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Peptidyl-prolyl-*cis*/*trans*-isomerase activity may be necessary for memory formation

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Abstract At present, evidence for a plethora of physiological roles for the different classes of peptidyl-prolyl-*cis*/*trans*-isomerases (PPIases, EC 5.2.1.8) is emerging. Cyclosporin A (CyA) has been previously reported to disrupt memory formation in a temporally specific manner, when administered intracranially to day-old chicks trained on a single-trial, passive-avoidance task [Bennett, P.C., Zhao, W., Lawen, A. and Ng, K.T. (1996) Brain Res. 730, 107–117]. CyA is known to inhibit both the PPIase activity of cyclophilin and, indirectly, the protein phosphatase activity of calcineurin. Therefore to begin to distinguish between these two functions we studied the effects on memory formation of three non-immunosuppressive CyA analogues, in order to study the involvement of cyclophilins. These drugs retain the capacity to bind to and inhibit the PPIase activity of cyclophilin, but do not bind in the complex with cyclophilin to calcineurin and, therefore, do not inhibit its phosphatase activity. All three drugs exert effects on memory formation comparable to those induced by CyA, significantly inhibiting memory formation when injected intracranially (50 fmol per hemisphere) immediately following training. Brain extracts from chicks treated with [MeVal[4]]CyA show a strong inhibition of cyclophilin activity. These data show a requirement for the PPIase activity of a cyclophilin for successful memory formation and constitute the first set of data establishing a physiological role for a cyclophilin.

Key words: Cyclophilin; Non-immunosuppressive cyclosporin A analogue; Memory formation; Peptidyl-prolyl-*cis*/*trans*-isomerase

1. Introduction

The immunosuppressant cyclosporin A (CyA) impairs T cell proliferation by inhibiting the activity of the calcium/calmodulin-dependent protein serine/threonine phosphatase calcineurin (CaN) [1,2]. More specifically, CyA has been found to bind to a class of cytosolic receptor proteins called cyclophilins. The cyclophilin/CyA complex then binds to CaN, inhibiting the activity of this enzyme and preventing the calcium-dependent translocation of the p88 subunit of transcription factor NF-AT to the nucleus, where it is required for T-cell activation [3]. This finding has proven to be particularly important for molecular biologists since, in addition to elucidating one mechanism responsible for pharmacological immunosuppression, it also suggests that the cell-permeable CyA may be suitable as an agent with which to investigate other cellular functions of CaN, an enzyme postulated to have important functions in various tissues [4,5]. As a result, both CyA and an alternative immunosuppressant, FK506, which acts via a similar mechanism of first binding to a class of cytosolic receptor proteins and subsequently binding to and inhibiting CaN [1,2], have recently been used to probe the involvement of CaN in various aspects of cell biology [6]. Since the mechanism of action can be either the direct inhibition of cyclophilin or the indirect inhibition of CaN, employment of non-immunosuppressive CyA analogues has proved a powerful tool for discriminating between peptidyl-prolyl-*cis*/*trans*-isomerase (PPIase) and CaN inhibition [7,8].

Most of the reported effects of CyA so far have been attributed to its inhibition of CaN, upon binding to its receptor proteins, the cyclophilins. Evidence is emerging, however, which identifies various physiological roles for the cyclophilins themselves. Several effects of CyA have been found to be mediated by inhibition of PPIase activity rather than by inhibition of CaN activity, including neurotrophic action [9], transport into the mitochondrion [10], and inhibition of replication of HIV-1 [7,11]. It was previously reported that intracranial administration of nanomolar concentrations of CyA significantly impairs long-term memory formation in neonate chicks trained on an established, single-trial, passive-avoidance task [12]. Importantly, groups of chicks tested up to 60 min post training demonstrated normal retention levels, while all groups tested 80 min after training, or at any time later up to 24 h, displayed significant retention deficits. Thus, it would appear that administration of CyA, at the time and concentration employed, selectively disrupts only later stages of memory formation, leaving earlier stages intact. No retention deficits were apparent following administration of CyH, a CyA analogue which does not bind to cyclophilin nor inhibit CaN [12]. Because CyA could affect memory through inhibition of either CaN or cyclophilin activity, to begin to clarify the mechanism responsible for the amnestic effect of CyA, the effects of three non-immunosuppressive CyA analogues, namely [MeVal[4]]CyA, [MeIle[4]]CyA and [MeAla[6]]CyA, on memory formation in the chick were investigated. Each of these agents is reported to bind to cyclophilin and inhibit its PPIase activity, but the resulting complex has no effect on the activity of CaN [6]. These compounds therefore are only effective against the cyclophilin itself, without affecting calcineurin. All three compounds showed an amnestic effect at similar concentrations and times as CyA itself. While this

