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THE IMMUNOPHILINS, FK506 BINDING PROTEIN AND CYCLOPHILIN, ARE DISCRETELY LOCALIZED IN THE BRAIN: RELATIONSHIP TO CALCINEURIN

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Abstract—The immunosuppressant drugs cyclosporin A and FK506 bind to small, predominantly soluble proteins cyclophilin and FK506 binding protein, respectively, to mediate their pharmacological actions. The immunosuppressant actions of these drugs occur through binding of cyclophilin-cyclosporin A and FK506 binding protein–FK506 complexes to the calcium-calmodulin-dependent protein phosphatase, calcineurin, inhibiting phosphatase activity. Utilizing immunohistochemistry, *in situ* hybridization and autoradiography, we have localized protein and messenger RNA for FK506 binding protein, cyclophilin and calcineurin. All three proteins and/or messages exhibit a heterogenous distribution through the brain and spinal cord, with the majority of the localizations being neuronal. We observe a striking co-localization of FK506 binding protein and calcineurin in most brain regions and a close similarity between calcineurin and cyclophilin. FK506 binding protein and cyclophilin localizations largely correspond to those of calcineurin, although cyclophilin is enriched in some brain areas that lack calcineurin.

The dramatic similarities in localization of FK506 binding proteins and cyclophilins with calcineurin suggest related functions.

Cyclosporin A, the immunosuppressant drug widely used for organ transplantation, binds to a family of small soluble receptor proteins designated as cyclophilins.[29] The subsequently developed immunosuppressant, FK506, is substantially more potent than cyclosporin A but has a similar pharmacological profile.[15,26,39] FK506 binds to a family of binding proteins, designated FK506 binding proteins (FKBPs), which are distinct from the cyclophilins. Both the cyclophilins and the FKBPs display peptide prolyl isomerase activity, involved in folding of proteins, which had been suggested as the common mode of therapeutic action for the immunosuppressants. However, substantial discrepancies between effects on immunosuppression and affinity for FKBPs indicated that inhibition of peptide prolyl isomerase cannot explain the therapeutic actions of these drugs. Recently, the FK506–FKBP and cyclosporin A–cyclophilin complexes were shown to bind to the calcium-dependent phosphatase calcineurin, inhibiting phosphatase activity.[36] The relative potencies of drugs in inhibiting calcineurin activity, when interacting with the drug–immunophilin complex, correlate with their immunosuppressant actions, establishing that inhibition of calcineurin activity is responsible for the pharmacological actions of these agents.[6,32,42]

The great majority of research on the immunophilins has focused on lymphocytes and lymphocyte-containing tissues such as the thymus, which displays the highest density of immunophilins of peripheral tissues. Very recently we discovered that levels of the immunophilin, FKBP, in the brain are 10–40 times greater than those in lymphocyte-containing tissues and that FKBP is discretely localized to specific neuronal populations.[33] Moreover, limited localization studies indicated a close similarity in the distribution of calcineurin and FKBP, implying linked functions.

In the present study we have examined the detailed localizations of FKBP and cyclophilin in rat brain and show selective neuronal distributions for these two proteins. We have also compared these localizations with that of calcineurin, revealing dispositions that imply related functions.

EXPERIMENTAL PROCEDURES

Autoradiography

Adult male Sprague–Dawley rats (Charles River Labs) were deeply anaesthetized with sodium pentobarbital (100 mg/kg) and perfused with 200 ml ice-cold 50 mm phosphate-buffered saline. The brain was rapidly removed and frozen in isopentane (−80°C). Serial sagittal and coronal sections, 20 μm thick, of rat brain were cut on a cryostat

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Abbreviations: FKBP, FK506 binding protein; GAP, growth-associated protein; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; P, postnatal day; TX-100, Triton X-100; TBS, Tris-buffered saline.

(Microm) and thaw-mounted on to chrome–alum–gelatin-coated slides. Slide mounted tissue sections were preincubated for 60 min in a buffer containing 50 mM HEPES, 2 mg/ml bovine serum albumin, 0.02% Tween-20, 5K ethanol, pH 7.4. This was followed by incubating sections for 60 min in fresh buffer with 1 nM [^{3}H]FK-506 (85.5 Ci/mmol; Dupont-NEN, Boston). Non-specific binding was defined by the addition of 1 μM FK-506. Following incubation, the slide-mounted tissue sections were rinsed for $\times$ 5 min in fresh buffer (4°C) and dried. The radiolabeled sections were juxtaposed to tritium-sensitive film for two to three weeks. Under these conditions, non-specific binding was routinely at background levels.

In situ *hybridization*

In situ hybridization was performed as described previously.[22] Antisense oligonucleotides were end-labeled with [^{35}S]dATP. FKBP-12 *in situ* hybridization employed antisense oligonucleotides, complementary to nucleotides 70–114, ATA AAA TTT CTT TCC ATC TTC AAG CAT CCC GGT GTA GTG CAC CAD, 214–258 AGT GGC ACC ATA GGC ATA ATC TGG AGA TAT AGT CAG TTT GGC TCT; 441–485, GAG TGA CAG AAC ACA TTC AGT CAG GGC AGA TGT CTA TAC AAA GTG of the cloned cDNA.[13,35] For calcineurin Aα antisense oligonucleotides, complementary to the nucleotides 1363–1410, TCG GAT CTT GTT CCT GAT GAC CTC CTT CCG GGC TGC AGC CGT GGC TCC and 1711–1758, GCT ATT ACT GCC GTT GCT GTC TGT GCC GTT AGT CTC TGA GGC GAG AGC;[23] and for calcineurin Aβ 1339–1386, TCC TCC AGC CAA CAC TCC ACT AGG CAA CAT CCC TGT GGG AGT CAG GCC and 1569–1616, CTG GGC ACT ATG GTT GCC AGT CCC GTG GTT CTC AGT GCT ATG TGC[20] were used. For cyclophilin antisense oligonucleotides were complementary to nucleotides 171–218, CCT GGA ATA ATT CTG AGT AAG GAG GAA CCC TTA TAG CCA AAT CCT TTC; 301–348, ATT TGC CAT GGA CAA GAT GCC AGG ACC TGT ATG CTT CAG GAT GAA GTT and 682–729, AAT CAT AAA CTT AAC TTT GCA ATC CTG CTA GAC TTG AAG GGG AAT GAG of the cloned cDNA.[30]

