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Localization of alpha type II calcium calmodulin-dependent protein kinase at glutamatergic but not γ-aminobutyric acid (GABAergic) synapses in thalamus and cerebral cortex

(excitatory synapses/inhibitory synapses/longterm synaptic plasticity)

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ABSTRACT **The alpha subunit of type II calcium/calmodulin-dependent protein kinase (CAM II kinase-α) plays an important role in longterm synaptic plasticity. We applied preembedding immunocytochemistry (for CAM II kinase-α) and postembedding immunogold labeling [for glutamate or γ-aminobutyric acid (GABA)] to explore the subcellular relationships between transmitter-defined axon terminals and the kinase at excitatory and inhibitory synapses in thalamus and cerebral cortex. Many (but not all) axon terminals ending in asymmetric synapses contained presynaptic CAM II kinase-α immunoreactivity; GABAergic terminals ending in symmetric synapses did not. Postsynaptically, CAM II kinase-α immunoreactivity was associated with postsynaptic densities of many (but not all) glutamatergic axon terminals ending on excitatory neurons. CAM II kinase-α immunoreactivity was absent at postsynaptic densities of all GABAergic synapses. The findings show that CAM II kinase-α is selectively expressed in subpopulations of excitatory neurons and, to our knowledge, demonstrate for the first time that it is only associated with glutamatergic terminals pre- and postsynaptically. CAM II kinase-α is unlikely to play a role in plasticity at GABAergic synapses.**

Type II calcium/calmodulin-dependent protein kinase (CAM II kinase), plays important roles in cellular mechanisms of learning and memory (1–9). By phosphorylating synapsin I, the alpha subunit of CAM II kinase (CAM II kinase-α) facilitates vesicle movement and transmitter release (10–16). The degree of phosphorylation of CAM II kinase may be a detector of the frequency of Ca^{2+} spikes (17–19).

CAM II kinase exerts its effect by modulating synaptic strength; at excitatory, glutamatergic synapses it phosphorylates non-*N*-methyl-D-aspartate (NMDA)-type glutamate receptors, which augment NMDA receptors (5, 20, 21). Forebrain synaptosomal fractions, enriched for asymmetrical, excitatory synapses, suggest that the kinase forms the major postsynaptic density protein (22, 23), and electron microscopic immunocytochemistry shows localization at asymmetrical synapses (24), but whether it is found at every excitatory synapse and its relationship to symmetrical, inhibitory γ-aminobutyric acid (GABA)ergic synapses are unknown.

In the central nervous system, genes for CAM II kinase-α and 67-kDa glutamic acid decarboxylase, the enzyme involved in GABA synthesis, are expressed in different neuronal populations (25–27): the first in glutamatergic, excitatory neurons and the second, in GABAergic, inhibitory neurons. The role of CAM II kinase-α at GABAergic synapses is thus uncertain. *In vitro* studies show it can phosphorylate intracellular loops of $GABA_A$ receptor subunit polypeptides (28–30). If this occurs *in vivo*, it could mediate plasticity at GABAergic synapses (31). High-resolution localization is therefore needed.

MATERIALS AND METHODS

Wistar rats (200–250 g) anesthetized with Nembutal (Abbott) were perfused with 4% paraformaldehyde/0.1–0.2% glutaraldehyde in 0.1 M phosphate buffer. Cerebral cortex and thalamus were cut on a vibratome at 50–80 μm and collected in phosphate buffer.

Sections were incubated in 3% normal horse serum, washed, and reincubated in a mouse monoclonal antibody to CAM II kinase-α (1, 27). Sections were washed and incubated in horse anti-mouse immunoglobulins for 2–4 hr and then reincubated in avidin–biotin–peroxidase complex for 1 hr, followed by 3,3′-diaminobenzidine·4(HCl) and H_2O_2. Control sections were stained by omitting the primary antibody.

Stained sections containing somatosensory cortex and ventral posterior thalamus were osmicated, embedded in Araldite (EM Science, Ft. Washington, PA), and resectioned at 60–70 nm. Serial thin sections were processed separately as follows: (*i*) without further labeling, (*ii*) by additional postembedding immunogold labeling for GABA; and (*iii*) by additional postembedding immunogold labeling for glutamate.