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Abbreviations: ANOVA, analysis of variance; CaN, calcineurin; Cy, cyclosporin; CyA, cyclosporin A; CyH, cyclosporin H; DMSO, dimethyl sulfoxide; DR, discrimination ratio; FKBP, FK506 binding protein; HIV, human immunodeficiency virus; LTD, long-term depression; PPIase, peptidyl-prolyl-*cis*/*trans*-isomerase

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does not eliminate the possibility that CaN may also play a role in memory formation, our data provide the first available evidence of an important, physiological role for cyclophilin activity.

2. Materials and methods

2.1. Animals

In all experiments chicks were trained using a passive-avoidance task described in detail elsewhere [13]. Briefly, groups of 20 chicks with two chicks per training box were pre-trained to peck at various colored beads. Each chick was then presented with a red bead coated with an aversive chemical, methylanthranilate. After pecking at this bead all chicks typically demonstrated a strong disgust reaction, emitting distress calls and wiping their beaks on the floor of the cage. At specified times following the aversive training experience, each chick was administered the required concentration of the appropriate agent. All drugs were dissolved in EtOH and diluted in physiological saline before being administered freehand (10 μl per hemisphere) bilaterally into the hyperstriatal/neostriatal complex of the chick brain, using a Hamilton repeating dispenser syringe fitted with a plastic sleeve to limit injection depth to 3.5 mm. This area of the chick brain is known to be metabolically active following passive-avoidance training [14,15]. Random histological checks were used to confirm the accuracy of all injections (>85% within 1 mm of the target site), with the 10 μl volume being selected on the basis of many previous experiments showing that this volume is most suited to the loose architecture of the neonate chick brain. Control groups were administered a 5 nM concentration of CyH (=50 fmol), a CyA analogue which does not bind to cyclophilin nor inhibit CaN [12], which was also dissolved in EtOH and diluted in saline. Retention for the aversive training experience was tested at the required time following training by presenting each chick with a red bead for 10 s, followed by a blue bead for 10 s. The number of pecks at each bead was recorded for each chick. Data from chicks which failed to train or peck the blue bead at test were eliminated before a discrimination ratio (DR), defined as the ratio between the number of pecks at the blue bead and the number of pecks at both red and blue beads, was calculated for each chick. The mean DR for each group of chicks was calculated, with good discrimination between the two colored beads leading to a mean DR approaching 1.0 and poor discrimination leading to a mean DR approaching 0.5. All experiments were carried out in accordance with the guidelines of the Australian Code for the Care and Use of Animals for Scientific Purposes.

2.2. In vitro PPIase assay

Three groups of 20 chicks were trained as described above. One group was injected with 10 μl of a 5 nM CyH solution (MeA-trained/CyH), the second group was injected with 10 μl of a 5 nM [MeVal^4]CyA solution (MeA-trained/[MeVal^4]CyA) in the left and right hemisphere immediately after training. Since all drugs used for injection were prepared from a 1 mM stock solution of the drug dissolved in DMSO, the third group was injected with 10 μl of a 0.000005% DMSO/saline (MeA-trained/DMSO).

