NMDA Receptor Activation in Differentiating Cerebellar Cell Cultures Regulates the Expression of a New POU Gene, *Cns-1*

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POU/homeobox genes encode transcription regulatory proteins that are important in defining cellular phenotypes. Expression of these genes may be critical for to the regulation of CNS cellular differentiation. We have identified a cDNA corresponding to a new member of the POU/homeobox gene family. Expression of RNA encoded by this new gene occurs predominantly in the CNS. Thus, this new gene was designated *Cns-1*. *Cns-1* transcripts are expressed in differentiating cells cultured from the early postnatal cerebellum. Treatment of these cultured cells with NMDA results in an increase in the level of *Cns-1* RNA. This increase is blocked by simultaneous treatment with the specific NMDA receptor antagonist amino-5-phosphonovaleric acid. Continued activation of the NMDA receptor allows maintenance of this new steady state level of *Cns-1* mRNA for at least 5 d in these cultured cells. A transcription runoff assay suggests that this increase in the level of RNA is due, at least in part, to an increase in transcription from the *Cns-1* gene. The NMDA-induced increase in *Cns-1* mRNA was reduced by pretreatment with calcium chelators EGTA or 1,2-bis(2-aminophenoxy)ethane-*N,N,N,N*-tetraacetic acid (BAPTA) tetrakis(acetoxymethyl). These studies suggest that specific activation of the NMDA receptor in cultures of differentiating cerebellar cells increases *Cns-1* gene expression and that calcium entry through the NMDA channel may be required for this response. This change in *Cns-1* expression may modify phenotypic characteristics of these cultured cells.

[Key words: NMDA receptor, CNS development, RNA, gene expression, POU gene, cerebellar cell culture]

The mammalian CNS contains a precisely integrated network of many distinct cellular phenotypes. During CNS development these diverse cell types arise from multipotent precursor cells present in the neural tube. Clonal analysis of CNS precursors suggests that, even up to their final cell division, an individual precursor can give rise to multiple cell types (Turner and Cepko, 1987; Luskin et al., 1988; Wetts and Fraser, 1988; Walsh and Cepko, 1992). It is likely that the specification of individual cell

fates requires local interactions involving environmental signals (Kessler, 1986; Adler and Hatlee, 1989; Iacovitti et al., 1989; McConnell and Kaznowski, 1991). These instructive signals direct the expression of specific genes that are ultimately responsible for defining particular cell fates. Homeobox genes may be important components in this process. These genes encode transcription regulatory proteins that are likely to function in combination with other transcription factors to specify individual CNS cellular phenotypes (He and Rosenfeld, 1991). Homeobox genes have been well characterized in invertebrates, where their protein products function as key regulators of transcription involved in controlling development and defining cellular phenotypes (Akam, 1987; Scott and Carroll, 1987; Doe et al., 1988a,b; Finney et al., 1988; Ingham, 1988; Way and Chalfie, 1989). In mammals, the expression of homeobox genes occurs in both CNS precursors and mature CNS cell populations. The *Hox* genes (Kessel and Gruss, 1990), several POU/homeobox genes (He et al., 1989), *Pax-3* (Goulding et al., 1991), *Tes-1* (Porteus et al., 1991), and *Nkx-2.2* (Price et al., 1992) are expressed in cells within spatially restricted regions of the developing neural tube. In the adult nervous system *Hox 1.3* and the POU/homeobox genes *Brn-1*, *Brn-2*, *Tst-1*, *Oct-1*, and *Oct-2* are expressed in distinct but overlapping populations of mature CNS cells (Odenwald et al., 1987; He et al., 1989). This distinct distribution of gene expression suggests that homeobox genes may also regulate the differentiation of individual mammalian CNS cellular phenotypes.

In the developing CNS instructive signals may define individual cellular phenotypes through control of the expression of particular homeobox genes. Cells cultured from the early postnatal cerebellum may provide a useful system for studying the effect of instructive signals on the differentiation of an individual CNS neuronal phenotype. These postnatal cerebellar cultures contain cerebellar granule neurons and their precursors, other cerebellar interneurons, and cerebellar glia (Trenkner, 1991). Some aspects of the development and differentiation of granule neurons occur in these postnatal cerebellar cultures and can be modified by the addition of extracellular factors (Balázs et al., 1988a; Hatten et al., 1988; Kingsbury et al., 1989; Messer, 1989; Gao et al., 1991; Trenkner, 1991). We are interested in learning whether conditions that modify differentiation of these cells also alter expression of identified homeobox genes. Several studies report that activation of NMDA receptors in cultured cerebellar granule neurons can modify neurite outgrowth (Pearce et al., 1987; Cambray-Deakin and Burgoyne, 1992), survival (Balázs et al., 1988a,b), differentiation (Moran and Patel, 1989; Cox et al., 1990), and migration (Komuro and Rakic, 1992) of these cells. NMDA receptor activation also appears to mediate trophic

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responses in other CNS neurons and may regulate activity-dependent development of certain populations of CNS neurons (Kleinschmidt et al., 1987; Lipton and Kater, 1989; Scherer and Udin, 1989; Cline and Constantine-Paton, 1990; Kalb and Hockfield, 1990; Cline, 1991; Simon et al., 1992). Studies have also shown that spontaneously active NMDA receptors are present in the early developing CNS (Blanton et al., 1990; Lo Turco et al., 1991; Kalb et al., 1992). One mechanism by which NMDA receptor activation could control these developmental events is through the regulation of expression of particular homeobox genes.

In the present study we have identified a new member of the POU/homeobox gene family, *Cns-1*. *Cns-1* is expressed in cells cultured from the early postnatal cerebellum. Activation of NMDA receptors in these cultured cells results in a specific increase in the level of *Cns-1* mRNA. An increase in the level of transcription from the *Cns-1* gene appears, at least in part, responsible for this increase in the level of *Cns-1* mRNA. These experiments suggest that NMDA receptor activation in developing cells of the CNS can alter homeobox gene expression. This change in homeobox gene expression may ultimately modify the phenotypic properties of these cells.

Materials and Methods

Identification of POU/homeobox sequences by PCR. Total RNA was prepared from adult mouse cerebella using previously described methods (Chomczynski and Sacchi, 1987). PolyA⁺ RNA was isolated by chromatography on oligo-dT cellulose as described by Sambrook et al. (1989). We synthesized the first strand of cDNAs using polyA⁺ RNA primed with random hexamers and Moloney murine leukemia virus reverse transcriptase (U.S. Biochemicals, Cleveland, OH). Amplification by the polymerase chain reaction (PCR) was accomplished by the addition of polymerase buffer (50 mM KCl, 20 mM Tris pH 8.4, 2.5 mM MgCl₂, and 100 μ g/ml BSA), dNTPs, primers, and Taq DNA polymerase (U.S. Biochemicals) to the cDNA. The cycling progression involved denaturation for 2 min at 94°C, primer annealing for 2 min at 58°C, and primer extension for 3 min at 72°C. We repeated this cycling progression 35 times with the last 72°C incubation lasting 15 min. The primers used for PCR were degenerate oligonucleotides representing all possible codons for two regions of conserved amino acid sequence in eight POU/homeodomain proteins (He et al., 1989). The upstream primer was from a conserved region in the POU-B domain, T/S-T-I-C/S-R-F-E, and the downstream primer was from a conserved region in the homeodomain, V-I/V-R-V-W-F-C-N (see Fig. 2). We had the upstream primer synthesized with a 5' EcoRI restriction site and the downstream primer with a 5' XbaI restriction site. Following PCR amplification DNA products were separated on a 1% agarose gel and stained with ethidium bromide. The DNA bands migrating to a position corresponding to 320–350 base pairs (bp) (expected size of POU/homeobox sequences based on the position of the primers) were purified as a single pool. We digested this DNA with EcoRI and XbaI, and ligated into the EcoRI-XbaI sites of the pGEM7Z vector. This DNA was used to transform *Escherichia coli*. Individual clones were isolated and the plasmid DNA purified for DNA sequence analysis (Sanger et al., 1977).

