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Reduction of Calcineurin Activity in Brain by Antisense Oligonucleotides Leads to Persistent Phosphorylation of τ Protein at Thr^{181} and Thr^{231}

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ABSTRACT

Phosphorylation of τ protein promotes stability of the axonal cytoskeleton; aberrant τ phosphorylation is implicated in the biogenesis of paired helical filaments (PHF) seen in Alzheimer's disease. Protein kinases and phosphatases that modulate τ phosphorylation have been identified using in vitro techniques; however, the role of these enzymes in vivo has not been determined. We used intraventricular infusions of antisense oligodeoxynucleotides (ODNs) directed against the major brain isoforms of the Ca^{2+}/calmodulin-dependent phosphatase calcineurin to determine how reduced activity of this enzyme would affect τ dephosphorylation. Five-day infusions of antisense ODNs (5 and 10 nmol/day) in rats decreased immunoreactive levels and activity of calcineurin throughout the brain; sense ODNs, scrambled ODNs, and infusion vehicle alone had no effect. When neocortical slices were prepared from antisense ODN-treated rats and incubated for 1 to 2 h in vitro, τ protein remained phosphorylated as determined by using the phosphorylation-sensitive monoclonal antibodies AT-180 (Thr^{231}) and AT-270 (Thr^{181}). In contrast, AT-180 and AT-270 sites were completely dephosphorylated during incubation of neocortical slices from vehicle-infused controls and sense ODN-treated rats. Neocortical slices from antisense-treated rats were incubated with the phosphatase inhibitors okadaic acid (100 nM; 10 μM) and FK-520 (5 μM); these preparations showed enhanced τ phosphorylation, consistent with a significant loss of calcineurin activity. Thus, we conclude that phosphorylation of at least two sites on τ protein, namely, Thr^{181} and Thr^{231}, is regulated by calcineurin.

τ Protein promotes the stability of the neuronal cytoskeleton via its binding to microtubules. The extent of τ phosphorylation is a major determinant of its ability to bind and therefore regulate microtubule assembly (Goedert and Jakes, 1990). Dephosphorylated τ readily binds microtubules, whereas phosphorylated τ, particularly at sites in or adjacent to microtubule binding domains (e.g., Ser^{396}), has a reduced affinity for microtubules (Bramblett et al., 1993). Highly phosphorylated forms of τ are primary constituents of the paired helical filaments (PHFs) of the neurofibrillary lesions seen in Alzheimer's disease (Grundke-Iqbal et al., 1986; Kosik et al., 1988). In PHF preparations from Alzheimer brain, phosphorylation sites variably include (using the numbering convention of the longest human isoform of τ, ht-40) Ser^{46}, Thr^{181}, Ser^{202}, Thr^{231}, Ser^{235}, Ser^{262}, Ser^{396}, and a site between residues 191 and 225 (Goedert et al., 1989; Hasegawa et al., 1993). Phosphorylation of Ser^{262} in the microtubule binding repeat was shown to alter the microtubule binding activity of τ; the protein kinase responsible for phosphorylating this site was recently characterized (Drewes et al., 1995). Numerous other protein kinases, including proline-directed and cyclin-dependent kinases, phosphorylate τ protein in vitro (Steiner et al., 1990; Biernat et al., 1992; Ishiguro et al., 1992; Lew et al., 1992; Vulliet et al., 1992). The diversity of phosphorylated isoforms of τ is further increased via alternative mRNA splicing (Goedert et al., 1992a).

Dephosphorylation of τ is crucial for normal function; protein phosphatases 2A (PP2A) and calcineurin (PP2B) dephosphorylate τ in vitro (Goto et al., 1985; Goedert et al., 1992b). Recent evidence has demonstrated that τ is phosphorylated in normal-appearing human brain at many of the sites previously thought to be specific for Alzheimer brain. During the initial 1- to 2-h postmortem period, endogenous neuronal phosphatase activity continued to dephosphorylate τ at numerous sites as determined using phosphorylation-sensitive antibodies (Garver et al., 1994; Matsuo et al., 1994). Incubation of human brain slices, rat brain slices, or cultured rat

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ABBREVIATIONS: PHF, paired helical filament; ODN, oligodeoxynucleotide; aCSF, artificial cerebrospinal fluid; PP1, protein phosphatase 1; PP2, protein phosphatase 2.

cortical neurons with okadaic acid (at micromolar levels) and other phosphatase inhibitors resulted in accumulation of phosphorylated forms of τ (Arias et al., 1993; Harris et al., 1993; Garver et al., 1995). These studies with phosphatase inhibitors in brain slice preparations suggested that PP2A and PP2B dephosphorylate τ. Agents such as nerve growth factor increased p42 mitogen-activated protein kinase activity and τ phosphorylation and enhanced the effects of phosphatase inhibitors. A current summary of the known phosphorylation sites on τ, the antibodies that recognize them, and the putative phosphatases that act on these sites in vitro is given in a recent review and shown in Fig. 1 (Billingsley and Kincaid, 1997).

This project was designed to investigate whether altered in vivo levels of calcineurin can lead to accumulation of phosphorylated τ ex vivo. Intraventricular infusion of antisense oligonucleotides (ODNs) directed against the catalytic (Aα, Aβ) and regulatory (B) subunits of calcineurin produced a significant decline in the levels and activity of this phosphatase. τ Phosphorylation was studied in neocortical slices from control and sense ODN- and antisense ODN-treated rats, using previously described brain slice paradigms (Harris et al., 1993; Garver et al., 1994, 1995). We now report that reduction in calcineurin levels and activity results in a selective and persistent phosphorylation of τ at two sites: Thr^{181} and Thr^{231}.

Materials and Methods

Preparation of Sense and Antisense ODNs. Eight different 18-base phosphorothioated ODNs were synthesized on a Milligen Expedite DNA synthesizer (Macromolecular Core Facility, Pennsylvania State University College of Medicine), using the Beaucage reagent for phosphorothioate formation at each residue. ODNs were purified using ethanol precipitation, and amounts were determined spectrophotometrically. Each ODN was designed using the Oligo 4.0 primer analysis software package (National Biosciences, Plymouth, MN). Sequences were determined from published reports of rat calcineurin isoforms (Kincaid et al., 1990; Kuno et al., 1992). The translation initiation site is underlined in each sense strand: calcineurin A1 (Aα), sense 5′-TGA CTG GAG ATG TCC GAG-3′, and antisense 3′-ACT GAC CTC TAC AGG CTC-5′; calcineurin A2 (Aβ), sense 5′-AGC ATG GCC GCC CCG GAG-3′, and antisense 3′-TCG TAC CGG CGG GGC CTC-5′; calcineurin B, sense 5′-G AGC AAA ATG GGA AAT GA-3′, antisense 3′-C TCG TTT TAC CCT TTA CT-5′; and scrambled, no. 1 5′-ATA TAC GGC TTC TGG-3′, and no. 2 5′-ACT ACT ACT TTC CTT-3′.

Intraventricular Delivery of Sense and Antisense ODNs. Male Sprague-Dawley rats (250–350 g) were anesthetized (45 mg/kg sodium pentobarbital i.p.) and placed in a Kopf stereotaxic instrument. A micro-osmotic pump (model 1007D; Alza, Inc.) was filled and implanted s.c. between the scapulae of the animal, according to the manufacturer's instructions. Polyethylene catheter tubing (PE-60) connected the osmotic pump to a cannula that was lowered to a depth of 5 mm into the lateral ventricle. Rats received a continuous 5-day infusion of one of the following three treatments: 1) artificial cerebrospinal fluid (aCSF; 60 μl total), which served as a vehicle control; 2) a calcineurin antisense ODN cocktail consisting of CNA1 (Aα), CNA2 (Aβ), and CNB antisense ODNs at either 5 or 10 nmol/day each; 3) a calcineurin sense ODN cocktail consisting of CNA1 (Aα) and CNA2 (Aβ) sense ODNs at 10 nmol/day each; and 4) a scrambled ODN cocktail consisting of scrambled no. 1 and no. 2 ODNs at 10 nmol/day each. After the infusion period, rats were sacrificed by decapitation.

