

Alterations in the Balance of Protein Kinase/Phosphatase Activities Parallel Reduced Synaptic Strength During Aging

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Norris, Christopher M., Shelley Halpain, and Thomas C. Foster. Alterations in the balance of protein kinase/phosphatase activities parallel reduced synaptic strength during aging. *J. Neurophysiol.* 80: 1567–1570, 1998. The current research examined the regulation of synaptic strength by protein phosphorylation during aging. Bath application of the protein phosphatase 1 and 2A (PP1 and PP2A) inhibitor calyculin A (1 μ M) enhanced CA3–CA1 synaptic strength in hippocampal slices from aged male (20–24 mo) but not from young adult male (4–6 mo) Fischer 344 rats. Similarly, injection of the PP1 and PP2A inhibitor microcystin–L,R (5 μ M) into CA1 cells caused an increase in the intracellular synaptic response only in slices from aged rats. In contrast, bath application of the serine/threonine kinase inhibitor H-7 (10 μ M) induced a decrease in synaptic strength only in slices from the young adult group. These results demonstrate that phosphorylation-dependent regulation of intrinsic synaptic efficacy changes during aging.

INTRODUCTION

Several recent reports suggest that hippocampal synaptic strength is regulated by protein phosphorylation. Activation of protein kinases causes an increase in synaptic efficacy, similar to long-term potentiation (LTP) (Lledo et al. 1995; Wang and Kelly 1995). Protein phosphatase activation, in contrast, may limit increments in synaptic strength and/or promote synaptic depression (Mulkey et al. 1993; Wang and Kelly 1996). However, whether naturally occurring changes in synaptic efficacy result from a shift in the balance of protein kinase and phosphatase activities is not known.

The well-characterized decline in hippocampal synaptic strength observed in rodents during aging (Foster and Norris 1997) provides a unique model system in which to examine the role of protein kinases and phosphatases in mediating naturally occurring synaptic strength changes. The results presented suggest that differences in basal synaptic efficacy across aged and adult hippocampal slices are in part attributable to discordant basal protein kinase and phosphatase activities.

METHODS

Methods for tissue preparation and collecting intra- and extracellular recordings were in accord with our previous work (Norris et al. 1998). Briefly, hippocampal slices from 22 aged (20–24 mo) and 14 young adult (4–6 mo) male Fischer 344 rats were harvested

in ice-cold artificial cerebrospinal fluid (ACSF) containing a reduced CaCl₂ concentration (0.5 mM). Slices were maintained in a holding chamber in oxygenated ACSF (23°C) containing (in mM) 124 NaCl, 2 KCl, 1.25 KH₂PO₄, 2 MgSO₄, 2 CaCl₂, 26 NaHCO₃, and 10 dextrose, pH ~7.4 and transferred to an interface recording chamber at 30°C as needed. Once in the interface chamber, slices were perfused with oxygenated ACSF.

Schaffer collaterals were activated with a bipolar stimulating electrode and the associated CA3–CA1 excitatory postsynaptic potential (EPSP) was recorded in stratum radiatum with the use of a glass pipette (1–6 M Ω) filled with ACSF. Baseline stimulation (at 0.033 Hz) was delivered at an intensity sufficient to elicit a 1-mV EPSP. Before drug application, a stable baseline (≥ 20 min) was collected for each slice followed by a full input/output (I/O) curve constructed from seven stimulus intensities. The mean EPSP slope calculated at each fiber volley level was used to determine synaptic strength. Drugs (RBI, Natick, MA) were added to the perfusion buffer and diluted to final concentrations of 1 μ M (calyculin A) and 10 μ M (H-7). Changes in synaptic strength were calculated by comparing the mean EPSP slope collected during the 5 min immediately preceding drug application with the mean EPSP slope collected from 10 successive sweeps at 60 min after drug wash-in.

Intracellular CA1 recordings by sharp microelectrodes (70–100 M Ω) filled with 3 M potassium acetate were selected on the basis of criteria specified by Norris et al. 1998. Orthodromic stimulation (0.1 Hz) was interleaved with the delivery of negative current pulses (–0.2 to –0.4 nA, 100-ms duration) through the recording electrode to monitor input resistance. Electrode tips were backfilled with microcystin–L,R (5 μ M in 0.5% ethanol solvent) or solvent alone. On cell entry, hyperpolarizing current was applied until the membrane potential stabilized (i.e., it did not drift). Then stimulation intensity was adjusted to elicit an intracellular synaptic response (i.e., 5–10 mV), which was below spike threshold. Baseline stimulation began ≤ 3 min after impalement. EPSP slope was measured to calculate synaptic response magnitude. No age differences in resting membrane potential, Na⁺ spike amplitude, input resistance, or initial EPSP slope were observed. Changes in synaptic strength were determined by comparing the mean EPSP slope collected during the first minute of recording with the mean of 12 successive EPSPs collected 20 min after impalement.