Immunohistochemistry

Adult male Sprague–Dawley rats were deeply anesthetized with sodium pentobarbital (100 mg/kg) and perfused transcardially with ice-cold phosphate-buffered saline followed by perfusion with 4% freshly depolymerized paraformaldehyde in 0.1 M phosphate buffer (PB). The brains were removed and post fixed in 4% paraformaldehyde 0.1 M PB for 2 h and then cryoprotected in 20% glycerol/0.1 M PB (v/v) overnight at 4°C. Free-floating 40-μm-thick sections were cut on a sliding microtome (Microm) and were stored in Tris (50 mM Tris-HCl)-buffered saline (TBS). The sections were permeabilized with 0.2% (v/v) Triton X-100 (TX-100) in TBS for 30 min followed by blocking with 4% (v/v) normal goat serum 0.1% (v/v) TX-100 in TBS (25°C). The sections were then incubated in TBS (4°C) containing 2% (v/v) normal goat serum; 0.1% (v/x) TX-100 and affinity purified calcineurin antiserum (1:50; gift from C. Klee, NIH). The sections were stained with an avidin–biotin–peroxidase system (Vector Labs) with diaminobenzidine as the chromagen.

Mutant mice

Mice were obtained from Jackson Laboratories (Bar Harbor, ME). The mutants were Lurcher and Reeler.[14,18] Sections of mouse brain were processed for autoradiography as described above.

RESULTS

FK506 binding protein is discretely localized in rat brain

Our preliminary studies indicated that [^{3}H]FK506 binding to FKBP, assessed by autoradiography, and FKBP-12 mRNA, monitored by *in situ* hybridization, display different densities throughout rat brain. To explore localizations of FKBP in greater detail, we have examined coronal sections of rat brain (Fig. 1).

Abbreviations used in the figures

5	motor nucleus of trigeminal nerve	MM	medial mammillary nucleus
Ao	anterior olfactory nucleus	Mol	molecular layer
Acb	accumbens nucleus	MP	medial posterior hypothalamic nucleus
BS	brainstem	OB	olfactory bulb
BST	bed nucleus of the stria terminalis	OC	occipital cortex
CA1–CA4	fields of Ammon's horn of the hippocampus	pH	posterior hypothalamus
CB	cerebellum	Pi	pineal gland
cc	corpus collusum	Pn	pons
CG	central gray	Pn	pontine nucleus
CP	caudate–putamen	PVT	paraventricular thalamac nuclei
CTx	cortex	RLi	rostral linear nucleus of raphe
DG	dentate gyrus	RS	retrosplenial cortex
Ent	enthorhinal cortex	S	subiculum
FP	frontoparietal cortex	sc	spinal cord
Fr	frontal cortex	SC	superior colliculus
GP	globus pallidus	Sch	suprachiasmatic nuclei
Gr	granule cell layer	SG	substantia gelatinosa
HC	hippocampus	SNC	substantia nigra compacta
IC	inferior colliculus	SNR	substantia nigra reticulata
IGr	internal granule cell layer	SO	supraoptic nuclei
La	lateral amygdaloid nucleus	T	thalamus
LG	lateral geniculate nucleus	Tu	olfactory tubercle
MG	medial geniculate necleus	VL	ventrolateral nucleus
MHb	medial habenula nucleus	VMH	ventromedial hypothalamic nucleus
Mi	mitral cell layer	VP	ventroposterior thalamic nucleus

Fig. 1. Dark-field photomicrographs illustrating the distribution of [^{3}H]FK506 in rat brain and spinal cord. In these images the densest labeling appears white against a black background. Scale bar = 5 mm.

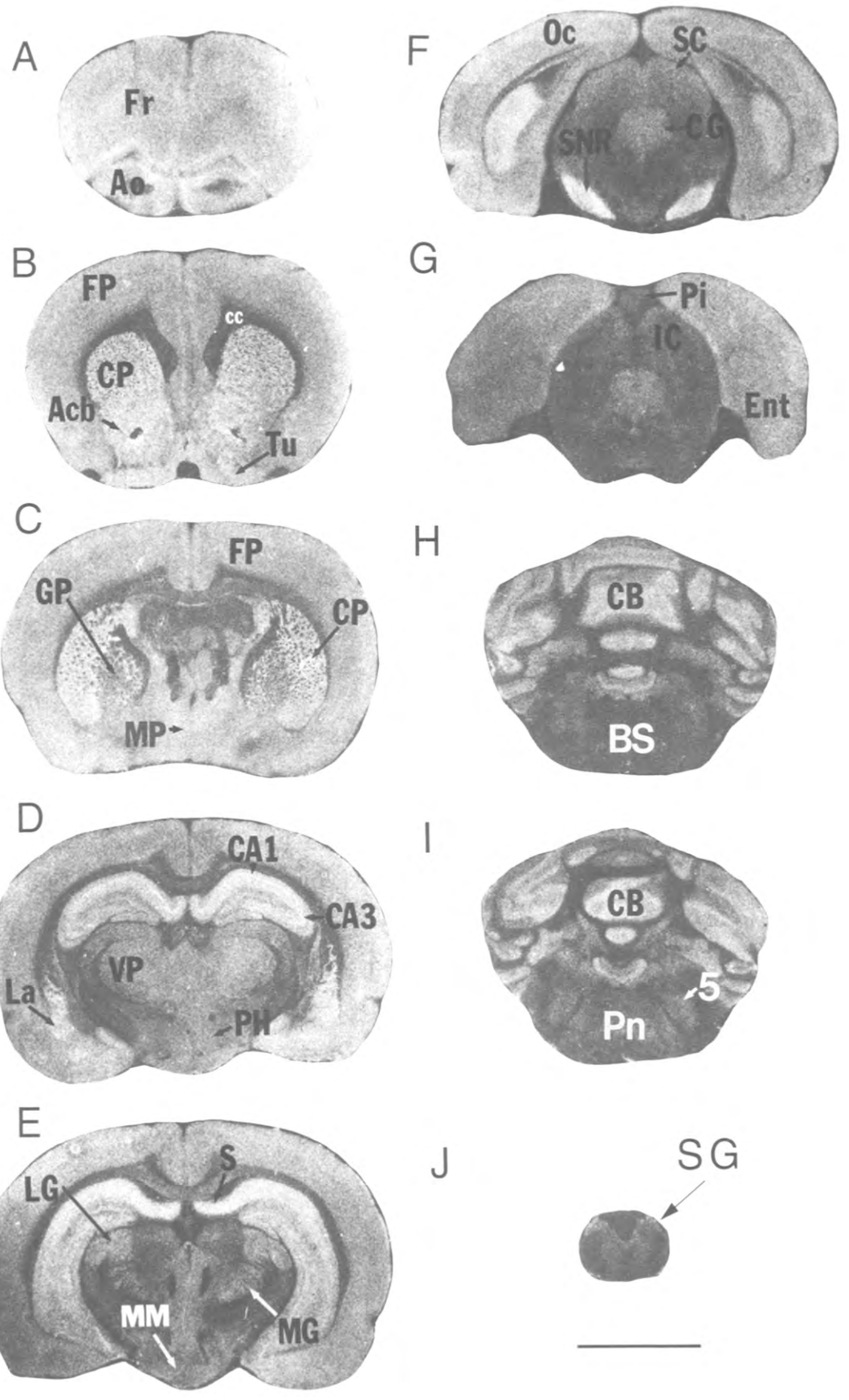
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Fig. 1.

