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Perspective

Immunophilins: Beyond Immunosuppression

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Introduction

In 1984, Gunther Fischer, a German biochemist studying protein folding, described an 18-kDa protein isolated from porcine kidney which catalyzed the interconversion of cis and trans rotamers of amide bonds adjacent to proline residues in peptidic substrates (Figure 1).[1,2] Fischer termed this enzymatic activity peptidylprolyl cis−trans isomerase activity, and the enzyme became known as PPIase. In that same year, Handschumacher and colleagues investigating the cellular actions of the immunosuppressant drug cyclosporin A (CsA) (Figure 2) isolated a protein from calf thymus that was the principal binding protein for CsA.[3] They dubbed this protein cyclophilin (CyP). By 1989, it became clear that PPIase and cyclophilin were the same protein.[4,5] At the same time, Stuart Schreiber, as well as workers at Vertex Pharmaceuticals, reported the identification and characterization of the cytosolic binding protein for another immunosuppressant drug, the macrolide antibiotic FK506 (Figure 2).[6,7] This protein also tightly bound the structurally related immunosuppressant drug rapamycin (RAPA). Although this new 12-kDa protein, called FKBP (for FK506 binding protein), had no sequence homology to cyclophilin, it too was shown to possess PPIase activity. All three drugs bound to the proline-binding site of their respective PPIase partners and potently inhibited their enzymatic activity. As it turns out, inhibition of PPIase activity is irrelevant for the immunosuppressive actions of CsA, FK506, and RAPA. It is the complex of the drugs with their cognate immunophilins that in each case is the active immunosuppressant species. As will be described in more detail shortly, the drug−immunophilin complexes interact with subsequent target proteins (the calmodulin-dependent phosphatase calcineurin in the case of FKBP/FK506 and CyP/CsA and the protein lipid kinase FRAP/RAFT in the case of FKBP/RAPA) to elicit immunosuppressive effects.[8] Nontheless, the finding that the biological activity of these two structurally distinct, clinically important classes of immunosuppressive drugs appeared to be mediated by two different proteins with a common enzymatic activity led to an explosion of interest and research on the PPIases. This research has demonstrated that cyclophilin and FKBP are each members of a large family of enzymes which are highly conserved and present in prokaryotic as well as eukaryotic organisms.[9,10]

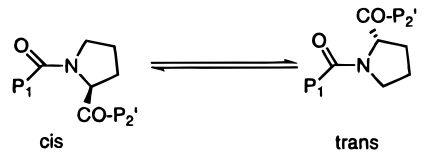

Figure 1. Cis−trans isomerization about peptidylprolyl bonds catalyzed by rotamases (immunophilins).

The Peptidylprolyl Isomerases

The PPIases were originally divided into two families or classes, the cyclophilins and the FKBPs, on the basis of their ability to bind either cyclosporin A or FK506, respectively. Members of these classes are generally referred to as the immunophilins, cyclophilin A (CyPA) and FKBP12 (the 12-kDa FKBP referred to above) being the archetypal members of their respective classes.[8,9] A third type of PPIase, which is neither a cyclophilin nor an FKBP, was discovered in 1994.[11,12] This enzyme, parvulin, has no sequence similarity to either cyclophilin A or FKBP and does not bind either cyclosporin A or

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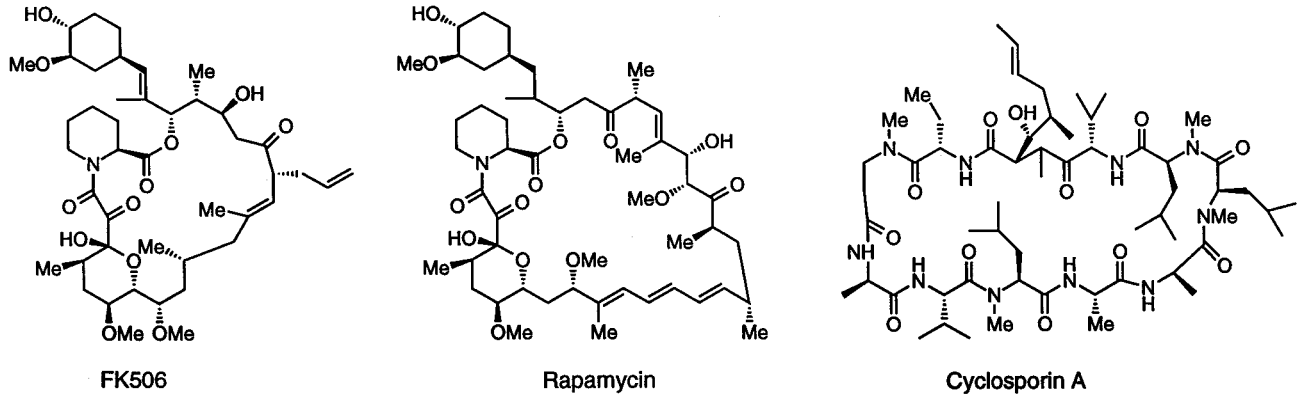


Figure 2. Immunosuppressant drugs which are selective inhibitors of the rotamase activity of the immunophilin FKBP12 (FK506 and rapamycin) or cyclophilin A (cyclosporin A).

Table 1. Properties of Known Human FKBPs

		K_d, nM					
name	M_r	FK506	RAPA	k_{cat}/K_m, μM^{-1} s^{-1}	domains	cellular localization	ref
FKBP12	11 820	0.4	0.2	4.3 (h) 82 (y)		cytosol	6, 7, 13, 14
FKBP12.6		0.55		0.62 (h)			15, 16
FKBP13	13 200	38	3.6	54 (y)		ER	17
FKBP25	25 325	160	0.9	0.8 (b)		cytosol, nucleus	18, 19
FKBP37		4.5	0.8		2 FKBP, 1 CyP		20
FKBP52	51 810	10	8	0.39 (h)	3 FKBP, tetratricopeptide	nucleus, cytosol	21, 22, 23

Table 2. Properties of Known Mammalian Cyclophilins

name	M_r	K_d, nM (CsA)	k_{cat}/K_m, μM^{-1} s^{-1}	cellular localization	ref
cyclophilin A	17 737	2	10	cytosol	6,24
cyclophilin B	23 500	84	6.3	ER	25
cyclophilin C	22 795	4		cytosol	26
cyclophilin D	19 981	3.6	0.9	mitochondria	27
cyclophilin-40	40 000	300	1.9	cytosol	28
cyclophilin-NK					29

FKBP but possesses PPIase activity comparable to that of cyclophilin A. Homology to parvulin has been found in other proteins,[12] suggesting that it may be the progenitor of another family of PPIases.

A large number of immunophilins belonging to the FKBP and cyclophilin families have been discovered in the past several years, including over 30 cyclophilins and more than 20 FKBPs. FKBP immunophilins known to be present in humans include FKBPs 12, 12.6, 13, 25, 37, and 52. (By convention, members of the FKBP family are named by appending to the prefix FKBP the apparent molecular weight in kilodaltons.) Cyclophilins found in human tissue include CyPs A, B, C, and D, CyP-40, and CyP-NK. Tables 1 and 2 summarize the enzymatic efficiency, immunosuppressant drug binding, and cellular localization of some of the better known immunophilins.

A spectrophotometric assay for monitoring PPIase activity was developed by Fischer.[1] α-Chymotrypsin is capable of liberating *p*-nitroaniline (pNA) from peptides such as succinyl-Ala-Xaa-Pro-Phe-*p*-nitroanilide only if the prolyl amide bond is in the trans conformation. Addition of α-chymotrypsin to such a peptide in solution results in rapid release of pNA from the population in the trans conformer, which can be quantitated spectrophotometrically. Release of additional pNA is dependent upon the slow isomerization of the remaining cis conformer to the trans, and this isomerization, and hence release of pNA, is enhanced by PPIase activity. A major problem with the originally described PPIase assay is that 90% of the tetrapeptide substrate is already in the trans rotamer in aqueous solution, giving a poor signal-to-noise ratio. Improvements in the assay, such as using lithium chloride and trichloroethanol to increase the proportion of the cis conformation, have been made.[30,31] These modified assays have allowed the measurement of kinetic constants of various PPIases and evaluation of their substrate specificities.[14,30,32] Other methods described for following PPIase activity include monitoring protein folding by fluorescence or circular dichroism[4,33−35] or use of NMR spectroscopy to follow the interconversion of cis and trans prolyl rotamers.[36,37]

Human CyPA is a very efficient enzyme (k_{cat}/K_m = 10 μM^{-1} s^{-1} with respect to the substrate succ-Ala-Ala-Pro-Phe-pNA) which is rather promiscuous; very little substrate specificity was observed in a series of tetrapeptides (succ-Ala-Xaa-Pro-Phe-pNA), except that a hydrophobic residue was preferred at the P1 (Xaa) position, with Ala being slightly more preferred than others.[32] By contrast, hFKBP12 has less catalytic efficiency but pronounced substrate specificity, with hydrophobic residues, and particularly residues with branched alkyl side chains, being most preferred.[14,32] Succ-Ala-Leu-Pro-Phe-pNA is the best substrate for FKBP12 in this series and is the standard substrate for characterization of the enzymatic activity of the FKBP family members. Succ-Ala-Ala-Pro-Phe-pNA is generally used for studies on the cyclophilin family.

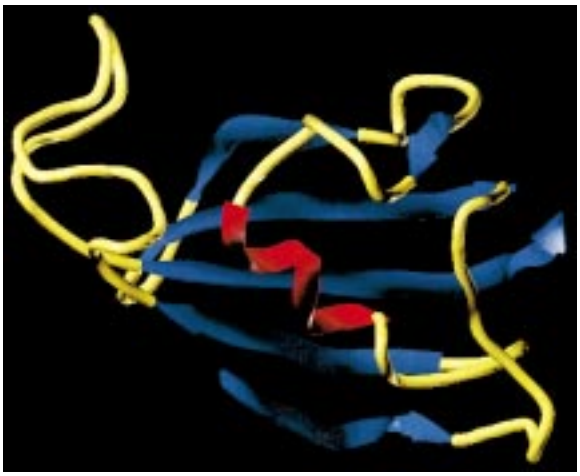


Figure 3. Ribbon diagram of FKBP12.

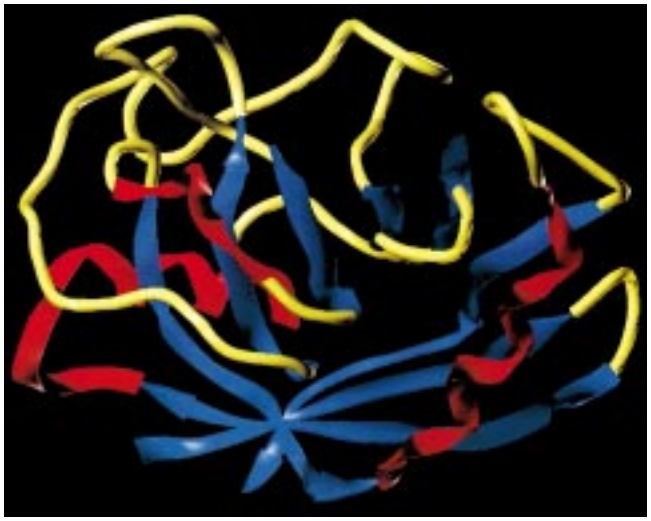


Figure 4. Ribbon diagram of cyclophilin A.

Structural studies of FKBP12 and cyclophilin A, and their complexes with assorted ligands, have provided a great deal of detailed information on the structure−activity functions in these proteins. The structures of human[38] and bovine[39] FKBP12 have been determined using NMR and X-ray crystallography, as has the structure of human cyclophilin A.[40,41] hFKBP12 contains a five-stranded antiparallel β-sheet wrapped with a right-handed twist around a short α-helix, together with flexible connecting loops (Figure 3). Twisting of the β-sheet results in the formation of hydrophobic concave and convex surfaces. The β-structure is composed of residues 2−8, 21−31, and 35−38 with 46−49, 71−78, and 97−107, the α-helix by residues 57−63. The loops comprising residues 39−45 (which connect the two parts of the third β-strand) and 84−91, termed the 40s and 80s loop, respectively, surround the rotamase domain and are rather disordered in the unliganded protein.

Cyclophilin A, on the other hand, comprises a β-barrel formed by eight β-strands connected by loops and α-helices at the top and bottom (Figure 4). Although cyclophilin's barrel has some similarity to the transport proteins β-lactoglobulin and retinol-binding protein, its topology is unique, and the center of the barrel is blocked with hydrophobic side chains, precluding ligand binding there.[41]

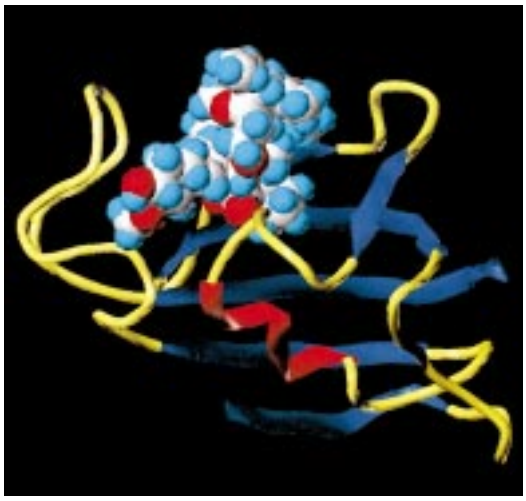


Figure 5. FK506 bound to FKBP12. FK506 is shown in a space-filling representation.

Binding of FK506 to FKBP12, or CsA to CyPA, does not cause significant change in the overall three-dimensional structure of either protein but does allow identification and analysis of the rotamase active sites.

The hFKBP12/FK506 complex has been solved using X-ray crystallography and NMR spectroscopy.[42−44] The FK506-binding domain of FKBP12 is a hydrophobic well, approximately 9 Å × 9 Å in area and 7 Å deep, formed by a convex portion of the β-sheet and three of the loops (Figure 5). A baker's dozen of residues in this binding domain, most of them hydrophobic, interact with FK506 in the bound complex: Tyr-26, Phe-36, Asp-37, Arg-42, Phe-46, Glu-54, Val-55, Ile-56, Trp-59, Tyr-82, His-87, Ile-91, and Phe-99. The FKBP12-binding portion of FK506 comprises the pipecolinyl ring together with the pendant pyranose ring and ketoamide linkage and a portion of the cyclohexylpropenyl ester side chain. Two hydrophobic pockets in the FKBP12-binding domain are occupied by FK506: (1) a pocket defined by Trp-59 (the "floor") and the side chains of Tyr-26, Phe-46, Phe-99, Val-55, and Ile-56 (the "walls"), into which the pipecolinyl ring binds deeply, and (2) another hydrophobic cavity formed by the side chains of Ile-90, Ile-91, His-87, Phe-36, Tyr-82, and Asp-37, into which the pyranose ring binds. Hydrogen bonds are formed between the ester carbonyl of FK506 and the backbone −NH of Ile-56 and between the amide carbonyl of the ketoamide linkage and the side chain −OH of Tyr-82. The cyclohexylpropyl side chain of FK506 lies in a shallow hydrophobic groove on the surface of the protein. In addition to the complex with FK506, the structures of FKBP12 complex with the related immunosuppressant drug ascomycin,[45] rapamycin,[44,46,47] a nonimmunosuppressive analogue of ascomycin, 18-hydroxyascomycin (or L-685,818),[48] and several small molecule inhibitors.[49,50] The FKBP-binding domain portions of all of these molecules are closely superimposable in all of these structures. The remaining portion of the macrocyclic compounds extends into the solvent-accessible region of the protein and is crucial for mediating the immunosuppressive effects of the drug−immunophilin complexes.

All of these ligands potently inhibit the PPIase activity of FKBP12 upon binding. It is believed that the pipecolinyl−ketoamide portion of the molecule functions

Table 3. FKBP-Binding Domain Residue Conservation in Human FKBPs

	residue no.												
protein	26	36	37	42	46	54	55	56	59	82	87	91	99
hFKBP12	Tyr	Phe	Asp	Arg	Phe	Glu	Val	Ile	Trp	Tyr	His	Ile	Phe
hFKBP12.6	●	●	●	●	●	●	●	●	Phe	●	●	●	●
hFKBP13	●	●	●	Gln	●	Gln	●	●	●	●	Ala	●	●
hFKBP25	●	●	●	Thr	Leu	Lys	●	●	●	●	Gln	●	●
hFKBP52-d1	●	●	●	●	●	●	●	●	●	●	Ser	●	●
hFKBP52-d2	Leu	●	●	●	Gly	Asp	Leu	Pro	Leu	Phe	Lys	●	Tyr
hFKBP52-d3	●	Tyr	Glu	Asn	Gln	Arg	Leu	Ala	Leu	Leu	Asn	Leu	Leu

as a transition-state mimic for the natural peptidylprolyl substrate. The initially proposed mechanism for FKBP12 rotamase activity—formation of a tetrahedral adduct[4]—was not consistent with subsequent experimental results.[51,52] Schreiber used radiolabeled FK506 to demonstrate that binding to FKBP12 was reversible and did not involve covalent bonding and proposed that the mechanism involved stabilization of a twisted prolyl amide bond intermediate.[53] Compounds such as FK506 and its relatives, with their α-ketoamide functionality, are ideally suited to act as transition-state mimics, since the ground-state orientation of the ketocarbonyl is orthogonal to the amide group. Computational studies of FKBP12 rotamase activity by molecular dynamics simulations[54] and molecular mechanics calculations[55] also suggest that the enzymatic catalysis involves stabilization of the twisted amide transition state.

The residues which make up the FK506 binding site of hFKBP12 define a common "FKBP domain" which is remarkably conserved both across species and in higher-molecular-weight members of the FKBP family.[10] Table 3 shows the preservation of these residues in the FKBP domains of human FKBP12.6, -13, -25, and -52; the latter contains three FKBP domains. Ten residues are strongly conserved, and Tyr-26, Phe-36, Asp-37, Val-55, Ile-56, Tyr-82, and Phe-99 are completely conserved in all known FKBPs (all species and isoforms) with significant PPIase activity.[10] The greatest variation in the domain is found at His-87, which is replaced by a variety of hydrophobic or hydrophilic residues in other FKBPs. Phe-46 may be replaced by other hydrophobic residues with little effect on rotamase activity. FKBP12.6 differs from FKBP12 in its rotamase domain only by replacement of Trp-59 with Phe, which does not alter appreciably either rotamase activity or FK506 binding. Mutagenesis experiments on hFKBP12 have demonstrated that rotamase activity and drug binding may be structurally dissected. The mutation F36Y was much more deleterious to rotamase activity than to macrolide binding,[56] as was the mutation Y82L;[57] the mutation W59A abolishes rotamase activity but not FK506 binding.[58] Replacement of Asp-37 with Val,[59] or Phe-99 with Tyr,[60] completely abolished both PPIase activity and FK506 binding. Other mutations, which affect the ability of the drug—immunophilin complex to interact with other protein targets, will be discussed in the next section.

After the discovery of FKBP12, the next FKBP immunophilin to be identified and sequenced was FKBP13.[17] hFKBP13 contains a hydrophobic N-terminal 21-amino acid sequence, indicative of secretion into the endoplasmic reticulum (ER), and a C-terminal RTEL tetrapeptide sequence which causes its retention in the pre-Golgi lumen.[61,62] The rotamase domain differs by the nonconservative changes of Gln for Arg-42 and Glu-54 and Ala for His-87 (Table 3). The Arg-42 residue of FKBP12 interacts with FK506, but not rapamycin, in the immunophilin—macrolide complexes, and the R42Q change may contribute to the fact that hFKBP13 binds rapamycin with 10-fold higher affinity than FK506. The X-ray structure of the hFKBP13/FK506 complex demonstrates that the FKBP domain of FKBP13 has a very similar conformation to that of FKBP12.[63] FKBP13 is widely expressed in all mammalian tissues examined.

FKBP12.6 has the same number of residues as FKBP12 and 18 amino acid substitutions, most of them conservative.[15,16] The only change in the FKBP domain is the substitution of Phe for Trp-59, which forms the floor or base of the binding domain. hFKBP12.6 has the same affinity as hFKBP12 for FK506, and the complexes of FKBP12.6 with FK506 or RAPA are potent inhibitors of calcineurin or RAFT/FRAP, respectively.[16] The mRNA for FKBP12.6 has been found in all tissues examined and is abundant in the brain.

FKBP25 differs from other FKBPs in two respects.[19] It contains an additional seven amino acids inserted into the 40s loop (the loop between the two parts of the third strand of the β-sheet, which contains R42 in hFKBP12). This change, together with the R42T substitution, may account for the 200-fold higher affinity of FKBP25 for RAPA over FK506. Aside from the additional changes of F46L, E54K, and H87Q, the FKBP binding domain is largely conserved. Another difference from other mammalian FKBPs is that FKBP25, alone among FKBP rotamases, prefers AAPF as a substrate, as does cyclophilin. FKBP25 is found in both the nuclear and cytosolic fractions of human T-cells.[64,65] It has been predicted that the N-terminal domain of the protein may form a helix—loop—helix structure, a common DNA-binding motif which suggests association of FKBP25 with DNA.[19] Lysine repeats in the C-terminal portion of the protein, including a KKKK sequence in the inserted sequence noted above, might function as a nuclear localization sequence for this immunophilin.