Isolation of *Cns-1* cDNAs. We synthesized a random-primed DNA probe from the PCR clone PCRPH3. This probe was used to screen an amplified adult C57Bl/6J mouse brain λ gt10 cDNA library (obtained from D. R. Burt, University of Maryland School of Medicine). We transferred 6×10^5 recombinants from this library to nitrocellulose filters by standard procedures (Sambrook et al., 1989). Filters were prehybridized for 2–4 hr at 42°C in H buffer containing $5.6 \times$ SSPE (0.84 M NaCl, 64 mM Na₂PO₄, 6 mM EDTA), 50% formamide, $5 \times$ Denhardt's solution (0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 1% SDS, and 200 μ g/ml tRNA. We performed hybridizations in H buffer containing $1-3 \times 10^6$ cpm/ml of PCRPH3 probe (specific activity of $1-2 \times 10^6$ cpm/ μ g) for 48 hr at 42°C. Filters were washed under high-stringency conditions [$0.1 \times$ SSC (15 mM NaCl, 1.5 mM sodium citrate), 1% SDS at 65°C] and exposed to Kodak RP x-ray film. We purified 10 positive clones and determined the cDNA insert size by restriction enzyme analysis. The longest clone (PH3,1-2)

contained an insert of ~4.6 kilobases (kb). The insert was subcloned into the plasmid pBluescript (Stratagene, La Jolla, CA) for restriction mapping and sequence analysis. We generated subclones for sequence analysis by restriction enzyme and exonuclease III digestion (Henikoff, 1984). We used double-stranded plasmid DNA for sequence analysis of both DNA strands (Sanger et al., 1977). The GenBank accession number for the *Cns-1* cDNA sequence is L13763.

Cell cultures from the early postnatal cerebellum. Cells from the cerebellum of postnatal day 7 (P7) CD-1 mice were dissociated into a single-cell suspension by previously described methods (Kingsbury et al., 1989); $1-2 \times 10^7$ cells were plated on 35 mm petri dishes in a chemically defined medium composed of minimal essential medium (without glutamic acid and glycine) containing 6 mg/ml glucose, 10 μ g/ml bovine serum albumin (Sigma, St. Louis, MO), 200 μ g/ml human transferrin (Sigma), 32 μ g/ml putrescine (Sigma), 10 ng/ml sodium selenite (Sigma), 10 μ g/ml insulin (Sigma), 1.25 ng/ml progesterone (Sigma), 20 ng/ml triiodothyronine (Sigma), 4 ng/ml corticosterone (Sigma), 2 mM glutamine (Sigma), and 100 μ g/ml gentamicin (GIBCO/Bethesda Research Labs, Gaithersburg, MD). This medium allows extended survival of cultured neurons without NMDA or depolarizing concentrations of KCl (Cox et al., 1990). The cultures were maintained for 24 hr at 37°C in 5% CO₂. Under these conditions the cells formed large aggregates with bundles of interconnecting cell processes similar to cerebellar cultures described previously (Trenkner, 1991). After 24 hr of culture we added KCl to each culture to bring the final concentration to 10 mM. Cultures were then treated with 10 μ M glycine (Sigma), 20 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Cambridge Research Biochemicals), 100 μ M amino-5-phosphonovaleric acid (APV; Sigma), 0.1–200 μ M *N*-methyl-D-aspartate (NMDA; Sigma), 10 μ M γ -aminobutyric acid (GABA; Sigma), 10 μ M somatostatin (Calbiochem, San Diego, CA), and/or basic fibroblast growth factor (Calbiochem). We added these compounds directly to the culture medium. Incubation of cultures was continued for 0–24 hr at 37°C in 5% CO₂. We treated some cultures containing 10 mM KCl and 10 μ M glycine for 2 hr with 4 or 8 mM MgSO₄, 1.5 or 2.5 mM ethylene glycol-bis(β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA; Sigma) or 20 μ M 1,2-bis(2-amino-phenoxylethane-*N,N,N',N'*-tetraacetic acid (BAPTA) tetrakis(acetoxymethyl) ester (Molecular Probes, Eugene, OR). We added NMDA (50 μ M) directly to the culture medium following pretreatment of cells with Mg²⁺ or EGTA. After BAPTA pretreatment we removed the BAPTA-containing medium from cells and replaced with the fresh chemically defined medium, minus BAPTA, containing 50 μ M NMDA. These cultures were then incubated for an additional 24 hr. The viability of cultured cells following these various treatment conditions was determined by trypan blue exclusion. Cells are loosely attached to the petri dish and can be removed and dissociated by trituration through a pipette tip. After dissociation, cell viability was determined by incubating cells in 0.08% trypan blue in PBS. Cells that excluded the dye and those that incorporated the dye were counted using a hemocytometer. Viability was expressed as the percentage of total cells that excluded the dye.

RNA blot analysis. We prepared total RNA from adult mouse tissues or from cultured cells using previously described methods (Chomczynski and Sacchi, 1987). We separated total RNA (20 μ g) from individual adult tissues or cultured cells by formaldehyde agarose gel electrophoresis (Sambrook et al., 1989). The gels were stained with ethidium bromide and the RNA visualized under UV light to confirm that similar amounts of RNA were loaded in each lane of the gel. We transferred the RNA to nylon membranes (Stratagene, La Jolla, CA) by standard capillary transfer (Sambrook et al., 1989). RNA was cross-linked to membranes using a UV cross-linker (Stratagene, La Jolla, CA). We hybridized RNA blots with a cDNA probe synthesized by random priming from a 3.7 kb EcoRI-HindIII fragment of PH3,1-2. RNA blots were prehybridized for 2–4 hr at 42°C in H buffer (as described above). Hybridization was accomplished in H buffer containing $1-3 \times 10^6$ cpm/ml of probe (specific activity of $1-2 \times 10^6$ cpm/ μ g) for 48–72 hr at 42°C. Blots were washed under high-stringency conditions ($0.1 \times$ SSC, 1% SDS at 65°C) and exposed to Kodak AR x-ray film for 1–3 d. After x-ray film exposure the probe was removed from blots by incubation in 25 mM Tris pH 7.5, $0.5 \times$ Denhardt's (0.01% ficoll, 0.01% polyvinylpyrrolidone, 0.01% BSA), 0.1% SDS for 30 min at 85°C. We hybridized the same blots under conditions described above with a probe synthesized from a 3 kb EcoRI fragment of an *En-2* cDNA obtained from A. L. Joyner, Mt. Sinai Hospital Research Institute, University of Toronto. Probes synthesized from other cDNAs were also used to rehybridize blots; an *Oct-1* cDNA was obtained from W. Herr, Cold Spring Harbor