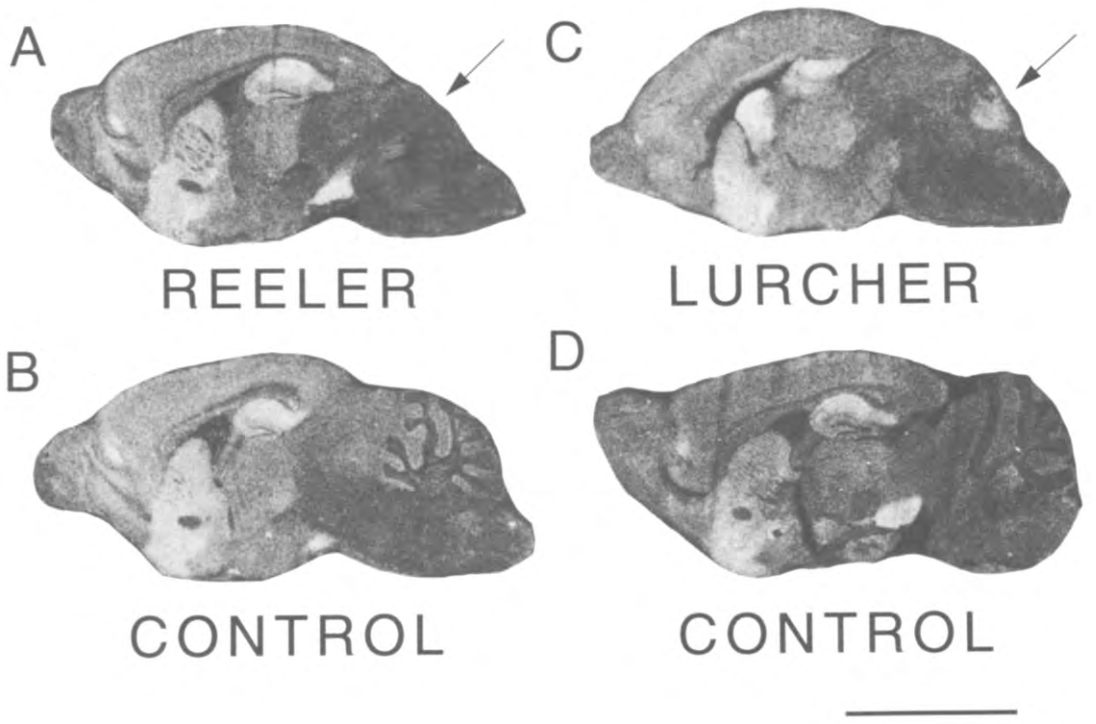

Fig. 2. [^{3}H]FK506 binding in sagittal sections of mutant mice. Parasagittal sections taken from Reeler (A, B) or Lurcher (C, D) mice were processed for [^{3}H]FK506 autoradiography. Littermate controls are shown below mutant mice. Note the complete absence of [^{3}H]FK506 binding in the cerebellum of Reeler mice and preservation of binding in Lurcher cerebellum. Scale bar = 5 mm.

As reported earlier,[33] [^{3}H]FK506 binding to brain slices on microscope slides is saturable and inhibited by drugs with potencies corresponding to their affinities for [^{3}H]FK506 binding to FKBP in brain and thymus extracts[33] (data not shown). Furthermore, the relative density of [^{3}H]FK506 binding determined autoradiographically parallels the relative density of [^{3}H]FK506 binding on membrane fractions[33] (data not shown). Substantial levels of FKBP are evident in the anterior olfactory nucleus and nearby cerebral cortex, while the olfactory track is unlabeled (Fig. 1). Higher levels of FKBP are evident in the caudate–putamen, accumbens nucleus and olfactory tubercle, with somewhat lower densities apparent in the adjacent globus pallidus. Our preliminary studies have indicated high levels of FKBP in the hippocampus.[33] These are discretely localized with highest levels in CA1 and lower amounts of FKBP in CA3 and the dentate gyrus. Very high densities are also evident in the amygdala, with the lateral amygdaloid nucleus particularly enriched.

The thalamus displays notable heterogenicity, with negligible levels of FKBP in the medical and lateral habenula nuclei, but much higher levels in the ventral posterior and lateral and medial geniculate nuclei. Moderate levels of FKBP are evident throughout the hypothalamus.

Very high densities of FKBP occur in the substantia nigra zona reticulata. FKBP here derives from cell bodies in the caudate–putamen, as we earlier established by quinolinic acid lesions of the caudate.[33] In other portions of the midbrain, low to moderate levels of FKBP are evident in the dorsal raphe nucleus and adjacent central gray, as well as the mammillary bodies. Low to moderate FKBP occur in the pineal gland and superior and inferior colliculli.