FKBP52 (also known as p59, FKBP59, and hsp56) is an example of a mammalian FKBP with multiple domains. A high-molecular-weight rotamase enzyme was isolated from human T-cells by FK506 and RAPA affinity columns.[66] Like most FKBPs, the protein had a migration rate on SDS—PAGE gel indicative of a higher molecular weight (56—59 kDa) than actually calculated from its sequence (51.8 kDa);[21] hence the confusion in nomenclature. It was subsequently found that FKBP52 was identical to a previously described heat-shock protein (hsp 56) found as a component of several unliganded steroid receptor complexes.[22,23,67] FKBP52 contains two FKBP domains with detectable

Figure 6. Cyclosporin A bound to cyclophilin A. Cyclosporin is shown in a space-filling representation.

rotamase activity and a third domain which contains three tetratricopeptide repeat motifs.[68] Following these three domains is a calmodulin-binding consensus sequence. The first N-terminal FKBP domain is highly conserved, and expression of the individual domains of hFKBP52 indicates that the rotamase activity of domain 1 is comparable to that of hFKBP12.[69] Domain 2 shows increased divergence away from the FKBP12 domain (Table 3), and this domain has only about 2% of the rotamase activity of the first domain. Interestingly, this second domain contains an ATP/GTP-binding site. The third domain bears little resemblance to FKBP12 and has no detectable rotamase activity.

Soldin et al. have reported isolation of 37- and 52-kDa immunophilins which apparently contain both FKBP and cyclophilin domains. The 52-kDa protein, which was isolated from human lymphoid cells and has no detectable rotamase activity toward a variety of peptide substrates, bound FK506, RAPA, and CsA with high affinity.[70] hFKBP37, isolated from the Jurkat T-cell line, also bound all three immunosuppressant drugs and in addition possessed glyceraldehyde 3-phosphate dehydrogenase activity.[20]

Many FKBPs are known in other eukaryotes, including yeast and fungi, as well as prokaryotes.[10] It seems likely that this family of ubiquitous proteins will continue to expand rapidly in the near term.

The structures of human cyclophilin A in complex with cyclosporin A,[71−75] as well as a variety of small peptides,[76−79] have been solved by X-ray crystallography or NMR spectroscopy. As is the case with FKBP12, the structure of CyPA is not radically altered upon ligand binding (Figure 6). The ligand-binding and rotamase active site is a groove formed by two loops (residues 101−110 and 69−74) on top of two β-strands (residues 60−63 and 111−113).[80] The residues comprising the active site of CyPA are Arg-55, Ile-57, Phe-60, M-61, Gln-63, Ala-101, N-102, Q-111, Phe-113, Leu-122, His-126, and Arg-148. The proline-binding pocket is formed by Phe-60, M-61, Leu-122, His-126, and Phe-113.

Cyclosporin A, however, undergoes a significant conformational change when binding to the protein. In the solution structure of free CsA in chloroform, the molecule adopts a compact antiparallel β-sheet conformation with four internal hydrogen bonds between Abu-2(NH)−Val-5(CO), Val-5(NH)−Abu-2(CO), Ala-7(NH)−MeVal-11(CO), and D-Ala-8(NH)−MeLeu-6(CO).[81,82] The amide bond between MeLeu-9 and MeLeu-10 is in the cis conformation. The bound structure of CsA, by contrast, is nearly turned inside out; all four hydrogen bonds are disrupted, and all amide bonds are trans. Similar to FK506, approximately half of CsA fits into the rotamase site of CyPA, with the remaining half protruding into the solvent-accessible region, "reminiscent of a coin that is partly inserted into a slot machine" [80] (Figure 6). Residues 1, 2, 3, 9, 10, and 11 of CsA make contact with cyclophilin, with the side chain of MeVal-11 buried in the proline-binding pocket. In addition to five direct hydrogen bonds between CsA and CyPA, intermolecular interactions are mediated by a set of active site water molecules.

Analogous to the case with FKBP, the 13 residues forming the rotamase active site and CsA-binding domain of CyPA define a cyclophilin domain which is highly conserved in other cyclophilins. Table 4 shows the conservation of this domain in other human cyclophilins relative to hCyPA.

The members of the cyclophilin B family differ from those of cyclophilin A by the presence of an ER-directed signal sequence. Comparison of hCyPB with other members of the family, which includes yeast CyPB, *Drosophilia* nina A, and rat cyclophilin-like protein, and hCyPA indicates that all of the proteins contain a highly conserved central rotamase domain which is flanked by variable N- and C-terminal domains.[25] The N-terminal domain in the CyPB group contains the signal sequence domain. The members of the CyPB family manifest potent PPIase activity which is inhibited by CsA. The X-ray structure of the complex of hCyPB with CsA shows that the rotamase domain has the same structure as in CyPA and that CsA binds in a nearly identical fashion.[83] Overall, CyPB was observed to have the same tertiary structure as hCyPA, with the exception of two of the loops and the C- and N-terminal domains. These differences may account for the fact that the CyPB/CsA complex is 10-fold more potent in inhibiting calcineurin (K_i < 21 nM) than the hCyPA/CsA complex (K_i = 270 nM).[84]

Cyclophilin C was originally isolated from a cDNA library from a stromal cell line derived from rat bone marrow.[85] The protein was observed to be expressed in a more restricted subset of tissues than was either CyPA or CyPB and was found in particularly high levels in rat kidney. These findings suggested that CyPC might mediate the well-known nephrotoxic properties of CsA. However, hCyPC was found to not be elevated in the kidney relative to other tissues (pancreas, skeletal muscle, heart, lung, and liver), arguing against this hypothesis. hCyPC is present in extremely low levels in the brain and in T-cells.[26] It binds CsA with an affinity comparable to that of hCyPA (Table 2). The crystal structure of rat CyPC bound to CsA has been solved.[86] mCyPC has the same β-barrel structure as hCyPA. The rotamase domain is essentially identical in the two isoforms, although significant differences are observed in three of the loops. CyPC binds a 77-kDa

Table 4. Cyclophilin-Binding Domain Residue Conservation in Human CyPs

	residue no.												
protein	55	60	61	63	72	101	102	103	111	113	121	122	126
hCyPA	Arg	Phe	Met	Gln	Gly	Ala	N	Ala	Gln	Phe	Trp	Leu	His
hCyPB	●	●	●	●	●	●	●	●	●	●	●	●	●
hCyPC	●	●	●	●	●	●	●	●	●	●	●	●	●
hCyPD	●	●	●	●	●	●	●	●	●	●	●	●	●
hCyP40	●	●	●	●	●	●	●	●	●	●	His	●	●
hCyP-NK	●	●	●	●	●	●	●	Arg	●	●	His	●	●

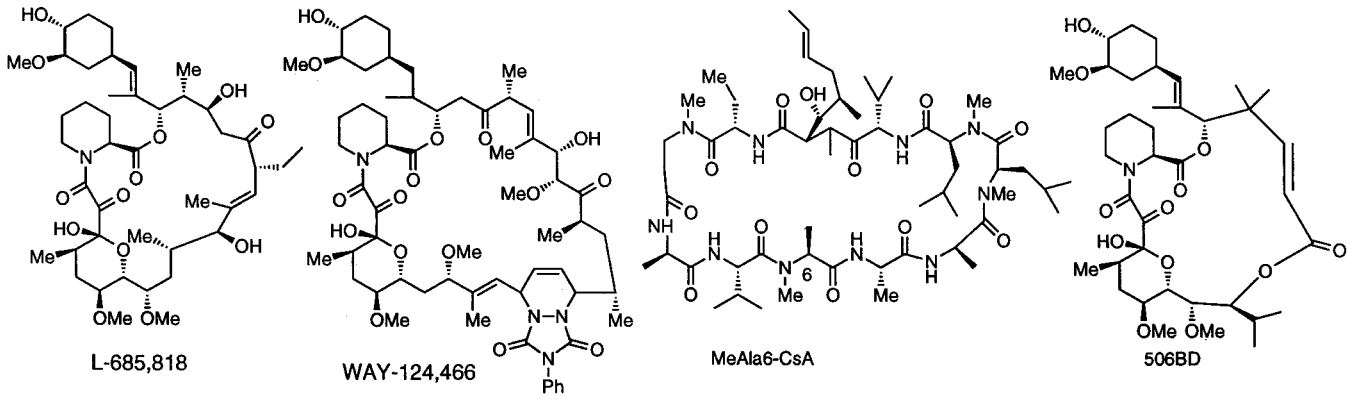


Figure 7. Examples of nonimmunosuppressive ligands for FKBP12 or cyclophilin A used to probe the relationship between rotamase inhibition and immunosuppression.

glycoprotein, named CypCAP for cyclophilin C-associated protein, in the absence of CsA; CsA is a competitive inhibitor of this interaction.[85,87] The physiological relevance of this protein−protein interaction is unknown.

CyPD is a mitochondrial matrix protein which plays a crucial role in the permeability transition of mitochondria.[27,88,89] Under conditions of elevated Ca^{2+} and oxidative stress, mitochondria in the heart, liver, and brain form large pores which are known to be blocked by CsA.[90] Studies in rats using photoactive CsA derivatives identified a mitochondrial protein labeled by CsA which possessed significant homology to hCyPD, suggesting that this cyclophilin mediates the pore blockade by CsA.[90]

A 40-kDa cyclophilin was isolated from calf brain and shown to possess PPIase activity that was inhibited by CsA, but with much less sensitivity than CyPA (Table 2).[28] Sequence alignments indicated that this protein contained two domains: an N-terminal rotamase domain and a C-terminal domain that was homologous to the C-terminus of FKBP52.[91] Like FKBP52, CyP-40 has been shown to be a component of the glucocorticoid receptor complex.[92]

As seen in Table 4, cyclophilins A−D possess conserved cyclophilin rotamase domains and are comparable in terms of enzymatic activity and CsA binding. Cyp-40 diverges from this pattern in that its rotamase activity is much less potently inhibited by CsA (K_i = 300 nM), suggesting it binds CsA less well. Molecular modeling and mutagenesis suggest that the weakened affinity for CsA arises from the presence of a histidine in position 141.[93] This His residue replaces a Trp in the corresponding position of CyPA that appears to be critical for CsA binding in cyclophilins.[94]

CyP-NK95 was identified as the product of a gene which is specific for natural killer (NK) cells in humans and mice. This multidomain protein appears to play a role in NK cell cytotoxicity. A large hydrophobic domain in the N-terminus is followed by a cyclophilin domain, which in turn is followed by hydrophilic domains. The function of the cyclophilin domain is presently unknown.

Immunophilins and Immunosuppressant Drugs

The mechanisms of immunophilin-mediated immunosuppression elicited by FK506, RAPA, and CsA have been extensively studied and reviewed.[8,80,96,97] Although FK506 and CsA are structurally dissimilar (Figure 2), while FK506 and RAPA are structural analogues of each other, FK506 and CsA share a similar pharmacology which is distinct from that of RAPA. Both FK506 and CsA inhibit Ca^{2+}-dependent signaling pathways in T-cells emanating from activated T-cell receptors and resulting in transcription of genes for interleukin-2 (IL-2) and its receptor. The discovery that both immunosuppressants bound to proteins that, although very different in sequence and structure, possessed the same enzymatic activity suggested that inhibition of rotamase activity was the pharmacologically relevant activity. However, RAPA, which like FK506 binds to FKBP12 and potently inhibits its rotamase activity, acts at a later, Ca^{2+}-independent stage in T-cell signaling, blocking IL-2-dependent entry of lymphocytes into the cell cycle and hence inhibiting cell proliferation.[98] These two mechanistic pathways are distinct from each other, and FK506 and RAPA are reciprocal antagonists of each other's pharmacological effects.[99] Schreiber designed 506BD (Figure 7) as a probe molecule that contained the common pipecolinyl-centered domain common to FK506 and RAPA but was truncated in the macrocyclic ring.[100] This compound potently inhibited FKBP12 rotamase activity but lacked immunosuppressant activity, demonstrating that PPIase inhibition was insufficient for immunosuppression.[101] Numerous nonimmunosuppressant analogues of the immunosuppressant drugs have been described (Figure 7). The compound 18-hydroxyascomycin (or L-685,818) is an analogue of

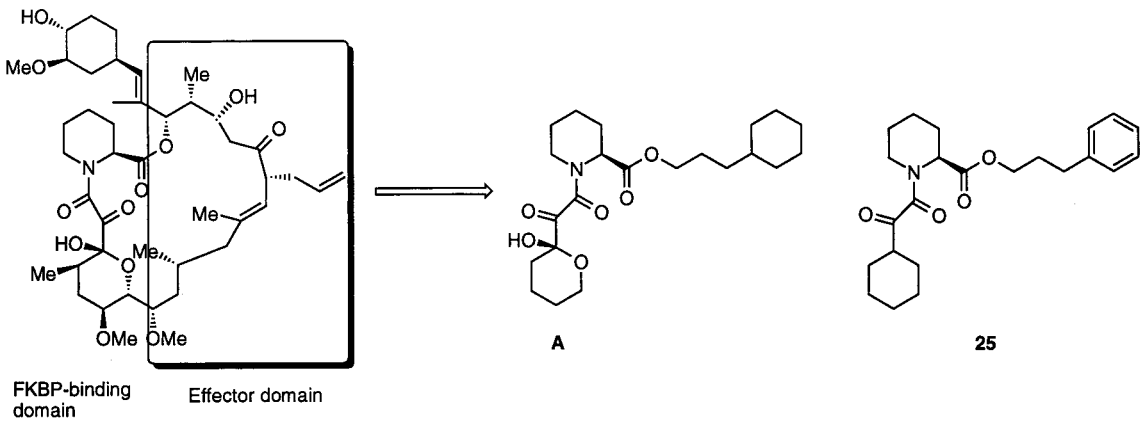


Figure 8. Immunophilin-binding immunosuppressant drugs possess two domains, as exemplified by FK506 (left): an immunophilin-binding domain and an "effector" domain. The minimal FKBP-binding domain (A) may be mimicked by simple molecules such as **25**.

FK506 with slight modification in the effector domain that lacks immunosuppressive activity.[102] MeAla-6-CsA is a CsA analogue which is likewise nonimmunosuppressive, though a potent CyPA inhibitor.[103] The compound WAY-124,466 is a rapamycin derivative modified in its effector domain which no longer blocks T-cell proliferation.[104] These results led to the formulation of the immunosuppressant drugs as possessing immunophilin-binding domains and "effector" domains, the latter responsible for mediating the immunosuppressive effects of the drug−immunophilin complexes (Figure 8). This picture suggests that the drugs do not work by inhibiting an activity of the immunophilins but by forming an activated complex which bestows a "gain of function".

The results with 506BD and related structures inspired a search for the putative target protein of the drug−immunophilin complexes. Using immunophilin fusion-protein affinity columns, two groups independently identified calcineurin as the target of both the FK506/FKBP12 and CsA/CyP complexes.[85,105] The phosphatase activity of this Ca^{2+}/calmodulin-dependent enzyme was demonstrated to be potently inhibited by either drug−immunophilin complex, though not appreciably by either drug or either immunophilin alone. Subsequent work has delineated in detail the signal transduction pathway involved in the immunosuppressive effects of the drugs. Nuclear factor of activated T-cells (NF-AT) is a transcription factor which regulates the transcription of the gene for IL-2.[106] Cytosolic NF-AT, which is phosphorylated, must be dephosphorylated in order to be translocated into the nucleus to activate transcription of the *IL2* gene. This dephosphorylation is effected by calcineurin, and thus inhibition of calcineurin blocks NF-AT translocation and subsequent production of IL-2 (Figure 9).[107] In addition to NF-AT, other transcription factors such as the NF-κB factors and Oct-1/OAp are affected by FK506 and CsA through this mechanism.[108]

Whereas CsA and FK506 operate by inhibiting IL-2 synthesis, RAPA blocks the IL-2-stimulated G1-to-S phase transition in T-cells, inhibiting lymphocyte proliferation. This activity was observed to correlate with decreased activity of several kinases: p70 S6 kinase, a 70-kDa protein which phosphorylates the S6 protein of the small ribosomal subunit,[109−111] and cyclin-dependent kinases weighing 33 and 34 kDa.[112,113] However, these proteins were not acted upon directly by the FKBP12/RAPA complex. It was also found that yeast cells possessing mutations in two proteins were resistant to rapamycin-induced lethality; these proteins were denoted TOR1 and TOR2 ("targets of rapamycin").[114] In a replay of the search for calcineurin described above, two groups independently discovered and characterized a protein which bound to FKBP12 only in the presence of RAPA. This protein was named RAFT1, for "rapamycin and FKBP12 target" (by Snyder's group, who isolated it from rat brain extracts),[115] or FRAP, for "FKBP12 and rapamycin-associated protein" (by Schreiber's group, who isolated the human protein).[116] Sequencing of this protein indicated that it was a mammalian homologue of the yeast TOR proteins.

RAFT1/FRAP possesses a serine/threonine kinase domain which has homology to phosphatidylinositol-3-OH kinase (PI3K). Using mutant FRAP proteins that did not complex with FKBP12/RAPA, Schreiber demonstrated that FRAP regulates S6 kinase in a rapamycin-sensitive manner in vivo and that FRAP's kinase activity is required for this regulation.[117] Autophosphorylation of FRAP in vitro was prevented by FKBP12/RAPA.

More recently, it has been reported that rapamycin potently inhibits the phosphorylation of a protein involved in regulation of the translation intiation factor eIF4E.[118] This protein, 4E-BPI, binds to eIF4E when dephosphorylated and is thought to inhibit initiation of translation.[119] To date, no direct interaction with, or phosphorylation of, either p70 S6 kinase or 4E-BPI has been demonstrated, and it seems likely that other, as yet unidentified, components of the pathways exist.

Thus, whereas CsA and FK506 affect transcriptional pathways through inhibition of a phosphatase (calcineurin), RAPA blocks translational signaling pathways through inhibition of a serine/threonine kinase (FRAP) (Figure 9). Rapamycin-induced inactivation of p70 S6 kinase causes dephosphorylation of the S6 protein of the 40S ribosomal subunit,[119] while inhibition of 4E-BP1 phosphorylation blocks activation of the elF4E initiation factor complex. In either case, the result is blockade of the translation of FRAP-dependent mRNAs.[120]

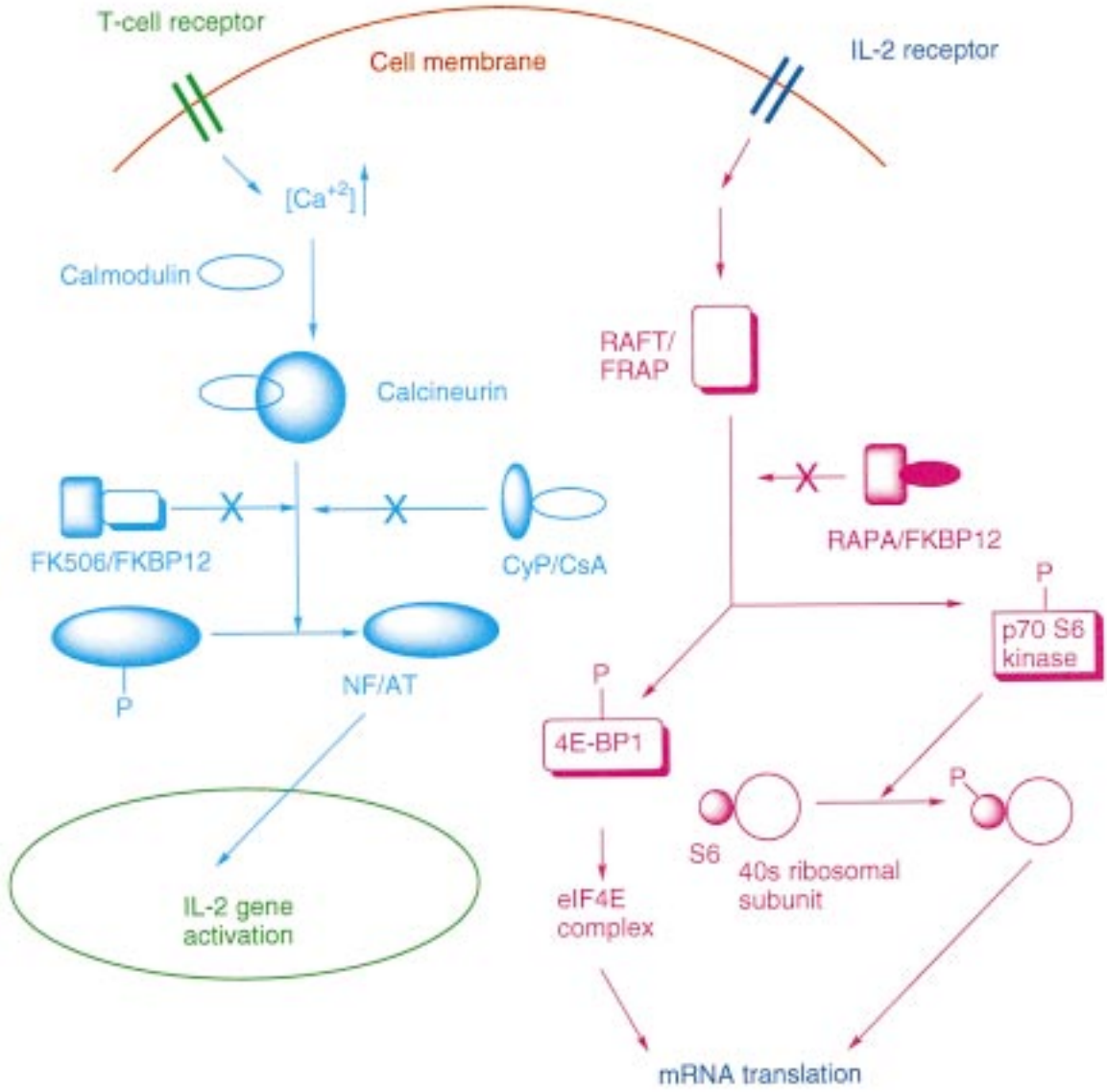


Figure 9. Transcriptional and translational signaling pathways in T-lymphocytes inhibited by immunophilin−immunosuppressant drug complexes.

