THE JOURNAL OF COMPARATIVE NEUROLOGY 468:264–276 (2004)

Calcyon Transcript Expression in Macaque Brain

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ABSTRACT

The anatomical distribution of the transcript encoding calcyon, a 24 kDa membrane protein associated with coupling D1-like dopamine receptor activation to potentiated intracellular calcium release, was examined using in situ hybridization in the macaque brain. Calcyon mRNA is found to be abundantly distributed throughout the primate brain. In neocortex, moderately dense, diffuse signal is found in all areas, with increased intensity present in a superficial isodense band corresponding generally to cortical layers II and III. Increased intensity of signal is also seen in the pyramidal cell layers of medial prefrontal and anterior cingulate cortex. Calcyon mRNA is present abundantly in subcortical limbic areas such as the nucleus accumbens, septum, hypothalamus, amygdala, and hippocampus. Moderate calcyon transcript expression is seen in caudate and putamen, with lower levels in globus pallidus. Thalamic nuclei, including the reticular nucleus, express low to moderate levels. Very dense expression is noted in the substantia nigra pars compacta. Numerous brainstem regions express this transcript, notably monoaminergic nuclei including the locus coeruleus and dorsal raphe. The cerebellum has detectable levels of expression in both cortex and deep nuclei. Although calcyon is hypothesized as a means for D1-like receptors to modulate "cross-talk" with other neurotransmitter receptor systems, it is notable that abundant calcyon transcript is detected in brain regions not associated with D1-like neurotransmission, particularly the substantia nigra pars compacta and other dopamine-synthesizing cell groups. A substantial proportion of this may relate to the reported association of calcyon with the D5 receptor, or in addition, may suggest that calcyon has a wider role as a regulator of intracellular signal transduction. J. Comp. Neurol. 468:264–276, 2004.

Indexing terms: Macaca receptors; dopamine D1; calcium signaling; GTP-binding proteins; RNA messenger

Calcyon is a 24 kDa membrane protein which was recently identified on the basis of its association with the D1 dopamine receptor in an in vitro assay (Lezcano et al., 2000). It has been shown that in the presence of calcyon, in vitro stimulation of cells with D1 agonists will potentiate the intracellular calcium flux stimulated by activation of the Gq/11 signaling pathways, such as that stimulated by muscarinic cholinergic and metabotropic glutamate receptors, provided that the latter mechanisms have already been "primed" by their respective agonists. This process has been hypothesized as a means by which dopamine binding at D1 receptors is able to modulate intracellular calcium ion concentrations in addition to the better-characterized Gs-dependent signal transduction mechanism of increasing intracellular cyclic AMP concentrations. Lezcano and Bergson (2002) demonstrate that this mechanism is able to regulate the excitability of dissociated hippocampal and neocortical neurons in a cAMP-independent manner when these cells have been previously stimulated with Gq/11-associated agonists. Calcyon appears to allow any of several Gq- or G11-coupled neurotransmitter receptors to modulate cellular responses to dopamine. Effective priming responses have been demonstrated with applications of muscarinic cholinergic,

Grant sponsor: National Institute of Mental Health (NIMH); Grant number: MH53327; Grant number: MH63742(J.H.M.-W.).

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Received 21 March 2003; Revised 24 July 2003; Accepted 20 August 2003
DOI 10.1002/cne.10993
Published online the week of November 17, 2003 in Wiley InterScience (www.interscience.wiley.com).

metabotropic glutamatergic, serotonergic, and alpha1 adrenergic agonists. The specific means by which this priming occurs remains unclear, but its inhibition by a protein kinase C inhibitor suggests the possibility of the diacylglycerol pathway being involved (Lezcano and Bergson, 2002).

The initial studies characterizing calcyon demonstrated the expression of calcyon protein in neurons of macaque striatum, prefrontal cortex, and the CA3 region of hippocampus (Lezcano et al., 2000). A more comprehensive survey of calcyon mRNA in rat brain by Zelenin et al. (2002) demonstrated its widespread distribution throughout the brain, including high levels of expression in the hypothalamus and cerebellum, in addition to striatal, cortical, and hippocampal regions. Clarification of its specific localization within these neural systems will be critical in determining its relevance to the function of the neural circuitry in these regions, particularly with respect to its ability to regulate "cross-talk" between disparate intracellular signaling pathways. In this study, we undertook a detailed survey of the distribution of the transcript encoding calcyon in macaque (*Macaca nemestrema*) brain as a prelude to studies of the role calcyon may play in pathophysiological processes in the human brain.

MATERIALS AND METHODS

Matched 10 μm frozen sections were obtained from the brains of four female macaques (*Macaca nemestrema*) ranging in age from 7–13 years at the time of death. These brains were obtained from the Regional Primate Research Center of the University of Washington. These animals had been sacrificed as part of protocols unrelated to the present study which did not require brain tissue. Protocols involving these animals were reviewed by the Washington Regional Primate Research Center Research Review Committee and by the University of Washington IACUC.

Each brain was blocked in the coronal plane into five 1.5 cm slabs comprising the full extent of the brain from the frontal pole through the cerebellum and occipital cortex. Blocks were stored at −80°C until cryostat sectioning. Sections were mounted onto labeled 2 × 3-inch poly-L-lysine-subbed glass microscope slides, desiccated, and stored at −80°C until their use in hybridization studies.

To generate a subclone for riboprobe synthesis, we amplified a unique region of calcyon (NCBI GenBank accession #AF225903, nucleotide coding region 120–509) from a human brain cDNA brain library (EdgeBiosystems, Gaithersburg, MD) using PCR. Amplified cDNA segments were extracted (QIAquick Gel Extraction Kit, Qiagen, Valencia, CA), subcloned (Zero Blunt TOPO PCR cloning kit, Invitrogen, Carlsbad, CA) and confirmed by nucleotide sequencing.

Riboprobes were synthesized using 100 μCi of dried [^{35}S]-UTP, 2.0 μl 5× transcription buffer, 1.0 μl 0.1M DTT, 1.0 μl each of 10 mM ATP, CTP, and GTP, 2.0 μl linearized plasmid DNA, 0.5 μl RNAse inhibitor, and 1.5 μl SP6 RNA polymerase for synthesis of antisense probe, or T7 RNA polymerase for synthesis of sense probe, then incubated for 2 hours at 37°C. After this incubation, 1.0 μl DNAse (RNAse-free) was added and incubated for 15 minutes at room temperature. The reaction mixture was then sieved through a 1 ml syringe containing G-50 Sephadex equilibrated in Tris buffer (100 mM Tris-HCl, pH 7.5, 12.5 mM EDTA, pH 8.0, 150 mM NaCl, and 0.2% SDS), and 100 μl fractions were eluted. DTT was added to each fraction to a final concentration of 0.01M.

Abbreviations

ac	anterior commissure
Acb	Nucleus accumbens
ACg	Anterior cingulate
AHA	Anterior hypothalamus
Amg	Amygdala
ArcH	Arcuate nucleus
CA1	CA1 pyramidal field
CA2	CA2 pyramidal field
CA3	CA3 pyramidal field
CA4	CA4 pyramidal field
Cb	Cerebellar cortex
Cd	Caudate
CG	Central grey
Cl	Claustrum
CM	Centromedial nucleus
DG	Dentate gyrus
DM	Dorsomedial hypothalamus
DpCb	Deep cerebellar nuclei
DR	Dorsal raphe
EC	Entorhinal cortex
FOG	Frontal orbital gyrus
GP	Globus Pallidus
GRe	Gyrus rectus
Hbn	Habenula
Hi	Hippocampus
IFG	Inferior frontal gyrus
ITG	Inferior temporal gyrus
Ins	Insula
IO	Inferior olivary complex
LC	Locus Coeruleus
LD	Laterodorsal thalamus
LH	Lateral Hypothalamus
LOG	Lateral orbital gyrus
MD	Mediodorsal thalamus
MFG	Medial frontal gyrus
MM	Mammillary nucleus
MTG	Medial temporal gyrus
opt	Optic tract
PA	Periamygdaloid area
Pa	Paraventricular nucleus of hypothalamus
PCg	Posterior cingulate gyrus
PH	Posterior hypothalamic area
PoCG	Post-central gyrus
PrCG	Pre-central gyrus
Pu	Putamen
Pul	Pulvinar
Rt	Reticular thalamic nucleus
S	Subiculum
SC	Superior colliculus
SI	Substantia innominata
SnC	Substantia nigra compacta
SnR	Substantia nigra reticulata
SO	Supraoptic nucleus
SFG	Superior frontal gyrus
STG	Superior temporal gyrus
STN	Subthalamic nucleus
StT	Nucleus of the stria terminalis
V1	Primary visual cortex
VA	Ventral Anterior thalamus
Ve	Vestibular nuclei
VMH	Ventromedial hypothalamus
VPL	Ventral posterior thalamus
VTA	Ventral tegmental area
VTM	Ventral tuberomammilary nucleus