The lower brain stem displays very little FKBP. In the spinal cord, a striking density of FKBP is apparent in the substantial gelatinosa, with very little FKBP in other portions of the gray or white matter of the spinal cord.

The cerebellum contains substantial FKBP, most concentrated in the molecular layer (Figs 1, 5). *In situ* hybridization reveals FKBP mRNA most enriched in the granule cells which presumably are the source of the FKBP protein in parallel fibers within the molecular layer[33] (Fig. 3). To further ascertain the cellular disposition of cerebullar FKBP, we have utilized mutant mice (Fig. 2). In reeler mice, which lack cerebellar granule cells, FKBP is absent from the cerebellum. By contrast, Lurcher mice, with a shrunken cerebellum devoid of Purkinje cells but with a relatively normal complement of granule cells, display intense labeling for FKBP. This established that the major if not sole localization of FKBP in the cerebellum involves the granule cells and their processes.

mRNA levels for cyclophilin and FI506 binding protein display notable similarities, but selective differences

Multiple forms of FKBP have been identified, but interactions with calcineurin are demonstrable only for FKBP-12,[36] which therefore is the form of FKBP for which we have conducted *in situ* hybridization (Figs. 3 and 4, Table 1). Several forms of cyclophin also exist. The 18,000 and 22,000 mol. wt cyclophilins have been cloned while the remaining forms have not yet been moderately cloned.[5,8,30] The sequences of the 18,000 and 22,000 mol. wt forms are extremely similar and the oligonucleotide probes we have employed for cyclophilin *in situ* hybridization recognises both 18,000 and 22,000 mol. wt forms. Together,

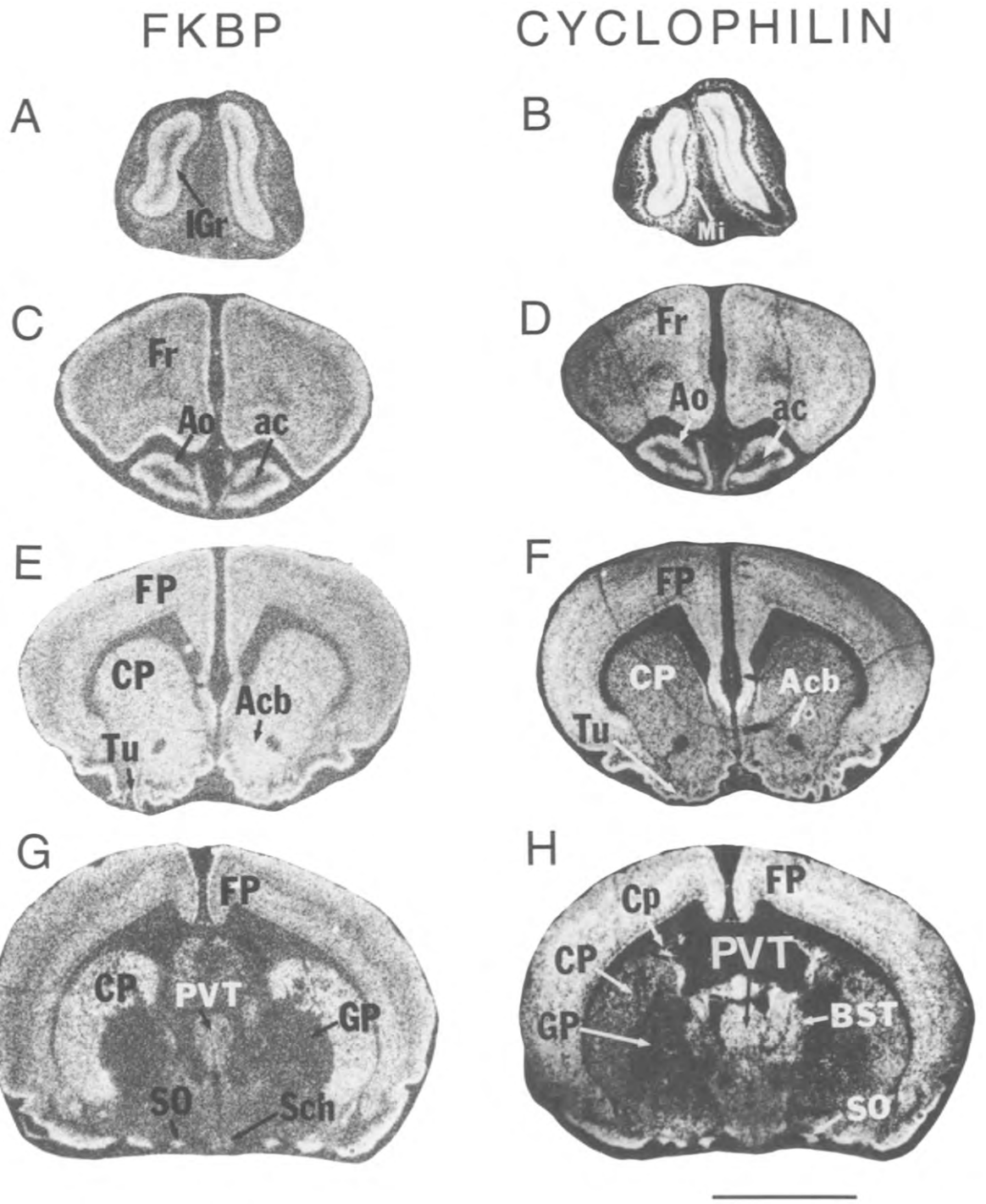

Fig. 3. (*part 1*).