After 90 min, the chicks were decapitated with their heads immediately dropped into liquid nitrogen for about 5 s. Five brains from the MeA-trained/CyH group (0.1791 g total frozen weight), from the MeA-trained/[MeVal^4]CyA group (0.198 g total frozen weight), and from the MeA-trained/DMSO group (0.194 g total frozen weight), were harvested. The frozen brains were then transferred onto dry ice and stored in a −80°C freezer. The forebrains were subsequently dissected out, and the IMHV/neostriatal, hippocampal regions, right to the surface of the forebrain (~7 mm by 5 mm by 5 mm), were cut out, for each of the two groups. Only the left hemisphere, previously shown to be most prominent in LTM formation, was analyzed for the PPIase activity.

Tissues from the two groups were rinsed very briefly (~5 s) in ice-cold washing buffer (0.32 M sucrose; 1 mM K_2HPO_4; 1 mM EDTA; 4 g/l of $MgCl_2$, 0.02% NaN_3, 1.2 mM EGTA, 1 mM $CaCl_2$, 1 mM NaF, 1 mM sodium vanadate, 10 μl/ml of protease inhibitor cocktail (Sigma P8340, Castle Hill, Australia)). Immediately thereafter, tissues were homogenized in 5 ml of washing buffer (containing 10 μl/ml of protease inhibitor cocktail) with 12 strokes in a mechanical rotatory glass-teflon homogenizer (John Morris Scientific Instruments, Melbourne, Australia) set at 1500 rpm. 1 ml of each extract was then mixed with 100 μl of Nonidet P-40 and re-homogenized using a manual glass-glass homogenizer (Kontes, NJ). The extract was cleared by centrifugation in an Eppendorf centrifuge for 20 min. Protein content from the cleared extracts was determined using both the using the Bio-Rad Bradford assay kit (Bio-Rad, Regent Park, Australia) and absorbance at 280 nM and then diluted to 100 μg protein/ml. These extracts were then used for the PPIase assays.

All enzymatic reactions were conducted at 10°C. Enzyme activity in the cleared extracts was measured essentially as described by [16]. In brief, a mixture of 50 μl of cleared extract was stirred with 1.5 ml of buffer (50 mM HEPES, 100 mM NaCl, pH 8.0) and 100 μl of α-chymotrypsin (dissolved in 1 mM HCl to a concentration of 25 mg/ml) in a quartz cuvette and cooled to 10°C. Three cuvettes of mixtures prepared from cleared extracts of two groups were prepared in any one experiment performed together with two cuvettes of control mixtures: without any extract and without any extract but with 50 μl of 200 mg/ml BSA solution. The reaction was started with the addition of 3 μl of peptide substrate and the change in absorbance at 390 nm was monitored every 3.75 s for each cuvette for 600 s, with the mixtures continuously being stirred. (The peptide substrate had been dissolved in 470 mM LiCl in trifluoroethanol to a concentration of 14.5 mg/ml and allowed to come to equilibrium for 24 h.) PPIase activity, which was not inhibitable by FK506, was measured by pre-incubating the extracts with 167 μM FK506 for 20 min before the assay.

To monitor the extent of inhibition of PPIases in the forebrain extracts by the injected drugs, brain extracts of each group were pre-incubated for 20 min with saturating concentrations of CyA (10 μl of 500 μM CyA per 50 μl extract) in addition to FK506 to inhibit most PPIase activity in the cleared extracts used.

3. Results

In an initial experiment groups of chicks were administered one of various concentrations of [MeVal^4]CyA or a 5 nM concentration of CyH or CyA immediately after training, and were tested for retention following an interval of 180 min. As shown in Fig. 1, various concentrations of [MeVal^4]CyA caused marked retention deficits. Differences between group means were analyzed using a one-way ANOVA [17], which revealed significant differences between groups ($F_{9,189} = 6.887$, $P < 0.001$). Post-hoc Dunnett's tests ($\alpha = 0.05$) [17], comparing each experimental group with the CyH-treated group, revealed a significant effect for all groups receiving a concentration of 0.1 nM [MeVal^4]CyA (=1 fmol) or greater and for the group which received 5 nM CyA (=50 fmol).