Age differences in synaptic strength during I/O curves were determined with the use of factorial analysis of variance (AN-OVA). Effects of drug, age, and/or recording configuration (intra- vs. extracellular) were determined by repeated measures ANOVAs. Post hoc analyses were conducted with the use of Scheffe's *F* test, with significance set at *P* < 0.05. Where stated, *n* represents the number of slices used in each experiment.

RESULTS

Figure 1A illustrates the EPSP slope plotted against the fiber volley amplitude for aged hippocampal slices (*n* = 13)

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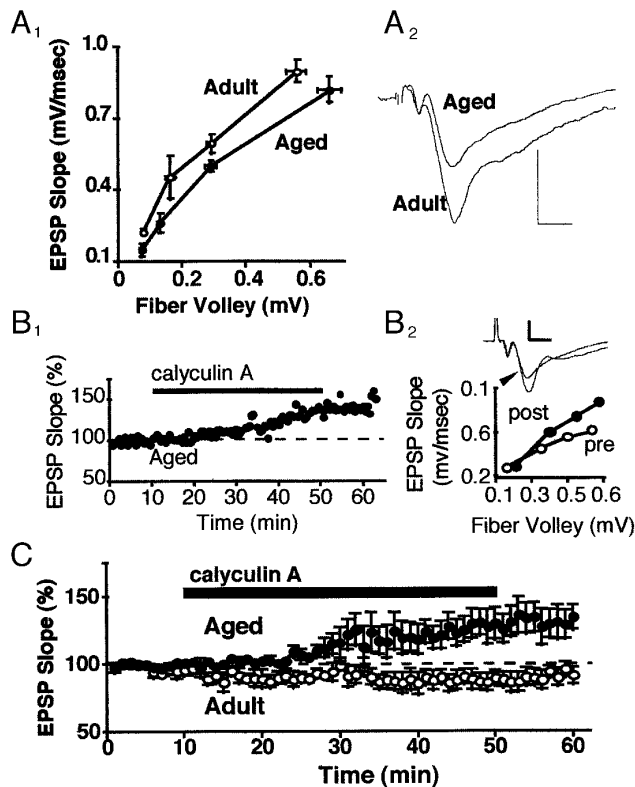


FIG. 1. Calyculin A increases synaptic strength in aged, but not adult slices. *A₁*: excitatory postsynaptic potential (EPSP) slope (mV/ms) plotted against fiber volley amplitude (mV) for aged ($n = 13$, filled circles) and young adult ($n = 15$, open circles) slices. *A₂*: overlay of means from 5 consecutive extracellular waveforms recorded from both a single aged and a single young adult slice that exhibited similar fiber volley amplitudes but markedly different EPSP slopes, illustrating the decrease in synaptic strength for aged slices. Calibration bars, 1 mV/5 ms. *B₁*: plot of normalized field CA3–CA1 EPSP slopes (percent of baseline) obtained from an aged rat slice perfused with calyculin A ($1 \mu\text{M}$) (bar). *B₂*, *top trace*: overlay of 10 consecutive responses collected from the slice in *B₁* immediately before and 60 min after (arrowhead) delivery of calyculin A. Calibration bars, 0.5 mV/5 ms. *B₂*, *bottom trace*: EPSP slope plotted against the fiber volley amplitude collected in the same slice before (open circle) and after (filled circle) calyculin A application. *C*: average data (mean \pm SE) from aged ($n = 6$, filled circle) and young adult ($n = 6$, open circle) slices exposed to calyculin A. For illustration purposes, the 2 EPSP slope values collected per minute from an individual slice were averaged, and these points were then averaged across slices.

and young adult slices ($n = 15$) during initial I/O curves. The EPSP-to-fiber volley curve was shifted to the right for the aged group ($P < 0.01$), confirming an age-related reduction in synaptic strength. Effects of the PP1 and PP2A inhibitor calyculin A ($1 \mu\text{M}$) on aged ($n = 6$) and young adult ($n = 6$) slices are illustrated in Fig. 1*B*. After drug application, only slices from the aged group exhibited an increase in the EPSP (aged, $130 \pm 11\%$; adult, $92 \pm 6\%$; $P < 0.05$). As delivery of calyculin A sometimes resulted in an increase in the fiber volley, small reductions in stimulus intensity were made to maintain fiber excitability at a constant level (Mulkey et al. 1993). An I/O curve collected before and after calyculin A wash-in showed that, for aged slices, the EPSP slope-to-fiber volley ratio was increased after drug application (Fig. 1*B₂*), indicating that calyculin A produces an increase in synaptic strength.