1	C	ACT	ACA	GCT	CCA	GTT	ATT	ACC	AAC	ACC	ATT	CCC	AGC	ATG	CCC	GGG	ATC	AGC	Met	Pro	Gly	Ile	Ser
6	Ser	Gln	Ile	Leu	Thr	Asn	Ala	Gln	Gly	Gln	Val	Ile	Gly	Ala	Leu	Pro	Trp	Val					
53	AGT	CAG	ATC	CTC	ACG	AAT	GCT	CAG	GGA	CAG	GTT	ATT	GGA	GCA	CTT	CCG	TGG	GTA					
24	Val	Asn	Ser	Ala	Ser	Val	Ala	Thr	Pro	Ala	Pro	Ala	Gln	Ser	Leu	Gln	Val	Gln					
107	GTG	AAC	TCA	GCT	AGC	GTG	GCC	ACA	CCA	GCA	CCG	GCA	CAG	AGC	CTG	CAG	GTC	CAA					
42	Ala	Val	Thr	Pro	Gln	Leu	Leu	Leu	Asn	Ala	Gln	Gly	Gln	Val	Ile	Ala	Thr	Leu					
161	GCC	GTG	ACT	CCC	CAG	CTC	TTG	TTG	AAT	GCC	CAG	GGC	CAG	GTG	ATC	GCA	ACC	CTA					
60	Ala	Ser	Ser	Pro	Leu	Pro	Gln	Pro	Val	Ala	Val	Arg	Lys	Pro	Asn	Thr	Pro	Glu					
215	GCC	AGC	AGC	CCC	CTG	CCT	CAG	CCT	GTG	GCT	GTC	AGG	AAG	CCA	AAC	ACA	CCG	GAG					
78	Ser	Pro	Ala	Lys	Ser	Glu	Val	Gln	Pro	Ile	Gln	Pro	Thr	Gln	Ala	Val	Pro	Gln					
269	TCC	CCT	GCT	AAG	AGT	GAG	GTG	CAG	CCT	ATC	CAG	CCG	ACA	CAA	GCC	GTG	CCC	CAG	SER, THR, PRO				
																			rich domain				
96	Pro	Ala	Val	Ile	Leu	Thr	Ser	Pro	Thr	Pro	Ala	Leu	Lys	Pro	Ser	Ala	Ala	Thr					
323	CCT	GCA	GTA	ATC	CTC	ACC	AGC	CCA	ACG	CCA	GCG	CTC	AAG	CCG	TCA	GCT	GCA	ACT					
114	Pro	Ile	Pro	Ile	Thr	Cys	Ser	Glu	Thr	Pro	Thr	Val	Ser	Gln	Leu	Val	Ser	Lys					
377	CCC	ATC	CCA	ATC	ACC	TGC	TCA	GAG	ACC	CCA	ACC	GTC	AGT	CAG	TTG	GTA	TCA	AAG					
132	Pro	His	Thr	Pro	Ser	Leu	Asp	Glu	Asp	Gly	Ile	Asn	Leu	Glu	Glu	Ile	Arg	Glu					
431	CCG	CAC	ACC	CCA	AGT	CTG	GAT	GAG	GAC	GGG	ATC	AAC	TTA	GAA	GAG	ATC	CGG	GAG					
150	Phe	Ala	Lys	Asn	Phe	Lys	Ile	Arg	Arg	Leu	Ser	Leu	Gly	Leu	Thr	Gln	Thr	Gln					
485	TTT	GCT	AAG	AAT	TTT	AAG	ATC	CGG	CGG	CTC	TCC	CTG	GGT	CTG	ACA	CAG	ACC	CAG	POU-specific				
																			domain				
168	Val	Gly	Gln	Ala	Leu	Thr	Ala	Thr	Glu	Gly	Pro	Ala	Tyr	Ser	Gln	Ser	Ala	Ile					
539	GTG	GGC	CAG	GCT	TTG	ACG	GCG	ACA	GAA	GGG	CCG	GCC	TAC	AGC	CAA	TCA	GCC	ATC					
186	Cys	Arg	Phe	Glu	Lys	Leu	Asp	Ile	Thr	Pro	Lys	Ser	Ala	Gln	Lys	Leu	Lys	Pro					
593	TGC	AGG	TTT	GAG	AAA	TTG	GAC	ATC	ACA	CCC	AAG	AGC	GCC	CAG	AAG	CTG	AAG	CCG					
204	Val	Leu	Glu	Lys	Trp	Leu	Met	Glu	Ala	Glu	Leu	Arg	Asn	Gln	Glu	Gly	Gln	Gln					
647	GTT	TTG	GAA	AAG	TGG	TTG	ATG	GAG	GCA	GAG	CTC	CGC	AAC	CAG	GAA	GGC	CAG	CAG					
222	Asn	Leu	Met	Glu	Phe	Val	Gly	Gly	Glu	Pro	Ser	Lys	Lys	Arg	Lys	Arg	Arg	Thr					
701	AAC	CTG	ATG	GAG	TTC	GTG	GGC	GGC	GAG	CCC	TCC	AAG	AAA	CGC	AAG	CGG	CGC	ACT					
240	Ser	Phe	Thr	Pro	Gln	Ala	Ile	Glu	Ala	Leu	Asn	Ala	Tyr	Phe	Glu	Lys	Asn	Pro					
755	TCC	TTC	ACA	CCG	CAG	GCC	ATA	GAG	GCT	CTC	AAT	GCC	TAC	TTT	GAG	AAA	AAC	CCC	Homeodomain				
258	Leu	Pro	Thr	Gly	Gln	Glu	Ile	Thr	Glu	Ile	Ala	Lys	Glu	Leu	Asn	Tyr	Asp	Arg					
809	CTG	CCC	ACC	GGC	CAG	GAG	ATC	ACG	GAG	ATC	GCC	AAG	GAG	CTC	AAC	TAC	GAC	CGG					
276	Glu	Val	Val	Arg	Val	Trp	Phe	Cys	Asn	Arg	Arg	Gln	Thr	Leu	Lys	Asn	Thr	Ser					
863	GAG	GTG	GTG	AGG	GTC	TGG	TTC	TGT	AAT	CGA	CGC	CAG	ACA	CTG	AAG	AAC	ACC	AGC					
294	Lys	Leu	Asn	Val	Phe	Gln	Ile	Pro															
917	AAG	CTG	AAC	GTC	TTT	CAG	ATC	CCG	TAG	GGC	TCA	GTG	TCA	GCG	TGT	GCC	GGC	GCA					

Figure 1. Part of the nucleotide sequence of the *Cns-1* cDNA is presented along with the predicted amino acid sequence of the Cns-1 protein. The open reading frame begins with an ATG that is consistent with Kozak's criteria for an initiation codon (Kozak, 1986, 1991). The predicted amino acid sequence of Cns-1 contains a POU-specific domain, a homeodomain, and a domain rich in serine, threonine, and proline residues. These domains are shown by the boxed sequences. The GenBank accession number for the *Cns-1* cDNA sequence is L13763.

Laboratory; *SCIP/Tst-1* and *Cer-1* cDNAs were isolated in our laboratory (R. F. Bulleit, unpublished observations). Repeated stripping of probes from blots reduced subsequent hybridization signals so that film exposure times frequently increased and varied from 2 to 12 d. Each experiment using cultured cells was repeated at least two times and the results of RNA blot analysis were consistently replicated in repeated experiments.

Nuclear runoff transcription assay. We measured transcriptional activity by isolating nuclei from cells ($\sim 3 \times 10^7$ cells) cultured in the presence or absence of 50 μM NMDA or 100 μM APV. Cells were lysed in 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl_2 , 5% NP40, and

nuclei were prepared as previously described (Greenberg and Ziff, 1984). *In vitro* nuclear transcription was carried out in the presence of ribonucleotides and ^{32}P -UTP at 30°C for 30 min (Greenberg and Ziff, 1984). We isolated labeled RNA by methods described previously (Groudine et al., 1981). Labeled transcripts produced in this manner were hybridized in $6\times$ SSC, $5\times$ Denhardt's, 1% SDS, 200 $\mu\text{g}/\text{ml}$ tRNA at 65°C for 36–48 hr to DNA immobilized on nitrocellulose filters. We hybridized each filter with an equal number of cpm and washed filters under stringent conditions ($0.1\times$ SSC, 1% SDS at 65°C). The filters were exposed to Kodak AR x-ray film for 5 d. Nitrocellulose filters were prepared using plasmid DNA (10 μg) linearized by restriction digestion and de-