Cellular Functions of Immunophilins

Rates of folding of proteins vary considerably, and fast- and slow-folding forms of unfolded proteins have been detected.[33] Isomerization about peptidylprolyl amide bonds is one of the slower steps in protein folding and thus may represent a rate-limiting step in protein folding and unfolding.[121] CyPA has been shown to accelerate the in vitro refolding of several proteins, including immunoglobulin chains,[33,122] carbonic anhydrase,[123] and RNase T1.[33,124] Human bradykinin is likewise a substrate for CyPA,[125] as is calcitonin.[126] The ability of CyPA to catalyze protein refolding is inhibited by CsA. FKBP12 has also been shown to catalyze the folding of carbonic anhydrase[127] and RNase T1,[128] with less efficiency than CyPA.

A considerable amount of research over the past several years has documented the growing role of the immunophilins in protein trafficking and as components of multisubunit complexes. The cyclophilins in particular have been observed to be involved in the translocation and maturation of proteins in the secretory pathway. The *ninA* gene in yeast encodes for a cyclophilin which is required for the transport of properly folded rhodopsin from the ER to the cell surface.[129,130] Cyclophilins have been identified in vertebrates that act as chaperones for opsin.[131] CsA is observed to slow the folding and secretion of transferrin from hepatocytes[132] and the formation of triple-helical collagen in fibroblasts.[133] Both of these processes take place in the ER and were unaffected by either FK506 or RAPA, suggesting the involvement of an ER-specific cyclophilin. CsA also inhibited the expression of functional nicotinic cholinergic and 5HT-3 serotonergic ion channels in *Xenopus* ooctyes, again suggesting mediation by a cyclophilin of the ER.[134] The trigger factor of *Escherichia coli* is a protein involved in translocation, which contains an FKBP domain.[135]

CyP-40[136] and FKBP52[137] were found to be associated with untransformed steroid receptor heterocomplexes. Both CyP-40 and FKBP52 bind to a common site on the steroid receptor-associated heat-shock protein hsp90.[138] Interaction of FKBP52 with hsp90 does not occur through its rotamase domain but instead through the tetratricopeptide repeats. FKBP52 is not required for

heterocomplex formation and is instead believed to be important for targeted movement of the receptor. Recent reports have described in more detail the chaperone functions of both CyP-40[139,140] and FKBP52[141] in hsp90-dependent signal transduction.

Immunophilins are involved in several aspects of calcium-related cell signaling. FKBP12 regulates intracellular calcium release by interactions with two calcium ion channels, the ryanodine receptor (RyR) and the inositol 1,4,5-trisphosphate receptor (IP_3R). RyR, localized to the sarcoplasmic reticulum (SR), is the calcium release channel involved in excitation−contraction coupling in muscle.[142] It is also found in other excitable tissue, including cardiac muscle and brain. It consists of four large (565-kDa) identical subunits which form the pore. Purification of RyR results in copurification of FKBP12, with one molecule of FKBP12 bound to each of the four subunits.[143] Association with FKBP12 stabilizes RyR, improving its Ca^{2+}-fluxing properties.[144] FKBP12 may be dissociated from RyR by high concentrations of FK506, implying that FKBP12 associates with RyR through its rotamase domain. Stripped of FKBP12, the calcium-fluxing properties of RyR degenerate, and the SR is diminished in its ability to accumulate calcium because Ca^{2+} pumped in leaks out through the RyR. Patch-clamp recordings from recombinant RyR indicate that FKBP12 binding has two effects on channel function: it stabilizes both the closed and open states, so that the channel is harder to open, but once open, the Ca^{2+} fluxing is optimized.[145]

Like RyR, the IP_3 receptor is a calcium release channel which is a tetramer of four identical subunits.[146] IP_3R is most commonly found in the ER and mediates calcium release from the ER, but some are found in the plasma membrane and regulate calcium entry into the cell. IP_3Rs respond to hormones and neurotransmitters that act at membrane-bound receptors and activate phospholipase C with resultant generation of IP_3.[147,148] Similar to the situation with RyR, each subunit of IP_3R is tightly associated with a molecule of FKBP12, and association with FKBP12 stabilizes the channel and improves the Ca^{2+}-gating properties.[149] As is the case with RyR, the association of FKBP12 with IP_3R can be disrupted by treatment with FK506 or rapamycin. Calcineurin is associated with both the FKBP12/RyR and FKBP12/IP3R complexes and in the IP3R complex modulates Ca^{2+} flux by controlling the phosphorylation state of the receptor.[150] The association of calcineurin with the complexes is disrupted by FK506, suggesting that FKBP12 acts as a docking module to facilitate assembly of the macromolecular complex.

Use of the yeast two-hybrid system to screen a cDNA library from neonatal rat heart uncovered another FKBP12 interaction. Transforming growth factor-β (TGF-β) binds to membrane-bound receptors that comprise heteromeric subunits, type I and type II, that possess serine/threonine kinase activity.[151] FKBP12 was found to interact in a specific fashion with type I but not type II TGF-β receptors.[152] This interaction with type I receptors was competitively inhibited by FK506. Subsequent work demonstrated that FKBP12 functions as an inhibitor of the type I receptor. The type I receptor can only bind TGF-β in the presence of the type II receptor, and it is believed that the type I receptor is responsible for initiating the downstream signaling, though the means by which type I is activated by type II have been unclear.[153] Wang et al. demonstrated that FKBP12 bound to the ligand-free type I receptor and induced phosphorylation of type I by type II-released FKBP12.[154] FK506, as well as nonimmunosuppressive analogues, displaced FKBP12 from type I, and functional responses elicited by TGF-β were enhanced by this displacement. These results suggest that FKBP12 functions as an inhibitor of TGF-β-mediated signaling and that the functional role of type II is to lift the blockade by phosphorylating type I, rendering it active. The G89P, I90K double mutant of FKBP12 was shown to be incapable of inhibiting signaling.[154] Since this mutation is known to abolish calcineurin inhibition, but not FK506 binding,[155] FKBP12 may serve to dock calcineurin to the type I receptor, similar to the RyR and IP3R complexes, and thus regulate its phosphorylation state.

A protein ligand for cyclophilin B in T-cells was also uncovered using the yeast two-hybrid system.[156,157] Named CAML, for calcium signal-modulating cyclophilin ligand, this protein appears to regulate calcium currents in T-cell signaling. The action of CAML is downstream of the T-cell receptor but upstream from calcineurin. The report of a chicken homologue of human CAML suggests that this immunophilin ligand may be an important, evolutionarily conserved signaling molecule.[158]

Cytokine-like actions have been observed for the immunophilins. FKBP12 activates Ca^{2+} signaling in neutrophils upon its release from mast cells.[159] Some cyclophilins may coregulate the expression of histamine and synthesis of the leukotriene C4.[160]

As seen by the examples of RyR, IP3R, TGF-β type I receptor, and steroid receptor, immunophilins may play a broad and important role in assembling and modulating multicomponent protein complexes. It has been suggested that the immunophilins produce their modulating effects via rotamase activity, for example, by changing the conformation of membrane-bound receptors.[161] However, it appears that in at least some instances this view is not entirely correct. Although in many cases this association appears to occur through the rotamase domain of the immunophilins, rotamase activity per se does not seem to necessarily be important. Timerman et al. demonstrated that FKBP12 could be displaced from RyR by mutant FKBP12s that lacked rotamase activity.[162] Substitution with these rotamase-inactive FKBPs for wild-type FKBP on RyR did not affect the Ca^{2+}-fluxing properties. Cameron et al. showed that mutant FKBPs devoid of rotamase activity served as functional components of the IP3R/FKBP12 complex just as well as the native protein.[163]

It is possible that in some roles the immunophilins function as adapter proteins, serving to couple together other macromolecules into assemblies. The rotamase domains may act as "molecular sockets" or recognition domains, with the rotamase activity itself in some cases an artifact. An analogy may be drawn between FKBP and cyclophilin domains and other known adapter molecule domains such as the SH2 and SH3 domains of Src.[164] The recent discovery of molecules that contain both FKBP and cyclophilin domains echoes adapter

molecules such as Grb2, which contain both SH2 and SH3 domains. It is possible that immunophilin-like domains will be found in other proteins not thought of as immunophilins. In this vein, it is interesting to note that the scaffolding protein AKAP78, which anchors both protein kinase A and calcineurin and targets them to subcellular sites, contains a putative calcineurin-binding domain which resembles FKBP12.[165]

The emerging role played by the immunophilins in the nervous system, and the potential therapeutic utility of these discoveries, will be the subject of the remainder of this review.

Immunophilins in the Nervous System

Because the initial interest on the immunophilins was in the context of understanding the mechanism of action of the immunosuppressant drugs, most of the early study of these proteins was in the cells and tissues of the immune system, although it was known by Northern blot analysis of mRNA that FKBP12 was present throughout the entire body, including the brain.[166] Using [^{3}H]FK506 binding experiments in brain homogenates, Solomon Snyder and colleagues at Johns Hopkins showed that FKBP12 levels in the brain were in fact as much as 40 times higher than in the immune system.[167] This finding suggested that FKBP12 and other immunophilins might play a role in neural functions. Cyclophilin A was likewise observed to be present at high levels in the brain, and both FKBP12 and CyPA were almost exclusively neuronal.[168] Furthermore, both proteins had a distribution in the brain with regional variations that closely paralleled those of calcineurin.[168] Cerebellar granule cells, the hippocampus, and the nigrostriatal dopaminergic pathway all contain particularly high levels of immunophilins. Although the distributions of FKBP12 and CyPA showed very substantial overlap, some areas of the brain contain predominately one over the other. The caudate nucleus, for example, is rich in FKBP12 and CN but not CyPA, whereas the brain stem contains much CyPA but little FKBP12 or CN.

The extensive colocalization of CN with FKBP12 and CyPA suggested a functional link between CN and the immunophilins in the CNS. Accordingly, Snyder's group screened brain homogenates for proteins whose phosphorylation levels were enhanced by treatment with FK506 or CsA, reasoning that calcineurin substrates would be so affected by the drug−immunophilin complexes. These experiments identified a number of such proteins, including growth-associated protein-43, or GAP-43,[167] and nitric oxide synthase, or NOS.[169]

GAP-43 has been linked to neuronal process elongation or neurite extension in neurons,[170] and mRNA levels for GAP-43 have been shown to increase in damaged facial or sciatic nerves undergoing regeneration.[171] The fact that this increase in GAP-43 levels correlated closely with increased FKBP12 levels, together with the observed high levels of FKBP12 in peripheral nerves and neonatal neuronal growth cones,[167] suggested that immunophilins might mediate nerve growth. The Hopkins group thus examined the effects of FK506 on neuronal process extension and found that the drug promoted neurite outgrowth in pheochromocytoma (PC12) cells (a neural crest-derived cell line which differentiates into a neuron-like phenotype in response to nerve growth factor treatment) and sensory ganglia at low-nanomolar and even subnanomolar concentrations.[172] Neurite outgrowth in PC12 cells was assessed by counting the percentage of cells with processes greater than 5 μm in length. The EC_{50} for FK506-dependent neurite outgrowth in PC12 cells was 500 pM in the presence of a submaximal concentration of nerve growth factor (NGF) (1 ng/mL). Rapamycin and CsA also produced neurotrophic effects in PC12 cells. FK506 failed to produce effects in PC12 cells in the absence of added NGF, indicating the ligand worked by increasing the sensitivity of the cells to the growth factor. In cultured chick dorsal root ganglia (DRGs), FK506 produced neurotrophic effects in the absence of exogenous NGF. However, as the effects of FK506 were partially blocked by an antibody to NGF, the drug may be acting to potentiate the effects of NGF produced by Schwann cells in the primary cultures.[161] The neurotrophic effects of FK506, CsA, and RAPA in chick DRGs were scored by counting the number of processes longer than the DRG explant diameter 48 h after drug treatment. FK506 and RAPA produced half-maximal effects at 500 pM concentration in the explants (Table 5). Cyclosporin A was considerably less potent but did increase neurite outgrowth with an EC_{50} of 50 nM. In contrast, Gold reported that CsA, at concentrations up to 1 mM, did not stimulate neurite outgrowth in neuroblastoma SH-SY5Y cells, while FKBP12 ligands enhanced process extension in these cells with ED_{50}'s of 1−10 nM, in the presence of 10 ng/M NGF.[173]

Table 5. In Vitro Neurotrophic Potencies and FKBP12 Rotamase Inhibition of Immunosuppressive Drugs and Nonimmunosuppressive Analogues

compd	K_i, nM	ED_{50}, nM
FK506	0.4	0.5
rapamycin	0.2	0.5
cyclosporin A	20.0	50.0
L-685,818	0.7	5.0
WAY 124,466	12.0	25.0
MeAla-6-CsA	50.0	50.0

Interestingly, Gold found that FK506 produced an inverted dose−response curve with respect to neurite outgrowth in SH-SY5Y cells, inhibiting NGF-induced neurite outgrowth at concentrations of 1 μM.[173] These results are consistent with a report that 50 μM FK506 inhibited neurite outgrowth in cell culture[174] and with in vivo dose−response experiments done by Gold's group (vide infra).

Although the initial investigations into the possible effects of FK506 on nerve growth were prompted by the correlation of increased expression levels for FKBP12 and GAP-43 in damaged neurons and the observation that phosphorylation levels of GAP-43 were affected by FK506 treatment, it quickly became clear that the latter changes were not relevant to the nerve regenerative properties of the drug. The fact that both FK506 and rapamycin produced neurite outgrowth effects of equal magnitude and potency, rather than RAPA antagonizing the effects of FK506, strongly suggested a calcineurin-independent mechanism for the neurotrophic effects. As will be discussed in the next section, Snyder's group and scientists at Guilford Pharmaceuticals demonstrated that the neurotrophic effects of these drugs are inde-

Table 6. Dose Dependency of FK506 To Promote Nerve Regeneration in Rats with Lesioned Sciatic Nerves[a]

FK506 dosage, mg/kg (daily, sc)	mean axonal area[b]	axonal regeneration rate[c]
1	67	16
2	83	nd
5	120	34
10	100	29

[a] Data taken from refs 176 and 177. See text for brief description of experimental protocol. [b] Axonal calibers of the soleus nerve were determined by electron microscopy and are expressed as percent increase from control levels. [c] Axonal regeneration rates were determined using radiolabeling techniques and are expressed as percent increase from control levels.

pendent of calcineurin (or FRAP) inhibition and thus unrelated to changes in GAP-43 phosphorylation.

The therapeutic relevance of the neurotrophic properties of FK506 was demonstrated by Gold et al., who showed that in rats with lesions of the sciatic nerve, treatment with FK506 enhanced both nerve regrowth and regain of neurological function.[175,176] Axotomy of the sciatic nerve in young rats was performed by crushing the nerve at the level of the hip with forceps. Functional recovery was assessed by monitoring the number of days ensuing before the animals demonstrated the onset of ability to right the foot and move the toes and the number of days until the animals were able to walk on its hind feet and toes. Morphological recovery was evaluated by measuring axonal calibers in the tibial nerve branch to the soleus muscle by electron microscopy and by measuring rates of axonal regeneration by radiolabeling the L5 dorsal root ganglion.

In control animals, onset of the ability to right the foot first occurred between days 17 and 18, whereas animals receiving 1 mg/kg FK506 daily subcutaneously were walking almost normally by day 18. FK506 thus accelerated functional recovery in these animals with lesioned sciatic nerves. Morphological improvement was likewise observed on day 18 of the experiments. Axonal calibers were increased in animals receiving FK506, and reinnervation of intrafusal fibers was evident in the most distal muscles. The rate of axonal regeneration in FK506-treated animals was increased 16% relative to controls (Table 6). The Snyder group reported that FK506 likewise promoted regeneration of crushed facial nerves in rat pups.[161]

Recently, Gold's group has studied the dose dependency of FK506's nerve regenerative effects in the sciatic nerve-lesioned rat.[173,177] In these experiments, FK506 was administered once a day subcutaneously at doses of 2, 5, and 10 mg/kg for 18 days. Animals receiving 5 mg/kg FK506 showed the most rapid functional recovery and the greatest extent of morphological recovery. The morphological results, summarized in Table 6, demonstrate that FK506 produces a bell-shaped dose−response curve for nerve regeneration, consistent with the in vitro cell culture experiments described above.

The calcium−calmodulin-dependent enzyme NOS, another known calcineurin substrate, was also affected by treatment with FK506.[169] NOS generates nitric oxide (NO) by oxidatively cleaving arginine to citrulline and NO. NO has been found to play several roles in the body, acting as a short-lived but rapidly diffusing messenger molecule.[178,179] In the brain, NO plays the unusual role of a gaseous neurotransmitter. Phosphorylated NOS is inactive, and therefore calcineurin can regulate NOS activity in a calcium-dependent manner via its phosphatase activity.[180] Increased Ca^{2+} levels from activation of the *N*-methyl-D-aspartate (NMDA) subtype of the glutamate receptor result in activation of NOS and subsequent stimulation of cGMP formation.[181] The well-known neurotoxic properties of excess glutamate[182] appear to involve NOS. Glutamate neurotoxicity is blocked by inhibitors of NOS,[183] as well as in neuronal NOS gene knockout mice.[169] NMDA receptor-mediated neurotoxicity is thought to play a major role in stroke-related ischemic brain damage. The complexes of FK506 with FKBP12, or CsA with CyPA, should block activation of NOS by inhibiting calcineurin, keeping NOS in its inactive hyperphosphorylated state. This suggests a potential therapeutic utility of the drugs in stroke patients. Snyder's group demonstrated that both FK506 and CsA potently blocked NMDA neurotoxicity in cortical cultures.[184] Rapamycin antagonized the neuroprotective effects of FK506, consistent with these effects being mediated by a calcineurin-dependent mechanism. In a rat model of stroke in which the middle cerebral artery was occluded, FK506 significantly reduced stroke damage in the cerebral cortex when given 1 h post-occlusion.[185] Again, rapamycin itself was ineffective as a neuroprotectant, but rapamycin blocked the neuroprotection of FK506. FK506 was also an effective neuroprotectant in the gerbil model of transient global ischemia.[186,187] However, it cannot be concluded that the neuroprotective effects of FK506 in these models are due to blockade of glutamate-mediated neurotoxicity. Despite the demonstration of protection against glutamate toxicity in vitro afforded by FK506, a recent study fails to substantiate this as the mechanism of protection in the focal ischemia model.[188] The report that cyclosporin A, in contrast to FK506, fails to protect hippocampal CA1 and cerebral neurons against transient global ischemia further underscores the likelihood that the neuroprotective effects of FK506 in ischemic injury are mediated by a calcineurin-independent pathway.[189]

A number of other observed neuronal effects of FK506 are likely to arise from inhibition of calcineurin phosphatase activity. The report that FK506 blocks NMDA receptor desensitization is consistent with the known role of calcineurin in regulating this phenomenon.[190] FK506 has been reported to block both long-term potentiation (LTP) and long-term depression (LTD) in the visual cortex.[191,192] In the hippocampus, it facilitates LTP[193] and inhibits LTD.[194] The generation of LTP and LTD both require NO generation,[195−199] suggesting the effects of FK506 in this regard are due to inhibiting calcineurin-dependent NOS activation. FK506 also affects neurotransmitter release, producing both an inhibition of NMDA-induced neurotransmitter release and an enhancement of depolarization-induced neurotransmitter release.[200] These opposite effects have been ascribed to FK506-induced hyperphosphorylation of two calcineurin substrates: NOS and synapsin I, respectively.

As the foregoing discussion makes clear, immunophilins such as FKBP12 play multiple roles in the nervous system, and various immunophilin ligands demonstrate neuroprotective and neurotrophic properties.[201] Both calcineurin-dependent and calcineurin-independent mechanisms appear to be in play. The remarkable ability of

FK506 to promote regeneration of damaged nerves has led to extensive investigations of this aspect of its neuronal actions.

Separation of Neurotrophic and Immunosuppressant Effects of Immunosuppressant Drugs and Delineation of the Structural Basis for FKBP12/Ligand-Mediated Nerve Growth

The results with FK506, RAPA, and CsA described above suggested an exciting new therapeutic utility for immunophilin-binding drugs. The in vivo results with FK506 held forth the promise that related agents might be clinically useful for the treatment of disorders involving degeneration of nerves. Small molecule drugs with oral bioavailability capable of penetrating the central nervous system and promoting the protection and regeneration of damaged nerves would constitute a breakthrough in the development of new drugs for the treatment of neurodegenerative diseases. However, the immunosuppressive effects of FK506, RAPA, and CsA represent a severe liability in the context of developing drugs for treating nervous system disorders. In addition, FK506 and CsA are extremely toxic.[202,203] This toxicity appears to be directly tied to their calcineurin-based mechanism of action.[102,103] A critical question is then whether the neurotrophic properties of the immunosuppressant drugs are mechanistically linked to their immunosuppressive actions, or operate by separate pathways.

As described previously, various nonimmunosuppressant analogues of FK506, RAPA, and CsA have been described by groups studying the mechanism of immunosuppression. These compounds, which bind to their respective immunophilins and inhibit their PPIase activity but lack the ability to interact with CN or FRAP, thus can serve as probes for the relationship between immunosuppression and neurotrophism. Steiner et al. evaluated the neurotrophic properties of three such compounds: 18-hydroxyascomycin (L-685,818, an analogue of FK506), WAY 124,466 (an analogue of rapamycin), and MeAla-6-CsA, an analogue of CsA.[204]

In PC12 cells, 18-hydroxyascomycin produced neurotrophic effects, as measured by stimulated neurite outgrowth, in a dose-dependent fashion, with maximal effects comparable to that of FK506. The potency of the nonimmunosuppressive analogue was about 10-fold less, corresponding to its lower affinity for FKBP12. In a similar manner, WAY 124,466 is a less potent inhibitor of FKBP12 than is RAPA. WAY 124,466 produced neurotrophic effects in PC12 cells with a potency relative to that of RAPA comparable to the differences in their affinities for FKBP12.

