

# Calcineurin controls the expression of numerous genes in cerebellar granule cells



Available online at www.sciencedirect.com
SCIENCE DIRECT®
ACADEMIC PRESS
Molecular and Cellular Neuroscience 23 (2003) 325–330
MCN Molecular and Cellular Neuroscience
www.elsevier.com/locate/ymcne

# Calcineurin controls the expression of numerous genes in cerebellar granule cells

Dana Kramer,[a] Luigia Fresu,[b] Dominique S. Ashby,[a] Tom C. Freeman,[c] and Armando A. Genazzani[a,*]

[a] *Department of Pharmacology, Tennis Court Road, Cambridge, CB2 1PD, UK*
[b] *Department of Pharmacology, Gynaecology and Obstetrics, University of Sassari, Italy*
[c] *Microarray Group, MRC HGMP-RC, Wellcome Trust Genome Campus, Hinxton, CB10 1SB, UK*

Received 31 October 2002; revised 7 January 2003; accepted 20 January 2003

**Abstract**

The $Ca^{2+}$/calmodulin-dependent phosphatase calcineurin plays a crucial role in gene expression in different cell types such as T-lymphocytes, cardiac myocytes, and smooth muscle cells. A possible role for calcineurin in gene expression was recently found in neurons, where calcineurin regulates the expression of several genes involved in $Ca^{2+}$ homeostasis. To detect additional genes regulated in a calcineurin-dependent way in neurons we analysed gene expression profiles of cerebellar granule cells cultured in depolarising conditions in the presence or absence of the calcineurin inhibitory agents FK506 and CsA. Using oligonucleotide arrays we identified 34 genes that are differentially expressed between the samples and confirmed the calcineurin-dependent regulation of some of these genes by RT-PCR. Therefore, our results, which are likely not to be comprehensive, suggest that calcineurin plays a fundamental role in neuronal gene expression by either activating or repressing the expression of genes such as receptors, transcription factors, and signalling molecules.


## Introduction

$Ca^{2+}$-signalling plays a central role in neuronal functions and mediates important events such as neurotransmitter release, activity-dependent survival, and long-term potentiation (Berridge et al., 2000). Alongside the short-term events associated with $Ca^{2+}$ rises in neurons, e.g., modulation of the activity of $Ca^{2+}$-dependent enzymes, one of the important consequences arising from increases in cytosolic $Ca^{2+}$ levels is change in gene expression. This can occur via at least two mechanisms (Mellstrom and Naranjo, 2001b): (1) $Ca^{2+}$ can activate enzymes, such as kinases and phosphatases, and these in turn can modulate the activity of transcription factors; and (2) $Ca^{2+}$ can directly bind to transcriptional regulators and change their affinity for DNA. For example, in neurons the cAMP-response element binding protein (CREB) activity can be regulated by $Ca^{2+}$/calmodulin kinase II and IV (West et al., 2001), while the downstream regulatory element DREAM loses affinity for DNA when $Ca^{2+}$-bound (Mellstrom and Naranjo, 2001a). This in turn allows its repressor effect to be relieved on the target gene.

In T lymphocytes, rises in $Ca^{2+}$ activate the $Ca^{2+}$/calmodulin phosphatase calcineurin and this leads to subsequent dephosphorylation of the transcription factor NFAT (nuclear factor of activated T cells) in the cytoplasm (Rao et al., 1997). In turn, the nuclear import sequence on the NFAT protein is exposed and the transcription factor can reach the target DNA sequence. This pathway is the site of action of the immunosuppressants FK506 and cyclosporin A (CsA), which, by inhibiting calcineurin, block the gene expression-dependent activation of lymphocytes (Clipstone and Crabtree, 1992). Furthermore, other cellular systems, such as cardiac and smooth muscle myocytes, have been shown to utilise this pathway in development (Graef et al., 2001a, 2001b) and hypertrophy (Molkentin et al., 1998).

Although calcineurin is abundant in neurons, accounting for more than 1% of the total protein (Klee et al., 1998), its

* Corresponding author. Department of Pharmacology, Tennis Court Road, CB2 1PD, Cambridge, UK. Fax: +01223-334040.
*E-mail address:* aag25@cam.ac.uk (A. Genazzani).