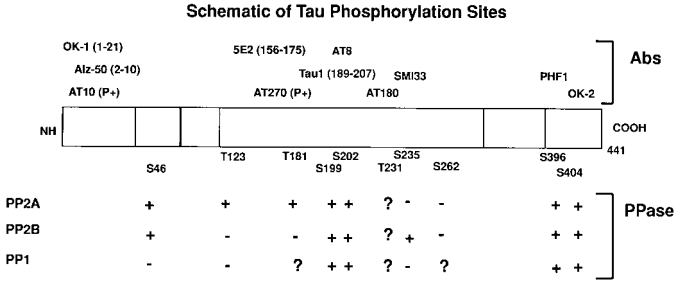

Fig. 1. Schematic showing several known phosphorylation sites on τ (e.g., Ser^{46}), the antibodies that recognize τ epitopes (Abs), and the putative Ser/Thr phosphatases (PPases) that act in vitro to dephosphorylate specific sites. P+ denotes antibodies that recognize specific phosphoepitopes.

The temporal neocortex was removed, and viable brain slices were prepared as previously described (Garver et al., 1994, 1995). The hippocampus, striatum, cerebellum, frontal cortex, and midbrain were also removed, with half of each region homogenized in 500 to 1000 μl of buffer A [50 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.0, containing 2 mM $Mg(C_2H_3O_2)_2$, 2 mM $Mn(C_2H_3O_2)_2$, 15 mM β-mercaptoethanol, 2 mM $CaCl_2$, and the protease inhibitors leupeptin (100 μM), soybean trypsin inhibitor (100 μg/ml), aprotinin (100 μM), and phenylmethylsulfonyl fluoride (100 μM)], centrifuged at 13,000*g* for 5 min, and kept at −20°C. The other half of each region was immersed in 4% paraformaldehyde and used for immunocytochemistry. The micro-osmotic pump was weighed after animal sacrifice to determine the efficiency of pump evacuation.

Preparation and Treatment of Rat Brain Slices. Rat temporal neocortex was removed using a brain mold (Activational Systems, Warren, MI.), with care taken to eliminate residual hippocampus. Immediately after excision, temporal neocortical sections (225 μm) were prepared with a Sorvall tissue slicer (DuPont, Inc., Wilmington, DE) and immediately immersed in ice-cold buffer B (10 mM HEPES, pH 7.4, containing 125 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 15 mM $MgSO_4$, and 10 mM glucose, oxygenated with 95%/5% O_2/CO_2); this buffer lacks Ca^{2+} and has relatively high levels of Mg^{2+} to minimize ischemic damage to the tissue (Harris et al., 1993). Sections were then divided randomly into groups of six to eight slices and incubated (30 min, 37°C) in oxygenated buffer B. This solution was removed with a Pasteur pipette and replaced with oxygenated buffer C (10 mM HEPES, pH 7.4, containing 125 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 5 mM $MgSO_4$, 1.5 mM $CaCl_2$, and 10 mM glucose). After 45 min in buffer C, slices were treated for 30 min with one or both of the following pharmacological agents: okadaic acid (100 nM or 10 μM; GIBCO BRL) or the FK-506 analog FK-520 (5 μM; generous gift from Dr. Nolan Sigal, Merck, Rahway, NJ). Both drugs were dissolved as concentrated stocks in dimethyl sulfoxide; this vehicle was included in control incubations (0.1%) and was without effect on any of the measured variables. Previous experiments have demonstrated that the phosphatase inhibitors at the concentrations used significantly inhibit PP2A (100 nM okadaic acid) or calcineurin (5 μM FK-520 or 10 μM okadaic acid).

Reactions were terminated by first removing buffer C and adding 200 μl of buffer A containing 2% SDS, 25 mM K_2HPO_4, 2 mM EGTA, 10 mM EDTA, and 50 mM NaF; tissues were disrupted by sonication while kept on ice, followed by heating at 90°C for 10 min. Protein was measured using the bicinchoninic acid reagent (Pierce Chemical Co.) according to the manufacturer's instructions; samples were stored at −20°C. After thawing, samples were heated at 90°C for 5 min and centrifuged for 5 min at 13,000*g* before electrophoresis. Samples used in calcineurin activity assays were homogenized in buffer A without the additions of phosphatase inhibitors and centrifuged as described but were not heated.

SDS-PAGE and Immunoblotting Procedures. Samples from rat brain (prepared as described above) were subjected to one-dimensional, 10% SDS-PAGE and transferred to nitrocellulose (Garver et al., 1994). Nonspecific binding sites on the membrane were blocked by incubating the blots with blocking buffer (Tris-buffered saline containing 5% nonfat dry milk). Blots were then incubated with one of the following τ-specific primary antibodies diluted in blocking

buffer: monoclonal antibody AT-270 (20 μg/ml) or AT-180 (20 μg/ml) or polyclonal τ antisera OK-1 or OK-2, both diluted 1:500 in blocking buffer (Garver et al., 1994, 1995). AT-270 and AT-180 were generous gifts of Dr. A. Van der Woorde of Innogenetics (Zwijndrecht, Belgium). Tau-1 monoclonal antibody was purchased from Boeringher-Mannheim and was used at concentrations of 10 μg/ml.

Other immunoblots were incubated with one of the following affinity-purified antipeptide calcineurin antibodies, all diluted at 1:1000 in blocking buffer: pan-A (Aα/Aβ) specific (Parsons et al., 1994), Aα (A1) specific, or Aβ (A2) specific. For monoclonal antibodies AT-270 and AT-180, immune complexes were detected using alkaline phosphatase-conjugated rabbit anti-mouse secondary antibody (1:1000 in blocking buffer; Jackson Immunoresearch, West Chester, PA). Affinity-purified rabbit polyclonal antibodies (OK-1, OK-2, or the calcineurin antibodies) were detected using alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (1:1000 in blocking buffer; Jackson Immunoresearch). Both preparations were visualized with 5-bromo-4-chloro-3-indoyl-phosphate/nitroblue tetrazolium chromogen system as previously described (Garver et al., 1995).

Blots were quantitatively analyzed by laser densitometry using a Molecular Dynamics densitometer coupled to digital processing software from PDI, Inc. Results were evaluated using two-way ANOVA. Significant F ratios were assessed using Scheffé's test. Densitometric data from Tau-1 immunoblots were analyzed using Student's unpaired t test.

Preparation of Phosphorylated R_{II} Peptide. The R_{II} peptide (bovine cardiac cAMP-dependent protein kinase regulatory subunit, amino acids 81–99 NH-DLDVPIPGRFDRRVSVCAE-COOH) was synthesized using a Milligen 9500 Peptide Synthesizer with Fmoc chemistry at the Macromolecular Core Facility (Pennsylvania State University College of Medicine) and used as a substrate for calcineurin assays. Purified R_{II} was phosphorylated using the catalytic subunit of cAMP-dependent protein kinase, and free radioactivity was removed as previously described (Blumenthal et al., 1986).