For immunogold staining, grids were pretreated with 1% $NaIO_4$ and 1% HIO_4, incubated in 5% normal goat serum followed by 1:1000 rabbit anti-GABA antiserum or 1:2000–1:3000 rabbit anti-glutamate antiserum (Sigma). After washing, grids were reincubated in goat anti-rabbit IgG conjugated to 10- or 15-nm gold particles (Biocell Laboratories), washed, stained with uranyl acetate and lead citrate, and examined with electron microscopy.

Neuronal profiles were considered immunopositive when electron-dense immunoperoxidase reaction product could be detected in at least three serial sections or when in 3–5 serial thin sections, the number of gold particles was at least three times higher than background. Three hundred fifty-five serially sectioned synapses were examined, 128 in cortex and 227 in thalamus.

RESULTS

In somatosensory cortex, CAM II kinase-α immunoreactivity is observed in many pyramidal neurons in layers II–VI (25, 27) and in many small round neurons in layer IV. In ventral posterior thalamus, most neurons are CAM II kinase-α-positive (26).

By electron microscopy, immunoreactivity for CAM II kinase-α was found in somata and dendrites. In labeled

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Abbreviations: CAM II kinase, type II calcium/calmodulin-dependent protein kinase; CAM II kinase-α, alpha subunit of CAM II kinase; GABA, γ-aminobutyric acid; RS, cortical; RL, lemniscal.

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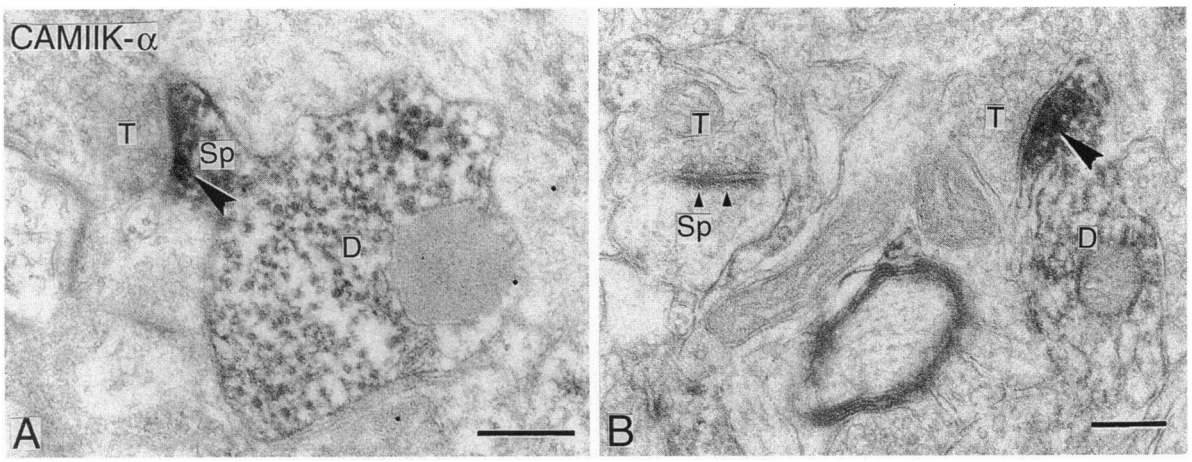

FIG. 1. Cerebral cortex. Preembedding immunostaining for CAM II kinase-α. *A* and *B* show dense immunoperoxidase reaction product in dendrites (D). Sp in *A* is a dendritic spine. The labeled profiles are contacted by nonimmunostained axon terminals (T) ending in asymmetrical synapses, the postsynaptic densities of which (large arrowheads) are strongly immunoreactive for CAM II kinase-α. In *B*, a nonimmunoreactive dendritic spine (Sp) and terminal (T) form an asymmetrical synapse (smaller arrowheads), which is also unlabeled. (Bars = 0.25 μm.)