1044-7431/03/$ – see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S1044-7431(03)00057-5

role in gene expression has not been investigated until recently, when the expression of certain isoforms of the inositol trisphosphate receptor ($IP_3R$), the plasma membrane $Ca^{2+}$-ATPases (PMCA), and the $Na^+/Ca^{2+}$ exchanger (NCX) in cultured neurons (Genazzani et al., 1999; Graef et al., 1999; Guerini et al., 2000; Li et al., 2000) have been shown to be regulated by this phosphatase. $IP_3R1$ has been shown to undergo upregulation upon calcineurin activation while PMCA4 and NCX2 undergo downregulation. Furthermore, the regulation of these genes differs in their time course, cycloheximide sensitivity, and sole requirement for calcineurin activation (Carafoli et al., 1999).

All the genes that have been found so far to be regulated by calcineurin in neurons appear to be involved in $Ca^{2+}$ homeostasis, thereby providing some kind of activity-dependent reorganisation of $Ca^{2+}$-signalling at the transcriptional level. In the present paper we have investigated whether genes involved in other functions might be controlled by calcineurin as well. To answer this question, we used cerebellar granule cells, where it is well-recognised that activity-dependent survival is dependent on $Ca^{2+}$ influx (Gallo et al., 1987; Franklin and Johnson, 1992), and have screened rat oligonucleotide arrays with RNA from cultured cells grown for 48 h in the presence or absence of the specific calcineurin antagonists FK506 or cyclosporin A. Of the approximately 8800 genes and ESTs present on the arrays, at least 34 were differentially expressed in our cultures. Our data therefore strongly suggest that calcineurin plays a central role in $Ca^{2+}$- and activity-dependent gene expression in neurons.

## Results and discussion

To investigate target genes for calcineurin-dependent pathways in cerebellar granule cells, we treated cells with 25 mM KCl (K25) in the presence or absence of 100 nM FK506 or 1 $\mu$M cyclosporin A for 48 h. This concentration of KCl induces a well-documented and long-lasting increase in resting $Ca^{2+}$ concentrations in cerebellar granule cells (Franklin and Johnson, 1992; Guerini et al., 1999). To increase the sensitivity of our assay and reduce false positives we performed two separate experiments: in the first, RNA extracted from cells grown in depolarising conditions (K25) in the presence or absence of FK506 was compared. In the second, cells were grown under depolarising conditions in the absence or presence of either FK506 or cyclosporin A. Two separate cell cultures were grown for each treatment and the RNA was pooled. To confirm the reliability of our experiments, aliquots of RNA were examined by gel electrophoresis or were used to perform RT-PCR on two genes known to be regulated by calcineurin in this model. In accord with previous reports (Genazzani et al., 1999; Guerini et al., 2000), $IP_3R1$ RNA was downregulated by FK506 or CsA treatment, while the NCX2 gene was upregulated (data not shown). Once the quality of the RNA was confirmed, it was used to produce biotin-labelled cRNA, which was fragmented and hybridised to rat Affymetrix oligonucleotide arrays. Results were computer-analysed with the algorithm provided in Microarray Suite 5.

Table 1
Genes downregulated by inhibition of calcineurin

| Fold change | Accession No. | Gene symbol | Gene name | PCR |
|---|---|---|---|---|
| 3.2 | M25890 | Sst, Srif | Somatostatin | √ |
| 2.9 | J05510 | $IP_3R1$ | $P_3$ receptor | √ |
| 2.7 | J04563 | Pde4 | cAMP phosphodiesterase | |
| 2.1 | M25350 | Pde4 | cAMP phosphodiesterase | √ |
| 1.5 | AA799729 | Pde4 | cAMP phosphodiesterase | |
| 2.5 | M58364 | Gch | GTP cyclohydrolase | |
| 2.1 | AA800908 | | Unknown | |
| 2.1 | U72620 | Lot1 | Lost on transformation 1 | √ |
| 1.9 | AA900750 | Lot1 | Lost on transformation 1 | |
| 1.7 | M59980 | Kcnd2 | Voltage-gated potassium channel | |
| 1.7 | AA875084 | | Unknown | |
| 1.3 | X86003 | Nor-2 | Neuron-derived orphan receptor | |
| 1.3 | AI230211 | Kcnd3 | Voltage-gated potassium channel | |
| 1.3 | X66845 | Dncic1 | Dynein | |
| 1.2 | X06769 | c-fos | c-fos | √ |
| 1.0 | X06769 | c-fos | c-fos | |
| 1.2 | AI030286 | Bdnf | Brain-derived neurotrophic factor | √ |
| 1.1 | M93669 | Scg2 | Secretogranin | |
| 1.0 | D37880 | Tyro3 | Receptor tyrosine kinase (Sky) | √ |