Calcineurin Activity Assay. Dephosphorylation of phosphorylated R_{II} peptide was used to determine changes in calcineurin activity as previously described (Blumenthal et al., 1985). Brain slices were homogenized in 200 μl of buffer A, and 50 μg of protein was used for each reaction. Calmodulin (10 μg) was added, and each reaction was incubated for 5 min at 30°C. Reactions were terminated by adding 10% trichloroacetic acid and 500 mg/ml BSA, followed by incubation on ice for 30 min. After centrifugation for 10 min at 13,000g, ^{32}P released in

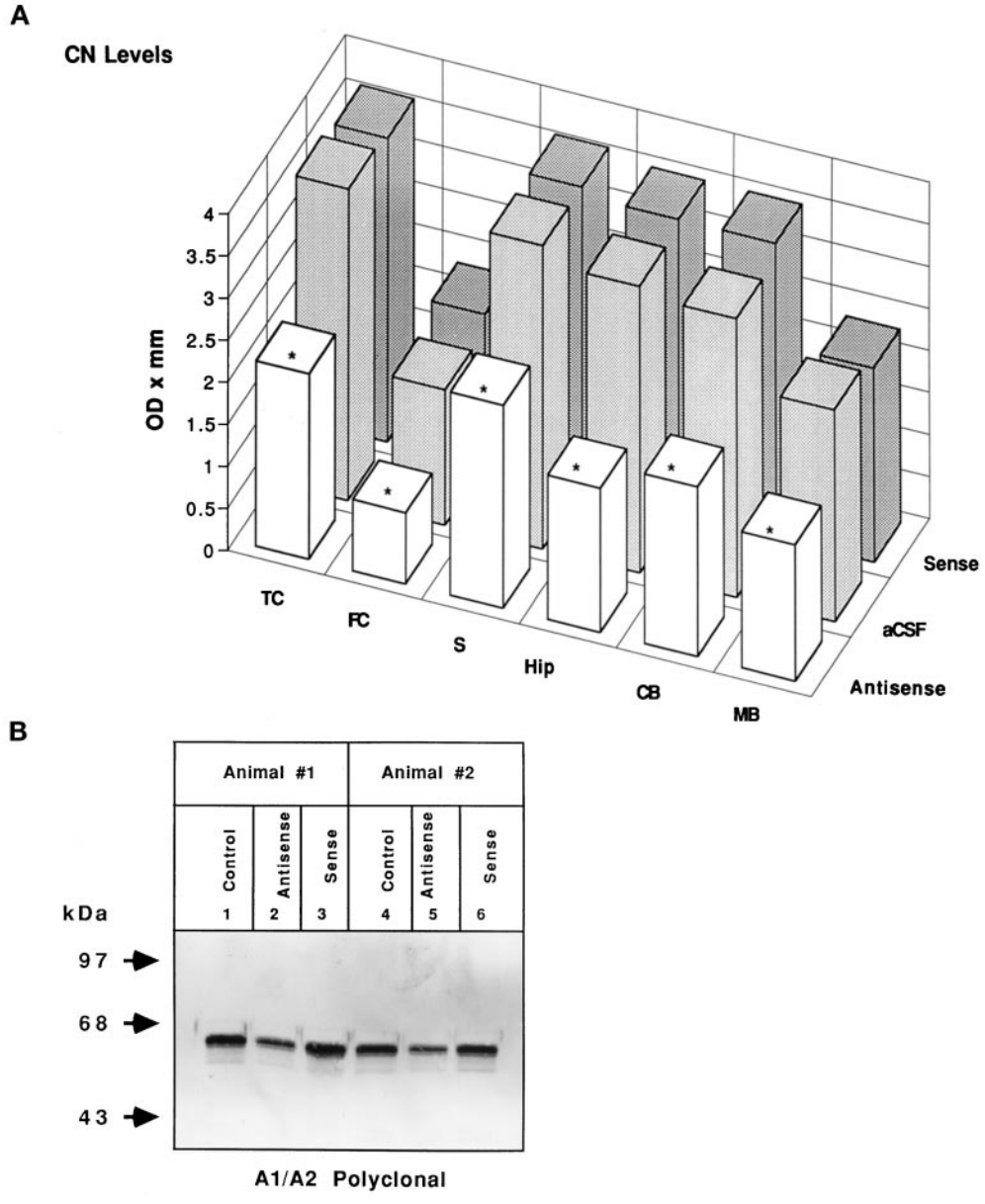

Fig. 2. Calcineurin immunoreactive protein levels (A1/A2) in antisense ODN-infused animals (10 nmol/day) versus aCSF- and sense ODN-infused animals. A, brain homogenates (50 μg) were electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose, and incubated with the A1/A2 polyclonal antisera. Immunoblots were subjected to laser densitometry, and the results are plotted as OD $\times$ mm $\pm$ S.E.M for each treatment group. The A1/A2 antisera indicated that calcineurin catalytic isozymes decreased 30 to 50% in antisense-treated versus aCSF- and sense-treated animals in all brain regions investigated. $^*P < .01$; two-way ANOVA with Scheffé's test. FC, frontal cortex; TC, temporal cortex; S, striatum; Hip, hippocampus; CB, cerebellum; MB, midbrain. B, temporal neocortex brain homogenates (50 μg) from six representative rats were electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose, and incubated with the calcineurin A1/A2 polyclonal antisera. This representative immunoblot illustrates the decrease in protein levels in antisense ODN-infused versus aCSF- (control) and sense ODN-infused animals.

the supernatant was quantitated via liquid scintillation spectrometry. Three duplicate assays were performed for each brain region from each animal. Results were analyzed using two-way ANOVA and Scheffé's test. Intra-assay variation was less that 10%.

Immunocytochemistry. After immersion fixation in 4% paraformaldehyde, neocortex from control and sense ODN- and antisense ODN-treated rats was processed into 25-μm free-floating sections using a vibratome for detection of calcineurin immunoreactivity as previously described (Polli et al., 1991). Primary calcineurin antibodies were incubated with the sections (1:1000) overnight at 4°C, and detection was performed using secondary antisera (1:2500) directly coupled to horseradish peroxidase (Jackson Immunoresearch). Immune complexes were visualized using diaminobenzidine as a chromogen. Photomicrographs were taken on an Olympus BH-2 microscope, using Kodak Ectachrome film.

Results

Effects of Antisense Calcineurin ODN Infusion on Calcineurin Protein Levels. To address the potential in vivo role of calcineurin as a bona fide regulator of τ phosphorylation, a protocol was developed for intraventricular infusion of calcineurin antisense ODNs into rat brain. Pilot experiments were conducted comparing doses of ODNs and bolus versus continuous administration. From these studies,