dendrites, concentrations of reaction product were found in postsynaptic densities of asymmetrical synapses (Figs. 1–5). In distal dendrites and dendritic spines, this represented most of the reaction product (Fig. 1*A*, 2*A*, and 4*A*). In somata, immunoreactivity was also associated with rough endoplasmic reticulum, Golgi apparatus, and outer membranes of mitochondria (data not shown); in proximal dendrites, reaction product was also distributed inside the plasmalemma and along microtubules. In thalamus and cerebral cortex, most synapses on labeled somata had symmetrical membrane thickenings not associated with CAM II kinase-α reaction product. CAM II kinase-α-immunoreactive axon terminals invariably ended in asymmetrical synapses (Fig. 3*C*) on both CAM II kinase-α-positive and -negative dendrites. The postsynaptic densities of many of these synapses were strongly immunoreactive for CAM II kinase-α. Unlabeled terminals and unlabeled asymmetrical synapses were found in small numbers (Fig. 1*B*).

In thalamus, CAM II kinase-α immunoreactivity in dendrites was weakly concentrated in postsynaptic densities associated with lemniscal (RL) (Fig. 4*A*) and densely concentrated in those associated with cortical (RS) axon terminals (Figs. 4*B*–5*C*). CAM II kinase-α-positive axon terminals were all RS, although not all RS terminals (109 of 184) were labeled. RL terminals (42 of 42) were not CAM II kinase-α-positive.

In double-stained thin sections, from cerebral cortex (Figs. 2 and 3), CAM II kinase-α immunoreactivity was found only in axon terminals immunoreactive for glutamate and forming asymmetrical synapses. These terminals ended on both CAM II kinase-α-immunopositive and CAM II kinase-α-immunonegative somata and dendrites. Many CAM II kinase-α-negative postsynaptic profiles were GABA-immunopositive (Fig. 3*C*). CAM II kinase-α immunostaining was invariably absent from postsynaptic membrane densities of symmetrical synapses formed by GABA-labeled terminals, even those ending on CAM II kinase-α-immunolabeled somata and dendrites (Figs. 3 *A–C* and 4 *C* and *D*). No profiles contained both CAM II kinase-α and GABA immunoreactivity. In thalamus, all RL and RS terminals were glutamate-positive and ended in asymmetrical synapses, all on CAM II kinase-α-immunoreactive profiles (Figs. 4 and 5). There are no intrinsic GABAergic neurons in this part of rodent thalamus (25, 26).

DISCUSSION

This is the first morphological evidence for a specific relationship of CAM II kinase-α with a large subset of glutamatergic synapses. CAM II kinase-α immunoreactivity is only associated with the postsynaptic membrane thickenings of asymmetrical synaptic contacts in which the presynaptic terminals

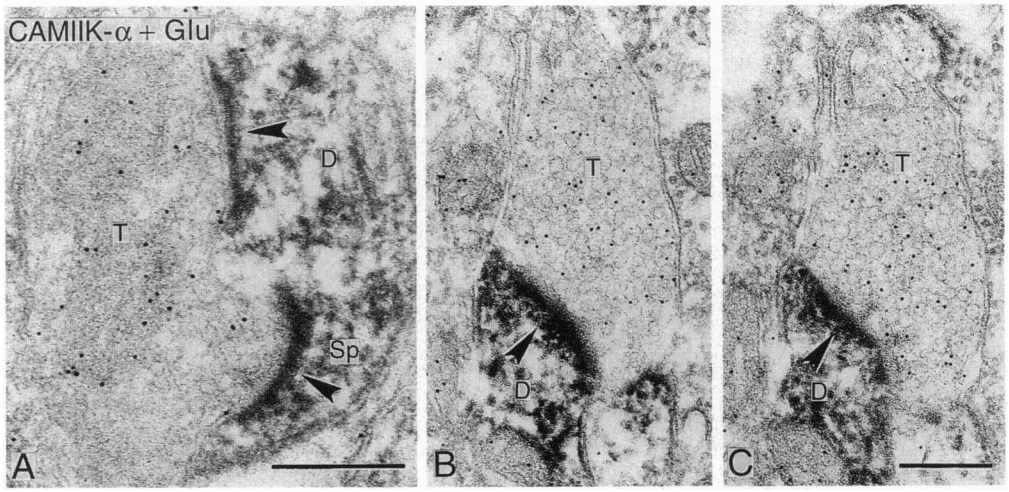

FIG. 2. Cerebral cortex. (*A–C*) Dendrites (D) and a spine (Sp) stained by preembedding immunostaining for CAM II kinase-α and excitatory axon terminals (T) stained by postembedding immunostaining for glutamate. *B* and *C* are part of a series of sections through the same profiles. Postsynaptic densities (arrowheads) of asymmetrical synapses are densely immunoreactive for CAM II kinase-α. (Bars = 0.25 μm.)