To increase the stringency of our results, we pooled together all the results and excluded all genes that were called absent in all samples, which left us with 4941 genes of the 8800 genes represented on the chip. We then compared each FK506 or CsA sample to its respective control (K25) and selected the genes that were consistently changed in all samples. Thus, we obtained 94 hits that corresponded to 86 genes, since some of the genes are replicated on the microarray. To produce a more reliable list, we then excluded genes whose hybridisation intensity was below 40 units and set an average threshold change of 1 as a limiting factor. This left us with 15 genes that are downregulated by calcineurin inhibition (Table 1) and 19 that were upregulated by FK506 and CsA treatment (Table 2).

To substantiate the results obtained with the oligonucleotide array, we randomly chose genes and analysed their expression by RT-PCR on different RNA samples. Neurons were cultured in either the presence or absence of depolarising concentrations of potassium to strengthen the claim that calcineurin is activated under depolarising conditions. Furthermore, FK506 and CsA were used to confirm the results of the array. The tyrosine kinase receptor Sky, the neurotrophic factor BDNF (brain-derived neurotrophic factor), and the neuropeptide transmitter somatostatin (SRIF) were expressed at higher levels in chronically depolarised (K25) cells, while cells that were not stimulated (K5) or were stimulated in the presence of calcineurin inhibitors displayed no expression or lower levels of expression, respectively (Fig. 1). Similar behaviours were observed with Lot-1 and the cAMP phosphodiesterase Pde4 genes (Lori,

Table 2
Genes upregulated by inhibition of calcineurin

| Fold change | Accession No. | Gene symbol | Gene name | PCR |
|---|---|---|---|---|
| 3.9 | M93273 | Sst2 | Somatostatin receptor | √ |
| 3.5 | AI145494 | Syn2 | Synapsin | |
| 3.0 | X62840 | Kcnc1 | Voltage-dependent potassium channel | |
| 2.9 | X55812 | Cnr1/Skr6 | Cannabinoid receptor | √ |
| 1.3 | U92564 | Roaz | Olf-1/EBF-associated zinc finger protein | √ |
| 2.7 | AA892511 | | Tescalcin | √ |
| 2.7 | U35099 | Cplx2 | Complexin | |
| 2.0 | X12589 | Kcna1 | Voltage-gated potassium channel | |
| 1.7 | M26161 | Kcna1 | Voltage-gated potassium channel | |
| 1.7 | X04139 | Prkcb1 | Protein kinase C | |
| 1.6 | K03486 | Prkcb1 | Protein kinase C | |
| 1.4 | U90261 | HRVF-1 | Hypertension-regulated vascular factor 1 | |
| 1.4 | AF013144 | Cpg21 | MAP kinase phosphatase | |
| 1.3 | M24852 | Pcp4 | Neuron-specific protein PEP-19 | |
| 1.2 | AB020504 | Pmf31 | PMF32 protein | |
| 1.2 | U16655 | Plcd4 | Phospholipase C | |
| 1.1 | M31725 | Tax, TAG-1 | Transient axonal glycoprotein 1 | √ |
| 1.0 | AF058795 | Gpr51 Gb2 | GABA-B R2 receptor | √ |
| 1.0 | AA866439 | | Unknown | |
| 1.0 | AA900505 | Arhb | RhoB | |
| 1.0 | M55291 | Ntrk2 | TrkB | √ |