From each animal, 12–15 mounted, frozen sections, selected to represent the entire rostrocaudal extent of the brain at ~0.5 mm intervals, were removed from −80°C storage and placed in 4% (weight:vol) formaldehyde at room temperature for 1 hour. The slides were briefly washed in 2× SSC (300 mM NaCl/30 mM sodium citrate, pH 7.2) three times. The slides were placed in 0.1M triethanolamine, pH 8.0/acetic anhydride, 400:1 (vol:vol), on a stir plate for 10 minutes. The final wash was in 2× SSC buffer for 5 minutes, followed by dehydration through graded alcohols and air drying. A coverslip with 0.4 ml radiolabeled riboprobe (5×10^6 cpm), 50% formamide buffer (50% formamide, 10% dextran sulfate, 3× SSC, 50 mM Na_2HPO_4 (pH 7.4), 1× Denhardt's solution, 100 μg/ml yeast tRNA) and 0.01M DTT was placed on each coronal section. Slides were placed in a covered tray lined with filter paper saturated with 50% formamide and incubated at 55°C overnight. As technical controls, an additional set of slides containing samples from prefrontal cortex, thalamus, striatum, hippocampus, or cerebellum were processed parallel to the above. Each of these slides was treated with radiolabeled riboprobe as above, half of the slides receiving sense riboprobe and adjacent sections receiving antisense riboprobe.

The next day the coverslips were removed and the slides were placed in 2× SSC for 5 minutes, followed by RNAse (200 μg/ml in 10 mM Tris-HCl, pH 8.0/0.5 M NaCl) at 37°C for 30 minutes. The slides then underwent the following washes: 2× SSC at room temperature for 10 minutes; 1× SSC for 10 minutes at room temperature; 0.5× SSC for 10 minutes at room temperature; 0.1× SSC at 55°C for 60 minutes; a second wash in 0.1× SSC at 55°C for 60 minutes; and 0.5× SSC for 10 minutes at room temperature. Sections were dehydrated in graded alcohols and apposed to Kodak BIOMAX MR film for 5 days, as determined by results from hybridized test slides run in parallel. Films were developed automatically in a Kodak X-omat automatic processor. Macroscopic images were acquired from films using a CCD camera via NIH Image software, v. 1.61.

Selected hybridized slides, including technical controls hybridized with labeled sense probe, were dipped in Kodak NTB-2 emulsion diluted 1:1 with sterile water and warmed to 40°C. Dipped slides were stored in darkness at 4°C. Test slides containing hybridized tissue were also emulsion-dipped on the same day and stored in individual slide boxes to be developed at weekly intervals for a determination of exposure time. After 34 days, all slides were developed by immersion in Kodak D19 developer for 2 minutes, rinsed, fixed in Kodak Rapid Fix for 3 minutes, and washed in running deionized water. The developed slides were then counterstained by immersion in cresyl violet (pH 4.2), followed by decolorization in a series of ethanol and xylene washes. Slides were coverslipped in Permount and viewed with a Zeiss microscope. Digital images were acquired using a CCD camera.

Macroscopic images were individually compared to plates and diagrams from two macaque atlases (Paxinos et al., 2000; Martin and Bowden, 2000) for identification of structures. Using Scion Image software, optical density measurements relative to background were obtained for regions of interest in each animal, then averaged across animals for each region. Average optical density by region was divided into quartiles and a rating of relative optical density assigned to each region on a scale of 1 (lowest) to 4 (highest) by quartile. Original images were exported to Adobe PhotoShop (San Jose, CA) for minor adjustments of brightness and contrast in final processing of the images contained herein.

To confirm that the riboprobe specifically identified a single species of calcyon transcript, a Northern blot containing mRNA from specific brain regions was hybridized with radiolabeled probe prepared as above, except that 83 μCi of [^{32}P]-UTP was used in place of [^{35}S]-UTP in the RNA polymerase reaction. Identified regions of macaque brain were dissected and frozen at −80°C until ready for use. The brain samples were weighed, then individually homogenized in 1 ml trizol per 100 mg tissue. Following centrifugation at 10,000 rpm for 15 minutes, the aqueous phase was mixed with chloroform and recentrifuged. RNA was precipitated from this aqueous phase with isopropanol and the resulting pellet washed in 75% ethanol, vortexed, recentrifuged at 5,000 rpm for 5 minutes, and resuspended in 200 μl formamide. After spectrophotometrically determining the concentration of RNA in each sample, 5 μg of RNA in a total volume of 30 μl was loaded onto a 1% agarose/formaldehyde gel and run for 60 minutes at 50 volts. RNA from the gel was then transferred to a Hybond membrane and fixed by baking the membrane at 80°C for 2 hours. The membrane was washed in 2× SSC and prehybridized at 65°C for 2 hours in 50% formamide buffer (50% formamide, 10% dextran sulfate, 3× SSC, 50 mM Na_2HPO_4 (pH 7.4), 1× Denhardt's solution, 100 μg/ml yeast tRNA) containing 10% SDS. The membrane was then incubated with [^{32}P]-labeled riboprobe (1×10^6 cpm per ml buffer) overnight at 65°C. The hybridized membrane was washed three times at 65°C in 1× SSC, 1× SSC containing 0.1% SDS, and 0.1× SSC containing 0.1% SDS. The membrane was dried and apposed to Kodak BIOMAX MR film with intensifying screens at −80°C for 6 hours.

RESULTS

Calcyon mRNA is found in a widespread distribution throughout the primate brain (Fig. 1A–L). Relative expression by specific region is summarized in Table 1. Northern blot analysis of calcyon expression in multiple regions of the brain confirms that the probe used identifies only a single mRNA species (Fig. 2A). Sections incubated with sense riboprobe have no significant labeling, while sections incubated with antisense probe demonstrate specific labeling (Fig. 2B,C).

In the cerebral cortex, calcyon mRNA is expressed in all areas and layers of cerebral cortex, with somewhat denser distribution observed in the anterior cingulate cortex, orbital cortex (Fig. 1B), and temporal regions, including entorhinal cortex (Fig. 1E,F). Although the expression appears to be present in the full thickness of cerebral cortex, its distribution in all cortical areas is noticeably denser in a superficial isodense band, corresponding primarily to layer II of neocortex. In granular cortex, such as primary visual cortex (Fig. 1L), deeper layers are also more intensely labeled. Deeper layers are also relatively more enriched in expression in associative cortical areas, namely, the anterior cingulate (Fig. 1B), precentral (Fig. 1C), and superior temporal gyri, but not in others, such as the medial temporal and postcentral gyri (Fig. 1J). There appears to be relatively less expression in an intermediate band corresponding to layer III in most areas, most no-