POU-SPECIFIC DOMAIN			
	POU _g -A	POU _g -B	LINKER
I	oct-1 EELEQFAKTFKQRRIKLGFTQGDVGLAMGKLYGND...SQTISRFEALNLSFKNMCKLPLLEKWLNDAE		NLSSDSSLSPSALNSPGIEGLS.
	oct-2 -----R-----		TMSVDSLSPNQLSSPSLGFEPA
II	pit-1 R-----NE--V-----Y--TN--E-LAAVH-SE-...-----C--N-Q-----A---AI-S---EE--		QVGALYNEKVGANE.....
III	cfla DD--A--Q-----A---L-T---V-...-----C---Q-----Q---EE-D		SSTGSPTSIDKIAAQ.....
	brn-1 DD-----Q-----A---L-T---V-...-----C---Q-----Q---EE-D		STTGSPTSIDKIAAQ.....
	brn-2 DD-----Q-----A---L-T---V-...-----C---Q-----Q---EE-D		SSSGSPTSIDKIAAQ.....
	brn-4 DD-----Q-----A---L-T---V-...-----C---Q-----N---EE-D		SSTGSPTSIDKIAAQ.....
	tst-1 DD-----Q-----A---L-T---V-...-----C---Q-----Q---EETD		SSSGSPTNLDKIAAQ.....
	ceh-6 DD--G--Q-----Y--A---L-T---I-...-----C---Q-----Q---EE-D		STTGSPTNSTFEKMTGQA.....
IV	unc-86 RQ--T--EH-----V--A--K-LAH-KMPGVGSL--S--C--S-T--HN--VA---I-HS--EK--		EAMKQKDTIGDINGILPNT....
	brn-3 R---A--ER-----V--A--S-LAN-KIPGVGSL--S--C--S-T--HN--IA---I-QA--EE--		GPQREKMNKPELFNG.....
	i-pou R---A--ER-----V--A--K-LAN-KLPGVGAV--S--C--S-T--HN--IA---I-QA--EE--		AQAKNKRDPDAPSVLPA.....
V	oct-3/4 K-----LL--K--T--Y--A---TL-V-F-KV-...-----C---Q--L-----R-----VEE-D		NNENLQEICKSETLVQA.....
VI	cns-1 --IRE--N--I--LS--L--TQ--Q-LTATE-PAY...--SA-C--K-DITP-SAQ---V-----ME--		LRNQEQQQLMEFVGGEPS.....
	----- Helix A -----	----- Helix B -----	
HOMEODOMAIN			
I	oct-1 .RRRKRTSIETNIRVALEKSFLENQKPTSEEITMIADQLNMEKEVIRVWFCNRRQKEKR		
	oct-2 G-----V-F-----A-----LL--E--H-----		
II	pit-1 .-K--R--T-SIAAKD---RH-G-HS--S-Q--MRM-EE--L---V-----R---		
III	cfla G-K-----VSVKG---QH-HKQP--SAQ---SL--S-QL---V-----		
	brn-1 G-K-----VSVKG---SH--KCP--S-Q---NL--S-QL---V-----		
	brn-2 G-K-----VSVKG---SH--KCP--SAQ---SL--S-QL---V-----		
	brn-4 G-K-----VSVKGV--TH--KCP--AAQ---SSL--S-QL---V-----		
	tst-1 G-K-----VGVKG---SH--KCP--SAH---GL--S-QL---V-----		
	ceh-6 G-K-----V-VKSR--FH-QS---NAQ---QV-ME-QL---V-----		
IV	unc-86 .DKKR-----AAPEKRE--QF-KQQR-SG-R-AS---R-DLK-N-V-----Q---Q--		
	brn-3 GEKKR-----AAPEKRS--AY-AVQPR-S--K-AA--EK-DLK-N-V-----Q---Q--		
	i-pou GEKK.AAPEKRS--AY-AVQPR-SG-K-AA--EK-DLK-N-V-----Q---Q--		
V	oct-3/4 ..-KR-----NRV-WS--TM--KCP--SLQQ--H--N--GL--D-V-----G---		
VI	cns-1 .KK--R---FTPOAIE--NAY-EK-PL--GQ---E--KE--YDR--V-----SL-N		
	----- Helix 1 -----	----- Helix 2 -----	----- Helix 3 -----

Figure 2. The predicted amino acid sequence of Cns-1 is similar to other POU/homeodomain proteins in the POU-specific domain and the homeodomain. All amino acid sequence information except that for Brn-4 and Cns-1 was as reported by Rosenfeld (1991). The amino acid sequence for Brn-4 was as reported by Le Moine and Young (1992). The amino acid sequence for Cns-1 is as reported in this publication. The sequences are grouped into six classes based on relative sequence similarity. A dash indicates identity of amino acid sequence with Oct-1. The solid black bars identify the position of the sequences used in designing PCR primers. Regions with α -helical structure are indicated as *Helix A* and *Helix B* in the POU-specific domain and *Helix 1*, *2*, and *3* in the homeodomain.

natured in 0.2 M NaOH for 30 min at room temperature followed by neutralization in 10 vol of 6 $\times$ SSC. We immobilized the DNA on nitrocellulose filters using a slot blot apparatus (Bio-Rad, Richmond, CA). Filters were baked for 2 hr at 80°C. The plasmid DNAs used in these experiments included a plasmid containing a 3.7 kb fragment of the *Cns-1* cDNA, a plasmid containing a 3 kb fragment of the *En-2* cDNA, and a control plasmid, pGEM11Z.

Densitometry. Densitometry (Molecular Dynamics densitometer, Sunnyvale, CA) of exposed x-ray films was used to quantitate the hybridization signals on RNA blots hybridized with *Cns-1* and *En-2* probes and in nuclear runoff transcription assays. Densitometric measurements (optical density, OD) were made on autoradiographs of RNA blots from three separate experiments in which cells were treated with glutamate receptor agonists or antagonists (see Fig. 4) or from three separate nuclear runoff transcription experiments (see Fig. 9). Background OD values were determined on each blot and subtracted from OD values for *Cns-1* and *En-2* hybridization signals. We recorded the mean OD values $\pm$ standard error of the mean for both *Cns-1* and *En-2* hybridization signals in RNA from cells treated under different conditions. Statistical analysis of the difference between the mean OD values for different treatment conditions was accomplished using a *t* test. We observed that

the level of the hybridization signal for *En-2* RNA didn't significantly change with any of the treatment conditions and was directly proportional to the level of 28S and 18S RNAs as visualized by ethidium staining of the RNA gels. Thus, *En-2* can provide a reasonable measure of the relative amount of RNA on each blot.

Results

Isolation of *Cns-1* cDNA clones

Reverse transcription of cerebellar RNA in combination with the PCR allowed identification POU/homeobox genes expressed in the cerebellum. PCR was accomplished using degenerate oligonucleotide primers representing all possible codons for two conserved regions (Fig. 2). PCR amplification yielded DNA fragments within the expected size range based on the position of the two primers. These PCR products were subcloned and individual clones were isolated and sequenced. Several different POU/homeobox cDNA clones were identified using this PCR protocol. The deduced amino acid sequence of one of these

Figure 3. *Cns-1* mRNA is expressed in the adult CNS. RNA was isolated from different adult brain regions and different adult tissues. Total RNA (20 μ g) from each of the indicated brain regions or tissues was separated in a 1% formaldehyde agarose gel and transferred to a nylon membrane. The membrane was incubated with a random-primed DNA probe synthesized from a 3.7 kb fragment of the *Cns-1* cDNA clone PH3,1-2. Probe hybridization and washing was accomplished as described in Materials and Methods. Following wash procedures, blots were exposed to Kodak AR x-ray film. Indicated on the left side are the positions of the 28S and 18S rRNAs.

clones (PCRPH3) suggested it encoded a new member of the POU/homeodomain protein family. We used a probe synthesized from the PCRPH3 clone to isolate full-length cDNAs from a mouse brain λ gt10 cDNA library. We isolated 10 clones that contained overlapping restriction maps. The largest clone (PH3,1-2) contained an insert of $\sim$ 4.6 kb. Sequence analysis of this clone revealed an open reading frame beginning with an ATG that is consistent with Kozak's criteria for an initiation codon (Kozak, 1986, 1991). The open reading frame is 903 base pairs long and encodes a 301 amino acid protein with a molecular weight of 32,850 (Fig. 1). This amino acid sequence contains regions that are similar but not identical to the POU-specific domain and the homeodomain of other POU/homeodomain proteins (Figs. 1, 2). We have designated this new sequence *Cns-1*.