Kramer, and Genazzani, unpublished). The transcription factor Roaz, the axonal glycoprotein TAG-1, and the cannabinoid receptor 1 displayed the opposite pattern, with RNA being higher in nonstimulated cells and in cells treated with calcineurin inhibitors (Fig. 1). A similar expression pattern was observed with somatostatin receptor 2 ($sst_2$), the $GABA_B$ receptor subunit 2 (Gb2), tescalcin, and neurotrophic factor receptor TrkB (Kramer, Sanna, Chowdhury, and Genazzani, unpublished). We therefore assume that Tables 1 and 2 are a true representation of genes that are regulated by calcineurin in cerebellar granule cells during development. The $IP_3$ receptor, the first protein to be described as regulated by calcineurin in neurons (Genazzani et al., 1999; Graef et al., 1999), is prominently represented in Table 1. Nonetheless, other proteins that have been subsequently shown to be regulated in this fashion, i.e., PMCA4 and NCX2, are absent (Carafoli et al., 1999). Reexamination of the data showed that the intensities yielded by these two genes were low and the genes excluded. This might not be surprising, due to the low levels of messenger and protein present at this stage of the culture (Carafoli et al., 1999). Nonetheless, from these omissions we can conclude that our list is not exhaustive and that other genes, including those present at lower levels, are also controlled by calcineurin. This is further supported by the fact that NGFI-B, a gene excluded from the final list since its fold change (0.6) was lower than the set threshold (1.0), was significantly changed in RT-PCR experiments (not shown).

Calcineurin has been shown to interfere with the activity of numerous plasma membrane ion channels via binding to FK binding proteins (Snyder et al., 1998), but this is unlikely to explain the effects described here. It has been previously shown that proteins that rely on $Ca^{2+}$ influx, but that are not controlled by calcineurin, are unaffected by FK506 or cyclosporin A treatment (Guerini et al., 1999, 2000; Carafoli et al., 1999; Li et al., 2000). Therefore, the ion channels responsible for $Ca^{2+}$-dependent gene expression are functional, independent of calcineurin inhibition, and the regulation we report here is downstream of channel activation.

Although it is possible that calcineurin controls RNA levels by regulating mRNA stability (Luo et al., 1999), it is more likely that calcineurin is involved in controlling the transcriptional machinery. Which transcription factors might be regulated in our model to initiate these events? It is well-established that the transactivating activity of the transcription factor MEF2 is controlled by calcineurin (Olson and Williams, 2000; Blaeser et al., 2000). RT-PCR with isoform-specific primers showed that MEF2A, MEF2C, and MEF2D (myocyte enhancer factor) are present in cerebellar granule cells at 3 DIV (data not shown), validating the hypothesis that this factor might be a possible regulator in our model. This result is in accord with the notion that MEF2 is responsible for calcium-dependent survival in cerebellar granule cells (Mao and Wiedmann, 1999). One of the known promoter targets for MEF2 appears to be NGFI-B (also known as Nur77 or Nr4a1; Blaeser et al., 2000) and it is suggestive that this gene is regulated in a calcineurin-dependent manner in our model. Interestingly, NGFI-B was also the only gene identified in a microarray screening of seizure-induced gene expression in the CA1

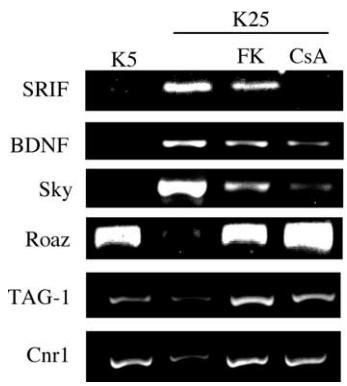


Fig. 1. RT-PCR analysis of calcineurin-regulated genes. mRNA levels were analysed by RT-PCR using gene-specific primers. Cells were grown for 48 h in the absence (K5) or presence (K25) of 25 mM KCl. Depolarised cells were additionally treated with the calcineurin inhibitors FK506 (FK; 100 nM) or cyclosporin A (CsA; 1 $\mu$M). SRIF, somatostatin; BDNF, brain-derived neurotrophic factor; TAG-1, transient axonal glycoprotein 1; Cnr1, cannabinoid receptor 1. All RNA samples were normalised against the housekeeping genes $G_3$PDH or actin.

region of the hippocampus (French et al., 2001). This opens the possibility that the $Ca^{2+}$ entry/calcineurin/MEF2 pathway is involved in neuropathological conditions as well as in development, paralleling the observation in the heart (Molkentin et al., 1998; Graef et al., 2001b).