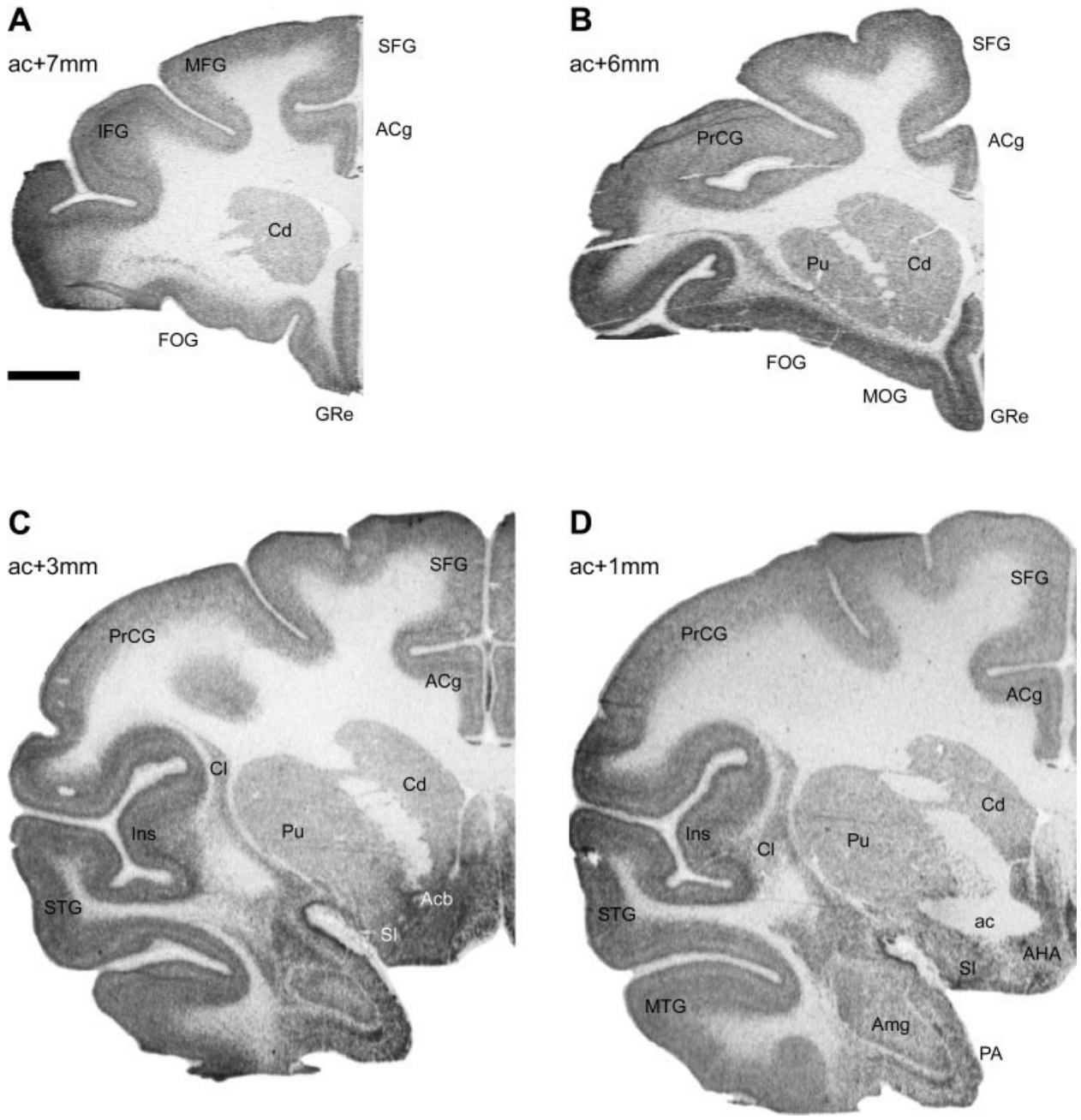

Fig. 1. **A–L:** Macroscopic images of film autoradiograms demonstrating distribution of calcyon transcript in representative coronal sections of macaque brain. Major structures are labeled according to the atlas of Martin and Bowden (2000). Scale bars = 5 mm in A,E,I.

ticeably in the anterior cingulate, precentral, and insular cortex (Fig. 1C).

In subcortical regions, the densest expression is found in the ventral striatum and associated limbic areas such as nucleus accumbens, substantia innominata, and nucleus of the stria terminalis. Expression is detected in the septal nuclei, particularly in the lateral septal nucleus, although less abundantly than in nucleus accumbens. Moderate calcyon mRNA expression is present throughout the caudate and putamen (Fig. 3), although it appears to be substantially less abundant relative to expression in neocortex and ventral striatal areas. Patchy, extremely light labeling is visible in the globus pallidus, although scattered isolated cells are intensely labeled on microscopic examination (Fig. 3C). The claustrum also expresses this transcript at moderate levels.

High levels of expression are visible in all nuclei of the amygdala, with relatively more expression in the lateral and medial nuclei. In the hippocampal formation, the dentate gyrus is found to have very high expression. Nearly

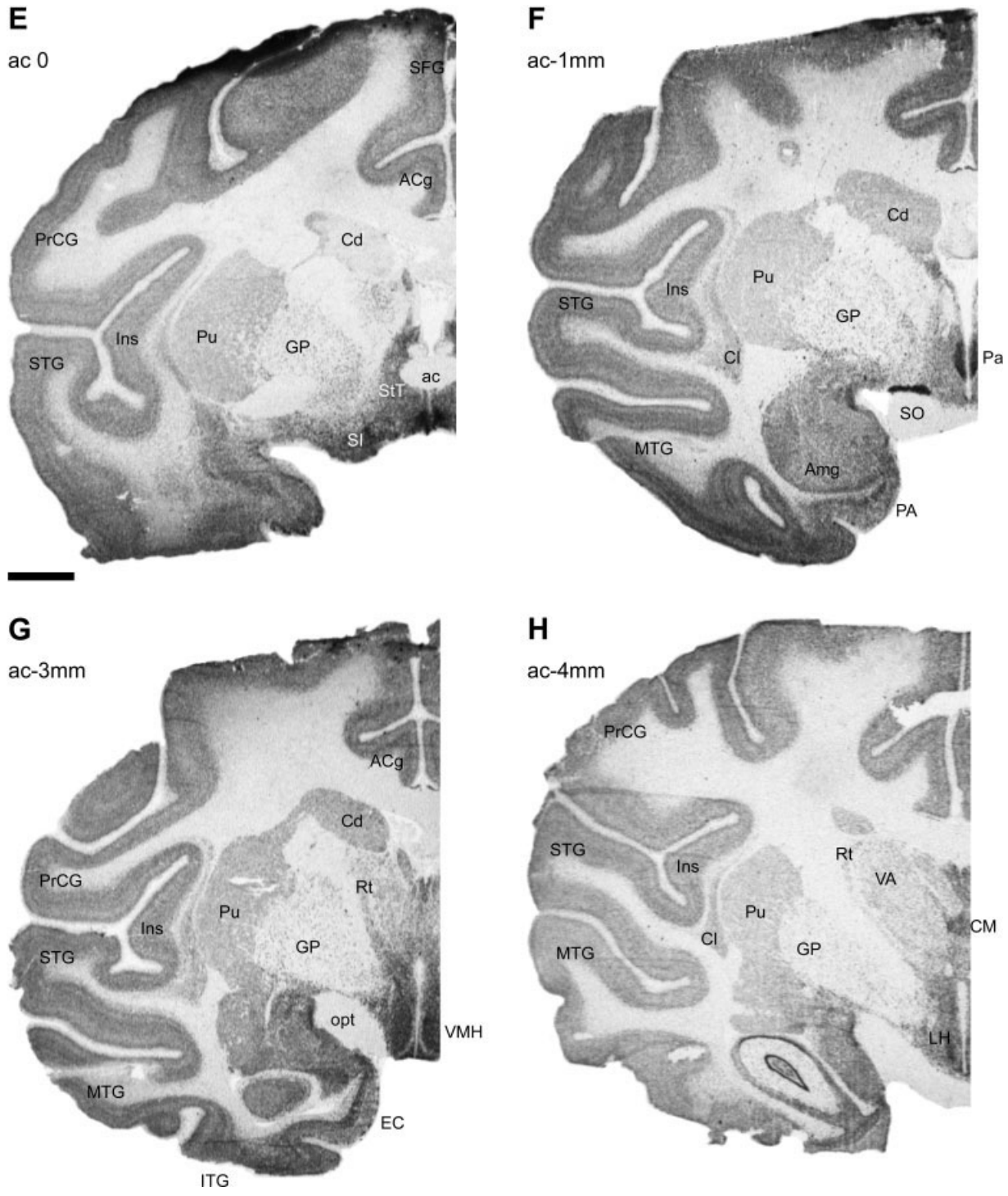

Figure 1 (Continued)

all granule cells in the dentate gyrus are labeled for calcyon on microscopic examination (Fig. 4B). All hippocampal pyramidal cell fields demonstrate high levels of calcyon expression (Fig. 4A), although there is a trend toward a higher level of expression in the CA2 pyramidal field relative to CA1 and CA3, and a minor trend toward relatively less expression in CA4.