An amino acid sequence comparison of the POU-specific domain and the homeodomain of *Cns-1* and 13 other POU/homeodomain proteins is presented in Figure 2. *Cns-1* is 48–57% identical to the other POU/homeodomain proteins within the POU-specific domain and 43–52% identical within the homeodomain. The greatest similarity was observed between *Cns-1* and Pit-1, where there was 57% identity in the POU-specific domain and 52% identity in the homeodomain. *Cns-1* and Pit-1 also have similar secondary structural features. Prediction of secondary structure in *Cns-1* (Chou and Fasman, 1978) suggests that within the POU-specific domain and the homeodomain there are five α -helical regions. In the POU-specific domain one helical domain resides in each of the POU subdomains (POU-A and POU-B) and three helical domains are present in the homeodomain (Fig. 2). The position of these helical domains is similar to the position of predicted helices in Pit-1 (Ingraham et al., 1990). We also observed that within the amino-terminal

half of *Cns-1* is a region rich in serine, threonine, and proline residues (Fig. 1). This domain consists of 25% serine and threonine residues and 21% proline residues. Similar regions rich in serine and threonine residues are also present in the POU/homeodomain proteins Pit-1 and Oct-2 (Ingraham et al., 1990; Tanaka and Herr, 1990).

Cns-1 is expressed in the CNS

We examined the tissue distribution of *Cns-1* mRNA. Total RNA was isolated from different adult brain regions and other tissues. We did RNA blot analysis under high-stringency conditions using a DNA probe synthesized from the *Cns-1* cDNA clone PH3,1-2. Under these conditions the probe hybridized to an $\sim$ 5 kb transcript (Fig. 3). This transcript was present in all regions of the CNS examined (forebrain, midbrain, hindbrain, cerebellum, and spinal cord). We also observed hybridization to RNA of the same size in RNA isolated from kidney tissue. We detected only faint hybridization signals in the other tissues examined (heart, lung, liver, spleen, and intestine). Thus, *Cns-1* mRNA is expressed predominantly although not exclusively in the CNS. We have also observed that *Cns-1* RNA is expressed in the developing postnatal cerebellum (P1–P21; data not shown). A comprehensive evaluation of the developmental expression of *Cns-1* in the CNS is currently under way.

Expression of Cns-1 is modulated by activation of NMDA receptors in cells cultured from the early postnatal cerebellum

Cultured established from the early postnatal cerebellum contain cerebellar granule cells and their precursors, other cerebellar interneurons, and cerebellar glia (Trenkner, 1991). Cells were cultured at high density in a chemically defined medium that allows extended survival of these cells without depolarizing concentrations of KCl (Cox et al., 1990). We cultured cells for 24 hr before the addition of glutamate receptor agonists and/or antagonists. RNA blot analysis shows that *Cns-1* mRNA is expressed in cultures under all treatment conditions (Fig. 4A). The *Cns-1* probe hybridized to two different-sized transcripts (Fig. 4A). The smaller of the two RNAs was the same size as the prominent *Cns-1* transcript observed in adult tissues (Fig. 3). This larger RNA was also observed in adult tissues if blots were exposed for longer periods of time (data not shown). Since all of the RNA blots were washed at high stringency, this larger RNA must be a transcript from a very closely related gene, a splicing variant of the *Cns-1* transcript or derived from the use of an alternate polyadenylation signal. An RNase protection experiment suggested that RNA from cultured cells could fully protect a 300 bp RNA fragment synthesized from the *Cns-1* cDNA. We observed no other partially protected fragments (data not shown). This observation suggests that the observed transcripts on RNA blots are likely to be derived from the *Cns-1* gene. However, further studies are necessary to determine which of several possibilities might account for the larger transcript.

The level of these *Cns-1* mRNAs changed in response to treatment with NMDA receptor agonists and antagonists. Treatment of cultures with glycine (10 μ M), which potentiates agonist response at the NMDA receptor (Johnson and Ascher, 1987), or 20 μ M CNQX, a non-NMDA glutamate receptor antagonist, did not result in a significant difference in the level of *Cns-1* RNA when compared to control cultures (Fig. 4A, lanes 1–3). Treatment with 100 μ M APV, a competitive antagonist at the NMDA receptor, appeared to reduced the level of *Cns-1* RNA when compared to treatment with 10 mM KCl (Fig. 4A, lane

Figure 4. NMDA receptor activation increases the expression of *Cns-1* mRNA in cultured cerebellar cells. Cells from the early postnatal cerebellum were cultured for 24 hr in a chemically defined medium containing 5 mM KCl. Cultures were continued for an additional 24 hr in medium containing (+) 10 mM potassium chloride (KCl), 10 μ M glycine (Gly), 20 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 100 μ M amino-5-phosphonovaleric acid (APV), and/or 50 μ M *N*-methyl-D-as-

partate (NMDA). However, this difference was not statistically significant when averaging the results from three separate experiments (Fig. 4A). Alternatively, treatment of cultures with 50 μ M NMDA resulted in a significant increase in the level of *Cns-1* mRNA (Fig. 4A, lane 5). Simultaneous addition of APV to cells treated with NMDA blocked the NMDA induced increase in the level of *Cns-1* mRNA (Fig. 4A, lane 7). However, CNQX did not affect this increase (Fig. 4A, lane 6). A comparison of the mean OD measurements from blots of three separate experiments showed that treatment of cells with NMDA or NMDA plus CNQX resulted in a 2.8–3.3-fold increase in the level of *Cns-1* mRNA when compared to APV-treated cultures and a 2.2–2.6-fold increase when compared to control cultures. These differences were statistically significant at p values < 0.03 . We observed a similar increase in the level of the larger *Cns-1* transcript following NMDA treatment. Treatment of these cultures with 20 μ M glutamate also resulted in an increase in the level of *Cns-1* mRNA that was blocked by cotreatment with APV (data not shown). We remove the *Cns-1* probe from the blots and rehybridized the blots with a probe to the homeobox gene *En-2* to determine if the levels of other homeobox gene transcripts were also increase in response to NMDA treatment. Densitometric measurements of the *En-2* hybridization signals showed that there was no significant change in the level of *En-2* RNA following treatment with any of NMDA receptor agonists or antagonists (Fig. 4B). Thus, NMDA treatment does not result in a general increase in the level of all homeobox gene transcripts. We also observed that the changes in the level of *En-2* RNA were consistent with changes in the level of RNAs as measured by absorbance at 260 nm and visualization by ethidium staining of the RNA gels (Fig. 4C). Thus, the *En-2* hybridization signal provides a measure of the relative amount of RNA in each lane of the blot and suggests that the increase in *Cns-1* mRNA is not the result of differences in the level of total RNA on the blots. Examination of RNAs encoded by several other homeobox genes suggested that the levels of mRNA for these genes were, for the most part, not altered by treatment with NMDA (Fig. 5). RNAs encoding *En-2*, the POU/homeobox gene *Oct-1*, and a newly identified homeobox gene related to the *Drosophila* *paired* gene, *Cer-1* (Bulleit, unpublished observations), are expressed at similar levels under all treatment conditions (Fig. 5). The exception was RNA encoded by the POU/homeobox gene *SCIP* (Monuki et al., 1989; also referred to as *Tst-1* or *Oct-6*), which also appeared to increase in response to NMDA treatment, but to a lesser degree than *Cns-1* mRNA. Thus, NMDA receptor activation specifically results in an increase in transcripts for the *Cns-1* gene and possibly the *SCIP/Tst-1* gene.