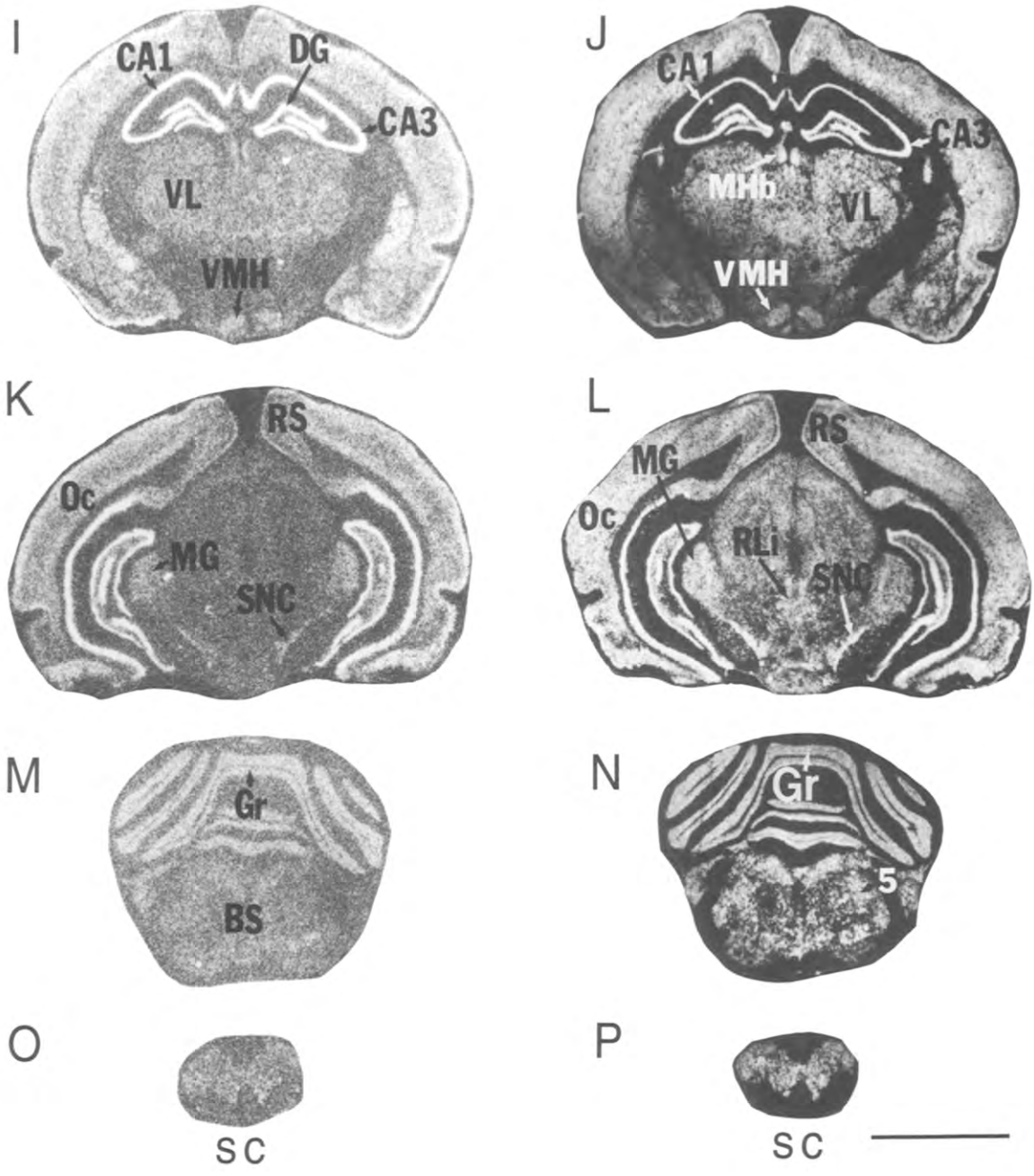

Fig. 3. (*part 2*). Coronal sections of rat brain and spinal cord processed for FKBP-12 and cyclophilin *in situ* hybrization to illustrate and compare the localization of mRNA for FKBP-12 and cyclophilin. Scale bar = 5 mm.

these two forms of cyclophilin are quantitatively the major ones in all body regions evaluated[5,8,30] and interactions with calcineurin are demonstrable only for these two forms.[6]

For *in situ* hybridization, we have constructed three distinct oligonucleotide probes each for FKBP-12 and cyclophilin. All three probes for FKBP-12 and cyclophilin, respectively, provide identical localizations, so that they have been pooled for *in situ* hybridization studies. The identical localizations observed with three discrete probes for FKBP-12 and cyclophilin, respectively, ensure that the observed localizations are specific. Additionally, we have employed sense probes for both FKBP-12 and cyclophilin, which provide no hybridization signal in brain, and RNAse treatment of tissue sections completely eliminates hydridization of anti-sense probes (data not shown).

mRNA localizations for FKBP-12 and cyclophilin display marked similarities, but a number of notable

Table 1. Brain distribution of FK506 binding protein, cyclophilin and calcineurin mRNA

	FKBP	Cyclophilin	Calcineurin
Olfactory bulb			
Internal granule cell layer	80	100	80
Mitral cell layer	40	100	40
Periglomerular cell layer	40	100	40
Anterior olfactory nucleus	80	100	80
Basal ganglia			
Caudate–putamen	100	60	80
Nucleus accumbens	100	60	80
Olfactory tubercle	100	80	80
Globus pallidus	40	20	40
Hippocampal formation			
Pyramidal cell layer CA1	100	100	100
Pyramidal cell layer CA2	100	100	100
Pyramidal cell layer CA3	100	100	100
Granule cell layer of dentate gyrus	100	100	100
Thalamus			
Paraventricular nucleus	60	100	60
Ventrolateral nucleus	60	100	60
Medial habenular nucleus	0	100	0
Medial geniculate nucleus	60	100	60
Hypothalamus			
Supraoptic nucleus	100	100	100
Suprachiasmatic nucleus	60	60	60
Ventromedial nucleus	80	60	80
Posterior nucleus	60	60	60
Cerebral cortex			
Layer 1	100	60	100
Layer 2	80	60	80
Layer 3	60	80	60
Layer 4	60	80	60
Layer 5	80	80	80
Layer 6	100	80	100
Cerebellum			
Molecular cell layer	0	0	0
Granule cell layer	100	100	100
Deep cerebellar nuclei	60	60	40
Mid brain			
Substantia nigra compacta	40	100	40
Substantia nigra reticulata	0	0	0
Superior colliculus	40	60	40
Linear raphe nucleus	20	100	20
Additional areas			
Reticular formation	40	100	40
Cranial nerve 5	20	100	20
Cranial nerve 7	20	100	20
Pontine nuclei	0	80	0
Subfornical organ	0	100	0
Bed nucleus stria terminalis	0	100	0
Choroid plexus	0	80	0
Spinal cord			
Substantia gelatinosa	40	60	40
Dorsal horn	40	60	40
Ventral horn	40	60	40