In the thalamus, most nuclei display only low levels of expression, with the prominent exceptions of the centromedial and paraventricular nuclei, which express the transcript abundantly. The thalamic reticular nucleus and lateral and medial geniculate nuclei also express detectable calcyon mRNA (Fig. 1G–J). The subthalamic nucleus contains a low but detectable level of expression.

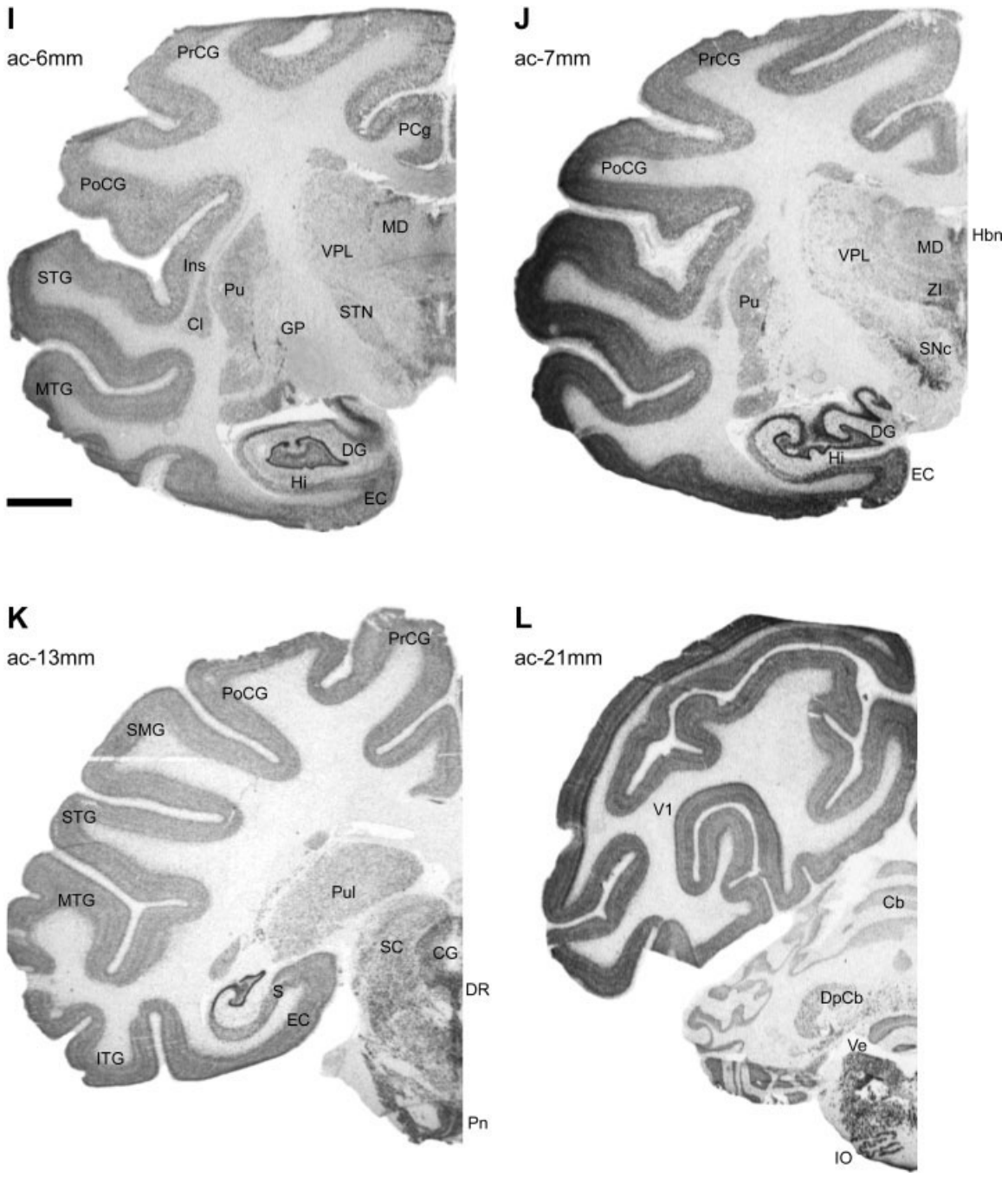

Figure 1 (Continued)

High levels of calcyon transcript are noted in the medial habenula and in the medial zona incerta, an area which contains neurons expressing tyrosine hydroxylase.

Particularly abundant mRNA expression is prominent throughout the hypothalamus, particularly in the supraoptic nucleus, arcuate nucleus, and paraventricular nucleus (Fig. 5), all of which are areas known to contain dopamine-synthesizing neurons. Abundant expression is also present in the mammillary nuclei, ventromedial nucleus, and anterior and lateral hypothalamic areas.

In the midbrain, the highest levels of expression are noted in the substantia nigra pars compacta, ventral tegmental area, and in the dorsal raphe nuclei (Fig. 6A–E). The central gray substance and reticular formation also express the transcript at moderate to high levels. The substantia nigra pars reticulata expresses only low mRNA levels.

Pontine and medullary regions expressing very high levels of calcyon mRNA include the locus coeruleus (Fig. 6F), peribrachial nuclei, pontine nuclei, vestibular nuclei,

TABLE 1. Relative Expression of Calcyon Transcript by Region

Cortex	
Anterior cingulate (ACg)	++
Gyrus Rectus (GRe)	+++
Frontoorbital gyrus (FOG)	++
Medial frontal gyrus (MFG)	++
Lateral orbital gyrus (LOrG)	+++
Pre-central gyrus (PrCG)	++
Insula (Ins)	++
Superior temporal gyrus (STG)	++
Middle temporal gyrus (MTG)	++
Inferior temporal gyrus (ITG)	+++
Periamygdaloid area (PA)	+++
Entorhinal (Ent)	+++
Post-central gyrus (PoCG)	++
Primary visual cortex (V1)	+
Subiculum (S)	+++
Hippocampus (Hi)	
CA1	+++
CA2	++++
CA3	++++
CA4	+++
Dentate gyrus (DG)	++++
Subcortex	
Nu. of Stria Terminalis (StT)	++++
Substantia Innominata (Sl)	++++
Lateral Septal Nu. (LS)	++
Medial Septal Nu. (MS)	++
Dorsal Septal Nu. (DS)	+
Nucleus Accumbens (Acb)	++++
Caudate (Cd)	+
Putamen (Pu)	+
Globus Pallidus (GP)	+
Claustrum (Cl)	++
Amygdala (Amg)	
Anterobasal nucleus	+++
Basal nucleus	+++
Central nucleus	++
Lateral nucleus	++
Diencephalon	
Mediodorsal Thalamus (MD)	++
Ventral Posterior Thalamus (VPL)	+
Ventral Anterior Thalamus (VA)	+
Centromedial Thalamus (CM)	++
Laterodorsal Thalamus (LD)	++
Paraventricular N. of Thalamus (PV)	+++
Lateral Geniculate (LGN)	+
Medial Geniculate (MGN)	+
Reticular N. Thalamus (Rt)	+
Pulvinar (Pul)	+
Zona Incerta (Zl)	+++
Habenula (Hbn)	+++
Subthalamic N. (STN)	+
Hypothalamus	
Anterior hypothalamus (AHA)	+++
Ventromedial hypothalamus (VMH)	++++
Supraoptic N. (SO)	++++
Lateral Hypothalamus (LH)	+++
Mammilary N. (MM)	++++
Arcuate N. (ArcH)	++++
Paraventricular N. (Pa)	++++
Mesencephalon	
Substantia nigra compacta (SNc)	++++
Substantia nigra reticulata (SNr)	+
Ventral tegmental area (VTA)	++++
Red Nucleus (RPC, RMC)	+
Central grey (CG)	+++
Dorsal raphe (DR)	++++
Pedunculopontine Nu. (PPT)	+++
Superior colliculus (SC)	++
Pons and Medulla	
Pontine Nuclei (Pn)	++++
Reticular fields (PnC, CMRT)	++
Locus Coeruleus (LC)	++++
Parabrachial nucleus (PBN)	+++
Vestibular nuclei (Ve)	+++
Inferior olivary complex (IO)	++
N. of solitary tract (NST)	++++
Spinal trigeminal nucleus (CSp5)	++++
Cerebellum	
Cerebellar Cortex (Lateral) (Cb)	+
Cerebellar Cortex (Vermis) (Cb)	+
Deep Nuclei (DpCb)	+

Key: ++++ highest signal intensity; +++ heavy signal; ++, moderate signal; +, light signal.

and inferior olivary complex. Cerebellar cortex expresses transcript at moderate levels throughout, although the vermis appears relatively more enriched in calcyon mRNA than do lateral cerebellar areas. The deep cerebellar nuclei express moderate amounts of transcript. Microscopic examination of cerebellar labeling reveals concentrations of silver grains over the Purkinje cells in cerebellar cortex (Fig. 7).