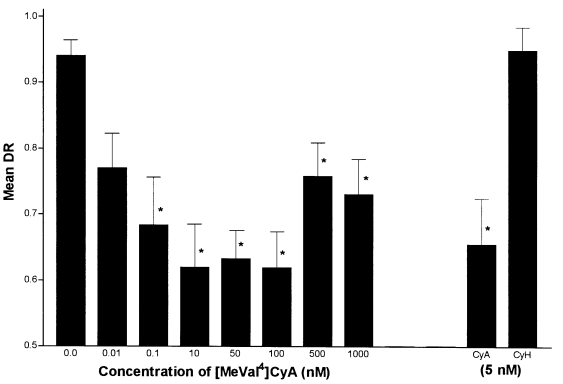

Fig. 1. Dose response for [MeVal^4]CyA. All concentrations of [MeVal^4]CyA were administered intracranially (10 μl/hemisphere) immediately following training, with chicks being tested for retention following an interval of 180 min. The results are compared against the effect of 5 nM CyA and 5 nM CyH (=50 fmol of cyclosporin administered). * Denotes those groups which differed significantly ($\alpha = 0.05$) from the CyH-treated chicks.

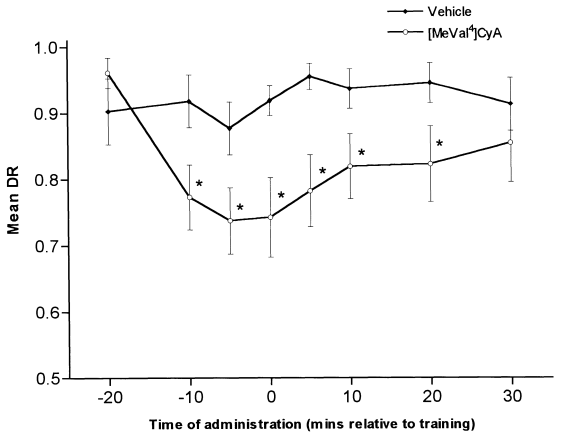

Fig. 2. Time of administration study for [MeVal^4]CyA. A 5 nM concentration of either [MeVal^4]CyA or the EtoH vehicle was administered intracranially (10 μl/hemisphere, 50 fmol of Cy) at various times relative to training, with all chicks being tested for retention after an interval of 180 min. * Denotes those [MeVal^4]CyA-treated groups which differed significantly ($\alpha = 0.05$) from the corresponding EtoH-treated group.

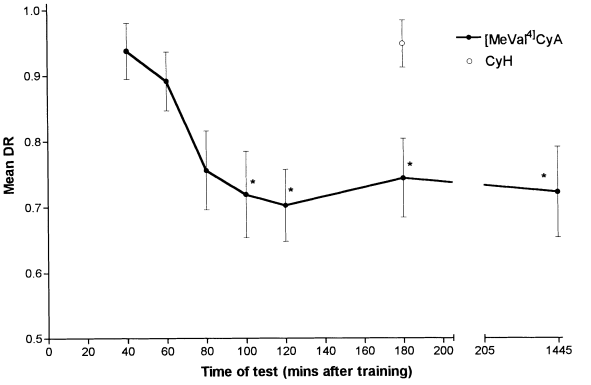

Fig. 3. Time course for the development of retention deficits following administration of [MeVal^4]CyA. A 5 nM concentration of either [MeVal^4]CyA or CyH was administered intracranially (10 μl/hemisphere, 50 fmol of Cy) immediately after training. Groups of [MeVal^4]CyA-treated chicks were tested for retention following various intervals, and are compared with the CyH-treated group, which was tested at 180 min post training. * Denotes those groups which differed significantly ($\alpha = 0.05$) from the CyH-treated chicks.