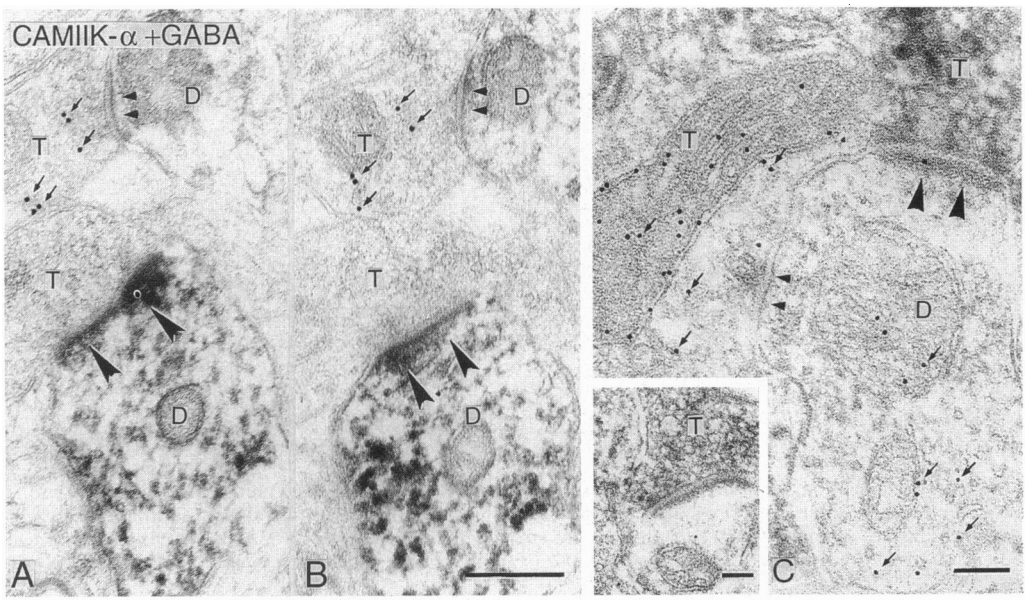

FIG. 3. Cerebral cortex. (*A–C*) Preembedding immunostaining for CAM II kinase-α and postembedding immunostaining for GABA. *A* and *B* are from a series through the same profiles. GABA-immunoreactive terminals (T) make symmetrical synapses (small arrowheads) on a small unlabeled dendrite (D) in *A* and *B* and on a GABA-immunoreactive dendrite (D) in *C*. The synapses are not immunoreactive for CAM II kinase-α. A, B, Postsynaptic densities (large arrowheads) of asymmetrical synapses formed by terminals not immunoreactive for CAM II kinase-α are densely immunoreactive for CAM II kinase-α. (*C*) Asymmetrical postsynaptic density (large arrowheads) formed by a CAM II kinase-α-immunoreactive terminal is not immunoreactive for CAM II kinase-α. Small arrows in *A–C* indicate immunogold particles. (*Inset*) CAM II kinase-α-positive terminal with a nonimmunostained postsynaptic density. [Bars = 0.25 μm (*A* and *B*), 0.1 μm (*C*).]

contain glutamate. Some but not all of these presynaptic terminals are themselves immunoreactive for CAM II kinase-α, and many, but not all, asymmetrical synapses are CAM II kinase-immunoreactive. CAM II kinase-α immunoreactivity