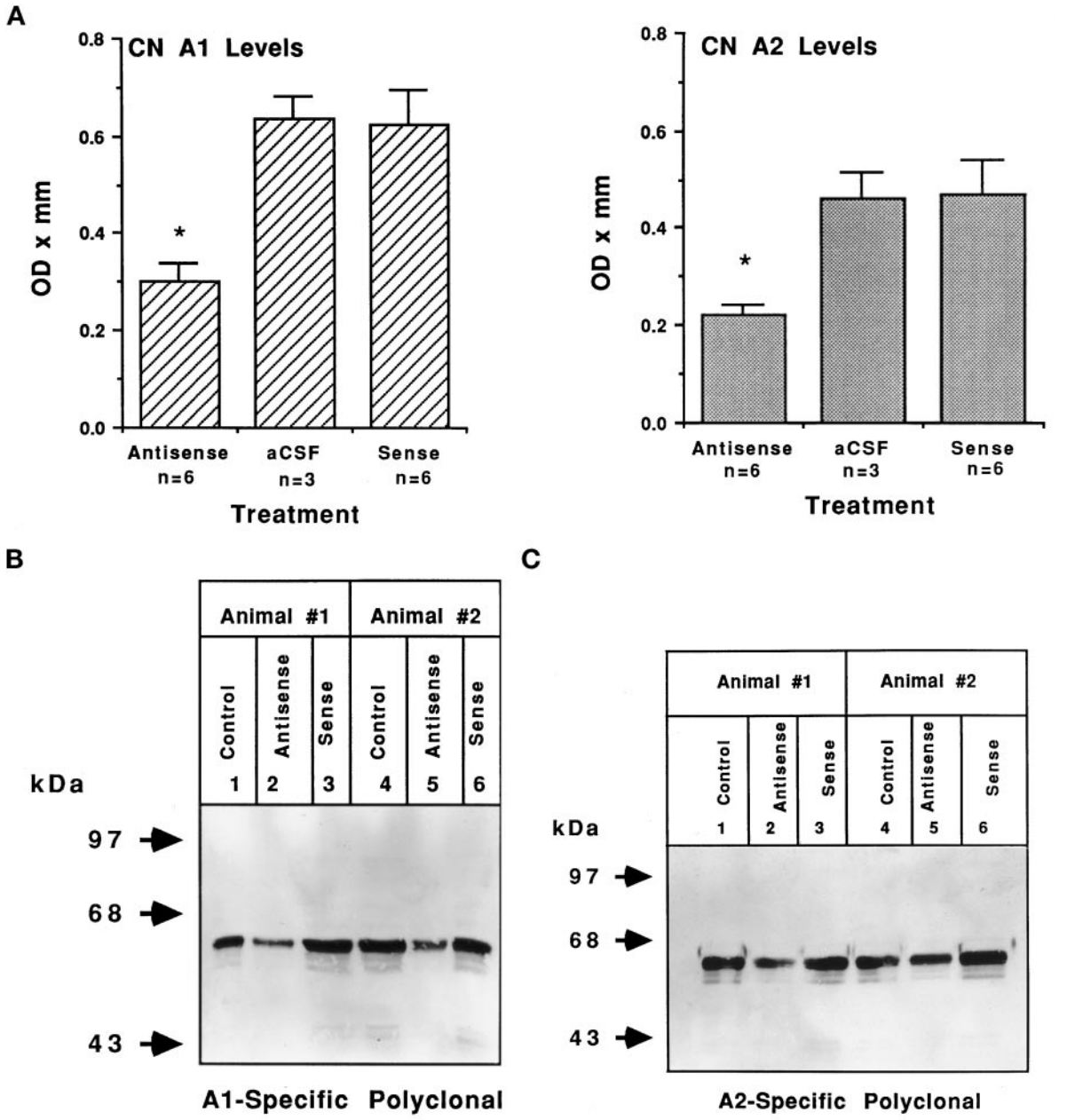

Fig. 3. Levels of the calcineurin catalytic subunit isozymes A1 and A2 levels in antisense ODN-infused (10 nmol/day) versus aCSF- and sense ODN-infused animals. A, brain homogenates (50 μg) were electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose, and incubated with either the A1- or A2-specific calcineurin polyclonal antisera. Immunoblots were subjected to laser densitometry, and the results are plotted as OD × mm ± S.E.M for each treatment group. Both the A1 (left) and A2 (right) calcineurin catalytic isoforms decreased 50 to 60% in antisense ODN-treated versus aCSF- and sense ODN-treated animals in all brain regions investigated. *$P < .05$, two-way ANOVA with Scheffé's test. B, representative immunoblot using A1-specific antisera. C, representative immunoblot using A2-specific antisera.

continuous intraventricular delivery of 5 or 10 nmol ODN/day was chosen for further study.

We first determined whether infusion of calcineurin antisense ODNs significantly diminished calcineurin protein levels and catalytic activity. Figure 2A shows the densitometric quantification of a series of immunoblots incubated with affinity-purified antipeptide antibodies that recognize a common peptide determinant (AVPFPPS/THRLT) near the amino terminus of the Aα and Aβ of calcineurin (Parsons et al., 1994). Calcineurin levels were markedly decreased ($P <$ .01) in all brain regions tested in rats infused with 10 nmol/day antisense calcineurin ODNs versus rats infused with either aCSF or sense calcineurin ODNs; the reduction in immunoreactive calcineurin protein ranged from 40 to 65%. Figure 2B is a representative immunoblot of neocortical samples from sense ODN-, antisense ODN-, and vehicle-treated rats after immunodetection with pan-calcineurin (i.e., Aα/Aβ) antibody. Decreased calcineurin levels were seen only in the antisense ODN treatment group.

The reduction in protein levels was not due to a selective effect of ODNs on a specific catalytic subunit isoform because isoform-specific antipeptide polyclonal antibodies showed similar patterns of decreased calcineurin protein levels in antisense animals. Figure 3A represents the densitometric scanning of calcineurin immunoblots (neocortex only) incubated with antibodies that distinguish either the A1 (Aα) or the A2 (Aβ) catalytic subunit. Levels of both calcineurin subunits were significantly decreased in rats infused with a 10 nmol/day antisense calcineurin ODN cocktail versus rats infused with either aCSF or sense ODNs. Figure 3, B and C, are representative blots showing decreased levels of both calcineurin catalytic subunit isoforms in antisense ODN-infused rats.

The decreased calcineurin levels produced by antisense ODNs was also observed using immunocytochemistry. Figure 4 shows a series of micrographs of calcineurin A1 (Aα) and A2 (Aβ) immunoreactivity in neocortex from vehicle-, sense ODN-, and antisense ODN-treated rats. There was a general decline in neuronal immunoreactivity of calcineurin after antisense ODN treatment rather than a loss of protein from selected neuronal populations. This suggested that antisense ODNs produced uniform effects throughout neurons of the neocortex.

Effects of Antisense Calcineurin ODN Infusion on Calcineurin Activity. The phosphatase activity of calcineurin was examined in all animals to see whether comparable changes occurred among the different treatment groups. Infusion of antisense ODNs against calcineurin caused a significant decrease in calmodulin-dependent phosphatase activity in all brain regions tested (Fig. 5). The loss of activity was in excellent agreement with the estimated reduction of immunoreactivity. Interestingly, some regions (e.g., hippocampus, neocortex) appeared to be slightly more sensitive to the antisense ODN infusion than other regions (e.g., midbrain). This may reflect differential bioavailability of the ODNs after intraventricular infusion.

Effects of Antisense Calcineurin ODN Infusion on τ Phosphorylation in Rat Temporal Neocortex Slices. The consequences of reduced calcineurin activity on τ dephosphorylation in temporal neocortex slices were investigated using shifts in τ mobility; previous experiments showed that inhibition of both PP2A and calcineurin leads to slowed electrophoretic mobility (Garver et al., 1994; Matsuo et al., 1994). Figure 6A shows a τ immunoblot pattern from the neocortex of a vehicle-treated rat after inhibition of phosphatases in brain slices. As previously reported (Harris et al., 1993; Garver et al., 1994, 1995), upward shifts in τ mobility and the appearance of a 68-kDa immunoreactive peptide were elicited only when both PP2A and PP2B were simultaneously inhibited during the slice experiment, using either 10 μM okadaic acid (lane 2) or a combination of 100 nM okadaic acid and 5 μM FK-520 (lane 5).

However, in animals infused with calcineurin antisense ODNs, an upward shift in τ mobility plus the appearance of the 68-kDa peptide were seen in slices treated with 100 nM okadaic acid (e.g., PP2A inhibition) alone (Fig. 6, C and D).