Alongside MEF2, the most commonly cited transcription factor activated by calcineurin is NFAT (Rao et al., 1997). It has been recently shown that NFAT might be responsible for $IP_3$ receptor regulation in hippocampal neurons (Graef et al., 1999). In our model, RT-PCR revealed that all four isoforms of NFAT are detectable at 3 DIV and immunoblotting revealed that NFATc2, NFATc3, and NFATc4 are present at the protein level (not shown).

Last, it has been reported that the phosphorylation of CREB can be regulated indirectly by calcineurin (Bito et al., 1996). Somatostatin, one of the genes identified in our screening, has been reported to be under the control of CREB in numerous cell systems (Montminy and Bilezikjian, 1987). Nonetheless, depolarisation, in contrast to forskolin treatment, was unable to induce any visible phosphorylation of CREB in cerebellar granule cells (data not shown). This result is paralleled by previous unsuccessful attempts at inducing CREB phosphorylation by 25 mM KCl in cerebellar granule cells (Guerini et al., 2000).

Taken together, these data suggest that more than one $Ca^{2+}$/calcineurin-dependent pathway is potentially active in cerebellar granule cells grown under depolarising conditions. Similarly, it is possible that some of the identified genes are regulated by other calcineurin-dependent pathways, such as NF$\kappa$B or Elk-1 (Sugimoto et al., 1997; Pons and Torres-Aleman, 2000). Alongside these transactivating factors, it is likely that calcineurin also regulates transcriptional suppressors, since more than half of the genes we identified as differentially expressed were present only when calcineurin activity was inhibited.

It is likely that not all the genes identified are controlled by calcineurin directly. Since the time point which was chosen for this investigation was relatively late (48 h), calcineurin might regulate the expression of genes which will in turn then trigger a wider secondary transcriptional rearrangement. This claim is substantiated by the facts that several transcription factors (e.g., NGFI-B, c-fos, Roaz, and Lot1) appear to be under the control of calcineurin and that it has been reported that the downregulation of PMCA4CII is dependent on de novo protein synthesis (Guerini et al., 2000).

It is interesting to note that TAG-1, an axonal glycoprotein that has been used as a late neuronal marker to determine the developmental stage of cerebellar granule cells (Kuhar et al., 1993), is present at higher levels when calcineurin is blocked. In vivo, migrating immature cerebellar granule cells express TAG-1 during their migration through the molecular and Purkinje cell layer, with decreased expression seen for cells arrived in the internal granule cell layer. From this, we can infer that in vivo calcineurin activation occurs once cells have reached their final destination, the internal granule layer. This would be highly likely, since the depolarising conditions used in culture are thought to mimic synaptic connections made between migrated granule neurons and mossy fibers (Gallo et al., 1987). A further indication of the potential role of calcineurin in cerebellar granule cell physiology can be drawn from the changed expression of somatostatin and somatostatin receptors (Yacubova and Komuro, 2002), as well as BDNF and TrkB receptors (Borghesani et al., 2002). For example, it has been shown that somatostatin, released from glia, is involved in the migration pattern of these cells (Yacubova and Komuro, 2002). Therefore, upon connecting to mossy fibers and subsequent glutamate signalling, the granule cells, via calcineurin activation, could downregulate the somatostatin receptor, preventing the cell from responding to further somatostatin and therefore signalling arrival at the final destination in the cerebellum. The simultaneous upregulation of somatostatin itself might play a role in communication with further incoming neurons. It would therefore appear that calcineurin acts as a cellular switch that rearranges gene expression in neurons changing from migrating to mature.

It is interesting to note that a similar screening was recently conducted in T cells (Cristillo and Bierer, 2002). Although the conclusion by Cristillo and Bierer (2002) was similar to ours, i.e., calcineurin affects the expression of numerous genes at the transcriptional level, the genes identified as differentially expressed were different from those found in our neuronal model. This suggests that, alongside calcineurin activation, other tissue-specific factors, not necessarily $Ca^{2+}$-dependent, determine the genes affected by calcineurin activation or inhibition.