The distributions of FKBP and calcineurin are virtually identical and the cyclophilin distribution is very similar to that of FKBP and calcineurin. The values for FKBP, cyclophilin and carcineurin are from the *in situ* hybridization presented and range from lowest (0%) to highest (100%) based on visual inspection of the average of three to six sets of *in situ* hybridization experiments.

differences (Fig. 3, Table 1). Both are enriched in the granule cell layer of the olfactory bulb, but cyclophilin is also concentrated in mitral cells and periglomerular cells, which contain low amounts of FKBP-12 message. Both immunophilins display enrichment in the anterior olfactory nucleus, with no message in the olfactory track. Both are enriched in the cerebral cortex, with FKBP-12 mRNA being most highly concentrated in superficial layers, a distribution not evident for cyclophilin.

The caudate–putamen, nucleus accumbens and olfactory tubercle are highly enriched in FKBP message, but have only modest levels of cyclophilin. By contrast, the paraventricular thalamic nucleus, the

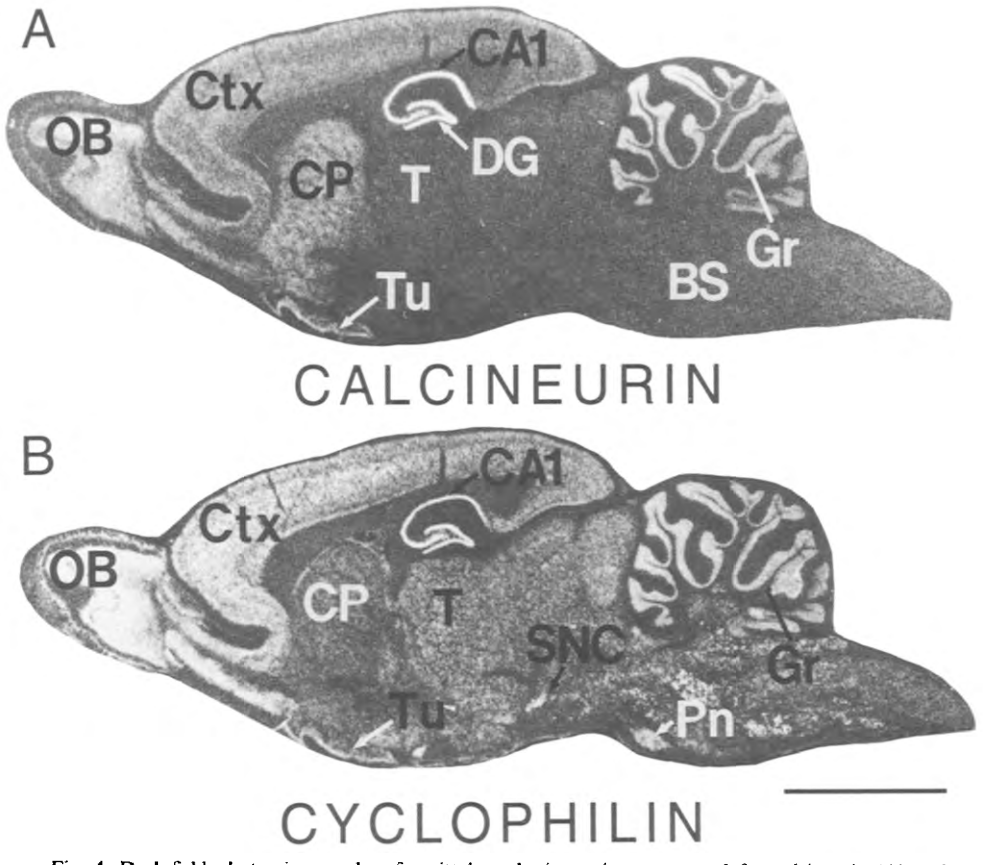

Fig. 4. Dark-field photomicrographs of sagittal rat brain sections processed for calcineurin (A) and cyclophilin (B) *in situ* hybridization to compare the distributions of mRNA for calcineurin and cyclophilin. Scale bar = 5 mm.

adjacent subfonical organ and the bed nucleus of the stria terminalis are highly enriched in cyclophilin message but relatively devoid of FKBP. Similarly, the choroid plexus displays very high densities of cyclophilin but not FKBP mRNA.

Message for both FKBP and cyclophilin is greatly enriched in all layers of the hippocampus and dentate gyrus. The medial habenula and related thalamic nuclei display substantially more cyclophilin than FKBP message.

While FKBP protein is highly concentrated in the substantial nigra zone reticulata, deriving from cell bodies in the caudate, no FKBP mRNA occurs in the zonta reticulata, while moderate levels are evident in the zona compacta. Cyclophilin messages are highly enriched in the zona compacta. Throughout the midbrain and lower brain stem, much higher levels of cyclophilin than FKBP message are evident, as is also true in the spinal cord.

We have not compared cyclophilin and FKBP protein localizations. We have been unsuccessful in detecting levels of specific [^{3}H]cyclosporin A binding autoradiographically that can be discriminated above non-specific binding, and all available antibodies to cyclophilin fail to provide adequate labeling for immunohistochemistry (data not shown).