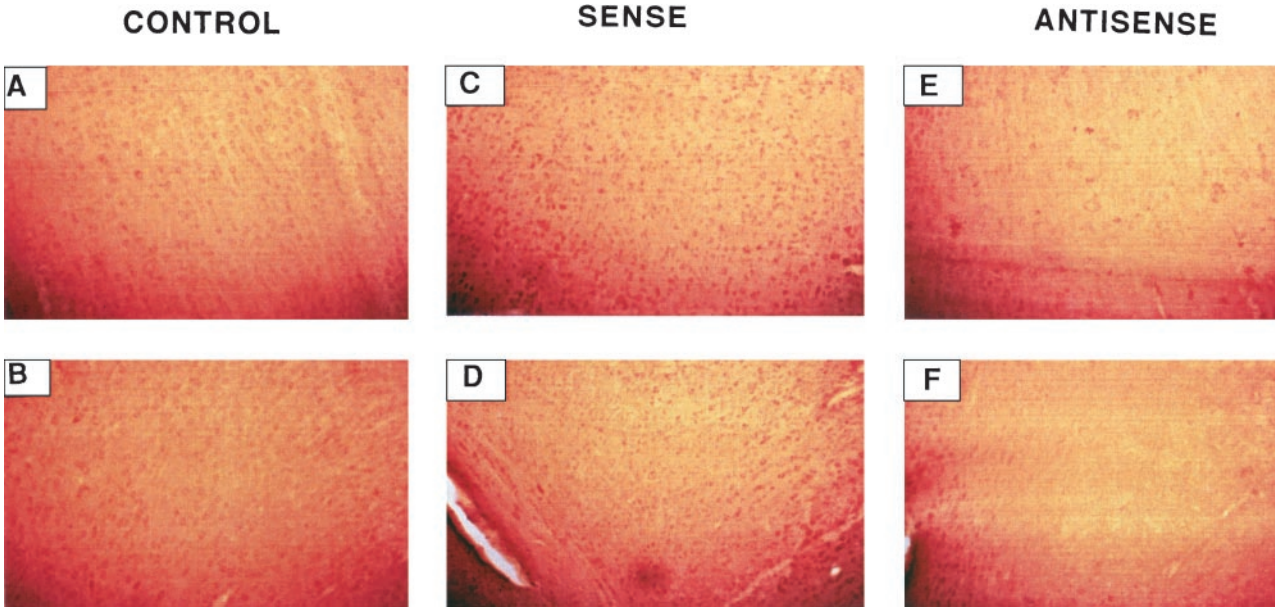

Fig. 4. Calcineurin immunoreactivity in neocortex. Rats were infused with aCSF vehicle (A and B), sense ODNs (C and D), or antisense ODNs (E and F), and the neocortex was removed and fixed in 4% paraformaldehyde. Each panel shows two independent regions of neocortex incubated with the A2-specific calcineurin antisera. Note the general loss of immunoreactivity throughout the neocortex in antisense ODN-treated rats. Magnification, 100×.

This effect was seen after antisense ODN infusions of either 5 or 10 nmol/day (Fig. 6A and data not shown). Both OK-1 and OK-2 anntisera detected similar shifts in τ mobility (Fig. 6, C and D). Infusion of sense calcineurin ODNs did not alter calcineurin levels or activity, and consequently, τ mobility shifts were seen only when both PP2A and calcineurin were simultaneously inhibited (Fig. 6B). These results suggest that calcineurin was sufficiently inhibited by antisense ODN treatment to cause mobility shifts in τ after the treatment of slices with 100 nM okadaic acid alone.

Effects of Antisense Calcineurin ODN Infusion on Specific τ Phosphorylation Sites. Most of the known sites of τ phosphorylation, including Thr^{231} and Thr^{181} (recognized by monoclonal antibodies AT-180 and AT-270, respectively), are variably phosphorylated in rapid biopsies from normal-appearing human, primate, and rodent brain (Matsuo et al., 1994). In non-Alzheimer brain, these sites of phosphorylation are rapidly (60–90 min) dephosphorylated by phosphatases in metabolically active brain slices and postmortem samples. Hence, an experiment was designed to determine whether inhibition of calcineurin in vivo altered the ex vivo persistence of phosphorylated τ. Site-specific dephosphorylation of τ was investigated in temporal neocortical slices from ODN- and vehicle-treated rats using AT-180 and AT-270 phosphorylation-sensitive monoclonal antibodies. Figure 7 shows replica τ immunoblots (top, AT-270; bottom, AT-180) from temporal neocortex after a 105-min postmortem incubation period. In animals (control) infused only with aCSF, immunoreactivity to AT-270 (Thr^{181}) was very weak and that toward AT-180 (Thr^{231}) was virtually absent. However, in all six calcineurin antisense ODN-treated rats, immunoreactivity to both AT-270 and AT-180 was clearly maintained after 105 min, presumably due to reduced calcineurin activity in the slice preparations. Thus, we suggest that calcineurin controls dephosphorylation of Thr^{181} and Thr^{231}.

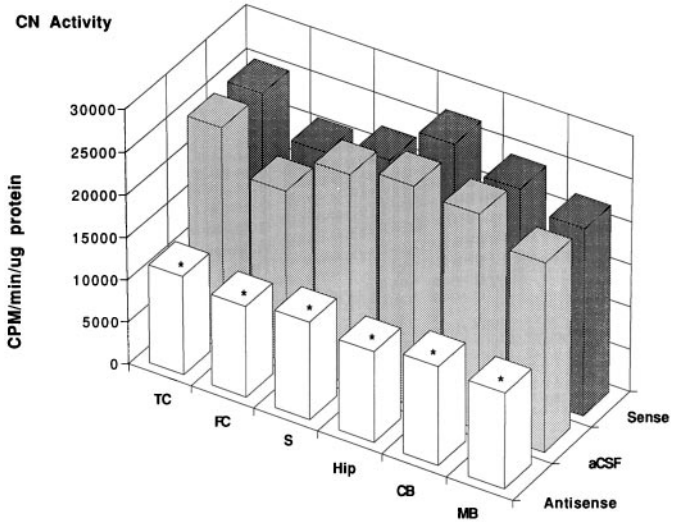

Fig. 5. Calcineurin activity in antisense ODN-treated animals (10 nmol/day) versus aCSF- and sense ODN-treated animals. Rat brain regions were isolated and homogenized as described in text, and 50 μg of total protein was added to each assay. Activity was measured as cpm ^{32}P released (mean ± S.E.M.) from the synthetic R_{II} peptide for each treatment group. Assays were performed in triplicate for each animal. Calcineurin activity decreased approximately 50% in calcineurin cocktail antisense ODN-treated animals versus aCSF- and sense-treated animals. $^*P < .01$, two-way ANOVA with Sheffé's test. Abbreviations are as described in the legend to Fig. 2.

Immediate biopsies under conditions that minimize phosphatase activity of sense- and antisense-treated rat hippocampus were analyzed for Tau-1 immunoreactivity. This antibody recognizes a dephosphorylated epitope between residues 189 and 207. As shown in Fig. 8, Tau-1 immunoreactivity was present in varying degrees in both sense- and antisense-treated rat hippocampus. Densitometric analysis of Tau-1 immunoreactivity revealed no significant differences between sense and antisense treatments and underscores the value of using in vitro dephosphorylation paradigms for analysis of τ dephosphorylation.

As a final control, scrambled ODNs were infused in three rats, and levels of calcineurin, AT-270, and AT-180 were determined in hippocampus and cortex. As shown in Fig. 9, there were no significant changes in the levels of either calcineurin or immunoreactive τ proteins as determined using Western blotting.

Discussion

Considerable in vitro evidence has accumulated that suggests that calcineurin dephosphorylates τ (Goto et al., 1985; Goedert et al.; 1992a; Harris et al., 1993; Garver et al.; 1995). To better assess whether calcineurin uses τ as a substrate, calcineurin antisense ODNs were infused into the lateral ventricles over a period of 5 days and changes in τ phosphorylation were monitored ex vivo. By several different criteria, antisense ODNs significantly and specifically reduced levels of functional calcineurin. Furthermore, patterns of τ phosphorylation were altered after ex vivo challenges with phosphatase inhibitors, and most interestingly, two specific sites, Thr^{181} and Thr^{231}, remained phosphorylated long after these sites were dephosphorylated in control and sense ODN-treated rats. A parsimonious explanation is that calcineurin directly dephosphorylates Thr^{181} and Thr^{232}, although an indirect effect of calcineurin on these sites cannot be excluded. Tau-1 immunoreactivity in hippocampus was not significantly different in sense- and antisense-treated rats. Two possible interpretations are that calcineurin is not the primary phosphatase for Tau-1 sites and/or that the 50% loss of calcineurin activity was not sufficient to alter dephosphorylation at this site. Indeed, PP2A has been suggested to dephosphorylate the Tau-1 site (Goedert et al., 1992b; Baumann et al., 1993).