CsA also produced neurite outgrowth in PC12 cells, though the effect was considerably less potent. Its nonimmunosuppressive analogue, MeAla-6-CsA, was considerably more potent and produced a greater maximal effect.

None of the compounds stimulated neurite outgrowth in PC12 cells in the absence of NGF, and it was shown that the compounds served to potentiate the effects of NGF in these cells. The drugs appear to enhance both the rate and extent of neurite outgrowth, though in no case did the outgrowth exceed that produced by maximally effective concentrations of NGF. In cultured chick DRGs, all of the drugs produced neurotrophic effects in the absence of added NGF. In these sensory neuronal cultures, all of the compounds displayed conventional dose−response curves, and the nonimmunosuppressive analogues were comparable in potency and maximal efficacy to their immunosuppressant parents. The combined in vitro data for the immunosuppressive and nonimmunosuppressive compounds is summarized in Table 5.

Table 7. Nonimmunosuppressive FKBP12 Ligand-Induced Regeneration of Lesioned Sciatic Nerves in Rats[a]

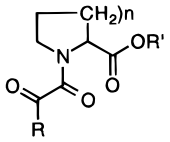
treatment	axonal diameter, mm	cross-sectional area, mm^2	myelination
sham	2.98 ± 0.221	9.14 ± 0.835	60.07
lesion/vehicle	1.56 ± 0.73	2.74 ± 0.357	5.30
lesion/FK506[b]	1.99 ± 0.192	4.16 ± 0.403	11.67
lesion/L-685,818[b]	1.87 ± 0.141	3.43 ± 0.295	16.68
sham	2.62 ± 0.179	8.69 ± 0.503	55.98
lesion/vehicle	1.40 ± 0.204	2.54 ± 0.376	2.95
lesion/compd **9**[c]	2.06 ± 0.360	5.04 ± 0.403	21.13
lesion/compd **10**[c]	1.81 ± 0.123	3.84 ± 0.281	nd
sham	3.46 ± 0.148	12.04 ± 0.949	63.44 ± 8.50
lesion/vehicle	1.94 ± 0.071	4.61 ± 0.324	3.18 ± 1.59
lesion/compd **41** (3 mg/kg)[d]	2.66 ± 0.079	8.62 ± 0.452	21.66 ± 5.94
lesion/compd **41** (10 mg/kg)[d]	2.74 ± 0.042	8.87 ± 0.337	24.31 ± 7.61

[a] Animals lesioned by sciatic nerve crush were treated as described for individual drugs and sacrificed on day 18 of the experiment. Diameter and cross-sectional area of axons were quantitated by antineurofilament staining. Myelin levels were quantitated by myelin basic protein-immunoreactive stain density. [b] FK506 or L-685,818 (1 mg/kg in intralipid) were administered in Gelfoam directly to the site of lesion, once a day for 18 days. [c] Compounds **9** and **10** were administered at 30 mg/kg sc, once a day for 18 days. [d] Compound **41** was administered sc once a day for 18 days.

Gold's experiments demonstrating the ability of FK506 to enhance morphological and functional recovery in rats with sciatic nerve lesions have been described above. When compared side-by-side in a similar sciatic nerve crush experiment in rats, the nonimmunosuppressive analogue of FK506, 18-hydroxyascomycin, produced positive effects equal to or greater than that of FK506 (Table 7). Animals receiving either compound had more large axons and axons with a larger average diameter than vehicle-treated animals. The degree of myelination was also strongly augmented in the drug-treated animals. Functional recovery of the injured hindlimb was found to be markedly accelerated. On day 14 of the experiment, all animals (control and drug-treated) showed the same toe spread distance, indicating that drug-treated animals experienced the same degree of degeneration as controls. By day 18, animals receiving FK506 or L-685,818 had almost completely regained the ability to bear weight on the injured limb, indicating an acceleration of functional recovery produced by both the immunosuppressive and nonimmunosuppressive compounds.

The results of this work demonstrated that inhibition of calcineurin and FRAP is not responsible for the neurotrophic actions of the drugs and suggested that the immunosuppressive and neurotrophic actions of the immunosuppressants were separable. However, the nonimmunosuppressant compounds used in this study were themselves large macrocyclic structures only subtly different from the parent compounds, and thus

Table 8. In Vitro Neurotrophic Effects of *N*-Glyoxylpipecolate and *N*-Glyoxylprolyl Esters



compd	*n*	R	R′	K_i, nM	ED_{50}, nM
1	2	OMe	Et	>10000	>10000
2	2	OMe	benzyl	8000	>10000
3	2	OMe	4-cyclohexylbutyl	6000	>10000
4	2	OMe	3-cyclopentylpropyl	35000	>10000
5	2	OMe	3-cyclohexylpropyl	20000	>10000
6	2	Me	Et	>10000	>10000
7	2	phenyl	Me	>10000	>10000
8	2	1,1-dimethylpropyl	Et	1300	5000
9	2	1,1-dimethylpropyl	3-phenylpropyl	250	300
10	2	1,1-dimethylpropyl	(3,4,5-trimethoxyphenyl)propyl	25	80
11	2	1,1-dimethylpropyl	3-(3-pyridyl)propyl	130	8.5
12	2	1,1-dimethylpropyl	1,7-diphenyl-4-heptyl	30	5
13	2	1,1-dimethylpropyl	4-(*p*-methoxyphenyl)-6-phenyl-3-hexyl	15	0.17
14	2	1,1-dimethylpropyl	1,5-diphenyl-3-pentyl	20	0.016
15	2	1,1-dimethylpropyl	3,3-diphenylpropyl	8.3	0.029
16	2	1,1-dimethylpropyl	4-(*p*-methoxyphenyl)-1-butyl	60	43
17	2	1,1-dimethylpropyl	3-(2,5-dimethoxyphenyl)propyl	12	2.5
18	2	1,1-dimethylpropyl	3-cyclohexylpropyl	170	1
19	2	1,1-dimethylpropyl	(1*R*)-1,3-diphenylpropyl	17	0.30
20	2	*tert*-butyl	3-phenylpropyl	2	0.085
21	2	*tert*-butyl	4-(*p*-methoxyphenyl)-1-butyl	24	0.002
22	2	*tert*-butyl	1,7-diphenyl-4-heptyl	5	0.085
23	2	*tert*-butyl	2-adamantyl	78	0.11
24	2	*tert*-butyl	3-cyclohexylpropyl	32	0.29
25	2	cyclohexyl	3-phenylpropyl	210	0.82
26	2	cyclohexyl	3-cyclohexylpropyl	760	1
27	2	cyclohexyl	4-(*p*-methoxyphenyl)-1-butyl	103	6
28	2	phenyl	3-(3-pyridyl)propyl	725	0.8
29	2	phenyl	1,7-diphenyl-4-heptyl	3	0.15
30	2	phenyl	1-phenyl-7-(2-pyridyl)-4-heptyl	50	0.063
31	2	3,4,5-trimethoxyphenyl	4-cyclohexylbutyl	40	0.031
32	2	3,4,5-trimethoxyphenyl	4-(*p*-methoxyphenyl)-1-butyl	13	0.03
33	2	3,4,5-trimethoxyphenyl	(3-phenoxy)benzyl	18	0.18
34	2	3,4,5-trimethoxyphenyl	3-(3-indolyl)propyl	17	0.06
35	2	3,4,5-trimethoxyphenyl	1,7-diphenyl-4-heptyl	1	0.61
36	2	3,4,5-trimethoxyphenyl	1-phenyl-6-(3-pyridyl)-4-hexyl	3	0.95
37	2	3,4,5-trimethoxyphenyl	1-phenyl-7-(2-pyridyl)-4-heptyl	1	0.05
38	2	3,4,5-trimethoxyphenyl	1-phenyl-7-(3-pyridyl)-4-heptyl	0.5	nd
39	1	1,1-dimethylpropyl	3-phenylpropyl	42	nd
40	1	1,1-dimethylpropyl	3-cyclohexylpropyl	194	0.257
41	1	1,1-dimethylpropyl	3-(3-pyridyl)propyl	7.5	0.05
42	1	1,1-dimethylpropyl	3-(2-pyridyl)propyl	195	0.075
43	1	1,1-dimethylpropyl	3-(2,5-dimethoxyphenyl)propyl	250	2
44	1	1,1-dimethylpropyl	(3,4,5-trimethoxyphenyl)ethyl	120	0.015
45	1	1,1-dimethylpropyl	3-(3,4-methylenedioxyphenyl)propyl	170	10
46	1	1,1-dimethylpropyl	3-(2,5-dimethoxyphenyl)prop-2-enyl	450	0.8
47	1	cyclohexyl	3-phenylpropyl	82	0.13
48	1	cyclohexyl	3-(3-pyridyl)propyl	9	2
49	1	*tert*-butyl	3-phenylpropyl	95	0.025
50	1	*tert*-butyl	3-(3-pyridyl)propyl	3	0.014

interaction with other protein targets could not be ruled out. Furthermore, it was not possible to ascertain on the basis of these studies the specific structural basis for the elicitation of the neurotrophic effects. This information was provided by Hamilton et al., who showed that the neurotrophic effects of FK506 and rapamycin resided within their FKBP-binding domains.[205] Simple FKBP-binding domain analogues had previously been described by groups working in the immunosuppression area.[206] In particular, *N*-glyoxylpipecolate esters had been shown by several groups to be high-affinity ligands for and inhibitors of FKBP12.[49,50,207,208] A large number of such compounds were synthesized and tested for their ability to inhibit FKBP12 rotamase activity and to promote neurite outgrowth in vitro from cultured chick DRG sensory neurons. *N*-Glyoxylprolyl esters have also been explored. The results of these experiments from several sources are compiled in Table 8.[205,206,209] Many of these compounds were previously reported by others working in the immunosuppression field.

The data make clear that the ability of FK506 and related structures to elicit neurite outgrowth from neurons is not dependent upon the effector domain element. Very simple small molecule FKBP12 ligands such as those in Table 8 are extremely potent neurotrophic agents in vitro. Compounds which contain the structural elements necessary for interacting with the binding sites in the FKBP domain, as discussed above, were effective in this functional assay. Compounds **1**−**7**,

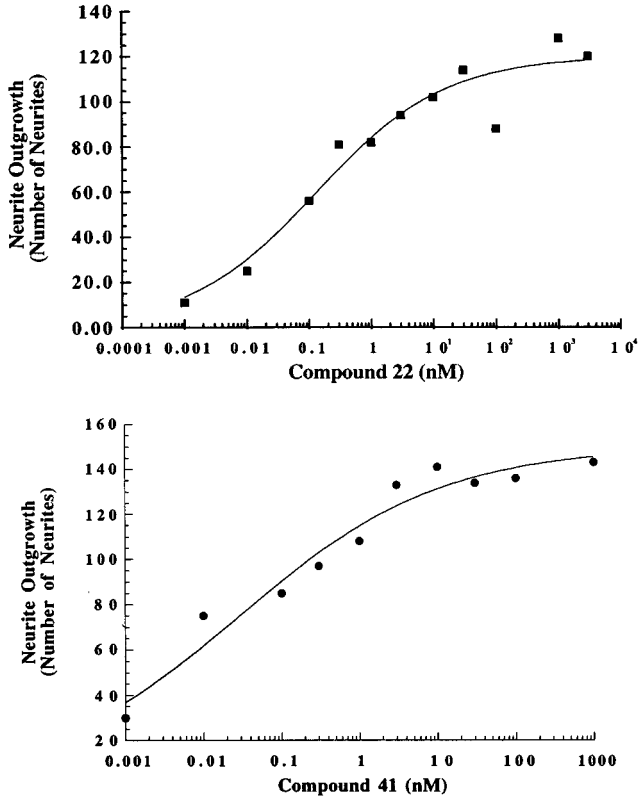


Figure 10. Dose−response curves for promotion of neurite outgrowth in cultured sensory neurons by compounds **22** and **41**.

which lack sufficient functionality to bind to FKBP12 with good affinity, were found to be ineffective as neurotrophic agents. These compounds are missing the hydrophobic alkyl functionality to interact with the pocket occupied by the pyranose ring of FK506. Incorporation of a dimethylpropyl moiety to mimic this portion of FK506 results in weak but detectable activity as an FKBP12 inhibitor and in vitro neurotrophic agent. Further addition of lipophilic arylalkyl ester side chains produced compounds which were quite potent neurotrophic agents. Many of these compounds are quite potent FKBP12 inhibitors, as previously reported by others, with single- or double-digit nanomolar inhibition constants. More strikingly, many are remarkably potent at promoting neurite outgrowth in cultured sensory neurons, in a dose-dependent fashion and in the absence of exogenous growth factors. The dose−response curves for compounds **22** and **41** are shown in Figure 10. The maximal effect of **41** in these cultures averaged about 140 processes per explant. This value is comparable to the maximal neurite outgrowth elicited in these cultures by treatment with 25−50 ng/mL NGF (ca. 140−150 processes per explant). The morphology exhibited by DRGs treated with the FKBP12 ligands is indistinguishable from NGF-treated explants; the neurites elicited by the drugs form a dense, highly arborized network, and both more processes and longer processes are elicited in a dose-dependent manner, analogous to NGF treatment.

While the neurotrophic ED_{50}'s for the macrocyclic immunosuppressive drugs and their nonimmunosuppressive analogues closely paralleled their IC_{50}'s for enzyme inhibition, this is not the case for the simpler compounds. Many of the compounds in Table 8 elicit neurite outgrowth with half-maximal responses observed at subnanomolar concentrations. Compound **41**, for example, has an ED_{50} of 58 pM. Many of the tested compounds have ED_{50}'s 10−100 times lower than their measured K_i's to inhibit FKBP12 rotamase activity. As will be discussed in a later section, we do not believe that inhibition of rotamase activity per se is the cause of the observed neurotrophic effects of these compounds.

The neurotrophic effects of small molecule FKBP ligands are not specific to *N*-glyoxyl esters. It has been reported in the literature that the ketoamide moiety of compounds such as those in Table 8 may be replaced by sulfonamide[207] or urea[210] linkages with the retention of FKBP binding and inhibition. The examples shown in Tables 9 and 10 (taken from ref 211) demonstrate that such compounds also retain the in vitro nerve growth ability of the ketoamide analogues, suggesting that structural changes consistent with known SAR for FKBP binding retain neurotrophic activity.

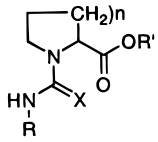
The nerve growth effects of FKBP ligands are not limited to cells derived from the peripheral nervous system. Gold has reported similar results for FK506 in both cortical cultures and hippocampal neurons.[173] As discussed in the next section, FKBP ligands have been shown to be effective in protecting and regenerating peripheral and central neurons of several types in vivo.

FKBP Ligands in Animal Models of Neurodegeneration

The original experiments demonstrating the nerve regenerative effects of FK506 in vitro were validated by the demonstration that FK506 promoted regeneration of damaged peripheral nerves in vivo, as described above. The discovery that simple FKBP-binding domain analogues of FK506 promote nerve growth in cell culture has recently been extended in a number of studies that demonstrate that these compounds have broad neuroprotective and neuroregenerative properties in a variety of neuronal populations.

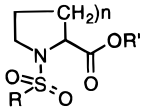
Peripheral Neurons. The results with FK506 and a nonimmunosuppressive analogue in rats with lesioned sciatic nerves were described in detail in the previous section. Several compounds from Table 8 were examined in this model by Steiner et al.[204,212,213] Subcutaneous administration of compounds **9**, **10**, and **41** to rats just prior to lesioning of the sciatic nerve, and daily for 18 days following the lesioning, augmented the regrowth and remyelination of lesioned sciatic nerves. Compounds **9** and **10**, at doses of 30 mg/kg sc, were equivalent to FK506 or L-685,818 in their actions (Table 5).[204] Compound **41** was tested at two different doses; at both 3 and 10 mg/kg sc, **41** significantly augmented the diameter and cross-sectional area of the recovering nerve fibers.[212] All three compounds produced a striking effect on myelin levels, as quantitated by anti-myelin basic protein Ig. Myelin sheaths were not evident in lesion/vehicle-treated animals, whereas animals receiving FKBP ligands showed a substantial myelin layering in injured nerves. Animals receiving **41** had myelin levels 7−8 times higher than lesioned animals receiving vehicle. Particularly significant was the finding that the compound did not appear to affect healthy peripheral nerves. Administration of **41** to normal rats (40 mg/kg/day sc for 18 days) did not result in abnormal axonal

Table 9. In Vitro Neurotrophic Effects of *N*-Carbamoylpipecolate and *N*-Carbamoylprolyl Esters



compd	*n*	X	R	R′	K_i, nM	ED_{50}, nM
51	1	O	1,1-dimethylpropyl	3-(3-pyridyl)propyl	742	1
52	1	O	cyclohexyl	3-(3-pyridyl)propyl	1482	nd
53	1	S	cyclohexyl	3-(3-pyridyl)propyl	131	0.30
54	1	S	2-adamantyl	3-(3-pyridyl)propyl	116	0.14
55	2	O	2-methylbutyl	3-(3-pyridyl)propyl	70	0.07

Table 10. In Vitro Neurotrophic Effects of *N*-Sulfonylpipecolate and *N*-Sulfonylprolyl Esters



compd	*n*	R	R′	K_i, nM	ED_{50}, nM
56	1	benzyl	3-(3-pyridyl)propyl	72	nd
57	2	benzyl	4-phenylbutyl	34	0.03
58	2	benzyl	1,5-diphenyl-4-pentyl	107	0.133
59	2	4-methylphenyl	1,7-diphenyl-4-heptyl	332	1
60	2	4-methylphenyl	4-phenylbutyl	504	nd
61	2	phenyl	4-phenylbutyl	470	nd

growth or altered myelination pattern in nonlesioned sciatic nerves.

Gold et al. evaluated compound **38** (Table 8) in the rat sciatic nerve crush model, evaluating functional and morphological recovery as described previously for FK506. Animals receiving 400 mg/kg sc daily of **38** showed a more rapid onset of functional recovery as compared to controls, and morphological analysis of the animals revealed larger size regenerating axons in the drug-treated animals.[214] In a preliminary report, **38** was shown to be effective in this model following oral administration at doses as low as 5 mg/kg daily.[215] Functional recovery in animals receiving **38** orally was reduced from 17 days in control animals to 13 days, a result superior to that produced by subcutaneous administration of FK506 in the earlier studies. Drug-treated animals also showed the presence of larger axonal calibers and more myelinated fibers distal to the site of lesion.

The data reviewed here document the remarkable ability of FKBP ligands to promote regeneration of damaged peripheral nerves in vivo. Such compounds may thus possess therapeutic utility for the treatment of peripheral neuropathies and other peripheral nerve disorders such as Bell's palsy and diabetic neuropathy. In addition, the remyelination effects observed with these compounds suggest further study in animal models of peripheral and central demyelination.

Central Neurons. Several studies demonstrate that the nerve regeneration properties of FKBP ligands appear to be quite general. In addition to peripheral sciatic nerves, the compounds are effective in promoting regeneration of lesioned dopaminergic, serotonergic, and cholinergic neurons, suggesting broad therapuetic potential.

1. Dopaminergic. The observation that the nigrostriatal pathway in the brain is particularly enriched in FKBP12 suggests potential utility for FKBP ligands in treating Parkinson's disease (PD). The corpus striatum is a region of the human brain involved in the regulation of motor behavior. Neurons (nerve cells) in this part of the brain use dopamine as their neurotransmitter. The cell bodies of the neurons that comprise the nigrostriatal pathway lie in a region of the brain called the substantia nigra pars compacta. The axonal fibers of these nerve cells traverse the medial forebrain bundle to terminate in the striatum. The motor abnormalities of PD are believed to be the result of decreased dopaminergic neurotransmission due to degeneration of the dopaminergic neurons of the nigrostriatal pathway.[216,217] *N*-Methyl-4-phenyltetrahydropyridine (MPTP) is a neurotoxin which selectively destroys dopaminergic neurons by free-radical oxidative processes.[218] MPTP has been shown to produce a parkinsonian state in humans,[219,220] and lesioning of the nigrostriatal pathway in animals with MPTP is a well-established animal model for PD. FKBP ligands have demonstrated potent neuroprotective and neuroregenerative effects in this PD model.

Scientists at Guilford Pharmaceuticals have reported on the activity of a number of FKBP ligands in the MPTP model of PD in CD1 mice following systemic and oral administration. The effects of the compounds have been evaluated in two distinct paradigms.

In a "concurrent" or protective protocol, compounds were administered together with MPTP for 5 days and then for an additional 5 days following cessation of MPTP treatment, to assess the ability of the immunophilin ligands to block the degeneration of dopaminergic neurons caused by the toxin. Animals were perfused after 18 days, and their brains were fixed, cryoprotected, and sectioned. Anti-tyrosine hydroxylase (TH) Ig immunostaining was performed on saggital and coronal brain sections in order to quantitate survival and recovery of dopaminergic neurons. Table 11 collects data for subcutaneous administration of FKBP ligands in this concurrent dosing model from several published sources.[205,206,212]

Treatment of animals with MPTP produces a 60−70% reduction of functional striatal dopaminergic terminals compared to control animals. Animals receiving systemic administration of a variety of FKBP ligands concurrently with MPTP displayed a striking neuroprotection, when assessed by recovery of TH-positive dopaminergic terminals relative to controls. In addition to the powerful protective/regenerative effects exerted on the striatal terminals by the compounds, substantial protection of the number of dopaminergic neurons in

Table 11. Recovery of Striatal TH Immunostaining in MPTP-Treated Mice by Concurrent Treatment with FKBP Ligands

compd	dosage, mg/kg sc	% recovery striatal innervation[a]
14	40	71.6 ± 4.90[b]
20	40	32.4 ± 3.98[b]
21	40	58.9 ± 4.93[b]
22	40	43.8 ± 8.65[b]
41	40	67.5 ± 4.30[c]
52	4	59.79 ± 4.57[d]
53	4	56.13 ± 4.74[d]
54	4	52.32 ± 3.89[d]
55	4	27.47 ± 2.65[d]
56	4	44.31 ± 4.73[d]
57	4	38.02 ± 4.00[d]
60	4	44.16 ± 3.37[d]
61	4	29.22 ± 3.00[d]

[a] Percent recovery was determined by subtracting from the experimental observation the mean density from the analogous lesioned/untreated group and dividing this by the calculated percent loss due to MPTP treatment. The extent of nigral lesions was assessed for each case. The total number of TH-positive neurons in each nigral section as well as the TH-positive neuronal density was determined for each section. [b] Data from ref 205. [c] Data from ref 212. [d] Data from refs 206 and 212.