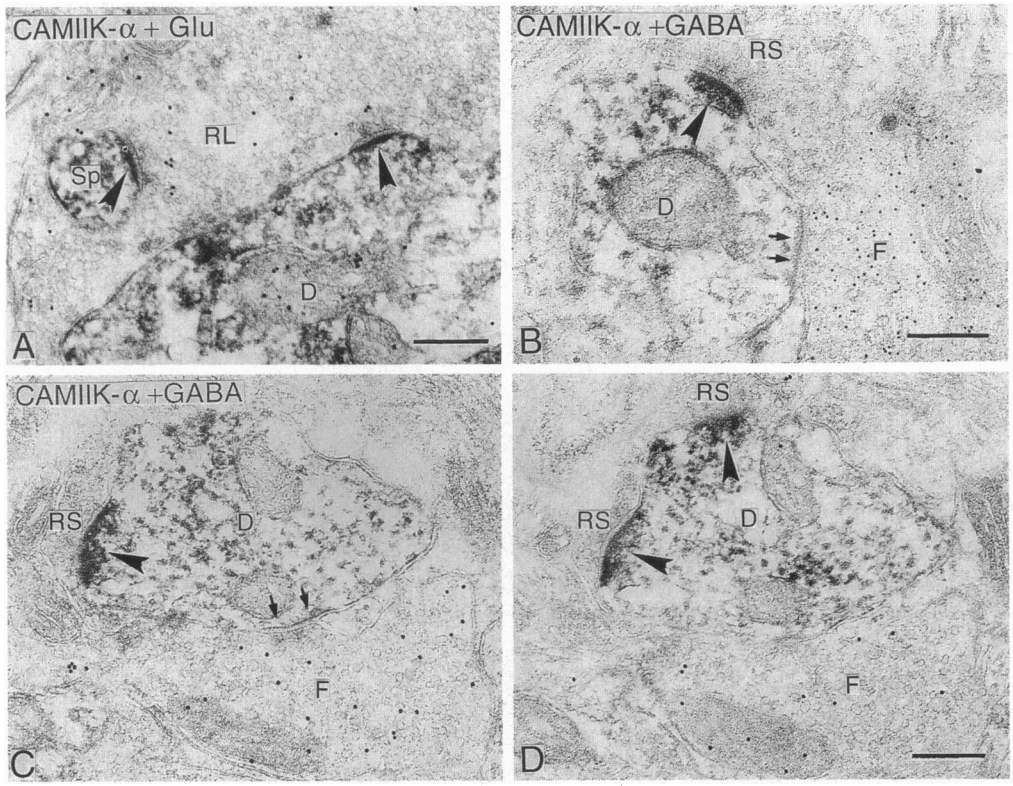

FIG. 4. Thalamus. Preembedding immunostaining for CAM II kinase-α and by postembedding immunostaining for glutamate (*A*) or GABA (*B–D*). (*A*) RL terminal, immunoreactive for glutamate but not for CAM II kinase-α, ending in asymmetrical synapses on a large, CAM II kinase-α-immunoreactive dendrite (D) and a spine-like dendritic appendage (Sp). Postsynaptic thickenings of these synapses (arrowheads) are immunoreactive for CAM II kinase-α. (*B* and *C*) GABA-immunoreactive terminals (F) ending in symmetrical synapses on CAM II kinase-α-immunoreactive dendrites (D). Postsynaptic thickenings (small arrows) of these symmetrical synapses are not associated with CAM II kinase-α immunoreactivity, by contrast with the heavy immunoreactivity of postsynaptic thickenings of asymmetrical synapses (arrowheads) made by RS terminals on the same dendrites. *C* and *D* are from a series through the same profiles. (Bars = 0.25 μm.)