For the purposes of comparison with earlier studies using 50 fmol of CyA [12] 50 fmol of [MeVal^4]CyA were administered bilaterally to groups of chicks at various times relative to training on the passive-avoidance task. Control groups were administered the EtOH vehicle at corresponding times, with retention in all groups being tested 180 min after the training event. The results are shown in Fig. 2. A two-way ANOVA revealed significant main effects for drug condition ($F_{1,362} = 19.86$, $P < 0.001$) and time of administration ($F_{9,362} = 2.27$, $P < 0.05$) and a significant drug condition by time of administration interaction effect ($F_{9,362} = 2.44$, $P < 0.05$). Post-hoc tests of simple main effects were significant ($P < 0.05$) across drug condition for groups injected at all times ranging from −10 min to +20 min relative to training, and, across times of administration, for groups of chicks administered [MeVal^4]CyA. These data demonstrate that memory formation in the chick is sensitive to [MeVal^4]CyA only within a specific window of administration. This window of sensitivity is similar to that previously obtained for CyA (−10 to +40 min), although CyA is effective for a longer time after training, perhaps indicating that it may act more rapidly than does [MeVal^4]CyA.

To once more facilitate direct comparison with earlier studies using 50 fmol of CyA [12] and 50 fmol of CyH, 50 fmol of [MeVal^4]CyA was administered bilaterally to groups of chicks immediately following training on the passive-avoidance task, with retention being tested following various intervals. To control for non-specific drug effects, one group of chicks was also administered 50 fmol of CyH and tested at 180 min post training. The results are shown in Fig. 3. A one-way ANOVA revealed significant differences in retention among the groups ($F_{7,136} = 3.405$, $P < 0.01$), with post-hoc Dunnett's tests ($\alpha = 0.05$), comparing each experimental group with the control group, administered 50 fmol of CyH and tested 180 min post training, revealing significant deficits for all time points after 80 min.

Finally, 50 fmol of either [MeAla^6]CyA or [MeIle^4]CyA was administered as described for the previous experiment. As shown in Fig. 4 both compounds were found to produce retention deficits similar to those induced by 50 fmol of [MeVal^4]CyA and CyA (also included in Fig. 4). A two-way ANOVA revealed a significant main effect for time of test ($F_{4,294} = 10.89$, $P < 0.001$), but no significant main effect for drug ($P = 0.238$) and no interaction effect between drug and time of test ($P = 0.968$). Hence, retention deficits induced by all three CyA analogues and by CyA are statistically indistinguishable and a non-specific drug effect is unlikely to account for the effect of any single agent.

Total PPIase activity was observed in the cleared extracts from both groups of chicks when compared with the control reaction (without addition of extracts). Fifty microliters of the cleared extracts with a protein concentration of 100 μg/ml each were used. Since the substrate used in this assay is isomerized by cyclophilins as well as by FKBPs, we performed these experiments after pre-incubation with FK506 (Fig. 5). The rate of reaction is expressed as the rate of absorbance

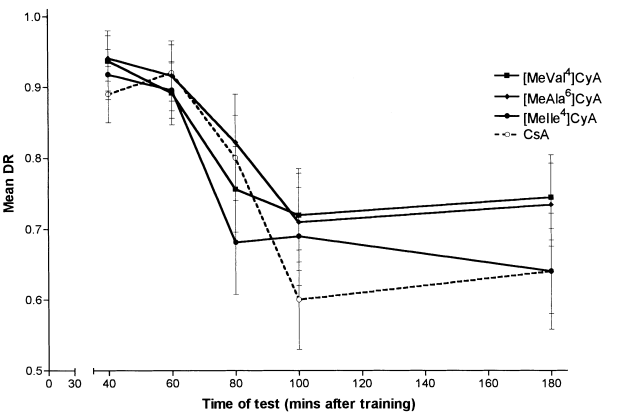

Fig. 4. Comparison of time courses obtained using various CyA analogues. Groups of chicks were administered a 5 nM concentration (50 fmol) of either [MeIle^4]CyA or [MeAla^6]CyA, immediately after training on the passive-avoidance task, with retention being tested following various intervals. Results obtained previously using CyA (5 nM) and [MeVal^4]CyA (5 nM) are included to facilitate comparison across drug conditions. No significant differences were obtained.