DISCUSSION

We examined the anatomical distribution of transcripts coding for calcyon in the macaque brain. Consistent with a previous report in the rat (Zelenin et al., 2002), we find calcyon to be distributed widely throughout the brain. As in the previous report, substantial areas of overlap exist between the distribution of calcyon and the previously reported distributions of the D1 and D5 receptor transcripts (Meador-Woodruff et al., 1991; Levey et al., 1993; Beischlag et al., 1995). However, our study also indicates that calcyon is expressed in brain areas not known to express either of these receptors.

Calcyon was initially identified and characterized using an in vitro assay that specifically sought molecules which interact with the D1 dopamine receptor (Lezcano et al., 2000). As a result of these experiments, calcyon has been presented in the literature as primarily associated with D1-like receptors (e.g., D1 and D5). The calcyon expression we observe in regions strongly associated with D1 dopamine signaling, such as the caudate and putamen, nucleus accumbens, neocortex, and hippocampus, is consistent with this view. We find moderate levels of calcyon in the caudate and putamen, with denser signal noted in the substantia innominata and nucleus accumbens. D1 receptors have long been shown to be abundantly expressed in these areas and are important mediators of synaptic plasticity in these structures (Kerr and Wickens, 2001). Prior to the identification of calcyon, it had been reported that D1 agonists may mediate inositol triphosphate formation in striatal neurons via the coupling of D1-like receptors to a Gq-linked mechanism (Wang et al., 1995). It now seems likely that calcyon has a role in mediating this process. We also find calcyon to be highly expressed in the amygdala and hippocampus, other regions in which dopamine signaling is implicated in processes of synaptic plasticity (Otmakhova and Lisman, 1996).

Although calcyon expression in the caudate and putamen is only moderate, it is noticeably more abundant in neocortex. Our results indicate a widespread, multilaminar expression of calcyon in cortex, with a somewhat greater density in an isodense band corresponding to layer II of neocortex. This stands in contrast to the reported predominance of D1 expression in deeper pyramidal cell layers in primate (Bergson et al., 1995) and human neocortex (Meador-Woodruff et al., 1996), although D5 transcripts are relatively more enriched in these middle layers (Meador-Woodruff et al., 1996). Dopamine neurotransmission in cortex has been demonstrated to have an important role in cognition and working memory functions (Williams and Goldman-Rakic, 1995). Evidence also exists that long-term depression in prefrontal cortical neurons requires coactivation of metabotropic glutamate and dopamine receptors (Otani and Connor, 1998). Calcyon may prove to be one of the underlying mechanisms required for

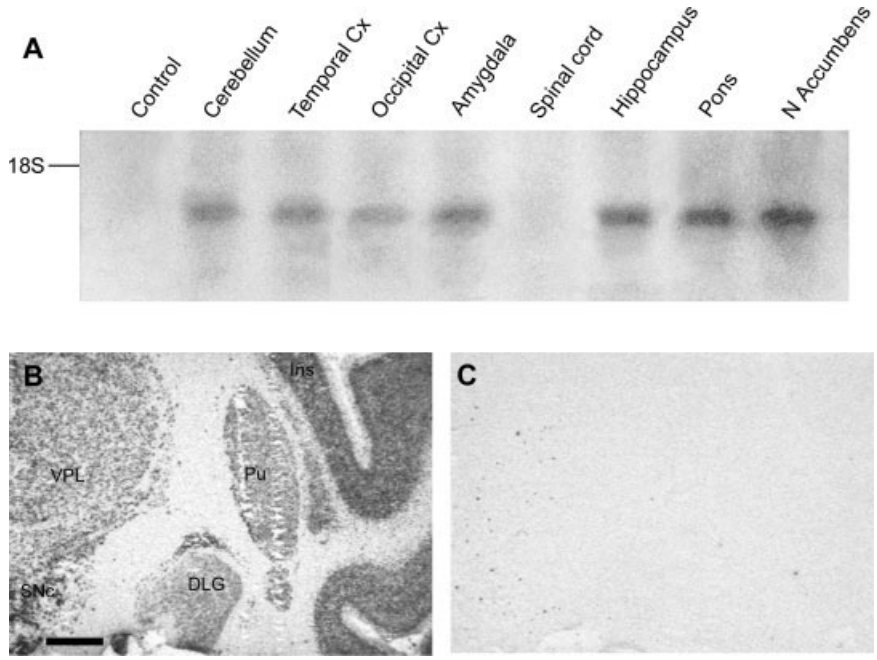

Fig. 2. Technical controls. **A:** Northern blot indicating identification of single species by radiolabeled antisense riboprobe in multiple brain regions. **B:** Film autoradiogram of section at the level of the thalamus (approx. ac –9 mm) treated with radiolabeled antisense riboprobe. **C:** Adjacent section treated with radiolabeled sense riboprobe. Pu, putamen; DLG, dorsal lateral geniculate nucleus; SNc, substantia nigra pars compacta; VPL, ventral posterior lateral nucleus; Ins, insula. Scale bar = 2 mm.

this instance of synaptic plasticity through its ability to allow other neurotransmitters, such as glutamate and acetylcholine, to regulate the nature of dopamine's intracellular effects. This modulation may well vary in a region-specific manner, depending on the relative abundance of the other receptors present. For example, the higher levels of calcyon expression we observe in cortex relative to caudate and putamen may indicate that calcyon-transduced signaling plays a relatively greater role in cortex. Further evidence for this may be seen in the observation that calcyon appears to affect calcium flux in dissociated neocortical and hippocampal neurons differently than in dissociated striatal neurons (Lezcano and Bergson, 2002).

In spite of the above instances of concordance with D1 receptor distribution, notable inconsistencies are also observed. In many areas the presence of calcyon transcripts in areas not associated with D1 signaling can be largely attributed to an overlap with D5. This is particularly true in cortex, where the presence of calcyon signal in middle cortical layers is similar to the reported distribution of D5 transcripts in human brain (Meador-Woodruff et al., 1996). It is also evident in the hippocampus and associated temporal cortical regions, where Meador-Woodruff et al. (1994) demonstrated a near-complete mismatch between the distributions of D1 and D5 transcripts in human brain. Although calcyon is expressed abundantly in the dentate gyrus, all hippocampal pyramidal fields, subiculum, and entorhinal and temporal cortices, D1 is primarily expressed in neocortex and faintly in subiculum and the CA1 field. In contrast, D5 is expressed at high levels in the dentate gyrus, CA2, CA3, and CA4 pyramidal fields, and entorhinal cortex, as well as faintly in CA1 and subiculum (Meador-Woodruff et al., 1994).