## Experimental methods

### *Cell culture*

Cerebellar granule cells were dissociated from the cerebella of 7-day-old Wistar rats as described (Gallo et al., 1987) and plated in DMEM Hepes modification (Invitrogen) 10% fetal calf serum (Sigma), 100 $\mu$g/ml gentamicin, 100 $\mu$g/ml pyruvate, on poly-L-lysine-treated plates at a density of approximately $3 \times 10^5$ cells per $cm^2$. After 24 h, 10 $\mu$M cytosine arabinofuranoside was added to the culture. All pharmacological treatments were done at this time in the culture medium.

### *RNA extraction and RT-PCR*

Total RNA was prepared from granule cells by using Trizol reagent according to the manufacturer's instructions (Invitrogen). Total RNA was either used to prepare labelled cRNA (see below) or was reverse-transcribed by using the Omniscript RT kit (Qiagen) and a poly (dT) primer (Amersham) according to the manufacturer's protocol.

The following nucleotides (written as 5′ to 3′) were used for PCR (Sigma Genosys): SRIFF: AGACTCCGTCAGTTTCT; R, GAAGAAGTTCTTGCAGCC; BDNFF: CCATGAAAGAAGCAAAGTC; R, GAAGTTGGTCTCGTTCATGGC; SkyF: TGGATGACCCTGACATACAC; R, TAAGGCTGTAGTTGGTGGC; RoazF: ACTCCCTCACTGGTTTCCGCTGTG; R, CGAAGGTCATCTGGCACTTGATGC; TAG-1F: AAGTACACGCTGCAAGCTCGTAC; R, GATTCATTTCGGAGAGGAACCAC; Cnr1F: ATCCTAGATGGCCTTGCAGAC; R, GTGTGGATGATGATGCTCTTC; MEF2F (all isoforms): TGATGAAGAAGGCTTATGARC; MEF2AR: CCTCTTCCTCCGAAAGTGG; MEF2BR: CTTGCACAGACTGCGG; MEF2CR: CGCCTGTGTTACCTGCAC; MEF2DR: GGAGTGACCAGAGAGCTAC. The NFAT primers are described in Stevenson et al. (2001).

### *Affymetrix oligonucleotide array*

To analyse the quality of total RNA, RT-PCR was performed (as described above) for selected genes prior to sample preparation for oligonucleotide arrays.

Total RNA was reverse-transcribed with the Superscript Choice system (Invitrogen) using a $T7(dT)_{24}$ primer, thus incorporating a T7 RNA polymerase promoter. Double-stranded cDNA was recovered and used to synthesise biotin-labelled cRNA using the BioArray high yield RNA transcript labelling kit (Enzo, Farmingdale, NY). Labelled cRNA was fragmented by incubation at 94°C for 35 min in fragmentation buffer and 15 $\mu$g of fragmented cRNA was hybridised for 16 h at 45°C to an RG-U34A array (Affymetrix, Santa Clara, CA). After hybridisation, a fluidics station was used to wash and stain the arrays with streptavidin–phycoerythrin. Finally, probe arrays were scanned and analysed using Affymetrix's Microarray Suite 5.0 and sorted by fold change.

### *Western blotting*

Cytosolic proteins were extracted from cells 24 h after treatment. Granule cells were washed once in phosphate-buffered saline and collected in a microfuge tube. Cells were then lysed in low-salt buffer (1.5 mM NaCl, 10 mM KCl, 0.5 mM DTT, 0.6% NP-40, 10 mM Hepes, pH 7.9) in the presence of a protease inhibitor cocktail. Cells were spun down for 60 s at 13,000 rpm and the cytosolic fraction was collected. Nuclear proteins were extracted by incubating the nuclei for 20 min at 4°C with intermittent vortexing in a high-salt solution (25% v/v glycerol, 1.5 mM $MgCl_2$, 1 mM DTT, 420 mM NaCl, 20 mM Hepes, pH 7.9) in the presence of a protease inhibitor cocktail. Nuclear membranes were centrifuged for 5 min at 13,000 rpm and the supernatant was collected. Proteins were separated by using 10% acrylamide gel (SDS–PAGE) and transferred to nitrocellulose membranes. Membranes were incubated with isoform-specific polyclonal antibodies against NFAT isoforms (Lyakh et al., 1997) kindly donated by Dr. Nancy Rice (Frederick, MD).