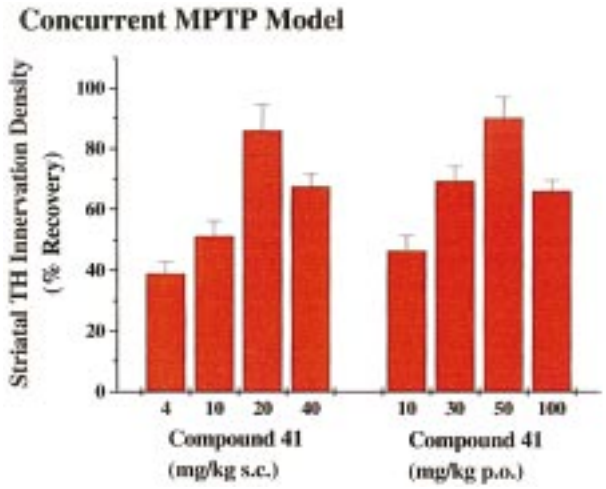


Figure 11. Dose-dependent protection of striatal TH immunostaining in MPTP-treated mice by concurrent systemic or oral treatment with compound **41**. Percent recovery was determined as described in Table 11. The data for sc and oral administration are from refs 212 and 221, respectively.

the substantia nigra pars compacta and the axonal tracts in the medial forebrain bundle was also reported.

Interestingly, dose−response studies on **41** revealed a bell-shaped response curve (Figure 11), in line with the results reported by Gold for FK506 in the sciatic nerve model. The maximum effect produced by **41** upon subcutaneous administration in the concurrent dosing model was observed at 20 mg/kg (86% recovery of striatal tyrosine hydroxylase immunostaining). Administration of **41** to normal mice (40 mg/kg/day sc for 10 days) did not produce intranigral sprouting of dopaminergic neurons or increased density of dopaminergic striatal innervation, again suggesting that the effects of the compound are exerted only upon damaged nerves.

The data compiled in Table 11 show that FKBP ligands of several different structural classes produce a remarkable sparing of dopaminergic neurons in MPTP-treated mice. *N*-Glyoxyl-, *N*-sulfonyl-, and *N*-carbamoylprolyl and -pipecolyl esters are all capable of effecting recovery of striatal dopaminergic innervation

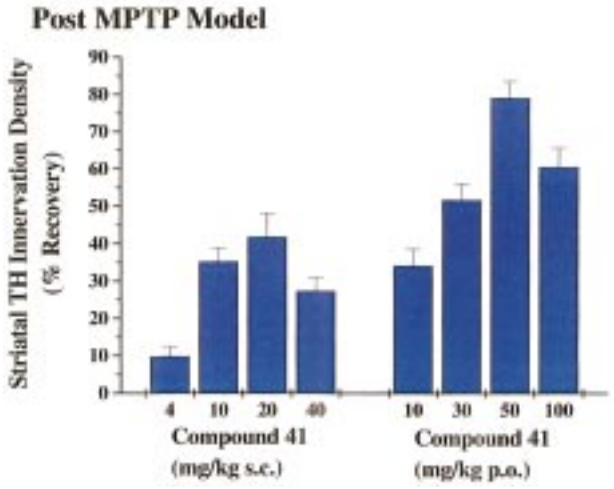


Figure 12. Dose-dependent recovery of striatal TH immunostaining in MPTP-treated mice by post-MPTP systemic or oral treatment with compound **41**. The data for sc and oral administration are from refs 212 and 221, respectively.

in an unprecedented manner following systemic administration. These results suggest that structural changes consistent with retention of affinity for FKBP12 retain the nerve regeneration properties originally observed for FK506.

Compound **41** was also shown to be effective in this model upon oral administration (Figure 11).[221] A dose of 50 mg/kg po produced a recovery of striatal TH immunostaining to greater than 90% of control levels. Similar results were subsequently reported for compound **38**.[222] The ability of FKBP ligands to produce such striking neuroprotective and regenerative effects upon oral administration suggests powerful therapeutic utility for these compounds in the treatment of neurodegenerative diseases.

The second paradigm in the MPTP PD model which has been investigated is a "post-MPTP" or regenerative protocol. In this paradigm, which more closely models the human disease state, treatment with FKBP ligands was begun only after maximal destruction of the dopaminergic pathway by MPTP had taken place. These experiments were done in order to assess whether immunophilin ligands were capable of stimulating axonal sprouting from spared projections and regenerating functional striatal terminals postlesioning, a result not previously seen with an orally active small molecule. In these experiments, mice received **41** for 5 days, beginning 3 days following the cessation of treatment with MPTP. Experiments were ended on day 18, and brain sections were analyzed for tyrosine hydroxylase immunostaining as described previously. Additional groups of animals were also analyzed by HPLC for striatal dopamine content and the dopamine metabolites DOPAC and homovanillic acid. Figure 12 collects the reported data for both systemic[212] (sc) and oral[221] dosing of compound **41** in this protocol. Upon either systemic or oral administration, post-MPTP lesioning, **41** produced a dose-dependent increase in striatal dopaminergic fiber innervation as visualized by tyrosine hydroxylase immunohistochemistry. At a dose of 50 mg/kg po, the compound caused a regeneration of striatal TH-positive innervation density to 80% of control levels.

Treatment with **41** also significantly restored striatal

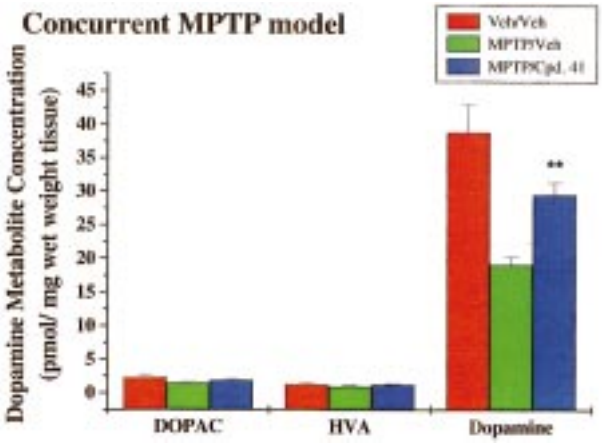


Figure 13. HPLC analysis of dopamine and metabolites following treatment of MPTP-lesioned mice with **41** in a concurrent treatment paradigm.

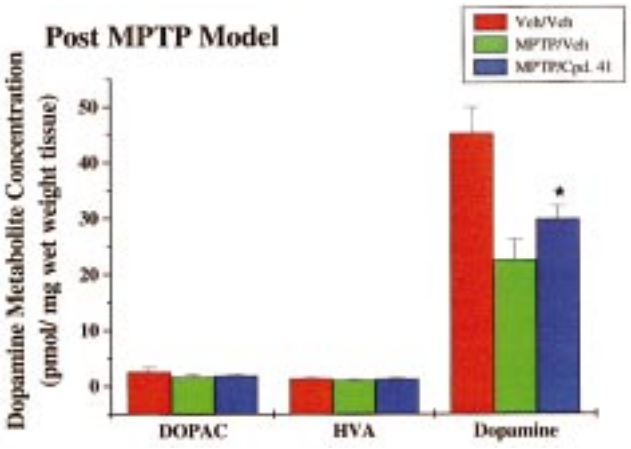


Figure 14. HPLC analysis of dopamine and metabolites following treatment of MPTP-lesioned mice with **41** in a post-MPTP treatment paradigm.

dopamine levels and dopamine metabolites, in both the concurrent and post-MPTP models (Figures 13 and 14). Animals lesioned with MPTP showed a ca. 50% depletion of striatal dopamine. Drug-treated animals showed a significant restoration of both striatal dopamine and dopamine metabolites, suggesting a regeneration of functional terminals.

In addition to the morphologic and neurochemical recovery in these lesioned animals, **41** elicited a behavioral recovery as well.[221] Administration of halperidol to MPTP-treated mice produces catalepsy and akinesia at doses which are subthreshold in normal mice. Treatment of MPTP-lesioned mice with **41** reversed the catalepsy and akinesia observed in MPTP-lesioned animals induced by haloperidol administration, again suggesting regeneration of functional dopaminergic terminal in the striatum after lesioning.

The effects of FKBP ligands have been studied in a rat model of PD.[212,223] Like MPTP, 6-hydroxydopamine (6HODA) is a severe neurotoxin which destroys dopaminergic neurons.[224] Intranigral injection of 6HODA into one side of the brains of rats produces a severe destruction of striatal dopaminergic innervation in the side ipsilateral to the lesion, leaving the contralateral side unaffected. Administration of amphetamine to animals with unilateral 6HODA lesions causes them to circle in a direction ipsilateral to the lesion.[225] Unilateral

Table 12. Regrowth of Striatal Dopamine Neurons in 6HODA-Lesioned Rats Following Treatment with **41**[a,b]

41-treated 6HODA-lesioned rats (postlesion)	% recovery of striatal TH innervation
1 h	34.22
7 days	25.47
28 days	28.13
60 days	17.28
120 days	19.50

[a] Percent recovery of striatal innervation was determined in the manner described in Table 11. [b] Data from refs 212 and 223.

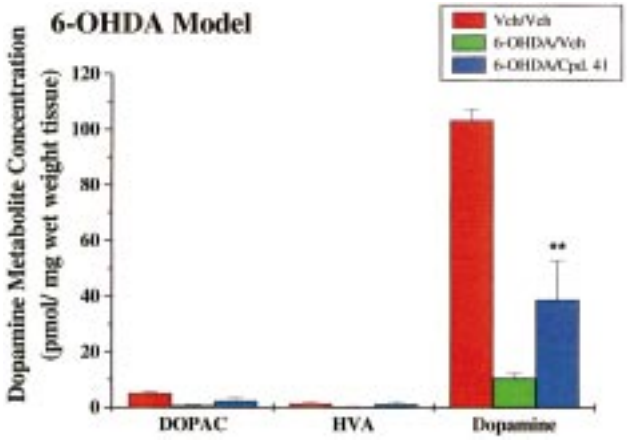


Figure 15. Restoration of dopamine levels and dopamine metabolite levels in 6HODA-lesioned rats following treatment with **41**. Data are taken from ref 223.

6HODA-lesioned rats thus provide a rodent model of lesioned nigrostriatal pathway with a documented functional deficit. The lesions produced by intranigral 6HODA are considerably more severe than those from MPTP, resulting in about 90% depletion of dopaminergic neurons in the pars compacta of the substantia nigra.

Adult male rats received unilateral 6HODA lesions and, following delays of 1 h to 4 months, were administered **41** sc at 10 mg/kg/ day for 5 days. Intranigral injection of 6HODA caused a reduction of striatal TH immunoreactive fiber density of 80−90% relative to controls. Systemic treatment with **41** in all cases significantly restored TH-positive fiber density in the striatum (Table 12).[212,223] Striatal TH-positive fiber density was increased 2.5−3.5 times in rats treated with **41** compared to vehicle-treated animals with analogous 80−90% loss of nigral neurons. Striatal reinnervation was seen in cases where administration of **41** was delayed up to 4 months following 6HODA lesion, was evident within 3 weeks of treatment onset, and remained intact 2 months after treatment cessation. The reinnervation was due to intrastriatal sprouting of spared fibers, partially from the substantia nigra compacta and partially from the VTA. Morphological recovery was accompanied by recovery of ∼30% of striatal catecholamine levels (Figure 15) and dramatic reductions in amphetamine-induced rotations (Figure 16). In addition to the results following systemic administration, **41** was orally active in stimulating intrastriatal sprouting.

2. Serotonergic. An impaired function of brain 5-HT systems has been implicated in a number of neurologic disturbances including the major depressive disorders, anxiety, and obsessive-compulsive behavior.[226] Significant loss of 5-HT neurons also has been reported in

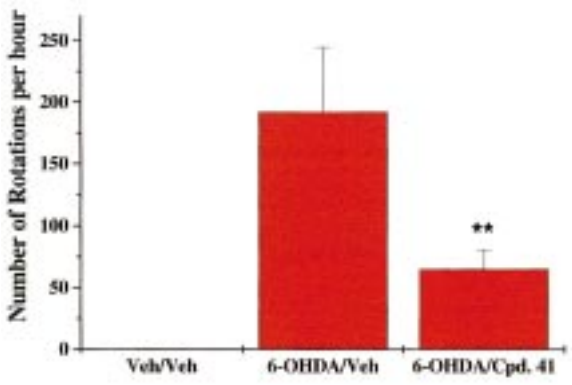


Figure 16. Reduction of motor abnormalities in 6HODA-lesioned rats by systemic treatment with **41**. Data are taken from ref 212.

Alzheimer's disease and PD.[227,228] *p*-Chloroamphetamine (PCA) produces marked destruction of serotonin neurons in the central nervous system. Systemic administration of PCA results in a rapid degeneration of most serotonergic axon terminals in the forebrain, while sparing 5-HT cell bodies in the raphe nuclei.[229,230] It has been shown previously that peptidic growth factors, such as BDNF, exert their neurotrophic effects on these serotonin neurons and induce sprouting following intracortical infusion of BDNF.[231] In a reported study, Guilford scientists examined whether systemic administration of immunophilin ligand **41** could prevent the severe loss of serotonergic axons observed 2 weeks after systemic administration of PCA.[232]

Serotonin neurons of adult male Sprague−Dawley rats were lesioned with systemic 10 mg/kg PCA. Rats received various doses of compound **41** (1−40 mg/kg sc) for 3 days, at which time PCA was administered, followed by the same dose of **41** daily for 14 days until sacrifice. Animals were sacrificed 21 days after PCA injection and perfused with 4% paraformaldehyde, and their brains were cut in 30-mm sagittal sections. One set of sections was processed using a monoclonal antibody to serotonin (5-HT). Corresponding regions of the frontal, parietal, and occipital cortices, CA3, CA1, and the dentate gyrus of the hippocampus were photographed and analyzed to determine the regional 5-HT-positive fiber density. In rats lesioned with PCA, cortical serotonin immunoreactivity is dramatically reduced by greater than 90%. PCA-lesioned animals receiving **41** displayed increased cortical serotonin innervation density, with a greater than 2-fold increase in the level of serotonin fiber staining in the somatosensory cortex. The effects of the FKBP12 ligand on serotonin innervation density were detected in all regions of the cortex, from frontal to parietal to occipital cortex. The protective effects of **41** were more robust in sparing serotonin-containing fibers in the frontal and motor cortex while exhibiting smaller protection in visual cortex. Drug treatment dose dependently spared the serotonin innervation of CA1 and CA3 hippocampus and had no effect on dentate gyrus 5-HT staining. However, no evidence of aberrant sprouting of neuronal processes was observed when **41** was administered to normal animals. These data indicate that FKBP ligands are neuroprotective for central serotonin neurons.

3. Cholinergic. Cholinergic neuronal loss from nucleus basalis and medial septal nucleus and the associated cholinergic deafferentation of cortical and hippocampal target regions are among the pathological hallmarks of Alzheimer's disease, and cholinergic deafferentation has been implicated in some aspects of cognitive impairment manifested by Alzheimer's victims.[233] Transection of the fimbria fornix in rats is an extensively used model of cholinergic deafferentation that has been used in neural morphological, biochemical, and behavioral studies to test the efficacy of transplantation, neurotrophic factors, and other strategies for reversing these deficits.[234]

It has been reported that systemic administration of **41** induces sprouting of residual cholinergic septohippocampal axons following partial transection of the fimbria fornix in adult rats.[235] Partial bilateral transection of the fimbria fornix was performed stereotactically. Compound **41**, 10 mg/kg/day, or vehicle was administered to these animals for 14 days, and animals were sacrificed 14 days after their last injection. The septohippocampal cholinergic system was visualized in these cases using ACHE histochemistry and CHAT immunohistochemistry on adjacent sections. The partial transections spared the medial and dorsal fornix but produced extensive (75−80%) deafferentation in the middle half of the hippocampal formation. Fimbria fornix transection produced extensive cholinergic deafferentation of all layers of CA1 and CA3 and the dentate gyrus, although a residual projection of CHAT-positive fibers and punctae remained. Treatment with **41** produced increased density of terminals in the CA3, CA1, and dentate gyrus. Treatment with the FKBP ligand induced a 4.2-fold sprouting of the spared residual processes in CA1, yielding a 62% recovery in CHAT-positive fiber density; a 2.8-fold sprouting of the spared residual processes in CA3, leading to a 48% recovery of the CHAT-positive fiber innervation density; and a 2.8-fold sprouting of the spared residual processes in the dentate, producing a 30% recovery in CHAT-positive fiber density. These results indicate that treatment with immunophilin ligands appears to stimulate partial recovery of central cholinergic systems which may hold promise for ameliorating cholinergic deficits present in Alzheimer's disease.

Conclusions and Outlook

The studies surveyed above document the remarkable ability of FKBP ligands to cause functional regeneration of a variety of damaged neuronal pathways. The ability of orally active small molecules to produce morphological and functional recovery in animal models of neurodegenerative disease raises the exciting possibility of a new and powerful approach to treating nervous system disorders which represents a major advance in neuroscience. FKBP ligands have been demonstrated to promote regrowth of damaged facial and sciatic nerves in the peripheral nervous system and the regeneration of damaged dopaminergic, serotonergic, and cholinergic neurons in the central nervous system. This broad scope of action suggests therapeutic utility in a variety of neurodegenerative disorders such as PD, Alzheimer's disease, diabetic and peripheral neuropathies, and spinal cord injuries.

Neuroimmunophilin ligands represent a significant advance over peptidic growth factors, many of which are

currently being evaluated in animal models of neurodegeneration. Growth factors such as BDNF, GDNF, NGF, and NT3 have been shown to promote nerve regrowth in vivo.[236−240] The therapeutic utility of these proteins is severely limited due to their lack of oral bioavailability and inability to cross the blood−brain barrier, necessitating their delivery directly to the site of injury. Abnormal neuronal sprouting has also been observed following administration of protein growth factors.[241] FKBP ligands, such as those discussed here, readily cross the blood−brain barrier and are active upon oral administration. In contrast to growth factors, no abnormal sprouting has been observed from administration of FKBP ligands to animals, in either the peripheral or central nervous system. This may be related to the finding that in damaged nerves, FKBP is rapidly synthesized and transported to the site of injury.

Many unanswered questions remain regarding the actions of these compounds. The mechanism whereby the nerve regenerative effects of FK506 and small FKBP ligands are exerted is unknown, and this area deserves much experimental attention. It does not appear that inhibition of FKBP rotamase activity per se is responsible. FKBP12 is present in high concentrations in neurons (up to 1 μM), and many compounds produce neurotrophic effects in vitro in concentrations at which less than 0.1% of the enzyme would be inhibited. In general, the neurotrophic potencies of the compounds reported do not correlate well with inhibition of PPIase activity (Tables 8−10).

It is possible that other FKBPs, present in lower concentrations in nerve cells, mediate the actions of these compounds. Extensive screening of compounds, such as those in Tables 8−10, for affinity to other FKBPs has not been reported to date. Compound **41**, whose in vivo actions have been described extensively, has been screened against other FKBPs with respect to rotamase inhibition, as well as a large number of receptor and enzymatic assays, and found to be highly selective for FKBP12. It was also shown not to inhibit monoamine oxidase B or dopamine uptake. Nontheless, there may be other proteins containing FKBP or FKBP-like domains which mediate the interactions of these molecules. Gold has suggested FKBP52 as a possibility, noting that subcutaneous injection of FK506 was found to increase expression of the heat-shock protein hsp-70 in neurons in the cortex, hippocampus, and amygdala of the brain, as well as in the spinal cord and dorsal root ganglion.[242] FK506 also has been demonstrated to increase GAP-43 mRNA levels in DRG neurons.[173]

The ability of neuroimmunophilin ligands to produce potent neurotrophic effects in vitro and in vivo at low concentrations, and the lack of correlation of these effects with inhibition of rotamase activity, suggests the possibility that interaction of the compounds with FKBP or an FKBP-like protein results in formation of an activated complex, leading to a gain of function for the FKBP. Although the original studies of the structure of FK506 bound to FKBP12 concluded that FKBP12 was essentially unchanged by ligand binding, more sophisticated studies have revealed that different ligands modulate the local conformation of FKBP in subtle but significant ways that may have functional implications.[47,243] Molecular dynamics simulations and NMR studies of the unliganded protein suggest that the 85−94 loop region may move as a hinged flap which becomes fixed upon FK506 binding. Movement of this flap may serve to conceal or expose portions of the binding domain. Wilson et al. have postulated the existence of an extended binding domain which may be the "footprint" of an endogenous protein ligand for FKBP.[47]

The historical parallel with the immunosuppressant story is intriguing. Once again, ligands for an immunophilin have been found to produce striking effects in a manner aparently unrelated to the enzyme activity of the protein target. In the immunosuppressant case, the search for the subsequent protein targets sheds considerable light on the signaling pathways involving calcineurin and FRAP in T-cells. Identification of the molecular targets of the FKBP/ligand complexes responsible for mediating the nerve regenerative effects of the compounds will undoubtedly result in a similar increase in knowledge in neurobiology. It is likely that application of modern tools for investigating protein−protein interactions, such as affinity methods and the yeast two- and three-hybrid genetic screening systems, will result in the identification of putative targets soon. It is most likely by identifying new protein targets for FKBP12, or new FKBP-related proteins which bind the ligands described in this Perspective, and by elucidating their roles in signal transduction pathways that we will determine the mechanism of the extraordinary neuroprotective and neuroregenerative actions of neuroimmunophilin ligands.