Calcyon is expressed at low to moderate levels in most thalamic nuclei, with a trend for higher levels of expression in midline nuclei, notably in the centromedial, paraventricular, and parafasicular nuclei. There is prominent calcyon expression in the thalamic reticular nucleus, as well as in sensory relay nuclei, such as the lateral and medial geniculate nuclei. Overall, this pattern of expression is not consistent with the distribution of mRNAs for D1 receptors, which are generally expressed at very low levels in thalamus (Meador-Woodruff, 1994). In addition, the moderate level of expression in cerebellar cortex, particularly in the Purkinje cells, is not consistent with previous findings showing relatively limited D1 mRNA expression in the cerebellum (Meador-Woodruff, 1994).

The most striking lack of concordance is the abundant calcyon expression in monoamine-synthesizing neurons, especially in the substantia nigra, where cell bodies in the pars compacta are intensely labeled for calcyon. Expression of D1 receptor mRNA has not been demonstrated in cell bodies of the substantia nigra pars compacta (SNc), although D1 receptors have been localized to the neuropil of the pars reticulata, where they likely are present on terminals of striatonigral projection neurons (Meador-Woodruff et al., 1991; Levey et al., 1993; Bergson et al., 1995). Again, the very high levels of calcyon mRNA expression in these cells may be indicative of an association between calcyon and D5 receptors, as suggested by Lezcano et al. (2000) and Zelenin et al. (2002). D5 receptor immunoreactivity and mRNA have been reported in cell bodies in the SNc in both monkey and human, although at somewhat lower levels (Beischlag et al., 1995; Bergson et al., 1995; Ciliax et al., 2000). We also find particularly high levels of calcyon expression in other monoaminergic nuclei, especially the dorsal raphe and locus coeruleus.

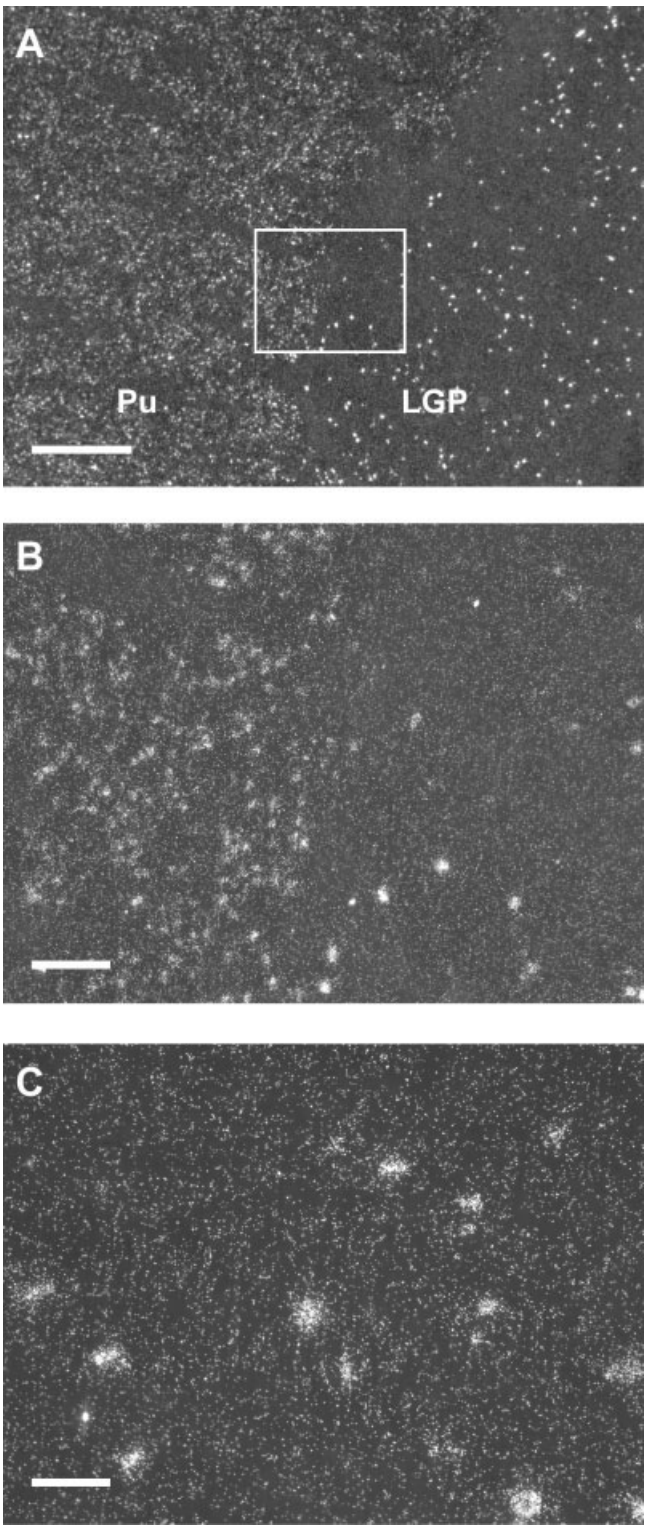

Fig. 3. Expression of calcyon mRNA in dorsal striatum and globus pallidus. **A:** Striatopallidal boundary at low power demonstrating widespread labeling in dorsal striatum and scattered large neurons in the globus pallidus. AP level approximately ac –4 mm. Scale bar = 1 mm. **B:** Highlighted region indicated by rectangle in A. Scale bar = 0.2 mm. **C:** Calcyon transcript expression in scattered large neurons in globus pallidus. Pu, putamen; LGP; lateral globus pallidus. Scale bars = 0.1 mm.

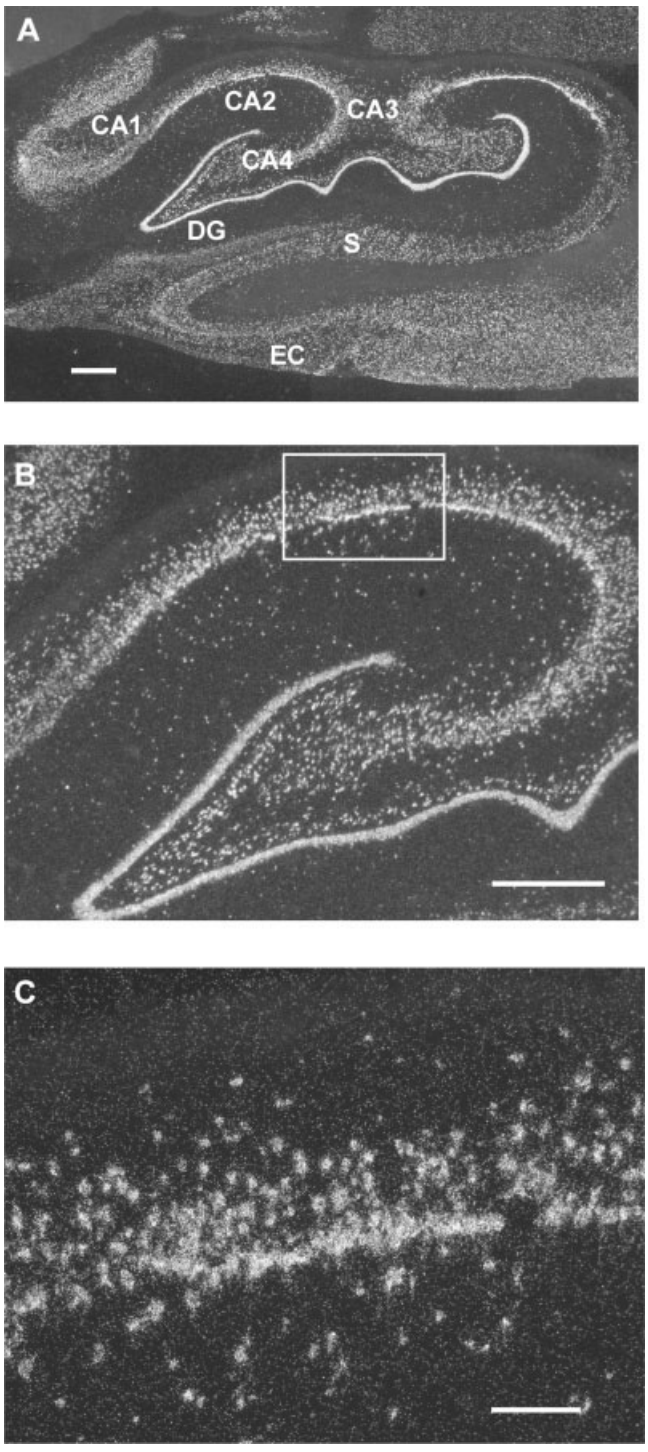

Fig. 4. **A:** Composite darkfield photomicrograph demonstrating distribution of calcyon in medial temporal lobe structures at AP level of ac –7 mm. Scale bar = 1 mm. **B:** Low-power view of high level of expression in dentate gyrus (DG), with moderate expression in hippocampal subfields (CA1-CA4), subiculum (S), and entorhinal cortex (EC). Scale bar = 1 mm. **C:** High-power view of CA2 hippocampal pyramidal field indicated by rectangle in B. Scale bars = 0.2 mm.