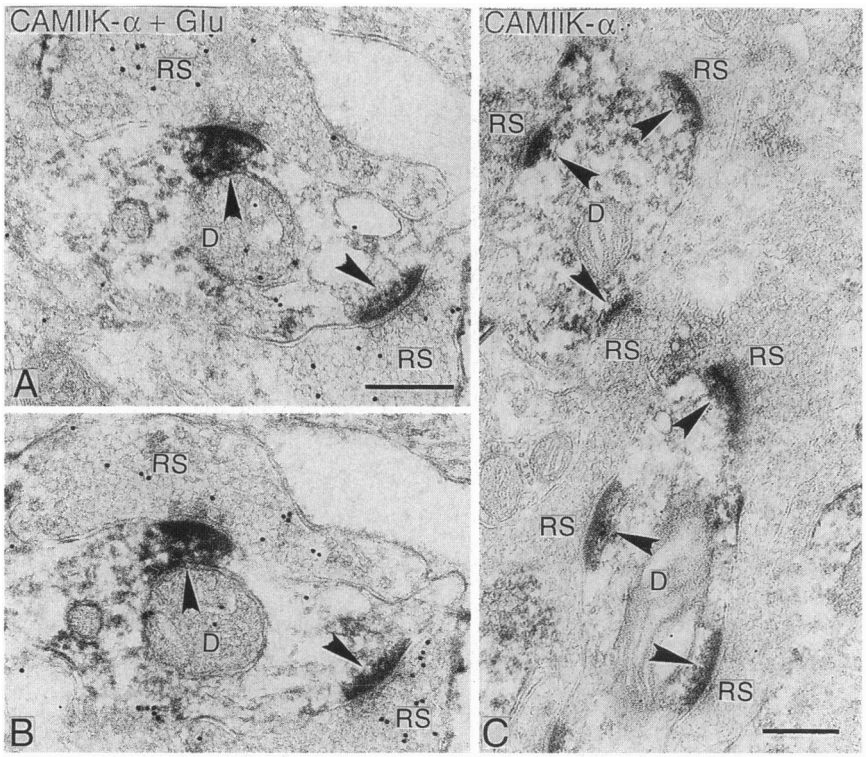

FIG. 5. Thalamus. Sections stained by preembedding immunostaining for CAM II kinase-α and by postembedding immunostaining for glutamate. Glutamate-immunoreactive RS terminals end on CAM II kinase-α-immunoreactive dendrites (D) in asymmetrical synapses, the postsynaptic membranes of which (arrowheads) are densely immunoreactive for CAM II kinase-α. *A* and *B* are from series of sections through the same profiles. (Bars = 0.25 μm.)

is not associated with the postsynaptic membrane thickenings of symmetrical synaptic contacts, in which the presynaptic terminals contain GABA, even when these end on CAM II kinase-α-positive cells. No presynaptic GABAergic terminals ending in symmetrical synapses are immunoreactive for CAM-II kinase-α. Although some cortical neurons are not CAM II kinase-α-immunoreactive, none of them are GABA-positive. It is not yet confirmed if these neurons express other less abundant forms (β, γ, and δ subunits) of CAM II kinase, but preliminary results suggest they do not (unpublished results).

CAM II kinase-α, when applied *in vitro* or injected into dissociated neurons, can phosphorylate intracellular domains of β1, γ2S, and γ2L $GABA_A$ receptor subunits (28–30), like other protein kinases (31–34). If the present observations in fixed tissue are representative of conditions *in vivo*, it appears that CAM II kinase-α is denied access to the postsynaptic sides of GABAergic synapses as well as to the presynaptic GABA terminals, and it is therefore unlikely to play a role in plasticity occurring at GABAergic synapses (35). Although it is possible that CAM II kinase-α accumulates at postsynaptic densities during fixation, this seems unlikely, given its total absence from the postsynaptic densities of symmetrical synapses. Whether soluble CAM II kinase-α is available to postsynaptic receptors at GABA synapses cannot be determined in fixed material and requires physiological investigation.

CAM II kinase-α-immunoreactive terminals in cerebral cortex may arise from thalamocortical, corticocortical, or commissural axons (26, 27, 36). The postsynaptic targets of these terminals were dendritic spines and shafts of CAM II kinase-α-immunopositive neurons and dendrites of GABAergic neurons, which is typical of the terminations of the three types of afferent fibers in rodents (37). CAM II kinase-α could form a mechanism for induction of longterm changes found at corticocortical synapses (38).

