluding Remarks

The regulation of *SLC8A3* promoter by CREB-mediated signals suggests that *SLC8A3* gene transcription is coordinately regulated with that of other genes, including the CRE, in their promoters, as the IEGs and BDNF *(185).* The regulatory features of the gene, together with the strong expression of NCX3 in specific regions of the hippocampus suggest that the NCX3 activity could be crucial in establishing the Ca^{2+} balance that controls synaptic plasticity and cognitive functions. The association between NCX3 activity and synaptic plasticity

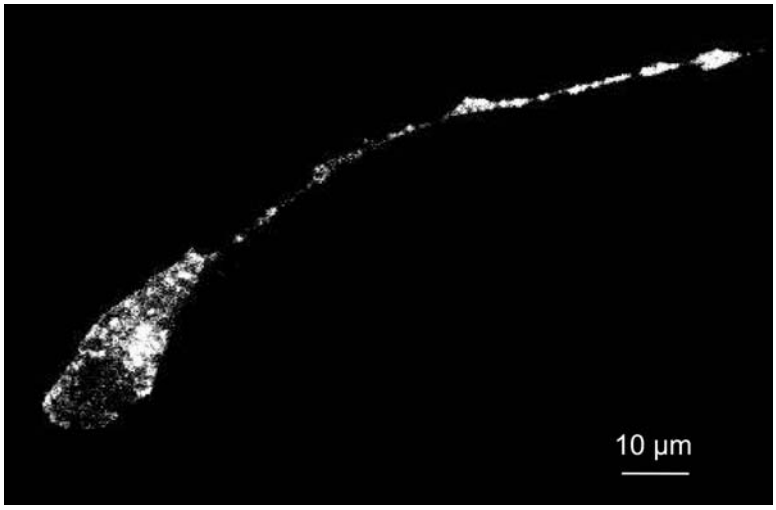

Fig. 6. Immunofluorescent NCX3 staining of differentiated SH-SY5Y cells. The localization of the NCX3 protein in differentiated SH-SY5Y cells exposed to BDNF for 4 d was analyzed by immunocytochemistry utilizing a specific anti-NCX3 antibody (kindly donated by Dr. K.D. Philipson). The confocal micrograph depicts the localization of the NCX3 protein in specific regions of the plasma membrane. Note immunolabeling in sites likely corresponding to the junctions of the plasma membrane with the endoplasmic reticulum, and the accumulation of the NCX3 protein in the growth cone.

is also suggested by the presence of the consensus element in the *SLC8A3* promoter for binding Egr-1 that becomes specifically activated by the NMDA signal. BDNF is crucial for the induction of LTP in hippocampus: the release of glutamate from Schaffer collaterals following repetitive activation of NMDA receptor causes the release of BDNF from postsynaptic CA1 pyramidal cells and induces LTP. BDNF stimulates the trkB receptor at both pre- and postsynaptic sites that facilitates synaptic function at CA1-CA3 regions. Since the NCX3 protein is strongly expressed in these regions, it may play an important role in the formation of LTP by modulating the intracellular Ca^{2+} concentration that triggers the release of BDNF. Furthermore, it may be important in the modulation of the response to the BDNF signal in postsynaptic cells.

As is the case of the *SLC8A3* promoter, transcription from the BDNF promoter (P1) is upregulated following Ca^{2+} increase via the CRE consensus sequence *(185).* Since the BDNF signal is coupled to gene transcription by CREB, BDNF upregulates it own synthesis via the CRE element, creating a positive feedback mechanism. The positive circuit created by Ca^{2+} and BDNF signals is underlined by the observation that high-frequency activation of glutamatergic synapses of hippocampal neurons trigger the release of BDNF from secretory granules by the activation of postsynaptic ionotropic glutamate receptors and resulting Ca^{2+} influx *(74).* The upregulation of transcription of *SLC8A3* gene appears to be coordinated with that of BDNF, since it is also induced by the BDNF signal and by the Ca^{2+} elevation via the CRE site. Neuronal Ca^{2+} signals reach the nucleus within seconds and trigger the activation of transcription factor CREB. Once Ca^{2+} concentration has returned to basal levels, transcription is rapidly inactivated *(186).* Since the Na^{+}/Ca^{2+} exchange activity is essential for the modulation of Ca^{2+} signals in neurons, the specific upregulation of NCX3 transcription by Ca^{2+} could produce an inhibitory feedback response by increasing NCX3 protein synthesis. In addition, elevated Ca^{2+} levels activate

PKC which can phosphorylate NCX3 thereby stimulating its Ca^{2+} efflux activity *(176)*. Furthermore, the inward current associated with NCX activity may be an important determinant of neuronal excitability *(181–183)*.

The parallel regulation of neuronal circuits involved in memory formation and in sensory processing has been indicated by the observation that BDNF also regulates the activity of pain synapses between primary sensory neurons and dorsal horn neurons *(185)*. In sensory neurons, NCX3 may modulate the Ca^{2+} transient that causes the glutamate, substance P, and BDNF release and induces LTP in postsynaptic dorsal horn neurons. The regulation of *SLC8A3* transcription by the Ca^{2+}-dependent transcriptional repressor DREAM, further suggests the functional implications of NCX3 protein in sensory pathways.

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