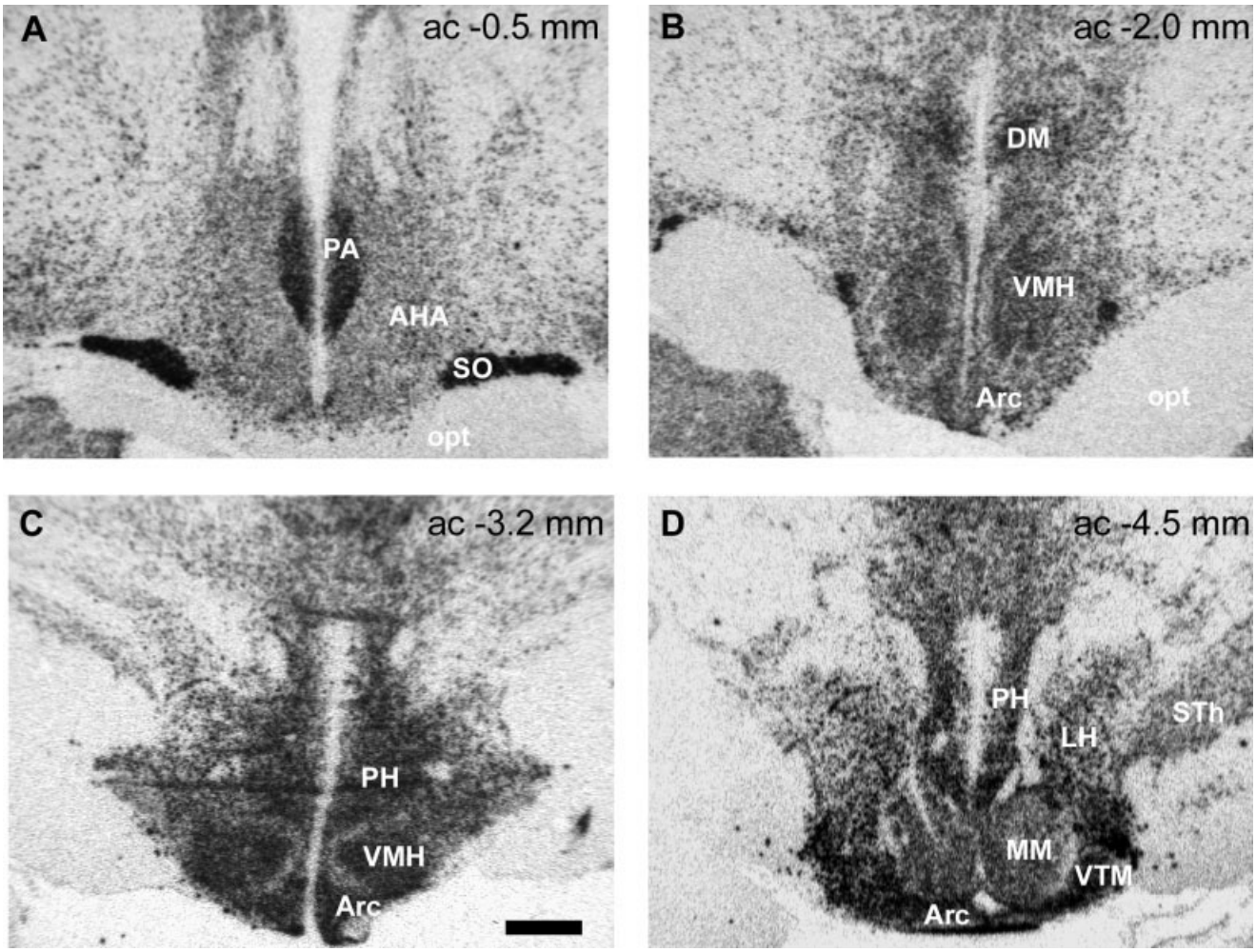

Fig. 5. Higher magnification macroscopic image displaying expression of calcyon mRNA in hypothalamic nuclei. Note very high mRNA expression in supraoptic (SO) and paraventricular (PA) nuclei (**A**) as well as abundant expression in ventromedial (VMH) (**B,C**) and arcuate (Arc) nuclei (**C,D**). Scale bar = 1 mm.

This also may relate to the association of calcyon with D5 receptors, based on reports of the localization of D5 mRNA and immunoreactivity in these regions in human, monkey, and rat (Beischlag et al., 1995; Khan et al., 1995; Ciliax et al., 2000), or could relate to the presence of other Gq/11-coupled monoamine receptors on these neurons.

The hypothalamus is also a region in which calcyon appears to overlap with D5 expression. Calcyon transcripts are abundantly expressed throughout the hypothalamus. Particularly dense labeling is present in the supraoptic nucleus (SO) and paraventricular nucleus (Pa), both areas which have been demonstrated to contain populations of tyrosine hydroxylase-expressing neurons (Swanson et al., 1981; Kawano and Daikoku, 1987; Ciliax et al., 2000). Evidence does exist for both D1 and D5 receptor expression in the supraoptic, suprachiasmatic, and paraventricular hypothalamic nuclei (Ciliax et al., 2000), and they are also sites in which c-fos can be induced by D1-type agonist infusion (Rivkees and Lachowicz, 1997). Similarly, the arcuate nucleus (ArcH) and ventromedial hypothalamus (VMH) also express calcyon mRNA at high levels. These areas are known to be areas in which dopamine acts via both D1 and D5 receptors to facilitate female reproductive behaviors in rats (Zhou et al., 1999), although a dopaminergic role in analogous primate behaviors remains to be elucidated. Calcyon may prove to play an important role in the integration of signaling in these processes, as the release of certain hypothalamic peptides, such as gonadotropin-releasing hormone (GnRH), is regulated by neurotransmitters such as acetylcholine, which can act to prime the interaction between calcyon and dopamine receptors. In addition, many of these peptides are themselves agonists at Gq/11-coupled receptors, leaving open the possibility that peptide neuromodulators can also "prime" the activation of calcyon for dopamine.

D5 receptor activation has also been implicated in the calcium-dependent induction of long-term potentiation of EPSPs in striatal aspiny cholinergic neurons (Suzuki et al., 2001), it is conceivable that calcyon coupling to D5 receptors could also provide a mechanism for this observation, and would be consistent with previous observations of D5 but not D1 receptor expression in cholinergic