NMDA caused a dose-dependent increase in the level of the *Cns-1* mRNA. We observed a response at concentrations of

←

partate (NMDA). **A**, RNA blot analysis using an *Cns-1* cDNA probe was accomplished as described in Figure 3 and Materials and Methods. The mean OD $\pm$ SE from densitometric measurements of the autoradiographs of three separate blots is presented along with a representative autoradiographic image. The asterisks (*) indicate that these mean OD values are statistically different ($P < 0.03$) than the mean OD values for any of the other treatment conditions. **B**, The *Cns-1* probe was removed from the blots and the blots were hybridized with an *En-2* cDNA probe (refer to Materials and Methods). The mean OD $\pm$ SE of the hybridization signals is presented along with the autoradiographic image from the same blot presented in **A**. **C**, Ethidium bromide staining of the RNA gel used for blot analysis to obtain the autoradiographic image presented in **A** and **B**.

Figure 5. NMDA receptor activation does not affect the expression of several homeobox gene transcripts in cultured cerebellar cells. Cells from the early postnatal cerebellum were cultured for 24 hr in a chemically defined medium containing 5 mM KCl. Cultures were continued for an additional 24 hr in medium containing 10 mM KCl and 10 μ M glycine plus (+) 20 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 100 μ M amino-5-phosphonovaleric acid (APV), and/or 50 μ M *N*-methyl-D-aspartate (NMDA). RNA blot analysis using an *Cns-1* cDNA probe was accomplished as described in Figure 3 and Materials and Methods. Probes to several other homeobox genes (*En-2*, *Oct-1*, *SCIP/Tst-1*, and *Cer-1*) were used for repeated rehybridization to the same blot (refer to Materials and Methods).

NMDA as low as 10 μ M, reaching a peak at 50–100 μ M NMDA (Fig. 6A). The increase was also apparent in cells treated with NMDA in physiologic magnesium (5 mM) without added glycine (Fig. 7). High concentrations of KCl (40 mM) also resulted in an increase in *Cns-1* transcript levels (Fig. 7). Treatment of cells with GABA, somatostatin, or basic fibroblast growth factor did not affect the level of *Cns-1* RNA (Fig. 7) although cultured granule cells express functional receptors for all three of these ligands (Hatten et al., 1988; Bovolenta et al., 1992; Gonzalez et al., 1992). An increase in the level of this mRNA was first apparent ~2 hr after the start of NMDA treatment (Fig. 6B). The level of mRNA continued to increase up to 24 hr following NMDA treatment (Fig. 6B). This increase in the level of *Cns-1* mRNA could be maintained for at least 5 d (Fig. 8). APV added after 4 d of NMDA treatment reduced the level of *Cns-1* mRNA to control levels over the next 24 hr of culture (Fig. 8). The above data suggest that the specific activation of the NMDA receptor results in an increase in the level of *Cns-1* mRNA and that the maintenance of this increased mRNA level requires that this receptor remain active.

The results of experiments described above suggest that activation of NMDA receptors in cultures of early postnatal cerebellar cells result in a specific increase in the level of *Cns-1*

Figure 6. NMDA receptor activation regulates the expression of *Cns-1* mRNA in a concentration- and time-dependent manner. Cells from the early postnatal cerebellum were cultured in a chemically defined medium containing 5 mM KCl. **A**, Cultures were continued for an additional 24 hr in medium containing 10 mM KCl and 10 μ M glycine plus 0–200 μ M *N*-methyl-D-aspartate (NMDA). **B**, Cultures were continued for additional time (0–24 hr) in medium containing 10 mM KCl and 10 μ M glycine plus 50 μ M NMDA. RNA blot analysis using an *Cns-1* cDNA probe was accomplished as described in Figure 3 and Materials and Methods.

mRNA. This increase in the steady state mRNA level could be the result of an increase in transcript synthesis or a decrease in transcript degradation. We measured the level of transcript synthesis using isolated nuclei from cultured cerebellar cells that had been treated with either APV or NMDA. A nuclear runoff transcription assay was used to measure the level of transcription from both the *Cns-1* and *En-2* genes (Greenberg and Ziff, 1984). Nuclei isolated from cultured cells after 24 hr of treatment with NMDA exhibited a 1.6-fold increase in transcription from the *Cns-1* gene when compared to nuclei of cells treated with APV (Fig. 9). These observations suggest that the NMDA induced change in *Cns-1* mRNA level is at least in part the result of an increase in the rate of transcription from the *Cns-1* gene. However, we cannot rule out that a change in the rate of transcript degradation is also partially involved in mediating this response since the increase in the level of transcription is not identical to the increase in the level of *Cns-1* RNA.

Calcium mediates the increase in the level of Cns-1 RNA in response to NMDA treatment

The NMDA receptor is a ligand-gated ion channel that when opened allows cations to move across the cell membrane, including Ca^{2+} . At physiologic Mg^{2+} concentrations there is a voltage-dependent Mg^{2+} block of the NMDA channel (Mayer et al., 1984). Depolarizing conditions release Mg^{2+} from the channel allowing Ca^{2+} to enter the cell. High Mg^{2+} concentrations will antagonize NMDA receptor activation by blocking the channel. Treatment of cultured cerebellar cells with high Mg^{2+} concentrations (4 mM) consistently reduced the increase in *Cns-1* mRNA in response to NMDA treatment (Fig. 10). Although higher concentrations of Mg^{2+} (8 mM) didn't reduce the viability of cultured cells (Fig. 11), they did alter the mor-

Figure 7. Treatment of cultured cerebellar cells with both NMDA and high concentrations of KCl results in an increase in the level of *Cns-1* mRNA. Cells from the early postnatal cerebellum were cultured for 24 hr in a chemically defined medium containing 5 mM KCl. Cultures were continued for an additional 24 hr in medium containing 5 mM potassium chloride (KCl) plus 10 μ M γ -aminobutyric acid (GABA), 10 μ M somatostatin, 20 μ M basic fibroblast growth factor (bFGF), 50 μ M *N*-methyl-D-aspartate (NMDA) or 40 mM KCl. RNA blot analysis using an *Cns-1* cDNA probe was accomplished as described in Figure 3 and Materials and Methods.

phology of the cultures, appearing to reduce cell aggregation. Thus, we didn't use higher concentration of Mg^{2+} in these experiments. The incomplete block by 4 mM Mg^{2+} may result from a reduced sensitivity of the NMDA receptor to Mg^{2+} in developing neurons (Bowe and Nadler, 1990; Morrisett et al., 1990). However, the observation that 4 mM Mg^{2+} could reduce the NMDA response suggests that an open NMDA receptor/channel is required to induce the change in *Cns-1* transcript levels. We also used Ca^{2+} chelators to determine if Ca^{2+} entry through the NMDA channel mediates the increase in the level of *Cns-1* RNA. Treatment of cells with 1.5 mM EGTA, a chelator of extracellular Ca^{2+} , reduced the NMDA mediated increase in *Cns-1* mRNA (Fig. 10). A membrane-permeant form of BAPTA (BAPTA acetoxymethyl ester) was used as an intracellular chelator of Ca^{2+} . Cells were pretreated for 2 hr with BAPTA and the BAPTA containing medium was removed before exposure to NMDA. Conversion of the BAPTA analog to the parent acid occurs inside the cell sequestering it inside the cellular compartment. Treatment with these calcium chelators reduced the level of *Cns-1* mRNA in response to NMDA (Fig. 10). The level of *En-2* RNA was the same under all these treatment conditions (data not shown). Thus, chelation of either extracellular and intracellular calcium reduced the response of

these cultured cells to NMDA. Only higher concentrations the chelator EGTA (2.5 mM) reduced cell viability in these cultures (Fig. 11). All other culture treatments did not reduce cell viability when compared to control cultures (Fig. 11). Except for

Figure 9. NMDA receptor activation increases the synthesis of transcripts from the *Cns-1* gene. Cells from the early postnatal cerebellum were cultured for 24 hr in a chemically defined medium containing 5 mM KCl. Cultures were continued for an additional 24 hr in medium containing (+) 10 mM potassium chloride (KCl) and 10 μ M glycine (Gly) plus 100 μ M amino-5-phosphonovaleric acid (APV), or 50 μ M *N*-methyl-D-aspartate (NMDA). Nuclei isolated from treated cells were used in a nuclear transcription runoff assay as described in Materials and Methods. RNA probes, synthesized from nuclei *in vitro* using 32 P-UTP, were incubated with a *Cns-1* cDNA, an *En-2* cDNA, or control plasmid DNA (pGEM 11Z) that had been immobilized to nitrocellulose membranes. Densitometric measurements (OD) of probe hybridization signals were made from autoradiographs in three separate experiments. OD values for hybridization of probes to pGEM11Z DNA were used as background and subtracted from OD values for hybridization to *Cns-1* cDNA and *En-2* cDNA. The ratio of the OD measurement for *Cns-1* to the OD measurement for *En-2* is presented as the mean $\pm$ standard error.