Calcineurin localizations resemble FK506 binding protein and cyclophilin

If the immunophilins act in the brain through regulating calcineurin, then their localizations should resemble those of calcineurin. In preliminary studies, we noted similarities between FKBP and calcineurin localizations.[33] In the present study we have compared the distribution of calcineurin with both cyclophilin and FKBP. Multiple isoforms of calcineurin have been identified from brain and represent alternative splicing of two distinct gene products designated calcineurin Aα and Aβ.[12,27,31,38] A testes-specific calcineurin has also been cloned.[2,25] We pooled oligonucleotide probes representing both calcineurin Aα and Aβ for *in situ* hybridization. Together these probes are quantitatively the major ones in the brain.[38]

In situ hybridization reveals notable similarities between the localization of mRNA for cyclophilin and calcineurin (Fig. 4, Table 1). Both are highly concentrated in granule cells of the cerebellum. Both are intensely stained in all areas of the hippocampus and dentate gyrus. Both are concentrated in the granule cell layer of the olfactory bulb. Additionally, cyclophilin message is evident in the mitral cells and

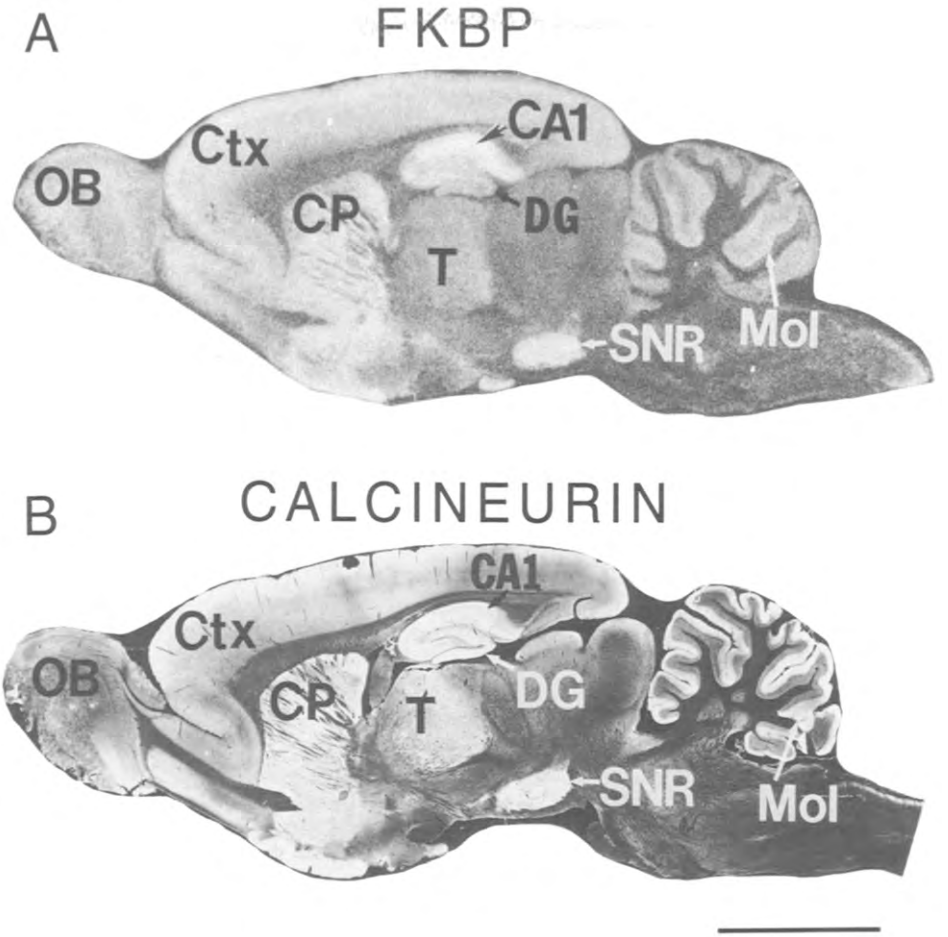

Fig. 5. Co-localization of FKBP and calcineurin. Sagittal sections of rat brain processed for [^{3}H]FK506 binding and calcineurin immunohistochemistry to illustrate the co-localization of FKBP and calcineurin. Scale bar = 5 mm.

periglomerular cells, which display much lower levels of calcineurin message. Similarly, cyclophilin message in the brain stem greatly exceeds levels for calcineurin. By contrast, calcineurin densities in the caudate greatly exceed those of cyclophilin. Thus, FKBP-12 appears to be the predominant immunophilin in the caudate–putamen.

Calcineurin localizations resemble those of FKBP more than cyclophilin (Fig. 5, Table 1). Thus, calcineurin and FKBP are both highly enriched in the caudate, nucleus accumbens and olfactory tubercle. Both display very low levels in the brain stem, but are highly concentrated in the zona reticulata of the substantially nigra. There are some notable differences. For instance, thalamic levels of the calcineurin substantially exceed those of FKBP and resemble the high densities of cyclophilin in the thalamus.

Calcineurin phosphatase activity is selective for a relatively limited number of proteins.[38] In our earlier study we showed that growth-associated protein 43 (GAP43) is one of the most prominent calcineurin substrates in the brain.[33] GAP43 is prominently associated with neuronal development with levels peaking seven to 10 days postnatally and diminishing markedly at later ages.[16,24] To assess whether FKBP disposition is intimately linked to GAP43, we monitored FKBP localization by autoradiography during ontogeny (Fig. 6). Negligible FKBP is evident at postnatal day (P) 5 with [^{3}H]FK506 binding becoming prominent at P14 and increasing to P30. The rate of development of FKBP is similar in all brain areas visualized. Interestingly, calcineurin levels develop with a time course essentially the same as that of FKBP.[21] Thus, the marked ontogenetic changes in GAP43 formation and function do not appear to be closely tied to FKBP.