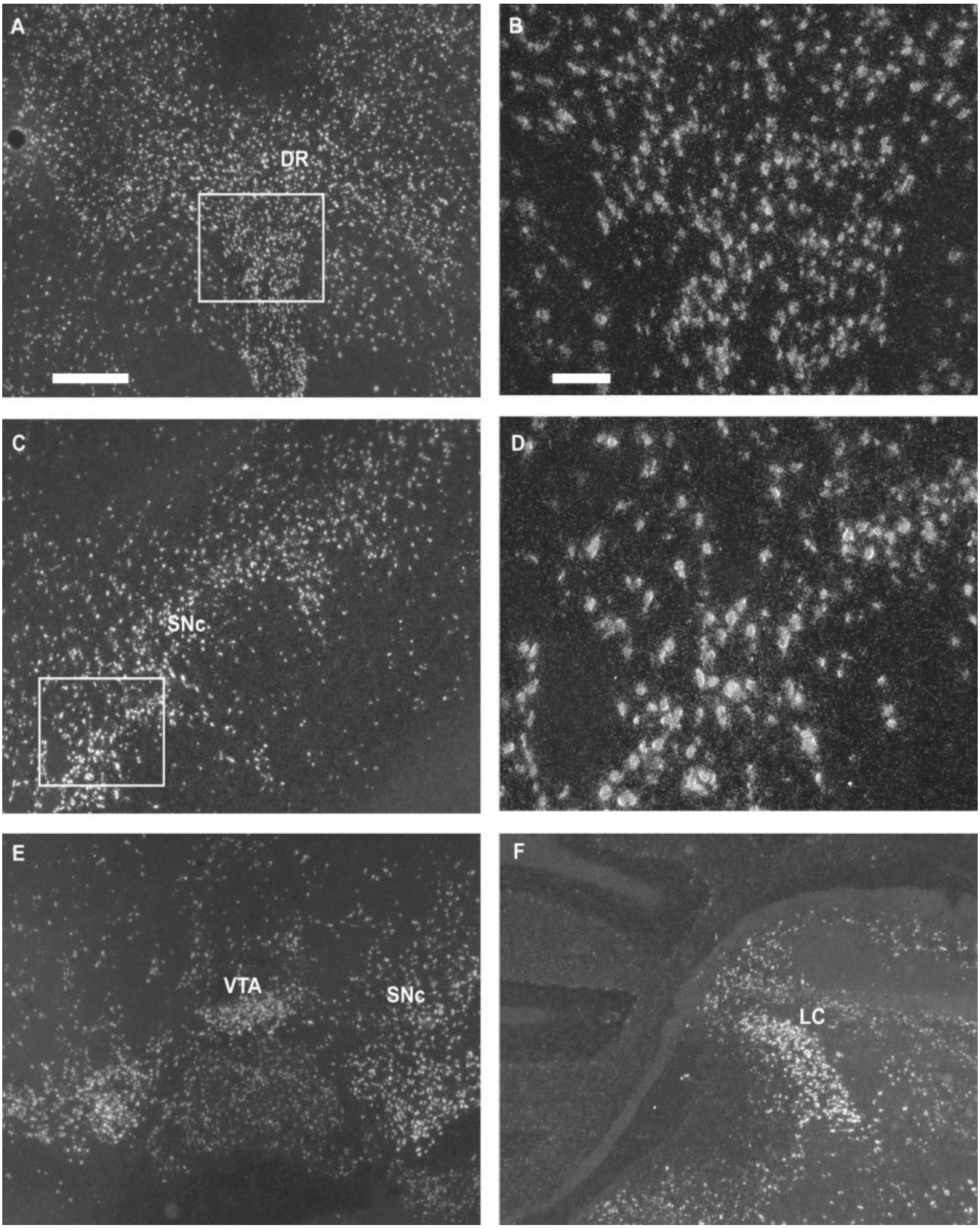

Fig. 6. Calcyon mRNA is abundantly expressed in multiple monoamine-synthesizing nuclei. **A,B:** Dorsal raphe nucleus (DR). AP level = ac –14 mm. **C,D:** Substantia nigra, pars compacta (SNc). AP level = ac –8 mm. **E:** Substantia nigra and ventral tegmental area (VTA). AP level = ac –10.5 mm. **F:** Locus coeruleus. AP level = ac –19 mm. Rectangles highlighted in A and C indicate area shown at higher power in B and D, respectively. Scale bar = 1 mm in A (applies to A,C,E,F); 0.2 mm in B (applies to B,D).

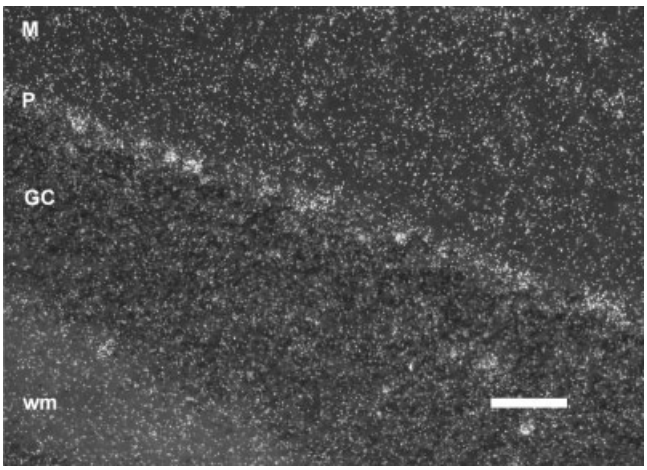

Fig. 7. High-power darkfield image demonstrating prominent Purkinje cell layer expression of calcyon mRNA in cerebellum. M, molecular layer; P, Purkinje cell layer; GC, granular cell layer; wm, white matter. Scale bar = 0.1 mm.

cells in this area (Rappaport et al., 1993; Bergson et al., 1995). Although we do not offer specific evidence to indicate the neurochemical identity of individual striatal cells, we do find scattered large calcyon-positive perikarya in the globus pallidus (GP) which are distributed similarly to the cholinergic cells in this region. As D1 receptors are not known to be expressed in the GP, whereas D5 receptors are present at low levels (Beischlag et al., 1995), it is possible that this represents a coexpression of calcyon with D5 receptors in this region. Further association of calcyon with the D5 receptor may also be evident in the high level of expression seen in the thalamic parafasicular nucleus, which is indicated by a previous study as the only thalamic nucleus which expresses appreciable D5 mRNA in rat brain (Meador-Woodruff et al., 1992).

As in an earlier report of calcyon distribution in rat brain (Zelenin et al., 2002), this study in primate reveals areas in which the distribution of calcyon overlaps with those reported for D1-like receptors, both D1 and D5, in brain. In many areas, the presence of calcyon transcripts in areas not associated with D1 transcripts can largely be attributed to an overlap with D5. Of these, it is striking that the most intense calcyon signals are observed in areas where D5 predominates, such as in the hippocampus and the monoaminergic nuclei. Nevertheless, we also note the presence of calcyon expression in some areas not associated with either D1 or D5 signaling. There are moderate to high levels of calcyon expressed in several brainstem regions, particularly the vestibular nuclei, nucleus of the solitary tract, and pontine and medullary reticular fields, which are not known to express D1 or D5 message. Likewise, in the cerebellum, although studies have reported D5 protein expression by Purkinje cells in rat and human (Ciliax et al., 2000; Khan et al., 2000), most D5 immunoreactivity has been observed in neuropil, suggestive of an origin in extracerebellar neurons such as the inferior olivary complex, which has been shown to express D5 protein.

We believe that further studies examining the colocalization of calcyon with specific Gq- or G11-coupled neurotransmitter receptors such as muscarinic acetylcholine, serotonin (5HT-1C, 5HT-2), and metabotropic glutamate receptors will highlight other specific regions or systems within the brain in which these interactions play an important physiologic role. It is interesting that comparing the distribution of calcyon to the reported distributions of the alpha subunits of Gq and G11 (Friberg et al., 1997; Mailleux et al., 1992) reveals a number of similarities: a widespread, multilaminar cortical expression, moderate striatal expression, abundant hypothalamic expression, and widespread brainstem expression, including localization in monoaminergic nuclei. This is not surprising, given the variety of neurotransmitters which couple to Gq or G11 as signal transduction mechanisms, but does call attention to the complexity of the neurotransmitter actions of which calcyon is a part.

Overall, our findings confirm the relationship between calcyon and dopaminergic signaling at D1 and D5 receptors, while suggesting the possibility of a wider role in intracellular mechanisms which coordinate and regulate signaling by multiple neurotransmitters, particularly neuromodulators. Whether this wider role involves associations with other G-protein-coupled monoaminergic or peptidergic receptors, or involvement in an alternative function such as protein trafficking, remains a question for further research. In general, the understanding of these mechanisms is likely to have important clinical implications. For example, it has become increasingly apparent that psychiatric diseases such as schizophrenia are best understood as dysregulations of multiple interacting neurotransmitter systems, rather than as a dysfunction of a single neurotransmitter or receptor. Koh et al. (2003), have recently shown that calcyon is measurably upregulated in the dorsolateral prefrontal cortex in schizophrenia. Future examination of the function and regulation of molecules such as calcyon will take on greater importance in our understanding of the complex pathophysiology of mental illness.

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