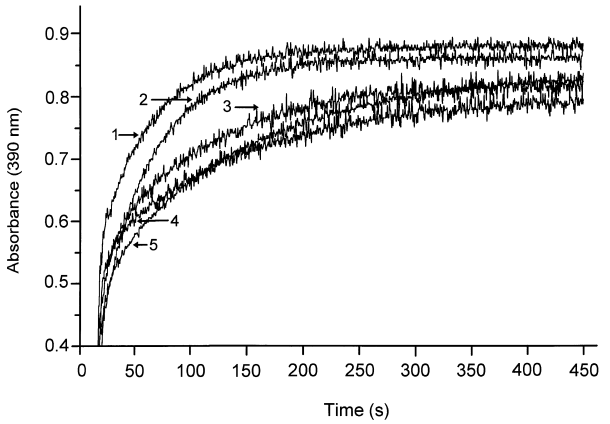

Fig. 5. In vitro cyclophilin PPIase activity of brain extracts. All extracts (50 μl each) were pre-incubated with 167 μM FK506 for 20 min on ice. The extracts were then mixed with 1.5 ml of buffer and 100 μl of α-chymotrypsin and allowed to reach 10°C. The reaction was started by the addition of 20 μl of substrate and was monitored at 390 nm every 0.5 s for 500 s. (1) Control assay with addition of brain extract from MeA-trained and CyH-treated chicken; (2) control assay with addition of brain extract from MeA-trained and DMSO-treated chicken; (3) assay with addition of brain extract from MeA-trained and [MeVal^4]CyA-treated chicken; (4) assay with addition of brain extract from MeA-trained and CyH-treated chicken that has undergone pre-incubation with saturating amounts of CyA; (5) control assay without addition of extract.

change as concentrations of all constituents in the different reaction mixtures were kept constant.

The rate of absorbance change in the MeA-trained/CyH group (0.0053 ΔA/min) was similar to that of the MeA-trained/saline group (0.0053 ΔA/min) and approximately 2 times the rate of absorbance change in the MeA-trained/[MeVal^4]CyA group (0.0039 ΔA/min). The rate of absorbance change in the MeA-trained/[MeVal^4]CyA group approached the rate measured for the control (0.0024 ΔA/min) strongly suggesting that administration of the drug led to inhibition of (a) Cy-sensitive PPIase(s). Fig. 5 depicts a representative of five independent experiments. The extract-free mixture containing BSA did not differ significantly in the rate from the extract-free mixture containing washing buffer. All the cleared extracts containing CyA had rates of absorbance change which closely matched the background rates of the controls, indicating total inhibition of PPIase activity in the mixtures (data not shown).

4. Discussion

The intracranial administration of various concentrations of [MeVal^4]CyA into the hyperstriatal/neostriatal complex was found to produce significant retention deficits in groups of chicks trained on an established, single-trial, passive-avoidance task. The effects were obtained only when the drug was administered within a temporally defined window of sensitivity surrounding the time of training. Further, when 10 μl of a 5 nM concentration of either [MeVal^4]CyA, [MeIle^4]CyA or [MeAla^6]CyA (50 fmol each) was administered immediately after training, retention deficits first became significant after 80 min and remained significant for at least 24 h. This time course is essentially identical to that induced by CyA [12]. Memory for the passive-avoidance task in the chick has been shown to involve three distinct stages: a short-term stage, formed by 5 min and decaying 10 min post training; an intermediate-term stage, formed by 20 min and decaying 50 min post training; and a long-term stage, available by 60 min post training [18–20]. Since retention levels following the administration of all agents remain high for at least 60 min after training, within the framework of this model both CyA and various non-immunosuppressive analogues would appear to selectively disrupt long-term memory, leaving earlier memory stages intact. Interestingly, recently obtained data suggest that the same effects can be obtained when the drugs are exclusively administered to the left hemisphere, whereas administration to the right hemisphere only does not lead to an amnestic effect (Bennett, Zhao, Lawen and Ng, unpublished results).