Another issue is whether cyclosporin is neurotrophic. Steiner et al. reported that CsA increased neurite outgrowth in both PC12 cells and chick DRG cells,[204] but Gold found that cyclosporin at concentrations up to 1 μM did not alter significantly neurite outgrowth in SH-SY5Y cells.[173] Gold also reported that CsA did not increase axonal regeneration rate in rats with lesioned sciatic nerves.[244] Yagita et al. reported that CsA treatment does not afford protection to hippocampal and cerebral neurons against transient global ischemia.[189] In a recent report, however, Boriongan et al. described experiments in rats with damaged blood−brain barriers. Animals receiving chronic ip injections of CsA for 9 days had significantly increased nigral dopaminergic neuronal outgrowth, suggesting the compound may have a trophic effect.[245] Clearly, much work needs to be done to clarify the nervous system effects of CsA.

Given the intensive interest and effort devoted to exploring the therapeutic utility of neuroimmunophilin ligands reviewed here, it is likely that this will be a major new area of medicinal chemistry and neuroscience research for years to come.

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Biographies

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References

(1) Fischer, G.; Bang, H.; Mech, C. Detection of enzyme catalysis for cis−trans isomerization of peptide bonds using proline containing peptides as substrates. *Biomed. Biochim. Acta* **1984**, *43*, 1101−1112.

(2) Fischer, G.; Bang, H. The refolding of urea-denatured ribonuclease A is catalyzed by peptidyl-prolyl *cis−trans* isomerase. *Biochim. Biophys. Acta* **1984**, *828*, 39−42.

(3) Handschumacher, R. E.; Harding, M. W.; Rice, J.; Drugge, R. J.; Speicher, D. W. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. *Science* **1984**, *226*, 544−546.

(4) Fischer, G.; Wittmann-Liebold, B.; Lang, K.; Kiefhaber, T.; Schmid, F. X. Cyclophilin and peptidyl-prolyl cis−trans isomerase are probably identical proteins. *Nature* **1989**, *337*, 476−478.

(5) Takahashi, N.; Hayano, T.; Suzuki, M. Peptidyl-prolyl cis−trans isomerase is the cyclosporin A-binding protein cyclophilin. *Nature* **1989**, *337*, 473−475.

(6) Harding, M. W.; Galat, A.; Uehling, D. E.; Schreiber, S. L. A receptor for the immuno-suppressant FK506 is a cis−trans peptidyl-prolyl isomerase. *Nature* **1989**, *341*, 761−763.

(7) Siekerka, J. J.; Hung, S. H. Y.; Poe, M.; Lin, S. C.; Sigal, N. H. A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. *Nature* **1989**, *341*, 755−757.

(8) Schreiber, S. L. Chemistry and biology of the immunophilins and their immunosuppressant ligands. *Science* **1991**, *251*, 283−287.

(9) Galat, A. Peptidylproline cis−trans isomerases: immunophilins. *Eur. J. Biochem.* **1993**, *216*, 689−707.

(10) Kay, J. E. Structure−function relationships in the FK506-binding protein (FKBP) family of peptidylprolyl cis−trans isomerases. *Biochem. J.* **1996**, *314*, 361−385.

(11) Rahfeld, J.-U.; Rucknagel, K. P.; Schelbert, B.; Ludwig, B.; Hacker, J.; Mann, K.; Fischer, G. Confirmation of the existence of a third family among peptidyl-prolyl cis/trans isomerases. Amino acid sequence and recombinant production of parvulin. *FEBS Lett.* **1994**, *352*, 180−184.

(12) Rudd, K. E.; Sofia, H. J.; Koonin, E. V.; Plunkett, G.; Lazar, S.; Rouviere, P. R. A new family of peptidyl-prolyl isomerases. *Trends Biochem. Sci.* **1995**, *20*, 12−14.

(13) Standaert, R. F.; Galat, A.; Verdine, G. L.; Schreiber, S. L. Molecular cloning and overexpression of the human FK506-binding protein, FKBP. *Nature* **1990**, *346*, 671−674.

(14) Albers, M. W.; Walsh, C. T.; Schreiber, S. L. Substrate specificity for the human rotamase FKBP: a view of FK506 and rapamycin as leucine-(twisted amide)-proline mimics. *J. Org. Chem.* **1990**, *55*, 4984−4986.

(15) Sewell, T.; Lam, E.; Martin, M.; Leszyk, J.; Weidner, J.; Calaycay, J.; Griffin, P.; Williams, H.; Hung, S.; Cryan, J.; Sigal, N.; Wiederrecht, G. Inhibition of calcineurin by a novel FK-506-binding protein. *J. Biol. Chem.* **1994**, *269*, 21094−21102.

(16) Lam, E.; Martin, M. M.; Timerman, A. P.; Sabers, C.; Fleischer, S.; Lukas, T.; Abraham, R. T.; O'Keefe, S. J.; O'Neill, E. A.; Weiderrecht, G. J. A novel FK506 binding protein can mediate the immunosuppressive effects of FK506 and is associated with the cardiac ryanodine receptor. *J. Biol. Chem.* **1995**, *270*, 26511−26522.

(17) Jin, Y.-J.; Albers, M. W.; Lane, W. S.; Bierer, B. E.; Schreiber, S. L.; Burakoff, S. J. Molecular cloning of a membrane-associated human FK506- and rapamycin-binding protein, FKBP-13. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6677−6681.

(18) Jin, Y.-J.; Burakoff, S. J.; Bierer, B. E. Molecular cloning of a 25-kDa high affinity rapamycin binding protein, FKBP25. *J. Biol. Chem.* **1992**, *267*, 10942−10945.

(19) Galat, A.; Lane, W. S.; Standaert, R. F.; Schreiber, S. L. A rapamycin-sensitive 25-kDa immunophilin. *Biochemistry* **1992**, *31*, 2427−2434.

(20) Murthy, J. N.; Goodyear, N.; Soldin, S. J. Identification of a 37 kDa tacrolimus, sirolimus and cyclosprine binding immunophilin possessing glyceraldehyde 3-phosphate dehydrogenase activity isolated from the Jurkat T cell line. *Clin. Biochem.* **1997**, *30*, 129−133.

(21) Peattie, D. A.; Harding, M. W.; Fleming, M. A.; DeCenzo, M. T.; Lippke, J. A.; Livingston, D. J.; Benasutti, M. Expression and characterization of human FKBP52, an immunophilin that associates with the 90-kDa heat shock protein and is a component of steroid receptor complex. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 10974−10978.

(22) Lebeau, M.-C.; Massol, N.; Herrick, J.; Faber, L. E.; Renoir, J.-M.; Radanyi, C.; Baulieu, E.-E. P59, an hsp90-binding protein. Cloning and sequencing of its cDNA and preparation of a peptide-derived polyclonal antibody. *J. Biol. Chem.* **1992**, *267*, 4281−4284.

(23) Yem, A. W.; Tommasselli, A. G.; Heinrikson, R. L.; Zurcher-Neely, H.; Ruff, V. A.; Johnson, D. A.; Deibel, M. R. The hsp56 component of steroid receptor complexes binds to immobilized FK-506 and shows homology to FKBP-12 and FKBP-13. *J. Biol. Chem.* **1992**, *267*, 2868−2871.

(24) Harding, M. W.; Handschumacher, R. E.; Speicher, D. W. Isolation and amino acid sequence of cyclophilin. *J. Biol. Chem.* **1986**, *261*, 8547−8555.

(25) Price, E. R.; Zydowsky, L. D.; Jin, M.; Baker, C. H.; McKeonand, F. D.; Walsh, C. T. Human cyclophilin B. A second cyclophilin gene encodes a peptidyl-prolyl isomerase with a signal sequence. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 1903−1907.

(26) Schneider, H.; Charara, N.; Schmitz, R.; Wehrli, S.; Mikol, V.; Zurini, M. G.; Quesniaux, V. F.; Movva, N. R. Human cyclophilin C: primary structure, tissue distribution, and determination of binding specificity for cyclosporins. *Biochemistry* **1994**, *33*, 8218−8224.

(27) Connern, C. P.; Halestrap, A. P. Purification and N-terminal sequencing of peptidyl-prolyl-cis−trans-isomerase from rat liver mitochondrial matrix reveals the existence of a distinct mitochondrial cyclophilin. *Biochem. J.* **1992**, *284*, 381−385.

(28) Kieffer, L. J.; Thalhammer, T.; Handschumacher, R. E. Isolation and characterization of a 40-kDa cyclophilin-related protein. *J. Biol. Chem.* **1992**, *267*, 5503−5507.

(29) Anderson, S. K.; Gallinger, S.; Roder, J.; Frey, J.; Young, H. A.; Ortaldo, J. R. A cyclophilin-related protein involved in the function of natural killer cells. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 542−546.

(30) Kofron, J. L.; Kuzmic, P.; Kishore, V.; Bonilla-Colon, E.; Rich, D. H. Determination of kinetic constants for peptidyl-prolyl cis−trans isomerases by an improved spectrophotometric assay. *Biochemistry* **1991**, *30*, 6127−6134.

(31) Kofron, J. L.; Kuzmic, P.; Kishore, V.; Gemmecker, G.; Fesik, S. W.; Rich, D. H. Lithium chloride perturbation of cis−trans peptide bond equilibria: effect on conformation equilibrium in cyclosporin A and on time-dependent inhibition of cyclophilin. *J. Am. Chem. Soc.* **1992**, *114*, 2670−2675.

(32) Harrison, R. K.; Stein, R. L. Substrate specificities of the peptidyl-prolyl cis−trans isomerase activities of cyclophilin and FK506-binding protein: evidence for the existence of a family of distinct enzymes. *Biochemistry* **1990**, *29*, 3813−3816.

(33) Lang, K.; Schmid, F. X.; Fischer, G. Catalysis of protein folding by prolyl isomerases. *Nature* **1987**, *329*, 268−270.

(34) Mucke, M.; Schmid, F. X. Enzymatic catalysis of prolyl isomerization in unfolding protein. *Biochemistry* **1992**, *31*, 7848−7854.

(35) Schonbrunner, E. R.; Mayer, S.; Tropschug, M.; Fischer, G.; Takahashi, N.; Schmid, F. X. Catalysis of protein folding by cyclophilins from different sources. *J. Biol. Chem.* **1991**, *266*, 3630−3635.

(36) Hsu, V. L.; Handschumacher, R. E.; Armitage, I. A. Peptidyl-prolyl cis−trans isomerase activity of cyclophilin studied by one-dimensional ^{1}H nuclear magnetic resonance spectroscopy. *J. Am. Chem. Soc.* **1990**, *112*, 6745−6747.

(37) Justice, R. M.; Kline, A. D.; Sluka, J. P.; Roeder, W. D.; Rodgers, G. H.; Roehm, N.; Mynderse, J. S. The detection of proline isomerase activity in FK506-binding protein by two-dimension ^{1}H NMR exchange spectroscopy. *Biochem. Biophys. Res. Commun.* **1990**, *171*, 445−450.

(38) Michnik, S. W.; Rosen, M. K.; Wandless, T. J.; Karplus, M.; Schreiber, S. L. Solution structure of FKBP, a rotamase enzyme and receptor for FK506 and rapamycin. *Science* **1991**, *252*, 836−839.

(39) Moore, J. M.; Peattie, D. A.; Fitzgibbon, M. J.; Thomson, J. A. Solution structure of the major binding protein for the immunosuppressant FK506. *Nature* **1991**, *351*, 248−250.

(40) Ke, H.; Zydowsky, L. D.; Liu, J.; Walsh, C. T. Crystal structure of recombinant human T-cell cyclophilin A at 2.5 Å resolution. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 9483.
(41) Ke, H. Similarities and differences between human cyclophilin A and other β-barrel structures. Structural refinement at 1.63 Å resolution. *J. Mol. Biol.* **1992**, *228*, 539−550.
(42) Van Duyne, G. D.; Standaert, R. F.; Karplus, P. A.; Schreiber, S. L.; Clardy, J. Atomic structure of FKBP−FK506, an immunophilin-immunosuppressant complex. *Science* **1991**, *252*, 839−842.
(43) Lepre, C. A.; Thomson, J. A.; Moore, J. M. Solution structure of FK506 bound to FKBP-12. *FEBS Lett.* **1992**, *302*, 89−96.
(44) Van Duyne, G. D.; Standaert, R. F.; Karplus, P. A.; Schreiber, S. L.; Clardy, J. Atomic structures of the human immunophilin FKBP-12 complexes with FK506 and rapamycin. *J. Mol. Biol.* **1993**, *229*, 105−124.
(45) Meadows, R. P.; Nettesheim, D. G.; Xu, R. X.; Olejniczak, E. T.; Petros, A. M.; Holzman, T. F.; Severin, J.; Gubbins, E.; Smith, H.; Fesik, S. W. Three-dimensional structure of the FK506 binding protein/ascomycin complex in solution by heteronuclear three- and four-dimensional NMR. *Biochemistry* **1993**, *32*, 754−765.
(46) Van Duyne, G. D.; Standaert, R. F.; Karplus, P. A.; Schreiber, S. L.; Clardy, J. Atomic structures of the human immunophilin FKBP-12 complexes with FK506 and rapamycin. *J. Mol. Biol.* **1993**, 229, 105−124.
(47) Wilson, K. P.; Yamashita, M. M.; Sintchak, M. D.; Rotstein, S. H.; Murcko, M. A.; Boger, J.; Thomson, J. A.; Fitzgibbon, M. J.; Black, J. R.; Navia, M. A. Comparative X-ray structures of the major binding protein for the immunosuppressant FK506 (Tacrolimus) in unliganded form and in complex with FK506 and rapamycin. *Acta Crystallogr.* **1995**, *D51*, 511−521.
(48) Becker, J. W.; Rotonda, J.; McKeever, B. M.; Chan, H. K.; Marcy, A. I.; Wiederrecht, G.; Hermes, J. D.; Springer, J. P. FK-506-binding protein: three-dimensional structure of the complex with the antagonist L-685,818. *J. Biol. Chem.* **1993**, *268*, 11335−11339.
(49) Holt, D. A.; Luengo, J. I.; Yamashita, D. S.; Oh, H.-J.; Konialian, A. L.; Yen, H.-K.; Rozamus, L. W.; Brandt, M.; Bossard, M. J.; Levy, M. A.; Eggleston, D. S.; Liang, J.; Schultz, L. W.; Stout, T. J.; Clardy, J. Design, synthesis, and kinetic evaluation of high-affinity FKBP ligands and the X-ray crystal structures of their complexes with FKBP12. *J. Am. Chem. Soc.* **1993**, *115*, 9925−9938.
(50) Armistead, D. M.; Badia, M. C.; Deininger, D. D.; Duffy, J. P.; Saunders, J. O.; Tung, R. D.; Thomson, J. A.; DeCenzo, M. T.; Futer, O.; Livingston, D. J.; Murcko, M. A.; Yamashita, M. M.; Navia, M. A. Design, synthesis and structure of nonmacrocyclic inhibitors of FKBP12, the major binding protein for the immunosuppressant FK506. *Acta Crystallogr.* **1995**, *D51*, 522−528.
(51) Liu, J.; Albers, M. W.; Chen, C. M.; Schreiber, S. L.; Walsh, C. T. Cloning, expression, and purification of human cyclophilin in *Escherichia coli* and assessment of the catalytic role of cysteines by site-directed mutagenesis. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 2304−2308.
(52) Harrison, R. K.; Stein, R. L. Mechanistic studies of peptidyl prolyl cis−trans isomerase: evidence for catalysis by distortion. *Biochemistry* **1990**, *29*, 1684−1689.
(53) Rosen, M. K.; Standaert, R. F.; Galat, A.; Nakatsuka, M.; Schreiber, S. L. Inhibition of FKBP rotamase activity by immunosuppressant FK506: twisted amide surrogate. *Science* **1990**, *248*, 863−866.
(54) Orozco, M.; Tirado-Rives, J.; Jorgensen, W. L. Mechanism for the rotamase activity of FK506 binding protein from molecular dynamics simulations. *Biochemistry* **1993**, *32*, 12864−12874.
(55) Fischer, S.; Michnik, S.; Karplus, M. A mechanism for rotamase catalysis by the FK506 binding protein. *Biochemistry* **1993**, *32*, 13830−13837.
(56) Wiederrecht, G.; Hung, S.; Chan, H. K.; Marcy, A.; Martin, M.; Calaycay, J.; Boulton, D.; Sigal, N.; Kincaid, R. L.; Siekierka, J. J. Characterization of high molecular weight FK-506 binding activities reveals a novel FK-506-binding protein as well as a protein complex. *J. Biol. Chem.* **1992**, *267*, 21753−21760.
(57) Bossard, M. J.; Bergsma, D. J.; Brandt, M.; Livi, G. P.; Eng, W.-K.; Johnson, R. K.; Levy, M. A. Catalytic and ligand binding properties of the FK506 binding protein FKBP12: effects of the single amino acid substitution of Tyr82 to Leu. *Biochem. J.* **1994**, *297*, 365−372.
(58) DeCenzo, M. T.; Park, S. T.; Jarrett, B. P.; Aldape, R. A.; Futer, O.; Murcko, M. A.; Livingston, D. J. FK506-binding protein mutational analysis: defining the active-site residue contributions to catalysis and the stability of ligand complexes. *Protein Eng.* **1996**, *9*, 173−180.
(59) Aldape, R. A.; Futer, O.; DeCenzo, M. T.; Jarrett, B. P.; Murcko, M. A.; Livingston, D. J. Charged surface residues of FKBP12 participate in formation of the FKBP12−FK506−calcineurin complex. *J. Biol. Chem.* **1992**, *267*, 16029−16032.
(60) Timerman, A. P.; Wiederrecht, G.; Marcy, A.; Fleischer, S. Characterization of an exchange reaction between soluble FKBP-12 and the FKBP-ryanodine receptor complex. Modulation by FKBP mutants deficient in peptidyl-prolyl isomerase activity. *J. Biol. Chem.* **1995**, *270*, 2451−2459.
(61) Nigam, S. K.; Jin, Y.-J.; Jin, M. J.; Bush, K. T.; Bierer, B. E.; Burakoff, S. J. Localization of the FK506-binding protein, FKBP 13, to the lumen of the endoplasmic reticulum. *Biochem. J.* **1993**, *294*, 511−515.
(62) Bush, K. T.; Hendrickson, B. A.; Nigam, S. K. Induction of the FK506-binding protein, FKBP13, under conditions which misfold proteins in the endoplasmic reticulum. *Biochem. J.* **1994**, *303*, 705−708.
(63) Schultz, L. W.; Martin, P. K.; Liang, J.; Schreiber, S. L.; Clardy, J. Atomic structure of the immunophilin FKBP13−FK506 complex: insights into the composite binding surface for calcineurin. *J. Am. Chem. Soc.* **1994**, *116*, 3129−3130.
(64) Riviere, S.; Menez, A.; Galat, A. On the localization of FKBP25 in T-lymphocytes. *FEBS Lett.* **1993**, *315*, 247−251.
(65) Jin, Y. J.; Burakoff, S. J. The 25-kDa FK506-binding protein is localized in the nucleus and associates with casein kinase II and nucleolin. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 7769−7773.
(66) Fretz, H.; Albers, M. A.; Galat, A.; Standaert, R. F.; Lane, W. S.; Burakoff, S. J.; Bierer, B. E.; Schreiber, S. L. Rapamycin and FK506 binding proteins (immunophilins). *J. Am. Chem. Soc.* **1991**, *113*, 1409−1410.
(67) Tai, P. K.-K.; Albers, M. A.; Chang, H.; Faber, L. E.; Schreiber, S. L. Association of a 59-kilodalton immunophilin with the glucocorticoid receptor complex. *Science* **1992**, *256*, 1315−1318.
(68) Callebaut, L.; Renoir, J.-M.; Lebeau, M.-C.; Massol, N.; Burny, R.; Baulieu, E.-E.; Mornon, J.-P. An immunophilin that binds M_r 90000 heat shock protein: structural features of a mammalian p59 protein. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6270−6274.
(69) Chambraud, B.; Rouviere-Fourmy, N.; Radanyl, C.; Hsiao, K.; Peattie, D. A.; Livingston, D. J.; Baulieu, E.-E. Overexpression of p59-HBI (FKBP59), full length and domains, and characterization of PPIase activity. *Biochem. Biophys. Res. Commun.* **1993**, *196*, 160−166.
(70) Donnelly, J. G.; Soldin, S. J. Partial characterization of a 52 kDa CsA/FK506/rapamycin binding protein. *Clin. Biochem.* **1994**, *27*, 367−372.
(71) Weber, C.; Wider, G.; Von Freyburg, B.; Traber, R.; Braun, W.; Widmer, H.; Wüthrich, K. The NMR structure of cyclosporin A bound to cyclophilin in aqueous solution. *Biochemistry* **1991**, *30*, 6563−6574.
(72) Theriault, Y.; Logan, T. M.; Meadows, R.; Yu, L.; Olejniczak, E. T.; Holzman, T. F.; Simmer, R. L.; Fesik, S. W. Solution structure of the cyclosporin A/cyclophilin complex by NMR. *Nature* **1993**, *361*, 88−91.
(73) Pfluegl, G.; Kallen, J.; Schirmer, T.; Jansonius, J. N.; Zurini, M. G.; Walkinshaw, M. D. X-ray structure of a decameric cyclophilin-cyclosporin crystal complex. *Nature* **1993**, *361*, 91−94.
(74) Mikol, V.; Kallen, J.; Pfluegl, G.; Walkinshaw, M. D. X-ray structure of monomeric cyclophilin A-cyclosporin A crystal complex at 2.1 Å resolution. *J. Mol. Biol.* **1993**, *234*, 1119−1130.
(75) Spitzfaden, C.; Braun, W.; Wider, G.; Widmer, H.; Wüthrich, K. Determination of the NMR solution structure of the cyclophilin-cyclosporin A complex. *J. Biomol. NMR* **1994**, *4*, 463−482.
(76) Kallen, J.; Walkinshaw, M. D. The X-ray structure of a tetrapeptide bound to the active site of human cyclophilin A. *FEBS Lett.* **1992**, *300*, 286−290.
(77) Ke, H.; Mayrose, D.; Cao, W. Crystal structure of cyclophilin A complexed with substrate Ala-Pro suggests a solvent-assisted mechanism of cis−trans isomerization. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 3324−3328.
(78) Zhao, Y.; Ke, H. Mechanistic implication of crystal structures of the cyclophilin-dipeptide complexes. *Biochemistry* **1996**, *35*, 7362−7368.
(79) Zhao, Y.; Ke, H. Crystal structure implies that cyclophilin predominately castalyzes the trans to cis isomerization. *Biochemistry* **1996**, *35*, 7356−7361.
(80) Braun, W.; Kallen, J.; Mikol, V.; Walkinshaw, M. D.; Wüthrich, K. Three-dimensional structure and actions of immunosuppressants and their immunophilins. *FASEB J.* **1995**, *9*, 63−72.
(81) Loosli, H.; Kessler, H.; Oschkinat, H.; Weber, H.; Petcher, T. J.; Widmer, A. The conformation of cyclosporin A in the crystal and in solution. *Helv. Chim. Acta* **1995**, *68*, 682−704.
(82) Kessler, H.; Gehrke, M.; Lautz, J.; Koeck, M.; Seebach, D.; Thaler, A. Complexation and medium effects on the conformation of cyclosporin A studied by NMR spectroscopy and molecular dynamics calculations. *Biochem. Pharmacol.* **1990**, *40*, 169−173.
(83) Mikol, V.; Kallen, J.; Walkinshaw, M. D. X-ray structure of a cyclophilin B/cyclosporin complex: Comparison with cyclophilin A and delineation of its calcineurin-binding domain. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 5183−5186.