To further examine the specificity of protein phosphatase

TABLE 1. Intracellular means

	Age Group, mo	
	4–6	20–24
EPSP 10–90% rise time, ms	4.49 ± 0.42	5.05 ± 0.51
Resting membrane potential, mV	-65.2 ± 0.9	-67.9 ± 3
Input resistance, $M\Omega$	37 ± 4	41.1 ± 6
Holding current, nA	-0.86 ± 0.1	-0.76 ± 0.1
Na^+ spike amplitude, mV	76.3 ± 0.8	78.6 ± 1

n for each group equals 12 slices.

inhibition on synaptic strength, intracellular recordings were obtained from CA1 cells by using microelectrodes backfilled with the PP1 and PP2A inhibitor microcystin-L,R ($5 \mu\text{M}$). Microcystin is structurally distinct from calyculin A and is membrane impermeant, making it well suited for intracellular delivery. Field potentials were simultaneously monitored in CA1 s. radiatum to insure that any changes in the intracellular EPSP were not attributable to changes in stimulus properties, slice viability, or other factors of the experimental preparation. Intracellular parameters for slices from aged and young adult rats were not different (see Table 1). In cells from aged rat slices ($n = 12$), microcystin injection produced a large increase in the intracellular EPSP but not in the field response (intracellular, $167 \pm 13\%$; extracellular, $96 \pm 9\%$, $P < 0.001$; Fig. 2). Potentiation occurred rapidly

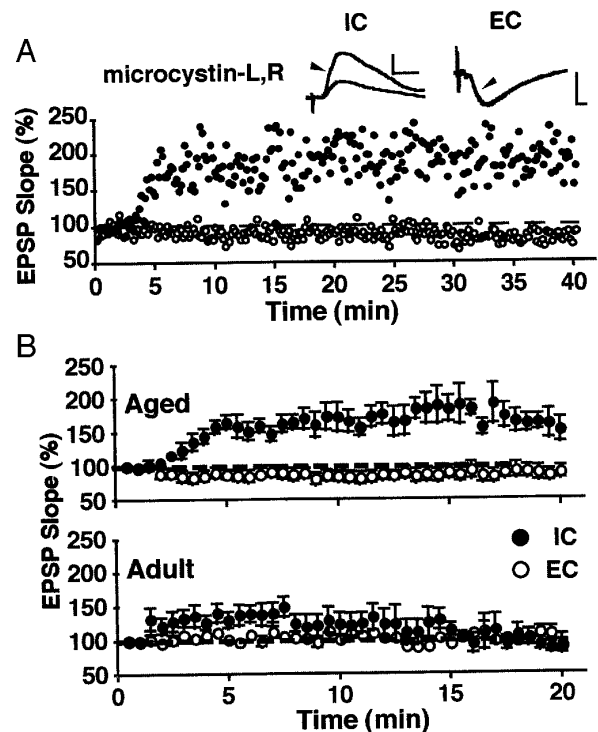


FIG. 2. Intracellular injection of microcystin enhances synaptic strength in an age-dependent fashion. *A*: plot of normalized intracellular (\bullet) and extracellular (\circ) EPSP slopes obtained from an aged rat slice. The intracellular recording pipette contained $5 \mu\text{M}$ microcystin-L,R. *Inset*: overlay of means from 10 consecutive responses collected intracellularly (IC) and extracellularly (EC) during the baseline and 40 min after (\blacktriangleright) impalement. Calibration bars, IC, 5 mV/10 ms; EC, 0.5 mV/5 ms. *B*: average data collected intracellularly (\bullet) and extracellularly (\circ) from 12 aged (*top trace*) and 12 adult (*bottom trace*) rat slices that received microcystin.