Figure 8. The maintenance of an increase in *Cns-1* mRNA is dependent on the continued activation of the NMDA receptor. Cells from the early postnatal cerebellum were cultured for 5 d in a chemically defined medium containing (+) 5 mM potassium chloride (KCl), 5 mM KCl plus 50 μ M *N*-methyl-D-aspartate (NMDA), or 5 mM KCl plus 50 μ M NMDA in which 100 μ M amino-5-phosphonovaleric acid (APV) was added for the last 24 hr. RNA blot analysis using an *Cns-1* cDNA probe was accomplished as described in Figure 3 and Materials and Methods.

Figure 10. Calcium chelators block the increase in expression of *Cns-1* mRNA in response to NMDA receptor activation. Cells from the early postnatal cerebellum were cultured for 24 hr in a chemically defined medium containing 5 mM KCl. Cultures were continued for an additional 2 hr in medium containing (+) 10 mM potassium chloride (KCl) and 10 μ M glycine (Gly) plus 4 mM $MgSO_4$ (Mg), 1.5 mM ethylene glycol-bis(β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), or 20 μ M 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) tetrakis(acetoxymethyl). Following the pretreatment with either magnesium or EGTA, 50 μ M *N*-methyl-D-aspartate (NMDA) was added directly to the culture medium. Following BAPTA pretreatment, the BAPTA-containing medium was removed and replaced with fresh medium, minus BAPTA, containing 50 μ M NMDA. The cultures were then incubated for an additional 24 hr. RNA blot analysis using an *Cns-1* cDNA probe was accomplished as described in Figure 3 and Materials and Methods.

treatment with 8 mM Mg^{2+} and 2.5 mM EGTA, these treatment conditions also did not appear to affect morphology of cultures, as examined under phase-contrast microscopy. Thus, cell death induced by the treatment conditions is not likely to account for the response to NMDA or the antagonism of this response by calcium chelators.

Discussion

The amino acid sequence of *Cns-1* is similar to the sequence of other POU/homeodomain proteins in the conserved POU-specific domain and homeodomain. Relative amino acid sequence similarity between these proteins in these two conserved domains separates them into five classes (Rosenfeld, 1991). There is an 80–98% identity between members of an individual POU/homeodomain protein class. *Cns-1* is at most 57% identical to any other POU/homeodomain protein in these two domains. Thus, *Cns-1* appears to belong to a sixth and new class of POU/homeodomain protein. The POU-specific domain and the homeodomain are involved in sequence specific DNA binding required for these proteins to function as transcriptional activators (Sturm and Herr, 1988; Ingraham et al., 1990). Outside the POU-specific domain and homeodomain, there are no conserved regions between *Cns-1* and other POU/homeodomain proteins. However, *Cns-1* contains a region in the amino-terminal half that is similar in amino acid composition to regions in other POU/homeodomain proteins. This region is rich in serine, threonine, and proline residues (Fig. 1). Regions with similar amino acid composition in Pit-1 and Oct-1 function as transcriptional activation domains (Theill et al., 1989; Ingraham et al., 1990; Tanaka and Herr, 1990). Thus, it is possible, given the sequence and structural similarity of *Cns-1* with other POU/homeodomain proteins, that *Cns-1* also functions as a tran-

Figure 11. Viability of postnatal cerebellar cells in culture. Cells from the early postnatal cerebellum were cultured for 24 hr in a chemically defined medium containing 5 mM KCl. Cultures were further incubated for 2 hr in medium containing 10 mM KCl and 10 μ M glycine. Some cultures also contained 4 or 8 mM $MgSO_4$ (Mg^{2+}), 1.5 or 2.5 mM ethylene glycol-bis(β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), or 20 μ M 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) tetrakis(acetoxymethyl). Except for cells treated with BAPTA, after the 2 hr pretreatment either 50 μ M *N*-methyl-D-aspartate (NMDA) or 100 μ M amino-5-phosphonovaleric acid (APV) was added directly to the culture medium. In the case of cells pretreated with BAPTA, the BAPTA-containing medium was removed and replaced with fresh medium, minus BAPTA, containing 50 μ M NMDA. All cultures were then incubated for an additional 24 hr in the presence of NMDA or APV. Cell viability was measured using a trypan blue exclusion assay described in Materials and Methods. Six separate viability measurements were done on cells treated with each condition. The mean percentage of viable cell $\pm$ standard error is presented for each treatment condition. The viability of cell treated in 2.5 mM EGTA was significantly reduced compared to cell under any other treatment condition ($P < 0.01$).

scriptional activator. However, further studies are necessary to determine definitively whether *Cns-1* functions in this manner.

POU/homeodomain and homeodomain proteins are important transcriptional regulators involved in controlling development and determining individual cellular phenotypes (Ingham, 1988; Rosenfeld, 1991; Ruvkun and Finney, 1991). As a member of the POU/homeodomain protein family, *Cns-1* may function similarly in the CNS, a tissue where it is predominantly expressed (Fig. 3). We have observed that *Cns-1* is expressed in reaggregate cultures of cells derived from the early postnatal cerebellum (Fig. 4). This *in vitro* preparation of CNS cells consists primarily of cerebellar granule cells but also contains other cerebellar cellular phenotypes including glia (Trenkner and Sidman, 1977; Trenkner, 1991). Several aspects of cerebellar granule neuron development may occur in these reaggregate cultures, including cell division of granule neuron precursors (Gao et al., 1991), neurite extension, and granule neuron migration (Trenkner and Sidman, 1977). *Cns-1* may be expressed in any of the

cell types present in these culture and thus may be involved in determining their phenotype(s). Determination of what cell types express *Cns-1* must come from experiments to localize its expression within individual cells.