(84) Etzkorn, F. A.; Chang, Z. Y.; Stolz, L. A.; Walsh, C. T. Cyclophilin residues that affect noncompetitive inhibition of the protein serine phosphatase activity of calcineurin by the cyclophilin-cyclosporin A complex. *Biochemistry* **1994**, *33*, 2380−2388.
(85) Friedman, J.; Weissman, I. Two cytoplasmic candidates for immunophilin action are revealed by affinity for a new cyclophilin: one in the presence and one in the absence of CsA. *Cell* **1991**, *66*, 799−806.
(86) Ke, H.; Zhao, Y.; Luo, F.; Weissman, I.; Friedman, J. Crystal structure of murine cyclophilin C complexed with immunosuppressant drug cyclosporin A. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11850−11854.
(87) Friedman, J.; Trahey, M.; Weissman, I. Cloning and characterization of cyclophilin C-associated protein: a candidate natural cellular ligand for cyclophilin C. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 6815−6819.
(88) Bergsma, D. J.; Eder, C.; Gross, M.; Kerster, H.; Sylvester, D.; Appelbaum, E.; Cusimano, D.; Livi, G. P.; McLaughlin, M. M.; Kasyan, K.; Porter, T. G.; Silverman, C.; Dunnington, D.; Hand, A.; Prichett, W. P.; Bossard, M. J.; Brandt, M.; Levy, M. A. The cyclophilin multigene family of peptidyl-prolyl isomerases. Characterization of three separate human isoforms. *J. Biol. Chem.* **1991**, *266*, 23204−23214.
(89) Woodfield, K. Y.; Price, N. T.; Halestrap, A. P. cDNA cloning of rat mitochondrial cyclophilin. *Biochim. Biophys. Acta* **1997**, *1351*, 27−30.
(90) Tanveer, A.; Virji, S.; Andreeva, L.; Totty, N. F.; Hsuan, J. J.; Ward, J. M.; Crompton, M. Involvement of cyclophilin D in the activation of a mitochondrial pore by Ca^{2+} and oxidant stress. *Eur. J. Biochem.* **1996**, *238*, 166−172.
(91) Kieffer, L.; Seng, T. W.; Li, W.; Osterman, D. G.; Handschumacher, R. E.; Bayney, R. M. Cyclophilin-40, a protein with homology to the p59 component of the steroid receptor complex. *J. Biol. Chem.* **1993**, *268*, 12303−12310.
(92) Ratajczak, T.; Carrello, A.; Mark, P. J.; Warner, B. J.; Simpson, R. J.; Moritz, R. L.; House, A. K. The cyclophilin component of the unactivated estrogen receptor contains a tetratricopeptide repeat domain and shares identity with p59 (FKBP59). *J. Biol. Chem.* **1993**, *268*, 13187−13192.
(93) Hoffmann, K.; Kakalis, L. T.; Anderson, K. S.; Armitage, I. A.; Handschumacher, R. E. Expression of human cyclophilin-40 and the effect of the His141→Trp mutation on catalysis and cyclosporin A binding. *Eur. J. Biochem.* **1995**, *229*, 188−193.
(94) Liu, J.; Chen, C.-M.; Walsh, C. T. Human and *Escherichia coli* cyclophilins: sensitivity to inhibition by the immunosuppressant cyclosporin A correlates with a specific tryptophan residue. *Biochemistry* **1991**, *30*, 2306−2310.
(95) Anderson, S. K.; Gallinger, S.; Roder, J.; Frey, J.; Young, H. A.; Ortaldo, J. R. A cyclophilin-related protein involved in the function of natural killer cells. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 542−546.
(96) Rosen, M. K.; Schreiber, S. L. Natural products as probes of cellular function: studies of immunophilins. *Angew. Chem., Int. Ed. Eng.* **1992**, *31*, 384−400.
(97) Armistead, D. M.; Harding, M. W. Immunophilins and immunosuppressive drug action. *Annu. Rep. Med. Chem.* **1993**, *28*, 207−215.
(98) Bierer, B. E.; Mattila, P. S.; Standaert, R. F.; Herzenberg, L. A.; Burakoff, S. J.; Crabtree, G.; Schreiber, S. L. Two distinct signal transmission pathways are inhibited in T-lymphocytes by complexes formed between an immunophilin and either FK506 or rapamycin. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9231−9235.
(99) Dumont, F. J.; Melino, M. R.; Staruch, M. J.; Koprak, S. L.; Fisher, P. A.; Sigal, N. H. The immunosuppressive macrolides FK-506 and rapamycin act as reciprocal antagonists in murine T-cells. *J. Immunol.* **1990**, *144*, 1418−1424.
(100) Somers, P. K.; Wandless, T. J.; Schreiber, S. L. Synthesis and analysis of 506BD, a high-affinity ligand for the immunophilin FKBP. *J. Am. Chem. Soc.* **1991**, *113*, 8045−8056.
(101) Bierer, B. E.; Somers, P. K.; Wandless, T. J.; Burakoff, S. J.; Schreiber, S. L. Probing immunosuppressant action with a nonnatural immunophilin ligand. *Science* **1990**, *250*, 556−559.
(102) Dumont, F. J.; Staruch, M. J.; Koprak, S. L.; Siekerka, J. J.; Lin, C. S.; Harrison, R.; Sewell, T.; Kindt, V.; Beattie, T. R.; Wyvratt, M.; Sigal, N. H. The immunosuppressive and toxic effects of FK-506 are mechanistically related: pharmacology of a novel antagonist of FK-506 and rapamycin. *J. Exp. Med.* **1992**, *176*, 751−760.
(103) Sigal, N. H.; Dumont, F.; Durette, P.; Siekerka, J. J.; Peterson, L.; Rich, D. H.; Dunlap, B. E.; Staruch, M. J.; Melino, M. R.; Koprak, S. L.; Williams, D.; Witzel, B.; Pisano, J. M. Is cyclophilin involved in the immunosuppressive and nephrotoxic mechanism of action of cyclosporin A? *J. Exp. Med.* **1991**, *173*, 619−628.
(104) Ocain, T. D.; Longhi, D.; Steffan, R. J.; Caccese, R. G.; Sehgal, S. N. A nonimmunosuppressive triene-modified rapamycin analogue is a potent inhibitor of peptidyl prolyl cis−trans isomerase. *Biochem. Biophys. Res. Commun.* **1993**, *192*, 1340−1346.
(105) Liu, J. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP−FK506 complexes. *Cell* **1991**, *66*, 807−815.
(106) Clipston, N. A.; Crabtree, G. R. Identification of calcineurin as a key signaling enzyme in T-lymphocyte activation. *Nature* **1992**, *357*, 695−697.
(107) O'Keefe, S. J.; Tamura, J.; Kincaid, R. F.; Tocci, M. J.; O'Neill, M. A. FK-506- and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. *Nature* **1992**, *357*, 692−694.
(108) Flanagan, W. M.; Corthesy, B.; Bram, R. J.; Crabtree, G. R. Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. *Nature* **1991**, *352*, 754−755.
(109) Kuo, C. J.; Chung, J.; Florentino, D. F.; Flanagan, W. M.; Blenis, J.; Crabtree, G. R. Rapamycin selectively inhibits interleukin-2 inhibition of p70 S6 kinase. *Nature* **1992**, *358*, 70−73.
(110) Chung, J.; Kuo, C. J.; Crabtree, G. R.; Blenis, J. Rapamycin-FKBP specifically blocks growth dependent activation of and signaling by the 70 kd S6 protein kinases. *Cell* **1992**, *69*, 1227−1236.
(111) Price, D. J.; Grove, J. R.; Calvo, V.; Avruch, J.; Bierer, B. E. Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase. *Science* **1992**, *257*, 973−977.
(112) Jayaraman, T.; Marks, A. R. Rapamycin-FKBP12 blocks proliferation, induces differentiation, and inhibits cdc2 kinase activity in a myogenic cell line. *J. Biol. Chem.* **1993**, *268*, 25385−25388.
(113) Morice, W. G.; Wiederrecht, G.; Brunn, G. J.; Siekerka, J. J.; Abraham, R. T. Rapamycin inhibition of interleukin-2-dependent p33cdk2 and p34cdc2 kinase activition in T lymphocytes. *J. Biol. Chem.* **1993**, *268*, 22737−22745.
(114) Kunz, J.; Henriquez, R.; Schneider, U.; Deuter-Reinhard, M.; Movva, N. R.; Hall, M. N. Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homologue required for G1 progression. *Cell* **1993**, *73*, 585−596.
(115) Sabatini, D. M.; Erdjument-Bromage, H.; Lui, M.; Tempst, P.; Snyder, S. H. RAFT1 (rapamycin and FKBP12 target): a mammalian protein that binds to FKBP12 in rapamycin-dependent fashion and is homologous to yeast TORs. *Cell* **1994**, *78*, 35−43.
(116) Brown, E. J.; Albers, M. W.; Shin, T. B.; Ichikawa, K.; Keith, C. T.; Lane, W. S.; Schreiber, S. L. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature* **1994**, *369*, 756−758.
(117) Brown, E. J.; Beal, P. A.; Keith, C. T.; Chen, J.; Shin, T. B.; Schreiber, S. L. Control of p70 S6 kinase by kinase activity of FRAP in vivo. *Nature* **1995**, *377*, 441−446.
(118) Beretta, L.; Gingras, A.-C.; Svitkin, Y. V.; Hall, M.; Sonenberg, N. Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. *EMBO J.* **1996**, *15*, 658−664.
(119) Chou, M. M.; Blenis, J. The 70 kDa S6 kinase: regulation of a kinase with multiple roles in mitogenic signaling. *Curr. Opin. Cell Biol.* **1995**, *7*, 806−814.
(120) Brown, E. J.; Schreiber, S. L. A signaling pathway to translational control. *Cell* **1996**, *86*, 517−520.
(121) Schmid, F. X.; Mayr, L.; Mucke, M.; Schonbrunner, E. R. Prolyl isomerases: the role in protein folding. *Adv. Protein Chem.* **1993**, *44*, 25−66.
(122) Lilie, H.; Rudolph, R.; Buchner, J. Association of antibody chains at different stages of folding: prolyl isomerization occurs after formation of quaternary structure. *J. Mol. Biol.* **1995**, *248*, 190−201.
(123) Freskgard, P.-O.; Bergenhem, N.; Jonsson, B.-H.; Svensson, M.; Carlson, U. Isomerase and chaperone activity of prolyl isomerase in the folding of carbonic anhydrase. *Science* **1992**, *258*, 466−468.
(124) Kiefhaber, T.; Quass, R.; Hahn, U.; Schmid, F. X. Folding of ribonuclease T1. 2. Kinetic models for the refolding and unfolding reactions. *Biochemistry* **1990**, *29*, 3061−3070.
(125) London, R. E.; Davis, D. G.; Vavrek, R. J.; Stewart, J. M.; Handschumacher, R. E. Bradykinin and its Gly6 analogue are substrates of cyclophilin: a fluorine-19 magnetization transfer study. *Biochemistry* **1990**, *29*, 10298−10302.
(126) Kern, D.; Drakenberg, T.; Wilkstrom, M.; Forsen, S.; Bang, H.; Fischer, G. The cis/trans interconversion of the calcium regulating hormone calcitonin is catalyzed by cyclophilin. *FEBS Lett.* **1993**, *323*, 198−202.
(127) Kern, G.; Kern, D.; Schmid, F. X.; Fischer, G. Reassessment of the putative chaperone function of prolyl-cis/trans-isomerases. *FEBS Lett.* **1994**, *348*, 145−148.
(128) Tropschug, M.; Wachter, E.; Mayer, S.; Schonbrunner, E. R.; Schmid, F. X. Isolation and sequence of an FK506-binding protein from *N. crassa* which catalyzes protein folding. *Nature* **1990**, *343*, 674−677.

(129) Colley, N. J.; Baker, E. K.; Stamnes, M. A.; Zuker, C. S. The cyclophilin homologue ninaA is required in the secretory pathway. *Cell* **1991**, *67*, 255−263.
(130) Ondek, B.; Hardy, R. W.; Baker, E. K.; Stamnes, M. A.; Shieh, B.-H.; Zuker, C. S. Genetic dissection of cyclophilin function. Saturation mutagenesis of the Drosophila cyclophilin homologue ninaA. *J. Biol. Chem.* **1992**, *267*, 16460−16466.
(131) Ferreira, P. A.; Nakayama, T. A.; Pak, W. L.; Travis, G. H. Cyclophilin-related protein RanBP2 acts as chaperone for red/green opsin. *Nature* **1996**, *383*, 637−640.
(132) Lodish, H. F.; Kong, N. Cyclosporin A inhibits an initial step in folding of transferrin within the endoplasmic reticulum. *J. Biol. Chem.* **1991**, *266*, 14835−14838.
(133) Steinmann, B.; Bruckner, P.; Furga-Supereti, A. Cyclosporin A slows collagen triple-helix formation in vivo: indirect evidence for a physiologic role of peptidyl-prolyl cis−trans isomerase. *J. Biol. Chem.* **1991**, *266*, 1299−1303.
(134) Helekar, S. A.; Char, D.; Neff, S.; Patrick, J. Prolyl isomerase requirement for the expression of functional homooligomeric ligand-gated ion channels. *Neuron* **1994**, *12*, 179−189.
(135) Callebaut, I.; Mornon, J. P. Trigger factor, one of the *Escherichia coli* chaperone proteins, is an original member of the FKBP family. *FEBS Lett.* **1995**, *374*, 211−215.
(136) Ratajczak, T.; Carrello, A.; Mark, P. J.; Warner, B. J.; Simpson, R. J.; Moritz, R. L.; House, A. K. The cyclophilin component of the unactivated estrogen receptor contains a tetratricopeptide repeat domain and shares identity with p59 (FKBP59). *J. Biol. Chem.* **1993**, *268*, 13187−13192.
(137) Ku, P.-K.; Albers, M. A.; Chang, H.; Faber, L. E.; Schreiber, S. L. Association of a 59-kilodalton immunophilin with the glucocorticoid receptor complex. *Science* **1992**, *256*, 1315−1318.
(138) Owens-Grillo, J. K.; Hoffmann, K.; Hutchison, K. A.; Yem, A. W.; Deibel, M. R.; Handschumacher, R. E.; Pratt, W. B. The cyclosporin A-binding immunophilin CyP-40 and the FK506-binding immunophilin hsp56 bind to a common site on hsp90 and exist in independent cytosolic heterocomplexes with the untransformed glucocorticoid receptor. *J. Biol. Chem.* **1995**, *270*, 20479−20484.
(139) Duina, A. A.; Chang, H.-C. J.; Marsh, J. A.; Lindquist, S.; Gaber, R. F. A cyclophilin function in hsp90-dependent sugnal transduction. *Science* **1996**, *274*, 1713−1715.
(140) Freeman, B. C.; Toft, D. O.; Morimoto, R. I. Molecular chaperone machines: chaperone activities of the cyclophilin Cyp-40 and the steroid aporeceptor-associated protein p23. *Science* **1996**, *274*, 1718−1720.
(141) Bose, S.; Weikl, T.; Bugl, H.; Buchner, J. Chaperone function of hsp90-associated proteins. *Science* **1996**, *274*, 1715−1718.
(142) Fleischer, S.; Inui, M. Biochemistry and biophysics of excitation-contraction coupling. *Annu. Rev. Biophys. Biophys. Chem.* **1989**, *18*, 333−364.
(143) Collins, J. H. Sequence analysis of the ryanodine receptor: possible association with a 12K, 506-binding immunophilin/protein kinase C inhibitor. *Biochem. Biophys. Res. Commun.* **1991**, *178*, 1288−1290.
(144) Jayraman, T.; Brillantes, A.-M.; Timerman, A. P.; Fleischer, S.; Erdjument-Bromage, H.; Tempst, P.; Marks, A. R. FK506 binding protein associated with the calcium release channel (Ryanodine receptor). *J. Biol. Chem.* **1992**, *267*, 9474−9477.
(145) Brillantes, A.-M.; Ondrias, K.; Scott, A.; Kobrinsky, E.; Ondriasova, E.; Moschella, M. C.; Jayraman, T.; Landers, M.; Ehrlich, B. E.; Marks, A. R. Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. *Cell* **1994**, *77*, 513−523.
(146) Furuichi, T. Primary structure and functional expression of the inositol 1,4,5-triphosphate-binding protein P400. *Nature* **1989**, *342*, 32−38.
(147) Berridge, M. J.; Irvine, R. F. Inositol phosphates and cell signaling. *Nature* **1989**, *341*, 197−204.
(148) Ferris, C. D.; Snyder, S. H. Inositol 1,4,5-triphosphate activated calcium channels. *Annu. Rev. Physiol.* **1991**, *54*, 469−488.
(149) Cameron, A. M.; Steiner, J. P.; Sabatini, D. M.; Kaplin, A. I.; Walensky, L. D.; Snyder, S. H. Immunophilin FK506 binding protein associated with inositol 1,4,5-trisphosphate receptor modulates calcium flux. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 1784−1788.
(150) Cameron, A. M.; Steiner, J. P.; Roskams, A. J.; Ali, S. M.; Ronnett, G. V.; Snyder, S. H. Calcineurin associated with the inositol 1,4,5-triphosphate receptor-FKBP12 complex modulates Ca^{2+} flux. *Cell* **1995**, *83*, 463−472.
(151) Massague, J.; Attisano, L.; Wrana, J. L. The TGF-β family and its composite receptors. *Trends Cell Biol.* **1994**, *4*, 172−178.
(152) Wang, T. W.; Donahoe, P. K.; Zervos, A. S. Specific interaction of type I receptors of the TGF-β family with the immunophilin FKBP12. *Science* **1994**, *265*, 674−676.
(153) Wrana, J. L.; Attisano, L.; Weiser, R.; Ventura, F.; Massague, J. Mechanism of activation of the TGF-β receptor. *Nature* **1994**, *370*, 341−347.
(154) Wang, T.; Li, B.-Y.; Danielson, P. D.; Shah, P. C.; Rockwell, S.; Lechleider, R. J.; Martin, J.; Manganaro, T.; Donahoe, P. K. The immunophilin FKBP12 functions as a common inhibitor of the TGF-β family type I receptors. *Cell* **1996**, *86*, 435−444.
(155) Yang, D.; Rosen, M. K.; Schreiber, S. L. A composite FKBP12−FK506 surface that contacts calcineurin. *J. Am. Chem. Soc.* **1993**, *115*, 819−820.
(156) Bram, R. J.; Crabtree, G. R. Calcium signaling in T cells stimulated by a cyclophilin B-binding protein. *Nature* **1994**, 371, 355−358.
(157) Halloway, M. P.; Bram, R. J. A hydrophobic domain of Ca+2-modulating cyclophilin ligand modulates calcium influx signaling in T lymphocytes. *J. Biol. Chem.* **1996**, *271*, 8549−8552.
(158) Bram, R. J. Isolation of a cDNA encoding the chicken homologue of the human calcium modulating cyclophilin ligand. *Gene* **1996**, *174*, 307−309.
(159) Bang, H.; Muller, W.; Hans, M.; Brune, K.; Swandulla, D. Activation of Ca2+ signaling in neutrophils by the mast cell-released immunophilin FKBP12. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 3435−3438.
(160) Cirillo, R.; de Paulis, A.; Ciccarelli, A.; Triggiani, M.; Marone, G. Cyclosporin A inhibits mediator release from human FcεRI+ cells by interacting with cyclophilin. *Int. Arch. Allergy Appl. Immunol.* **1991**, *94*, 76−77.
(161) Snyder, S. H.; Sabatini, D. M. Immunophilins and the nervous system. *Nature Med.* **1995**, *1*, 32−37.
(162) Timerman, A. P.; Wiederrecht, G.; Marcy, A.; Fleischer, S. Characterization of an exchange reaction between soluble FKBP12 and the FKBP-ryanodine receptor complex. Modulation by FKBP mutants deficient in peptidyl-prolyl isomerase activity. *J. Biol. Chem.* **1995**, *270*, 2451−2459.
(163) Cameron, A. M.; Nucifora, F. C.; Fung, E. T.; Livingston, D. J.; Aldape, R. A.; Ross, C. A.; Snyder, S. H. FKBP12 binds the inositol 1,4,5-triphosphate teceptor at leucine-proline (1400−1401) and anchors calcineurin to the FK506-like domain. *J. Biol. Chem.* **1997**, *272*, 27582−27588.
(164) Pawson, T. Protein modules and signaling networks. *Nature* **1995**, *373*, 573−580.
(165) Coghlan, V. M.; Perrino, B. A.; Howard, M.; Langeberg, L. K.; Hicks, J. B.; Gallatin, W. M.; Scott, J. D. Association of protein kinase A and protein phosphatase 2B with a common anchoring protein. *Science* **1995**, *267*, 108−111.
(166) Maki, N.; Sekiguchi, F.; Nishimaki, J.; Miwa, K.; Hayano, T.; Takahashi, N.; Suzuki, M. Complementary DNA encoding the human T-cell FK506-binding protein, a peptidylprolyl cis−trans isomerase distinct from cyclophilin. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 5440−5443.
(167) Steiner, J. P.; Dawson, T. M.; Fotuhi, M.; Glatt, C. E.; Snowman, A. M.; Cohen, N.; Snyder, S. H. High brain densities of the immunophilin FKBP colocalized with calcineurin. *Nature* **1992**, *358*, 584−587.
(168) Dawson, T. M.; Steiner, J. P.; Lyons, W. E.; Fotuhi, M.; Blue, M.; Snyder, S. H. The immunophilin, FK506-binding protein and cyclophilin, are discretely localized in the brain: relationship to calcineurin. *Neuroscience* **1994**, *62*, 569−580.
(169) Dawson, T. M.; Steiner, J. P.; Dawson, V. L.; Dinerman, J. L.; Uhl, G. R.; Snyder, S. H. Immunosuppressant, FK506, enhances phosphorylation of nitric oxide synthase and protects against glutamate neurotoxicity. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 9808−9812.
(170) Meiri, K. F.; Bickerstaff, L. E.; Schwob, J. E. Monoclonal antibodies show that kinase C phosphorylation of GAP-43 during axonogenesis is both spatially and temporally restricted in vivo. *J. Cell Biol.* **1991**, *112*, 991−1005.
(171) Lyons, W. E.; Steiner, J. P.; Snyder, S. H.; Dawson, T. M. Neuronal regeneration enhances the expression of the immunophilin FKBP. *J. Neurosci.* **1995**, *15*, 2985−2994.
(172) Lyons, W. E.; George, E. B.; Dawson, T. M.; Steiner, J. P.; Snyder, S. H. Immunosuppressant FK506 promotes neurite outgrowth in cultures of PC-12 cells and sensory ganglia. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 3191−3195.
(173) Gold, B. G. FK506 and the role of immunophilins in neuronal regeneration. *Mol. Neurobiol.* **1997**, *15*, 285−306.
(174) Chang, H. Y.; Takei, K.; Sydor, A. M.; Born, T.; Rusnak, F.; Jay, D. G. Asymmetric retraction of growth cone filopodia following focal inactivation of calcineurin. *Nature* **1995**, *376*, 686−690.
(175) Gold, B. G.; Storm-Dickerson, T.; Austin, D. R. The immunosuppressant FK506 increases functional recovery and nerve regeneration following peripheral nerve injury. *Restorative Neurol. Neurosci.* **1994**, *6*, 287.
(176) Gold, B. G.; Katoh, K.; Storm-Dickerson, T. The immunosuppressant FK506 increases the rate of axonal regeneration in rat sciatic nerve. *J. Neurosci.* **1995**, *15*, 7509−7516.
(177) Wang, M. S.; Zeleny-Pooley, M.; Gold, B. G. Comparative dose-dependence study of FK506 and cyclosporin A on the rate of axonal regeneration in the rat sciatic nerve. *J. Pharmacol. Exp. Ther.* **1997**, *282*, 1084−1093.