CyA is known to disrupt both the PPIase activity of cyclophilins and the phosphatase activity of CaN [1,2,21], and has also been reported to bind to a multidrug resistance (MDR)-related P-glycoprotein [22]. The temporal specificity of the data, as the low drug concentrations required, as well as the specific window of effective concentrations, would appear to exclude any non-specific toxicological effects of the drugs employed and, because CyH binds as well as CyA to the MDR-related P-glycoprotein [22], the failure of CyH to induce memory loss in this or our previous study [12] suggests that the effects of CyA are not mediated by binding of CyA to the P-glycoprotein. All literature data so far suggest that the non-immunosuppressive CyA analogues employed in this study do not inhibit calcineurin either in vitro or in vivo [6–8,23–27]. Administration of 50 fmol of [MeVal^4]CyA was sufficient to inhibit most of the CyA-sensitive PPIase activities in that area of the brain. As is evident from Fig. 5 by no means all cyclophilin activities are inhibited in our extracts. However, these data show some cyclophilin inhibition, including obviously at least one activity necessary for memory formation. Together with the amnestic effects obtained with the three drugs these data strongly support the conclusion that CyA-sensitive PPIase activities are required for the formation of a relatively late stage of memory in the day-old chick. While a concurrent role for CaN has not yet been adequately tested, due to the non-specificity of all available inhibitory agents, this supports previous claims that the ubiquitous distribution of cyclophilins in the brain denotes important but as yet largely unidentified roles independently of their pharmacologically-induced inhibition of CaN [28].

Besides its PPIase activity, cyclophilin A has also been suggested to exert a chaperone activity. This interpretation results from testing cyclophilin A in the in vitro folding of carbonic anhydrase [29,30] and from studies of mutants of the ninaA protein [31], although a recent report suggests that the observed effects can be contributed to PPIase activity [32]. Indirect evidence for its function as a chaperone is, however, the presence of the decameric unit of cyclophilin A-CyA complexes in crystals of the complex, in a manner reminiscent of other chaperones and heat shock proteins [33]. Therefore, we cannot totally exclude from our data a chaperone-like function of a cyclophilin in memory formation.

Our data, while providing strong evidence of a role for cyclophilin in memory formation, do not eliminate the possibility of a role for CaN in information storage processes. There is evidence that CaN is involved in the induction of LTD [34–36], an important cellular model of memory and, given evidence that other phosphatases contribute to memory

formation in *Drosophila* [37], rodents [38], and the neonate chick [39], it remains likely that evidence of a role for CaN in memory formation will be forthcoming. Further studies, currently under way in our laboratory, are presently addressing this issue using alternative inhibitors for CaN, such as FK506 and the pyrethroid deltamethrin. Preliminary evidence suggests that CaN may indeed play a role in memory formation, as may the class of immunophilins inhibited by FK506 (Bennett, Zhao, Lawen and Ng, unpublished results), but this is yet to be adequately confirmed and the temporal parameters of the effects are not yet established.

A role for cyclophilins in memory is consistent with unequivocal evidence that protein biosynthesis, and therefore folding and possibly transport, is essential for long-term memory formation in various species [40]. Further research will be needed to clarify the exact nature of the role played by cyclophilins in memory formation. Although cyclophilins have been reported to have a role in HIV infectivity [7,11] and in the folding of proteins imported into mitochondria [10], few physiological roles for these enzymes have been identified. It is of interest, however, that neurotrophic actions of CyA and FK506, previously attributed tentatively to the inhibition of CaN [41], have also recently been described to be mediated by inhibition of PPIase activity [9]. Additional roles for PPIase enzymes seem likely to emerge now that more specific inhibitory agents are available. Our laboratory is presently trying to identify the cyclophilin isozyme necessary for the formation of long-term memory.

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