For CREB Western blots total protein was obtained after 3 h of treatment. Cells were harvested on ice in cold solubilisation buffer [50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 mM $ZnCl_2$, 100 $\mu$M $Na_3VO_4$, 200 nM okadaic acid, 10 mM p-NPP, 1x protease cocktail (Sigma), 0.2 mM PMSF, 0.5 mM benzamidine, 1 mM DTT, 20 mM $\beta$-glycerophosphate, 1% Triton] and centrifuged at 13,000 rpm for 15 min at 4°C. Supernatants were collected and total protein amounts quantified using the Bradford assay. A total of 100 $\mu$g of protein was separated in a 10% SDS–polyacrylamide gel, transferred onto PVDF membranes, and incubated with CREB and p-CREB-specific antibodies (NEB).

## Acknowledgments

We thank Laurie Scott, Richard Stephens, and Mirna Lnenicek-Allen for help with Affymetrix, Prof. Colin Taylor and Ms. Tasmina Goraya for help with the CREB phosphorylation experiments, and Dr. Nancy Rice for the isoform-specific NFAT antibodies.

## References

Berridge, M.J., Lipp, P., Bootman, M.D., 2000. The versatility and universality of calcium signalling. Nature Rev. Mol. Cell Biol. 1, 11–21.

Bito, H., Deisseroth, K., Tsien, R.W., 1996. CREB phosphorylation and dephosphorylation: a $Ca^{2+}$- and stimulus duration-dependent switch for hippocampal gene expression. Cell 87, 1203–1214.

Blaeser, F., Ho, N., Prywes, R., Chatila, T.A., 2000. $Ca^{2+}$-dependent gene expression mediated by MEF2 transcription factors. J. Biol. Chem. 275, 197–209.

Borghesani, P.R., Peyrin, J.M., Klein, R., Rubin, J., Carter, A.R., Schwartz, P.M., Luster, A., Corfas, G., Segal, R.A., 2002. BDNF stimulates migration of cerebellar granule cells. Development 129, 1435–1442.

Carafoli, E., Genazzani, A., Guerini, D., 1999. Calcium controls the transcription of its own transporters and channels in developing neurons. Biochem. Biophys. Res. Commun. 266, 624–632.

Clipstone, N.A., Crabtree, G.R., 1992. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. Nature 357, 695–697.

Cristillo, A.D., Bierer, B.E., 2002. Identification of novel targets of immunosuppressive agents by cDNA-based microarray analysis. J. Biol. Chem. 277, 4465–4476.

Franklin, J.L., Johnson Jr., E.M., 1992. Suppression of programmed neuronal death by sustained elevation of cytoplasmic calcium. Trends Neurosci. 15, 501–508.

French, P.J., O'Connor, V., Voss, K., Stean, T., Hunt, S.P., Bliss, T.V., 2001. Seizure-induced gene expression in area CA1 of the mouse hippocampus. Eur. J. Neurosci. 14, 2037–2041.

Gallo, V., Kingsbury, A., Balazs, R., Jorgensen, O.S., 1987. The role of depolarization in the survival and differentiation of cerebellar granule cells in culture. J. Neurosci. 7, 2203–2213.

Genazzani, A.A., Carafoli, E., Guerini, D., 1999. Calcineurin controls inositol 1,4,5-trisphosphate type 1 receptor expression in neurons. Proc. Natl. Acad. Sci. USA 96, 5797–5801.

Graef, I.A., Chen, F., Chen, L., Kuo, A., Crabtree, G.R., 2001a. Signals transduced by $Ca^{2+}$/calcineurin and NFATc3/c4 pattern the developing vasculature. Cell 105, 863–875.

Graef, I.A., Chen, F., Crabtree, G.R., 2001b. NFAT signaling in vertebrate development. Curr. Opin. Genet. Dev. 11, 505–512.

Graef, I.A., Mermelstein, P.G., Stankunas, K., Neilson, J.R., Deisseroth, K., Tsien, R.W., Crabtree, G.R., 1999. L-type calcium channels and GSK-3 regulate the activity of NF-ATc4 in hippocampal neurons. Nature 401, 703–708.

Guerini, D., Garcia-Martin, E., Gerber, A., Volbracht, C., Leist, M., Merino, C.G., Carafoli, E., 1999. The expression of plasma membrane $Ca^{2+}$ pump isoforms in cerebellar granule neurons is modulated by $Ca^{2+}$. J. Biol. Chem. 274, 1667–1676.