We have made an interesting observation concerning expression of *Cns-1* in the cerebellar reaggregate cultures. Treatment of these cultures with NMDA at concentrations greater than 10 μM results in an increase in the level of *Cns-1* mRNA that is blocked by simultaneous treatment with APV, an NMDA receptor antagonist. We did not observe antagonism of this NMDA response by CNQX, an antagonist of non-NMDA glutamate receptors (Fig. 4A). Treatment of these cultures with APV alone consistently reduced the level of *Cns-1* mRNA. Although this was not statistically significant, it is possible that an endogenous activator of the NMDA receptor is present in these cultures. Since granule neurons are glutaminergic and can release glutamate under conditions of tissue culture, it is likely that this endogenous activator is glutamate (Gallo et al., 1982; Van Vliet et al., 1989). The addition of glycine, a coagonist at the NMDA receptor, might be expected to increase the level of *Cns-1* mRNA by potentiating the response of NMDA receptors to endogenous glutamate (Johnson and Ascher, 1987). However, we observed no significant change in the level of *Cns-1* mRNA in response to glycine. In cerebellar slice preparations from the early postnatal cerebellum, endogenous activity of the NMDA receptor on granule neurons is absent unless glycine is present (D'Angelo et al., 1990). Our reaggregate cultures respond to NMDA at physiologic concentration of KCl (5 mM) even without added glycine (Fig. 7). These observations suggest that endogenously release glycine may also be present in these cultures. Variability in the level of *Cns-1* RNA under control conditions may be the result of variability in the amount of endogenous NMDA receptor agonists present in the cultures. Thus, cultures treated with APV may provide a more accurate measure of the basal level of expression of *Cns-1* RNA. At resting membrane potentials there is a voltage-dependent Mg^{2+} block of the NMDA channel (Mayer et al., 1984). Activation of the channel in the presence of an agonist is dependent on depolarization of the cell that removes the Mg^{2+} block. At physiologic K^+ our cultures responded to NMDA (Figs. 7, 8). This may be due to endogenous glutamate activating non-NMDA glutamate receptors or spontaneous depolarizing activity in the cell providing a depolarizing response to remove the Mg^{2+} block. Alternatively, this result may be due to a difference in the NMDA receptor's Mg^{2+} sensitivity in developing neurons (Bowe and Nadler, 1990; Morrisett et al., 1990). The observation that CNQX did not affect the NMDA-induced increase in *Cns-1* RNA would suggest that activation of non-NMDA glutamate receptors was not involved in the response. The combined results of these pharmacologic experiments suggest that specific activation of the NMDA subtype of glutamate receptors in cultured cerebellar cells increases the level of *Cns-1* mRNA. Experiments using several other homeobox genes, *En-2*, *Oct-1*, *SCIP/Tst-1*, and *Cer-1*, suggest that NMDA receptor activation has a relatively specific effect on the level of *Cns-1* mRNA. Three of these sequences, *En-2*, *Oct-1*, and *SCIP/Tst-1*, have been previously shown to be expressed in the cerebellum (Davis and Joyner, 1988; Davis et al., 1988; He et al., 1989). Excepting *SCIP/Tst-1*, NMDA receptor activation did not alter the level of mRNA encoded by these genes (Fig. 8). However, the response is not limited to the *Cns-1* since the level of *SCIP/Tst-1* RNA also appears to increase in response to NMDA treatment.

An increase in the level of *Cns-1* mRNA in response to NMDA was first apparent about 2–4 hr after NMDA treatment (Fig. 6B). The level of *Cns-1* mRNA reached a new steady state level after about 24 hr of NMDA treatment. This new level was maintained as long as the NMDA receptor remained active. A transcription assay also suggests that, at least in part, this increase in the level of *Cns-1* mRNA is the result of an increase in transcription from the *Cns-1* gene (Fig. 9). The timing of the response would suggest that the synthesis of new genes and their protein products may be required for new *Cns-1* gene expression. The immediate-early genes may be candidates for regulators of *Cns-1* expression. Many of these genes are transcription regulators whose expression rapidly increases in the CNS in response to a variety of signals, including NMDA receptor activation (Cole et al., 1989; Szekely et al., 1989; Morgan and Curran, 1991). Further experiments will be required to test whether this interaction takes place.

Changes in the level of expression of individual homeobox genes regulate various aspects of development and cellular differentiation (Akam, 1987; Scott and Carroll, 1987; Ingham, 1988). Similarly, the observed increase in *Cns-1* expression may mediate developmental changes of cultured cerebellar cells in response to NMDA receptor activation (Pearce et al., 1987; Balázs et al., 1988a,b; Moran and Patel, 1989; Cox et al., 1990; Cambray-Deakin and Burgoyne, 1992). This increase in *Cns-1* expression may occur in granule cells that express functional NMDA receptors (Cull-Candy and Ogden, 1985; Sciancalepore et al., 1989; Cox et al., 1990), or alternatively, granule neuron activation by NMDA could secondarily modify *Cns-1* expression in other cell types, possibly glia. If the 2.8–3.3-fold increase in the level of *Cns-1* RNA is occurring in individual cells, is this change large enough to affect cellular differentiation? In *Drosophila* there are several studies that suggest there is a concentration dependence of homeodomain protein function. The homeodomain protein encoded by *bicoid* has a critical threshold concentration necessary to activate expression of a second developmental control gene *hunchback* (Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989). As little as a twofold difference in the concentration of the *bicoid* protein will determine whether *hunchback* gene expression is on or off (Struhl et al., 1989). In two loss-of-function mutants the normal two copies of the homeobox genes *Ultrabithorax* or *Sex combed reduced* are replaced by only a single copy of the gene. These mutants develop abnormally with a partial transformation of larval segments (Lewis, 1963; Kaufman et al., 1980). In *Drosophila*, gene dosage usually determines the concentration of the gene's product. Thus, as little as a twofold change in the level of expression of a homeodomain protein can initiate new gene expression, modifying cellular development and differentiation. Thus, it is possible that this increase in *Cns-1* mRNA level could also result in alterations in the differentiation of postnatal cerebellar cells in culture. However, it must first be demonstrated that the increase in *Cns-1* mRNA results in a comparable increase in the *Cns-1* protein and that this increase in *Cns-1* protein corresponds to a significant change in developmental gene expression.

Both NMDA and depolarizing concentrations of KCl have similar effects on the development and differentiation of cerebellar cells in culture (Gallo et al., 1987; Moran and Patel, 1989; Cox et al., 1990). The response to both these treatments occurs through calcium entry into the cells, either through the NMDA receptor/channel complex or through voltage-dependent L-type calcium channels (Gallo et al., 1987; Moran and Patel, 1989;

Cambray-Deakin and Burgoyne, 1992). Thus, changes in the concentration of intracellular calcium may be an important mechanism regulating cerebellar cell development and differentiation *in vivo*. We have observed that the increase in *Cns-1* expression, in response to NMDA, is also dependent on calcium. Both extracellular and intracellular chelation of calcium reduced the increase in *Cns-1* expression in response to NMDA (Fig. 10). This experiment might suggest that calcium entry through the NMDA channel is responsible for the increase in the level of *Cns-1* expression. However, we can't rule out that calcium released from intracellular stores is also involved in this response. We have tested the effect of several other agents on *Cns-1* expression in these postnatal cerebellar cultures. Only NMDA and high concentrations of KCl (40 mM) resulted in an increase in *Cns-1* transcript levels. Treatment of cells with GABA, somatostatin, or basic fibroblast growth factor did not affect the level of *Cns-1* RNA (Fig. 10) though cultured granule cells express functional receptors for all three of these ligands (Hatten et al., 1988; Bovolin et al., 1992; Gonzalez et al., 1992). An increase in the level of *Cns-1* mRNA in response to 40 mM KCl is not blocked by the addition of APV (X. Lin, unpublished observation). This observation suggests that the response to KCl is not due to K⁺-induced release of endogenous glutamate activating NMDA receptors. Thus, this response appears specific to NMDA receptor activation and KCl depolarization, both of which allow calcium to enter cells. Changes in *Cns-1* expression may be part of the mechanism by which Calcium regulates differentiation and development of cultured cerebellar cells.

Both NMDA receptor activation and changes in intracellular calcium can have important effects on development and plasticity in the CNS. Spontaneously active NMDA receptors are present in the early developing CNS (Blanton et al., 1990; Lo Turco et al., 1991). Activity-dependent development of certain CNS neurons requires NMDA receptor activation (Kleinschmidt et al., 1987; Scherer and Udin, 1989; Cline and Constantine-Paton, 1990; Kalb and Hockfield, 1990; Simon et al., 1992). Changes in the intracellular concentration of calcium have been shown to play an important role in regulating neuronal differentiation and neuronal growth cone behavior (Mattson et al., 1988; Holliday and Spitzer, 1990; Kater and Mills, 1991). The observation that *Cns-1* is expressed throughout the CNS suggests that NMDA receptor activation or changes in the intracellular calcium concentration may also modify its expression in other CNS cells. Thus, *Cns-1* could have several roles in regulating development and plasticity in the CNS.

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