Antisense ODN infusions may be suitable for the study of calcineurin actions in the adult brain relative to targeted gene disruptions for several important reasons (Albert and Morris, 1994). First, there are two major catalytic isoforms of calcineurin in brain, and both may have τ directed activity (Giri et al., 1992; Billingsley, 1995). Our infusion paradigm used a mixture of calcineurin antisense ODNs to avoid the possible redundancy of multiple catalytic subunit genes and also targeted the regulatory B subunit. A double-deletion would be needed for accurate interpretation of a targeted gene disruption model of calcineurin. Second, calcineurin plays an important role in the immune system, suggesting that targeted gene disruptions would be disruptive to this system. Direct brain infusion of antisense ODNs minimizes the systemic effects that would be seen in a targeted disruption model or after high-dose treatments with immunosuppressive agents such as cyclosporin A and FK-506. Third, calcineurin is likely to play an important role in axonal development (Ferreira et al., 1993). Targeted disruptions

may permanently affect the normal central nervous system developmental pattern, making interpretation of the subtle effects of calcineurin on τ function difficult. Antisense infusions can be administered to adult rats, thus mitigating the effects of ODN treatment on neuronal development.

However, antisense ODNs must be designed to avoid nonspecific effects and to minimize degradation by nucleases (Milligan et al., 1993). We used phosphorothioated ODNs to minimize nuclease actions, and each was designed to bind the complementary in vivo sense mRNA across the translational start codon to inhibit nascent de novo enzyme production and to enhance degradation of double-stranded RNA hybrids. Also, we used two control conditions, namely, sense ODN infusion and vehicle controls, to monitor the effects of antisense ODNs (Wagner, 1994). One situation that frequently occurs after in vivo use of antisense ODNs is incomplete inhibition of the target enzymes. In this experiment, we achieved significant inhibition of calcineurin activity and levels but did not obtain complete inhibition. This may be a reflection of turnover of the enzyme, delivered cellular dose of the ODN, and various compensatory mechanisms. Nevertheless, we were able to observe striking changes in τ dephosphorylation after antisense ODN treatment. This suggests that the high levels of calcineurin in brain are functionally important and that the conditions that lower activity by 40 to 60% may be deleterious. Moreover, the effectiveness of a 50% loss of calcineurin activity on τ phosphorylation suggests that this enzyme may act as a calcium-triggering mechanism. Such a mechanism could explain why there is no linear increase in τ phosphorylation after a 50% loss of calcineurin.

Other studies have used antisense ODNs against calcineurin to demonstrate changes in calcineurin-mediated processes. Using a similar phosphorothioate cocktail approach directed against both catalytic subunits of calcineurin, Ikegami et al. (1996) demonstrated that intraventricular infusion of antisense ODNs led to a 40 to 60% decrease in calcineurin protein levels and a concomitant decrease in the threshold for hippocampal long-term potentiation. Thus, the extent of reduction of calcineurin in the current study was in agreement with that seen by others using ODNs. Similarly, there were changes in τ phos-

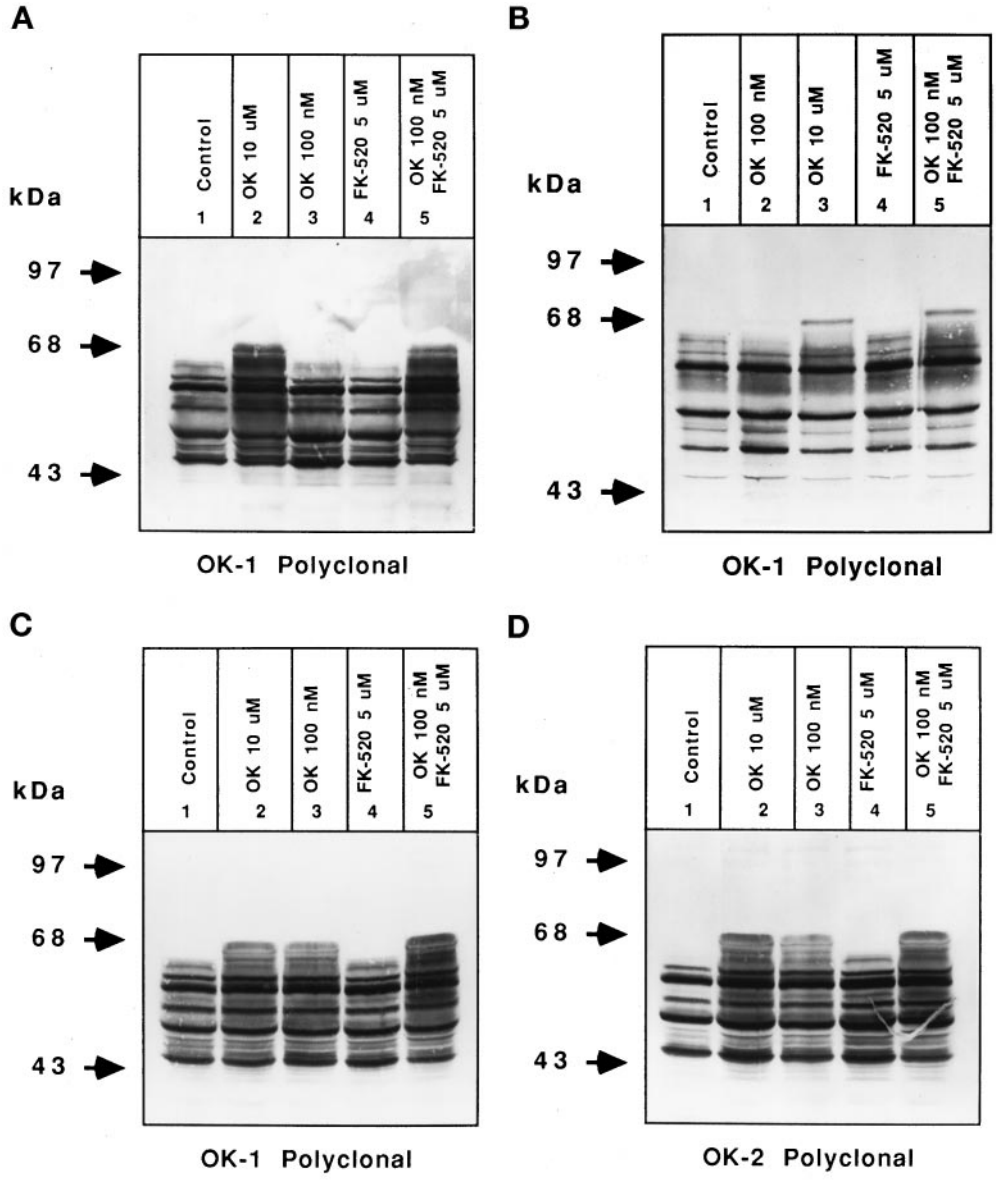

Fig. 6. Effects of aCSF, sense, and antisense ODN infusion on τ phosphorylation in rat temporal neocortex slices. A, slices of Spague-Dawley rat temporal neocortex were prepared and treated after a 5-day aCSF infusion, as described in the text. Samples (70 μg protein) were electrophoresed using 10% SDS-PAGE, and resolved proteins were transferred to nitrocellulose and incubated with the amino-terminal τ OK-1 polyclonal antisera. Immune complexes were visualized using an alkaline phosphatase-conjugated secondary antibody, using the 5-bromo-4-chloro-3-indoyl-phosphate/nitroblue tetrazolium chromogen system. Lane 1, DMSO vehicle control; lane 2, 10 μM okadaic acid (PP2A and PP2B inhibition); lane 3, 100 nM okadaic acid (PP2A inhibition); lane 4, 5 μM FK-520 (PP2B inhibition); and lane 5, combination of 100 nM okadaic acid and 5 μM FK-520 (PP2A and P2B inhibition). B, effects of sense calcineurin ODN infusion (10 nmol/day). Lane 1, DMSO vehicle control; lane 2, 100 nM okadaic acid (PP2A inhibition); lane 3, 10 μM okadaic acid (PP2A and PP2B inhibition); lane 4, 5 μM FK-520; and lane 5, combination of 100 nM okadaic acid and 5 μM FK-520 (PP2A and P2B inhibition). C, effects of antisense calcineurin ODN infusion (10 nmol/day); OK-1 antisera. D, effects of antisense calcineurin ODN infusion (10 nmol/day); OK-2 antisera.