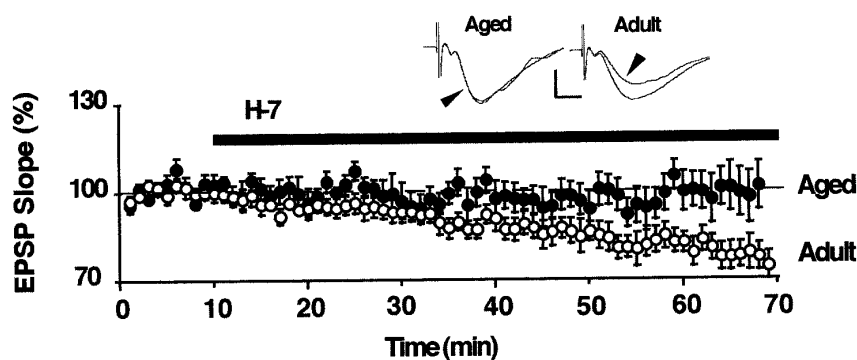


FIG. 3. H-7 depresses synaptic strength in slices from young adult, but not aged rats. Average data from aged ($n = 7$, ●) and adult ($n = 9$, ○) slices that received H-7. *Inset*: overlays of means from 10 consecutive responses collected from an individual slice from an aged and an adult rat immediately before and ~70 min after (▶) the delivery of H-7. Calibration bars, 0.5 mV/5 ms.

(within 5 min after impalement), persisted for ≥ 45 min (the longest time examined) and was not accompanied by changes in input resistance or Na^+ spike amplitude. Injection of aged cells with solvent (0.5% ethanol, $n = 6$) did not increase the intracellular EPSP ($96 \pm 8\%$). In cells from young adult rat slices ($n = 12$), the intracellular EPSP, measured 20 min after impalement, was not different from the initial baseline ($99 \pm 11\%$).

To determine whether regulation of synaptic strength by protein kinases changes as a function of the animals' age, aged slices ($n = 7$) and young adult slices ($n = 9$) were bathed in the broad-spectrum serine/threonine protein kinase inhibitor, H-7 ($10 \mu\text{M}$; Fig. 3). Similar to previous work (Grover and Teyler 1995; Hrabetva and Sacktor 1996), H-7 resulted in synaptic depression in slices from the young adult group ($79 \pm 5\%$, $P < 0.01$). Responses recorded from aged rat slices, however, exhibited no discernible effects of H-7 ($100 \pm 8\%$).

DISCUSSION

The results demonstrate that the regulation of synaptic transmission by protein phosphorylation changes with advanced age. Application of the protein phosphatase inhibitor calyculin A produced an increase in the synaptic response specific for slices from aged animals. The time course for this synaptic growth is similar to the time course for the reversal of long-term depression (LTD) by calyculin A (Mulkey et al. 1993). Likewise, intracellular injection of a structurally distinct phosphatase inhibitor microcystin resulted in a rapid and sustained increase in the EPSP reminiscent of the potentiation observed after the postsynaptic injection of kinase activators (Wang and Kelly 1995). Although microcystin produced an initial increase in the intracellular EPSP for young adult slices, this increase was short lasting. The reason for this transient effect for the younger group is unclear but may indicate the presence of a compensatory mechanism that is absent in the aged group. Finally, the serine/threonine protein kinase inhibitor H-7 only reduced synaptic strength for slices from young adults, suggesting that the balance of kinase/phosphatase activity is shifted in older animals.

Recent evidence indicates that manipulation of protein kinase and phosphatase activities results in changes in synaptic transmission that are mechanistically similar to LTP and LTD (Hrabetva and Sacktor 1996; Lledo et al. 1995; Mulkey et al. 1993; Wang and Kelly 1995), suggesting that these

enzymes may also be involved in regulating basal synaptic strength. However, in contrast to previous work examining stimulation-induced LTD, the current study is the first to demonstrate that a decrease in synaptic strength, which occurs naturally over the course of aging, is mediated through these same enzyme pathways. Age differences in the response to protein kinase and phosphatase inhibitors establish the plausibility of protein phosphorylation as a mechanism for regulating endogenous changes in synaptic strength.

Currently a dispute revolves around the relative contributions of pre- and postsynaptic mechanisms, including the possibility of synaptic loss, to reduced synaptic transmission for aged rodents (for reviews see Barnes 1994; Foster and Norris 1997). The results of the current study demonstrate that protein phosphorylation processes that are involved in synaptic plasticity mediate, at least in part, the decrease in synaptic strength that is characteristic of aged memory-impaired rodents. However, it is still unclear whether changes in phosphorylation state result in a reduction in the pre- or postsynaptic effectiveness of individual synapses or complete functional elimination (e.g., silencing) of synapses.