Guerini, D., Wang, X., Li, L., Genazzani, A., Carafoli, E., 2000. Calcineurin controls the expression of isoform 4CII of the plasma membrane $Ca^{2+}$ pumps in neurons. J. Biol. Chem. 275, 3706–3712.

Ho, S., Clipstone, N., Timmermann, L., Northrop, J., Graef, I., Fiorentino, D., Nourse, J., Crabtree, G.R., 1996. The mechanism of action of cyclosporin A and FK506. Clin. Immunol. Immunopathol. 80, S40–S45.

Klee, C.B., Ren, H., Wang, X., 1998. Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. J. Biol. Chem. 273, 13367–13370.

Kuhar, S.G., Feng, L., Vidan, S., Ross, M.E., Hatten, M.E., Heintz, N., 1993. Changing patterns of gene expression define four stages of cerebellar granule neuron differentiation. Development 117, 97–104.

Li, L., Guerini, D., Carafoli, E., 2000. Calcineurin controls the transcription of $Na^{+}/Ca^{2+}$ exchanger isoforms in developing cerebellar neurons. J. Biol. Chem. 275, 20903–20910.

Luo, Z.D., Wang, Y., Werlen, G., Camp, S., Chien, K.R., Taylor, P., 1999. Calcineurin enhances acetylcholinesterase mRNA stability during C2-C12 muscle cell differentiation. Mol. Pharmacol. 56, 886–894.

Lyakh, L., Ghosh, P., Rice, N.R., 1997. Expression of NFAT-family proteins in normal human T cells. Mol. Cell. Biol. 17, 2475–2484.

Mao, Z., Wiedmann, M., 1999. Calcineurin enhances MEF2 DNA binding activity in calcium-dependent survival of cerebellar granule neurons. J. Biol. Chem. 274, 31102–31107.

Mellstrom, B., Naranjo, J.R., 2001a. $Ca^{2+}$-dependent transcriptional repression and derepression: DREAM, a direct effector. Semin. Cell Dev. Biol. 12, 59–63.

Mellstrom, B., Naranjo, J.R., 2001b. Mechanisms of $Ca^{2+}$-dependent transcription. Curr. Opin. Neurobiol. 11, 312–319.

Molkentin, J.D., Lu, J.R., Antos, C.L., Markham, B., Richardson, J., Robbins, J., Grant, S.R., Olson, E.N., 1998. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 93, 215–228.

Montminy, M.R., Bilezikjian, L.M., 1987. Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature 328, 175–178.

Olson, E.N., Williams, R.S., 2000. Remodeling muscles with calcineurin. Bioessays 22, 510–519.

Pons, S., Torres-Aleman, I., 2000. Insulin-like growth factor-I stimulates dephosphorylation of IκB through the serine phosphatase calcineurin (protein phosphatase 2B). J. Biol. Chem. 275, 38620–38625.

Rao, A., Luo, C., Hogan, P.G., 1997. Transcription factors of the NFAT family: regulation and function. Annu. Rev. Immunol. 15, 707–747.

Snyder, S.H., Sabatini, D.M., Lai, M.M., Steiner, J.P., Hamilton, G.S., Suzdak, P.D., 1998. Neural actions of immunophilin ligands. Trends Pharmacol. Sci. 19, 21–26.

Stevenson, A.S., Gomez, M.F., Hill-Eubanks, D.C., Nelson, M.T., 2001. NFAT4 movements in native smooth muscle. J. Biol. Chem. 276, 15018–15024.

Sugimoto, T., Stewart, S., Guan, K.L., 1997. The calcium/calmodulin-dependent protein phosphatase calcineurin is the major Elk-1 phosphatase. J. Biol. Chem. 272, 29415–29418.

West, A.E., Chen, W.G., Dalva, M.B., Dolmetsch, R.E., Kornhauser, J.M., Shaywitz, A.J., Takasu, M.A., Tao, X., Greenberg, M.E., 2001. Calcium regulation of neuronal gene expression. Proc. Natl. Acad. Sci. USA 98, 11024–11031.

Yacubova, E., Komuro, H., 2002. Stage-specific control of neuronal migration by somatostatin. Nature 415, 77–81.