phorylation in brains of mice lacking calcineurin Aα, in that they showed a 33 to 36% increase in the staining intensity of AT180 and AT270 sites and a 220% increase in PHF-1 intensity (Kayyali et al., 1997). When phosphorylation-independent antibodies such as monoclonal 5E2 and polyclonal τ antisera were used, marked electrophoretic mobility changes were seen in τ from calcineurin Aα (−/−) mice, again suggestive of increased phosphorylation. Thus, taken together with the current and past ODN knockdown studies, it is likely that τ phosphorylation is specifically affected by a reduction in calcineurin activity.

Because τ is highly phosphorylated in vivo and to variable degrees, it is difficult to use direct biopsies to determine quantitative changes in τ phosphorylation resulting from ODN treatment. This was illustrated in the Tau-1 immunoblots performed on freshly biopsied hippocampus. Thus, we used several ex vivo dephosphorylation paradigms to determine the effects of ODN treatment on τ phosphorylation. Normal-appearing rat, monkey, and human brain undergoes rapid dephosphorylation of τ at most sites during a 1- to 2-h postmortem period (Garver et al., 1994; Matsuo et al., 1994). However, this apparently is not the case in the Alzheimer brain, in which highly phosphorylated τ persists throughout the postmortem period. This could reflect either altered phosphatase activity or impaired τ substrate availability. The pattern of AT-270 and AT-180 immunoreactivity after calcineurin antisense ODN treatment persisted during this postmortem period and was seen only in animals with significant declines in this enzyme. Thus, antisense ODN treatment resembles some features seen in the Alzheimer brain with respect to the persistence of phosphorylated epitopes on τ and has potential use as a model for testing the phosphatase theory of PHFtau formation.

The sites on τ dephosphorylated by calcineurin are likely to be phosphorylated by proline-directed kinases. The sequences around Thr^{181}, APKT(P), and Thr^{231}, VVRT(P), somewhat resemble other sites seen in calcineurin substrates (Spencer et al., 1992). Recognition sites in R_{II} peptide [RRVS(P)], crystallin [RLPS(P)], heat shock protein-25 [RSPSP], inhibitor-1 [RRPT(P)], phosphorylase kinase [RRLS(P)], and DARPP-32 [RRPT(P)] share a common proline and fit the consensus RXXS/T(P). Sites Thr^{181} and Thr^{231} of τ more closely resemble two putative calcineurin sites in GAP-43, which are at Ser^{96}, PATS(P), and Thr^{172}, AATT(P). In both τ and GAP-43, the T(P)

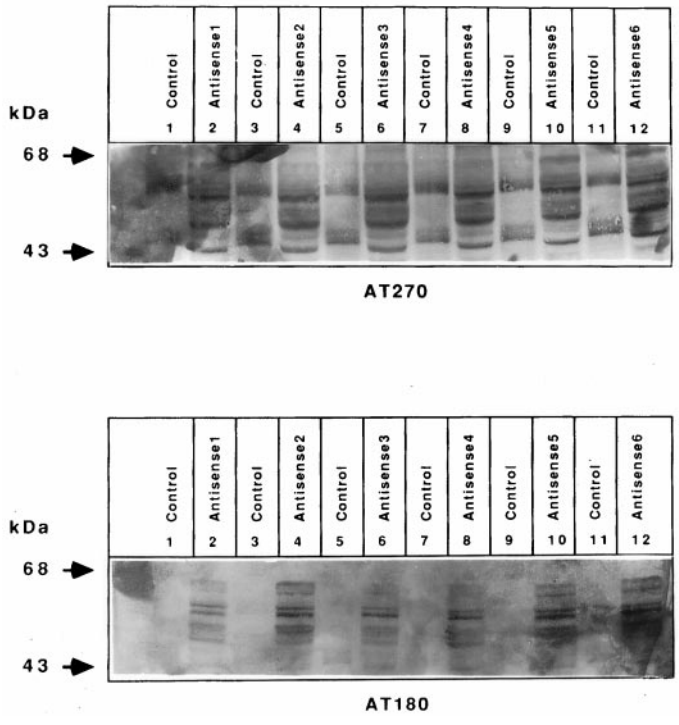

Fig. 7. Effects of calcineurin antisense ODN infusion on site-specific τ phosphorylation in rat temporal neocortex. Slices of rat temporal neocortex were prepared and treated after the infusion of either aCSF or calcineurin antisense ODNs (10 nmol/day), as described in the text. After slices were incubated for 105 min, samples (70 μg of protein) were electrophoresed as described, and immunoblots were incubated with either AT-270 (top) or AT-180 (bottom) phosphorylation-dependent, site-specific monoclonal antibodies. Lanes 1, 3, 5, 7, 9, and 11 in both immunoblots show a loss of τ immunoreactivity at both Thr^{181} (detected by AT-270) and Thr^{231} (detected by AT-180) in slices from rats infused with aCSF after a 105-min incubation period. Lanes 2, 4, 6, 8, 10, and 12 in both immunoblots show a maintenance of τ immunoreactivity at both Thr^{181} and Thr^{231} during the 105-min incubation in rats infused with calcineurin antisense ODN. Each lane represents a sample from an individual rat.