(178) Palmer, R. M.; Higgs, E. A. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.* **1991**, *43*, 109−142.
(179) Dawson, T. M.; Snyder, S. H. Gases as biological messengers: nitric oxide and carbon monoxide in the brain. *J. Neurosci.* **1994**, *14*, 5147−5159.
(180) Bredt, D. S.; Ferris, C. D.; Snyder, S. H. Nitric oxide synthase regulatory sites. *J. Biol. Chem.* **1992**, *267*, 10976−10981.
(181) Bredt, D. S.; Snyder, S. H. Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9030−9033.
(182) Choi, D. W. Bench to bedside: the glutamate connection. *Science* **1992**, *258*, 241−243.
(183) Dawson, V. L.; Dawson, T. M.; London, E. D.; Bredt, D. S.; Snyder, S. H. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6368−6371.
(184) Dawson, V. L.; Kizushi, V. M.; Huang, P. L.; Snyder, S. H.; Dawson, T. M. Resistance to neurotoxicity in cortical cultures from neuronal nitric oxide synthase-deficient mice. *J. Neurosci.* **1996**, *16*, 2479−2487.
(185) Sharkey, J.; Butcher, S. P. Immunophilins mediate the neuroprotective effects of FK506 in focal cerebral ischemia. *Nature* **1994**, *371*, 336−339.
(186) Tokime, T.; Nozaki, K.; Kikuchi, H. Neuroprotective effect of FK506, an immunosuppressant, on transient global ischemia in gerbil. *Neurosci. Lett.* **1996,** *206*, 81−84.
(187) Ide, T.; Morikawa, E.; Kirino, T. An immunosuppressant, FK506, protects hippocampal neurons from forebrain ischemia in the Mongolian gerbil. *Neurosci. Lett.* **1996**, *204*, 157−160.
(188) Butcher, S. P.; Henshall, D. C.; Teramura, Y.; Iwasaki, K.; Sharkey, J. Neuroprotective actions of FK506 in experimental stroke: in vivo evidence against an anti-excitotoxic mechanism. *J. Neurosci.* **1997**, *17*, 6939−6946.
(189) Yagita, Y.; Kitagawa, K.; Matsushita, K.; Taguchi, A.; Mabuchi, T.; Ohtsuki, T.; Yanagihara, T.; Matsumoto, M. Effect of immunosuppressant FK506 on ischemia-induced degeneration of hippocampal neurons in gerbils. *Life Sci.* **1996**, *59*, 1643−1650.
(190) Tong, G.; Shepherd, D.; Jahr, C. E. Synaptic desensitization of NMDA receptors by calcineurin. *Science* **1995**, *267*, 1510−1512.
(191) Funauchi, M.; Haruta, H.; Tsumoto, T. Effects of an inhibitor for calcium/calmodulin-dependent protein phosphatase, calcineurin, on induction of long-term potentiation in rat visual cortex. *Neurosci. Res.* **1994**, *19*, 269−278.
(192) Torii, N.; Kamishita, T.; Otsu, Y.; Tsumoto, T. An inhibitor for calcineurin, FK506, blocks induction of long-term depression in rat visual cortex. *Neuroscience* **1995**, *185*, 1−4.
(193) Ikegami, S.; Kato, A.; Kudo, Y.; Kuno, T.; Ozawa, F.; Inokuchi, K. A facilitatory effect on the induction of long-term potentiation in vivo by the chronic administration of antisense oligonucleotides against catalytic subunits of calcineurin. *Mol. Brain Res.* **1996**, *41*, 183−191.
(194) Hodgkiss, J. P.; Kelly, S. J. Only "de novo" long-term depression (LTD) in the rat hippocampus in vitro is blocked by the same low concentration of FK506 that blocks LTD in the visual cortex. *Brain Res.* **1995**, *705*, 241−246.
(195) Kantor, D. B.; Lanzrein, M.; Stary, S. J.; Sandoval, G. M.; Smith, W. B.; Sullivan, B. M.; Davidson, N.; Schuman, E. M. A role for endothelial NO synthase in LTP revealed by adenovirus-mediated inhibition and rescue. *Science* **1996**, *274*, 1744−1748.
(196) Schuman, E. M.; Madison, D. V. A requirement for the intercellular messenger nitric oxide in long-term potentiation. *Science* **1991**, *254*, 1503−1506.
(197) O'Dell, T. J.; Hawkins, R. D.; Kandel, E. R.; Arancio, O. Tests of the roles of two diffusible substances in long-term potentiation: evidence for nitric oxide as a possible early retrograde messenger. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 11285−11289.
(198) Shibuki, K.; Okada, D. Endogenous nitric oxide release required for long-term synaptic depression in the cerebellum. *Nature* **1996**, *349*, 326−328.
(199) Malen, P. L.; Chapman, P. F. Nitric oxide facilitates long-term potentiation, but not long-term depression. *J. Neurosci.* **1997**, *17*, 2645−2651.
(200) Steiner, J. P.; Dawson, T. M.; Fotuhi, M.; Snyder, S. H. Immunophilin regulation of neurotransmitter release. *Mol. Med.* **1996**, *2*, 325−333.
(201) Snyder, S. H.; Sabatini, D. M.; Lai, M. M.; Steiner, J. P.; Hamilton, G. S.; Suzdak, P. D. Neural actions of immunophilin ligands. *Trends Pharmacol. Sci.* **1998**, *19*, 21−26.
(202) The U.S. Multicenter FK506 Liver Study Group. A comparison of tacrolimus (FK506) and cyclosporin for immunosuppression in liver transplantation. *N. Engl. J. Med.* **1994**, *331*, 1154−1155.
(203) Mueller, A. R.; Platz, K. P.; Bechstein, W. O.; Schattenfroh, N. Neurotoxicity after orthotopic liver transplantation. A comparison between cyclosporin and FK506. *Transplantation* **1994**, *58*, 155−170.
(204) Steiner, J. P.; Connolly, M. A.; Valentine, H. L.; Hamilton, G. S.; Dawson, T. M.; Hester, L.; Snyder, S. H. Neurotrophic actions of nonimmunosuppressive analogues of immunosuppressive dtugs FK506, rapamycin and cyclosporin A. *Nature Med.* **1997**, *3*, 421−428.
(205) Hamilton, G. S.; Huang, W.; Connolly, M. A.; Ross, D. T.; Guo, H.; Valentine, H. L.; Suzdak, P. D.; Steiner, J. P. FKBP12-binding domain analogues of FK506 are potent, nonimmunosuppressive neurotrophic agents in vitro and promote recovery in a mouse model of Parkinson's Disease. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1785−1790.
(206) For a comprehensive review, see: Hamilton, G. S.; Steiner, J. P. Neuroimmunophilin ligands as novel therapeutics for the treatment of degenerative disorders of the nervous system. *Curr. Pharm. Des.* **1997**, *3*, 405−428.
(207) Holt, D. A.; Konialian-Beck, A. L.; Oh, H.-J.; Yen, H.-K.; Rozamus, L. W.; Krog, A. J.; Erhard, K. F.; Ortiz, E.; Levy, M. A.; Brandt, M.; Bossard, M. J.; Luengo, J. I. Structure−activity studies of synthetic FKBP ligands as peptidyl-prolyl isomerase inhibitors. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 315−320.
(208) Wang, G. T.; Lane, B.; Fesik, S. W.; Petros, A.; Luly, J.; Krafft, G. A. Synthesis and FKBP binding of small molecule mimics of the tricarbonyl region of FK506. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1161−1166.
(209) Steiner, J. P.; Snyder, S. H.; Hamilton, G. S. Inhibitors of rotamase enzyme activity effective at stimulating neuronal growth. U.S. Patent 5,696,135.
(210) Dragovich, P. S.; Barker, J. E.; French, J.; Imbacuan, M.; Kalish, V. J.; Kissinger, C. R.; Knighton, D. R.; Lewis, C. T.; Moomaw, E. W.; Parge, H. E.; Pelletier, L. A. K.; Prins, T. J.; Showalter, R. E.; Tatlock, J. H.; Tucker, K. D.; Villafranca, J. E. Structure-based design of novel, urea-containing FKBP12 inhibitors. *J. Med. Chem.* **1996**, *39*, 1872−1884.
(211) Li, G. S.; Hamilton, W.; Huang, M. A.; Connolly, D. T.; Ross, H.; Guo, H.; Valentine, J. P. Steiner. In vitro and in vivo neurotrophic effects of (N-sulfonyl) and (N-carbamoyl) pipecolate esters. 214th American Chemical Society National Meeting, 1997; Abstract MEDI 183.
(212) Steiner, J. P.; Hamilton, G. S.; Ross, D. T.; Valentine, H. L.; Guo, H.; Connolly, M. A.; Liang, S.; Ramsey, C.; Li, J.-H.; Huang, W.; Howorth, P.; Soni, R.; Fuller, M.; Sauer, H.; Nowotnick, A.; Suzdak, P. D. Neurotrophic immunophilin ligands stimulate structural and functional recovery in neurodegenerative animal models. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2019−2024.
(213) Valentine, H. L.; Liang, S.; Suzdak, P. D.; Hamilton, G. S.; Steiner, J. P. The neuroimmunophilin ligand GPI 1046 stimulates recovery following sciatic nerve injury. *Soc. Neurosci. Abstr.* **1997**, 677.9.
(214) Gold, B. G.; Zeleney-Pooley, M.; Wang, M.-S.; Chaturvedi, P.; Armistead, D. M. A nonimmunosuppressive FKBP-12 ligand increases nerve regeneration. *Exp. Neurobiol.* **1997**, *147*, 269−278.
(215) Gold, B. G.; Zeleney-Pooley, M.; Wang, M.-S.; Chaturvedi, P.; Armistead, D. M.; McCaffrey, P. G. FKBP ligands speed functional recovery and nerve regeneration in the rat sciatic nerve following oral administration. *Soc. Neurosci. Abstr.* **1997**, 449.12.
(216) Strange, P. G. *Brain Biochemistry and Brain Disorders*; Oxford University Press: Oxford, U.K., 1994.
(217) Agid, Y. Parkinson's Disease: pathophysiology. *Lancet* **1991**, *337*, 1321−1324.
(218) Gerlach, M.; Riederer, P.; Przuntek, H.; Youdim, M. B. MPTP mechanisms of neurotoxicity and their implications for Parkinson's Disease. *Eur. J. Pharmacol.* **1991**, *208*, 273−286.
(219) Davis, G. C.; Williams, A. C.; Markey, S. P.; Ebert, M. H.; Caine, E. D.; Reichert, C. M.; Kopin, I. J. Chronic Parkinsonism secondary to intravenous injection of meperidine analogues. *Psychiat. Res.* **1979**, *3*, 249−254.
(220) Langston, J. W.; Ballard, P.; Tetrud, J. W.; Irwin, I. Chronic Parkinsonism in humans due to a product of meperidine-analogue synthesis. *Science* **1983**, *219*, 979−980.
(221) Steiner, J. P.; Ross, D. T.; Valentine, H. L.; Liang, S.; Guo, H.; Huang, W.; Suzdak, P. D.; Hamilton, G. S. The orally active neuroimmunophilin ligand GPI 1046 promotes structural and functional recovery in the mouse MPTP model of Parkinson's Disease. *Soc. Neurosci. Abstr.* **1997**, 677.6.
(222) Constantini, L. C.; Chaturvedi, P.; McCaffrey, P.; Deacon, T. W.; Isacson, O. Neuroprotective and regenerative effects of immunophilin ligands in an animal model of Parkinson's Disease. *Soc. Neurosci. Abstr.* **1997**, 573.11.
(223) Ross, D. T.; Guo, H.; Howorth, P.; Fuller, M.; Nowotnick, A.; Hamilton, G. S.; Suzdak, P. D.; Steiner, J. P. The novel neuroimmunophilin ligand GPI 1046 stimulates morphological, biochemical, and behavioral recovery in the rat intranigral 6-OHDA Parkinson's Disease model. *Soc. Neurosci. Abstr.* **1997**, 677.7.
(224) Sachs, C.; Jonsson, G. Mechanisms of action of 6-hydroxydopamine. *Biochem. Pharmacol.* **1975**, *24*, 1−8.

(225) Ungerstedt, U.; Aburthnott, G. W. Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system. *Brain Res.* **1970**, *24*, 485−493.
(226) Whitaker-Azmita, P. M.; Peroutka, S. J. The neuropharmacology of serotonin. *Ann. N. Y. Acad. Sci.* **1990**, *600*.
(227) Agid, Y.; Javoy-Agid, F.; Ruberg, M. Biochemistry of neurotransmitters in Parkinson's Disease. In *Movement Disorders 2*; Marsden, C. D., Fahn, S., Eds.; Butterworths: New York, 1992; pp 160−230.
(228) Cross, A. J. Serotonin in Alzheimer-type dementia and other dementing illnesses. *Ann. N. Y. Acad. Sci.* **1990**, *600*, 405−417.
(229) Mamounas, L. A.; Molliner, M. E. Evidence for dual serotonergic projections to neocortex: axons from the dorsal and median raphe nuclei are differentially vulnerable to the neurotoxin *p*-chloroamphetamine (PCA). *Exp. Neurol.* **1988**, *102*, 23−36.
(230) Mamounas, L. A.; Mullen, C. A.; O'Hearn, E.; Molliner, M. E. Dual serotonergic projections to forebrain in the rat: morphologically distinct 5-HT axon terminals exhibit differential vulnerability to neurotoxic amphetamine derivatives. *J. Comp. Neurol.* **1991**, *314*, 558−586.
(231) Mamounas, L. A.; Blue, M. E.; Siuciak, J. A.; Altar, C. A. Brain-derived neurotrophic factor promotes the survival and sprouting of serotonergic axons in rat brain. *J. Neurosci.* **1995**, *15*, 7929−7939.
(232) Liang, S.; Valentine, H. L.; Ross, D. T.; Huang, W.; Suzdak, P. D.; Hamilton, G. S.; Steiner, J. P. Neuroimmunophilin ligands augment serotonin fiber protection following lesions with para-chloroamphetamine (PCA). *Soc. Neurosci. Abstr.* **1997**, 677.10.
(233) Bartus, R. T.; Dean, R. L.; Beer, B.; Lippa, A. S. The cholinergic hypothesis of geriatric memory dysfunction. *Science* **1992**, *217*, 408−417.
(234) Hefti, F. Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. *J. Neurosci.* **1986**, *6*, 2155−2162.
(235) Guo, H.; Spicer, D. M.; Howorth, P.; Hamilton, G. S.; Suzdak, P. D.; Steiner, J. P.; Ross, D. T. The novel small molecule immunophilin ligand GPI 1046 stimulates cholinergic reinnervation of deafferented hippocampal regions after fimbria-fornix transection. *Soc. Neurosci. Abstr.* **1997**, 677.12.
(236) Apfel, S. C.; Arezzo, J. C.; Brownlee, M.; Federoff, H.; Kessler, J. A. Nerve growth factor administration protects against experimental diabetic sensory neuropathy. *Brain Res.* **1994**, *634*, 7−12.
(237) Apfel, S. C.; Arezzo, J. C.; Lipson, L.; Kessler, J. A. Nerve growth factor prevents experimental cisplatin neuropathy. *Ann. Neurol.* **1992**, *31*, 76−80.
(238) McMahon, S. B.; Priestley, J. V. Peripheral neuropathies and neurotrophic factors: animal models and clinical perspectives. *Curr. Opin. Neurobiol.* **1995**, *5*, 616−624.
(239) Martinez-Serrano, A.; Lundberg, C.; Horellou, P.; Fischer, W.; Bentlage, C.; Campbell, K.; McKay, R. D.; Mallet, J.; Bjorklund, A. CNS-derived neural progenitor cells for gene transfer of nerve growth factor to the adult rat brain: complete rescue of axotomized cholinergic neurons after transplantation into the septum. *J. Neurosci.* **1995**, *15*, 5668−5680.
(240) Gash, D. M.; Zhang, Z.; Ovadia, A.; Cass, W. A.; Yi, A.; Simmerman, L.; Russell, D.; Martin D.; Lapchak, P. A.; Collins, F.; Hoffer, B. J.; Gerhardt, G. A. Functional recovery in parkinsonian monkeys treated with GDNF. *Nature* **1996**, *380*, 252−255.
(241) Woolf, C. J.; Ma, Q. P.; Allchorne, A.; Poole, S. J. Peripheral cell types contributing to the hyperalgesic action of nerve growth factor in inflammation. *J. Neurosci.* **1996**, *16*, 2716−2723.
(242) Goto, S.; Singer, W. Laminar and columnar organization of immunoreactivity for calcineurin, a calcium- and calmodulin-regulated protein phosphatase, in monkey striate cortex. *Cereb. Cortex* **1994**, *4*, 636−645.
(243) Ivery, M. T.; Weiler, L. Modeling the interaction between FK506 and FKBP12: a mechanism for the formation of the calcineurin inhibitory complex. *Bioorg. Med. Chem.* **1997**, *5*, 217−232.
(244) Wang, M. S.; Zeleny-Pooley, M.; Gold, B. G. Comparative dose-dependency study of FK506 and cyclosporin A on the rate of axonal regeneration in rat sciatic nerve. *J. Pharmacol. Exp. Ther.* **1997**, *282*, 1084−1093.
(245) Boriongan, C. V.; Stahl, C. E.; Fujisaki, T.; Porter, L. L.; Wtanabe, S. Cyclosporine A inhibits calcineurin activity and induces nigral dopamine neurite outgrowth, but does not impair memory retention in rats with damaged blood brain barrier. *Soc. Neurosci. Abstr.* **1998**, 778.16.

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