DISCUSSION

The major finding of the present study is that the immunophilins FKBP and cyclophilin are highly concentrated in discrete neuronal sites in the brain. Our localizations for cyclophilin and calcineurin confirm and extend previous observations.[3,4,8,10,17,19,21,28,40] It is likely that most, if not all, these localizations are neuronal. Under high

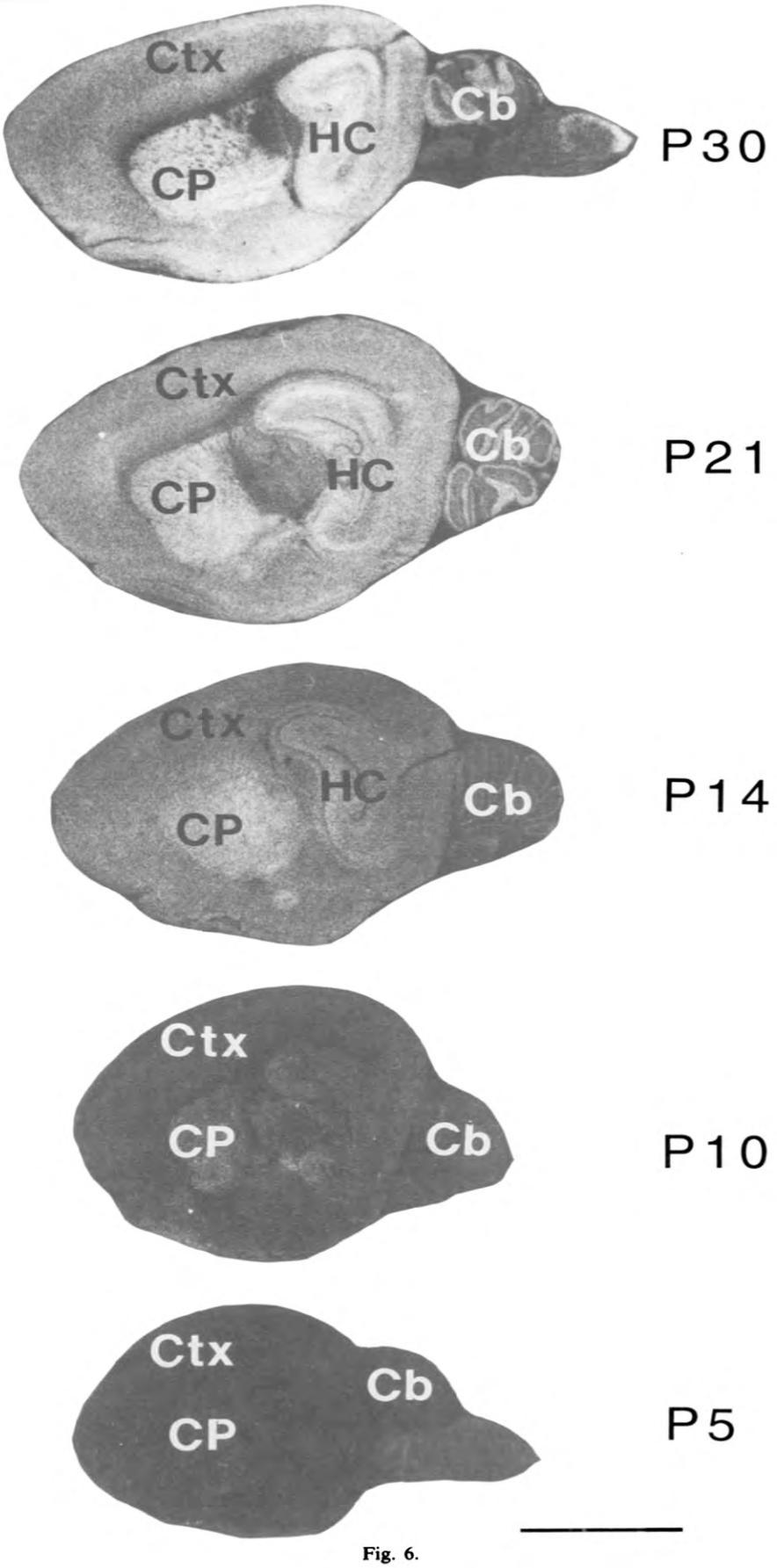
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Fig. 6.

magnification, *in situ* hybridization silver grains in all brain areas examined occur in neuronal cell bodies (data not shown). Earlier, we had reported high levels of FKBP in the brain that coincided with the distribution of calcineurin.[33] It is striking that the other immunophilin cyclophilin is also greatly enriched in the brain and in discrete neuronal populations.

In assessing the functions of immunophilins in the brain, specific localizations may be revealing. Localizations of FKBP and cyclophilin are similar, though there are a number of differences. Our present detailed localization studies fit with our preliminary evidence that FKBP and calcineurin are co-localized in most brain regions. We now find a similar co-localization for cyclophilin. These observations fit with the notion that immunophilins exert their major biological actions through inhibition of calcineurin activity. If this is the case and if cyclophilin and FKBP are responsible for calcineurin regulation in different neuronal populations, one might expect FKBP and cyclophilin to have similar localizations. For the most part all FKBP and cyclophilin localizations correspond to those for calcineurin. However, cyclophilin is enriched in some brain areas that lack calcineurin. Conceivably, our probes may interact with other forms of unidentified cyclophilin that do not act through calcineurin. Alternatively, there may be less abundant isoforms of calcineurin which more closely fit with the distribution of cyclophilin.

The very high levels of immunophilins in the brain and their striking localizations indicate some key regulatory function in the brain. For instance, FK-506 and cyclosporin A protect against glutamate-induced neurotoxicity in cortical cultures by preventing the calcineurin-mediated dephosphorylation of nitric oxide synthase.[11] Additionally, FK506 enhances neurotransmitter release by preventing calcineurin-mediated dephosphorylation of synaptic vesicle proteins[9] and FKBP-12 may influence neural regeneration.[37] Immunophilins influence calcineurin catalytic activity only when bound to immunosuppressant drugs. These observations strongly imply the existence of endogenous ligands for the immunophilins. This possibility has been suggested previously for cells of the immune system.[39] The much greater density of immunophilins in brain than in any other tissues implies that such ligands would be most enriched in the brain.

CONCLUSION

The neuronal associations of the immunophilins suggests that these postulated ligands might represent novel types of neuronal messengers, fitting with recent evidence for highly unusual neurotransmitter-like messenger such as nitric oxide,[7,34] carbon monoxide[41] and anandamide.[1]

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Fig. 6. Ontogeny of [^{3}H]FK506 binding. Parasagittal sections through rat brains were taken at postnatal (P) days 0, 2, 5, 10, 14, 21 and 30 and processed for [^{3}H]FK506 autoradiography. [^{3}H]FK506 binding is first detected at day 10. [^{3}H]FK506 binding increases until P30, which is equivalent to adult levels. Scale bar = 5 mm.

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