In the thalamus, CAM II kinase-α immunoreactivity was found in many of the glutamate-positive RS or corticothalamic (39) terminals. It was not found in glutamate-positive RL terminals, whose parent axons arise from non-CAM II kinase-α-expressing cells of the dorsal column and principal trigeminal nuclei (40). CAM II kinase-α immunoreactivity was present in postsynaptic densities associated with asymmetrical synapses made by RS and RL terminals but was much stronger at RS terminals. These corticothalamic terminals activate both metabotropic and ionotropic glutamate receptors, leading to a combination of both long and short excitatory postsynaptic potentials (41). Whether CAM II kinase-α is involved in phosphorylating both types of receptor has not been tested.

CAM II kinase enhances currents through α-amino-3-hydroxy-5-methyl-4-isoxazolepropidinic acid (AMPA) channels, and this enhancement is blocked if the CAM II kinase phosphorylation site is eliminated (20, 21, 42). CAM II kinase also enhances kainate currents associated with glutamate receptor subunit 6 and with glutamate receptor subunit 1, indicating that the CAM II kinase regulatory phosphorylation site is probably conserved in all AMPA/kainate receptors (43). Concentration of CAM II kinase-α immunoreactivity at postsynaptic densities of glutamatergic synapses is morphological support for the postsynaptic action of CAM II kinase-α on AMPA/kainate receptors at these synapses. Association with NMDA receptors is likely but not demonstrated. However, CAM II kinase-α is not necessarily associated with all glutamatergic terminals, because it is clearly absent from some excitatory cortical neurons postsynaptically and from certain presynaptic terminals—e.g. the terminals (RL) of the major ascending system to the ventral posterior nucleus. It will be interesting to determine how mismatches between pre- and postsynaptic CAM II kinase-α functionally express themselves and whether or not an absence of the alpha isoform is compensated for by the presence of other isoforms.

GABAergic terminals and the postsynaptic membranes of all symmetrical synaptic contacts lack CAM II kinase-α immunoreactivity. The presynaptic lack of CAM II kinase-α was anticipated from earlier gene expression studies (25–27). The present findings extend this to the postsynaptic side and make it unlikely that CAM II kinase-α is associated with GABAergic transmission or plasticity at GABAergic synapses in the cerebral cortex or thalamus.