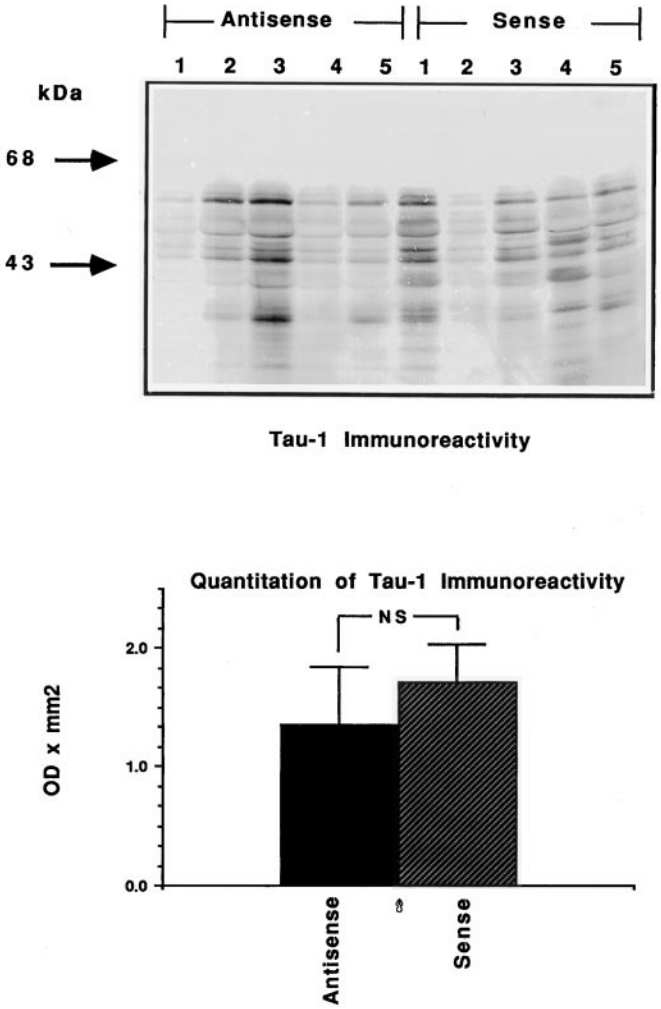

Fig. 8. Tau-1 Immunoreactivity in freshly biopsied rat hippocampus. Rats were infused with 10 nmol/day either sense or antisense ODNs as described in text. Samples of hippocampus were immediately homogenized under conditions designed to minimize alterations in phosphorylation, heated to 90°C, and electrophoresed on 10% SDS-PAGE. Immunoblots were incubated with Tau-1 monoclonal antibody. Each lane represents a sample from an individual rat. Total Tau-1 immunoreactivity was analyzed densitometrically; no statistically significant differences were found between the treatment groups (Student's unpaired t test; $P <$ .56).

site is preceded by one or two hybrophobic residues. Thus, calcineurin may recognize several sequence motifs surrounding S/T(P) sites.

Prior brain slice experiments indicated that both PP2A and calcineurin must be inhibited in slice experiments to generate slower mobility τ after SDS-PAGE (Garver et al., 1995). When both phosphatases are inhibited, the ability to dephosphorylate τ is lost while, simultaneously, a putative τ-di-

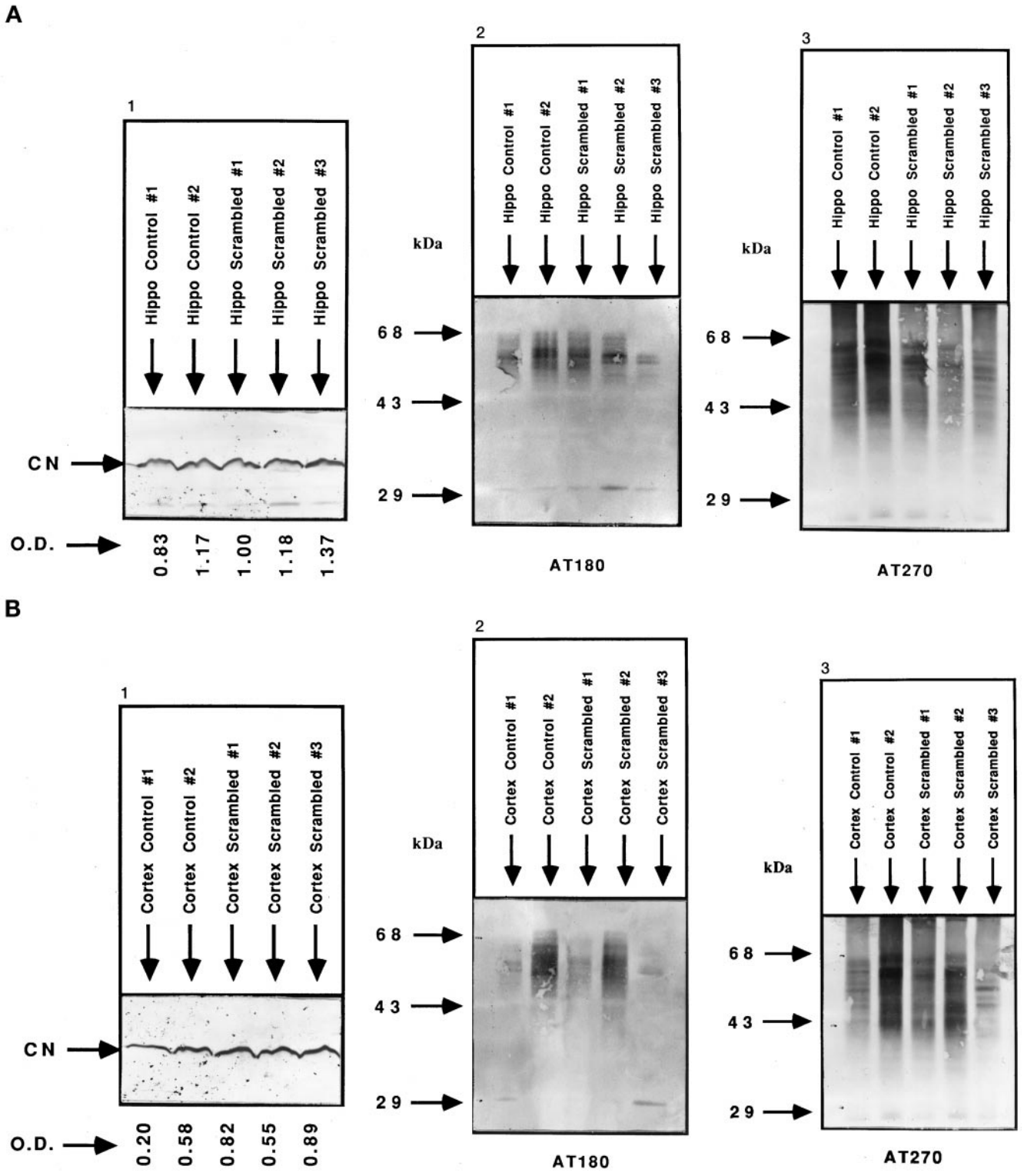

Fig. 9. Scrambled ODNs do not alter calcineurin levels or τ immunoreactivity. Rats were infused with 10 nmol/day scrambled ODN cocktail or with vehicle (control). After 7 days, samples of hippocampus and cortex were immediately processed to avoid dephosphorylation and subjected to SDS-PAGE and immunoblotting with antisera against calcineurin catalytic subunit AT-270 or AT-180. As can be seen in each panel, there were no apparent changes in calcineurin levels or AT-270/180 antibody staining as a result of ODN infusion. A, hippocampus. B, cortex. CN, calcineurin; OD, optical density determined via laser densitometry.

rected kinase (mitogen-activated protein kinase) is activated due to PP2A inhibition (Payne et al., 1991). In animals infused with the calcineurin antisense ODN cocktails, inhibition of PP2A alone (via incubation of slices with 100 nM okadaic acid) was sufficient to generate slower-electrophoretic-mobility, higher-molecular-weight isoforms of τ, including the 68-kDa peptide.

Although experiments using fresh brain biopsy specimens and metabolically active brain slices provide insight into the molecular pathways that regulate τ phosphorylation, they fall short of demonstrating which specific sites are regulated by specific phosphatases. Infusion of antisense ODNs provides a method to investigate τ-directed phosphatases and kinases and to test whether decrements in phosphatases can lead to accumulation of phosphorylated τ. It will be interesting to see whether long-term antisense ODN infusions produce patterns of neuropathology and behavioral changes that are similar to those seen in Alzheimer's disease. Such antisense ODN infusions may also serve as models to test the effects of specific therapeutic agents that alter τ phosphorylation. Importantly, direct intraventricular delivery of antisense compounds allows simultaneous targeting of multiple regulatory and catalytic subunits and avoids systemic barriers to absorption. The results presented in this study indicate that calcineurin dephosphorylates τ at two novel sites and that a partial (50%) inhibition of calcineurin leads to persistent τ phosphorylation ex vivo. Such persistence could lead to accretion of highly phosphorylated τ, thus initiating a cascade ultimately leading to tangle formation. Phosphatases may be an important focus of research in neurofibrillary tangle development in terms of understanding both the pathological mechanism of action and potential sites for therapeutic intervention.

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