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1. Erondu, N. E. & Kennedy, M. B. (1985) *J. Neurosci.* **5,** 3270–3277.
2. Hanson, P. I. & Schulman, H. (1992) *Annu. Rev. Biochem.* **61,** 559–601.
3. Miller, S. G. & Kennedy, M. B. (1986) *Cell* **44,** 861–870.
4. Soderling, T. R. (1995) *Advances Second Messenger Phosphoprotein Res.* **30,** 175–189.
5. Madison, D. V., Malenka, R. C. & Nicoll, R. A. (1991) *Annu. Rev. Neurosci.* **14,** 379–397.
6. Malinow, R., Schulman, H. & Tsien, R. W. (1989) *Science* **245,** 862–866.
7. Silva, A. J., Paylor, R., Wehner, J. M. & Tonegawa, S. (1992) *Science* **257,** 206–211.
8. Stevens, C. F., Tonegawa, S. & Wang, Y. (1994) *Curr. Biol.* **4,** 687–693.
9. Silva, A. J. Stevens, C. G., Tonegawa, S. & Wang, Y. (1992) *Science* **257,** 201–205.
10. Benfenati, F., Valtorta, F., Rubenstein, J. L., Gorelick, F. S., Greengard, P. & Czernick, A. J. (1992) *Nature (London)* **359,** 417–419.
11. Chapman, P. F., Frenguelli, B. G., Smith, A., Chen, C.-M. & Silva, A. J., (1995) *Neuron* **14,** 591–597.
12. Hackett, J. T., Cochran, S. L., Greenfield, L. J., Brosius, D. C. & Ueda, T. (1990) *J. Neurophysiol.* **63,** 701–706.
13. Lin, J.-W., Sugimori, M., Llinás, R. R., McGuinness, T. L. & Greengard, P. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 8257–8261.
14. Llinás, R., McGuinness, T. L., Leonard, C. S., Sugimori, M. & Greengard, P. (1984) *Proc. Natl. Acad. Sci. USA* **82,** 3035–3039.
15. McGuinness, T. L., Brady, S. T., Gruner, J. A., Sugimori, M., Llinás, R. & Greengard, P. (1989) *J. Neurosci.* **9,** 4138–4149.
16. Nichols, R. A., Sihran, T. S., Czernick, A. J., Nairn, A. C. & Greengard, P. (1990) *Nature (London)* **343,** 647–650.
17. Hanson, P. I. Meyer, M. T., Stryer, L. & Schulman, H. (1994) *Neuron* **12,** 943–956.
18. Lisman, J. (1994) *Trends Neurosci.* **17,** 406–412.
19. Meyer, T., Hanson, P. I., Stryer, L. & Schulman, H. (1992) *Science* **256,** 1190–1202.
20. McGlade-McCulloh, E., Yamomoto, H., Tan, S.-E., Brickley, D. A. & Soderling, T. R. (1993) *Nature (London)* **362,** 640–642.
21. Tan, S. E. M., Wenthold, R. J., & Soderling, T. R. (1994) *J. Neurosci.* **14,** 1123–1129.
22. Kelly, P. T. McGuinness, T. L. & Greengard, P. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 945–949.
23. Kennedy, M. B., Bennett, M. K. & Erondu, N. E. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 7357–7361.
24. Ouimet, C. C., McGuinness, T. & Greengard, P. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 5604–5608.
25. Benson, D. L. Isackson, P. J., Gall, C. M. & Jones, E. G. (1992) *Neuroscience* **46,** 825–850.
26. Benson, D. L., Isackson, P. J., Hendry, S. H. C. & Jones, E. G. (1991) *J. Neurosci.* **11,** 1540–1564.
27. Jones, E. G. Huntley, G. W. & Benson, D. L. (1994) *J. Neurosci.* **14,** 611–629.
28. Machu, T. K., Firestone, J. A. & Browning, M. D. (1993) *J. Neurochem.* **61,** 375–377.
29. McDonald, B. J., & Moss, S. J. (1994) *J. Biol. Chem.* **269,** 19111–18117.
30. Wang, R. A., Cheng, G., Kolaj, M. & Randic, M. (1995) *J. Neurophysiol.* **73,** 2099–2106.
31. Browning, M. D., Bureau, M., Dudek, E. M. & Olsen, R. W., (1990) *Proc. Natl. Acad. Sci USA* **87,** 1315–1318.
32. Kirkness, E. G., Bovenkerk, C. F., Ueda, T. & Turner, A. J. (1989) *Biochem. J.* **159,** 613–616.
33. Moss, S. J., Gorrie, G. H., Amato, A. & Smart, T. (1995) *Nature (London)* **377,** 344–348.
34. Sweetnam, P. M., Lloyd, J., Gallombardo, P., Malison, R. T., Gallager, D. W., Tallman, J. F. & Nestler, E. J. (1988) *J. Neurochem.* **51,** 1274–1284.
35. Komatsu, Y. (1994) *J. Neurosci.* **14,** 6488–6499.
36. Conti, F. A., Rustioni, A., Petrusz, P. & Towle, A. C. (1987) *J. Neurosci.* **7,** 1887–1901.
37. White, E. L. (1979) *Brain Res. Rev.* **1,** 275–312.
38. Iriki, A., Pavlides, C., Keller, A. & Asanuma, H. (1989) *Science* **245,** 1385–1387.
39. Jones, E. G. (1984) *Cerebral Cortex: Cellular Components of the Cerebral Cortex*, eds. Peters, A. & Jones, E. G. (Plenum, New York), Vol. 1, pp. 521–555.
40. Rausell, E., Cusick, C., Taub, E. & Jones, E. G. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 2571–2575.
41. McCormick, D. A. & Von Krosigk, M. (1992) *Proc. Natl. Acad. Sci.* **89,** 2774–2778.
42. Soderling, T. R., Tan, SEM, McGlade-McCulloh, E., Yamamoto, H. & Fukunaga, K. (1994) *J. Neurobiol.* **25,** 304–311.
43. Yakel, J. L., Vissajjhala, P., Derkach, V. A. Brickley, D. A. & Soderling, T. R. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 1376–1380.