Age-related alterations in the regulation of synaptic function by protein kinase and phosphatase activities parallel changes in the induction and expression of synaptic plasticity during aging as well as the phosphorylation patterns in LTP and/or LTD. Specifically, protein kinases and protein phosphatases are known to be essential to the expression of LTP and LTD, respectively (Malenka et al. 1989; Wang and Feng 1992). During senescence, LTP induction and maintenance are impaired, while the induction of LTD is enhanced (Foster and Norris 1997). Thus a shift in the susceptibility to synaptic plasticity in favor of synaptic depression appears to be a mechanism for diminished synaptic strength in aged rodents. It is still unclear whether changes in susceptibility to synaptic plasticity alters basal enzyme activities or whether changes in the enzymes modify the threshold for induction of synaptic plasticity.

The protein kinase and phosphatase cascades involved in synaptic plasticity are dependent on a rise in intracellular Ca^{2+} . In CA1 neurons of aged mammals, Ca^{2+} influx through L-type voltage-dependent Ca^{2+} channels is elevated (Disterhoft et al. 1993; Landfield 1996). A slight elevation in Ca^{2+} influx may preferentially activate protein phosphatases relative to protein kinases (Foster and Norris 1997). Indeed, increased influx of Ca^{2+} through L-channels is largely responsible for age-related changes in synaptic modifiability favoring LTD (Norris et al. 1998). However, it

should be noted that L-channel function can be regulated in turn by phosphorylation (Hell et al. 1993). As such, age differences in basal enzyme activities may represent either a cause or consequence of disrupted Ca^{2+} homeostatic mechanisms with age. Regardless, the results suggest that, like LTP and LTD, changes in protein phosphorylation are tightly coupled to altered synaptic function during aging.

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REFERENCES

- BARNES, C. A. Normal aging: regionally specific changes in hippocampal synaptic transmission. *Trends Neurosci.* 17: 13–18, 1994.
- DISTERHOFT, J. F., MOYER, J. R. JR., THOMPSON, L. T., AND KOWALSKA, M. Functional aspects of calcium-channel modulation. *Clin. Neuropharmacol.* 16: S12–S24, 1993.
- FOSTER, T. C. AND NORRIS, C. M. Age-associated changes in Ca^{2+} -dependent processes: relation to hippocampal synaptic plasticity. *Hippocampus* 7: 602–612, 1997.
- GROVER, L. M. AND TEYLER, T. J. Different mechanisms are required for maintenance of NMDA receptor dependent and independent LTP. *Synapse* 19: 121–133, 1995.
- HELL, J. W., YOKOYAMA, C. T., WONG, S. T., WARNER, C., SNUTCH, T. P., AND CATTERALL, W. A. Differential phosphorylation of two size forms of the neuronal class C L-type calcium channel 1 subunit. *J. Biol. Chem.* 268: 19451–19457, 1993.
- HRABETVA, S. AND SACKTOR, T. C. Bidirectional regulation of protein kinase M zeta in the maintenance of long-term potentiation and long-term depression. *J. Neurosci.* 16: 5324–5333, 1996.
- LANDFIELD, P. W. Aging-related increase in hippocampal calcium channels. *Life Sci.* 59: 399–404, 1996.
- LLEDO, P. M., HJELMSTAD, G. O., MUKHERJI, S., SODERLING, T. R., MALENKA, R. C., AND NICOLL, R. A. Calcium/calmodulin-dependent kinase II and long-term potentiation enhance synaptic transmission by the same mechanism. *Proc. Natl. Acad. Sci. USA* 92: 11175–11179, 1995.
- MALENKA, R. C., KAUER, J. A., PERKEL, D. J., MAUK, M. D., KELLY, P. T., NICOLL, R. A., AND WAXHAM, M. N. An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. *Nature* 340: 554–557, 1989.
- MULKEY, R. M., HERRON, C. E., AND MALENKA, R. C. An essential role for protein phosphatases in hippocampal long-term depression. *Science* 261: 1051–1055, 1993.
- NORRIS, C. M., HALPAIN, S., AND FOSTER, T. C. Reversal of age-related alterations in synaptic plasticity by blockade of L-type Ca^{2+} channels. *J. Neurosci.* 18: 3171–3179, 1998.
- WANG, J.-H. AND FENG, D. P. Postsynaptic protein kinase C essential to induction of long-term potentiation in the hippocampal CA1 region. *Proc. Natl. Acad. Sci. USA* 89: 2576–2580, 1992.
- WANG, J.-H. AND KELLY, P. T. Postsynaptic injection of Ca^{2+} /CaM induces synaptic potentiation requiring CaMKII and PKC activity. *Neuron* 15: 443–452, 1995.
- WANG, J.-H. AND KELLY, P. T. The balance between postsynaptic Ca^{2+} -dependent protein kinase and phosphatase activities controlling synaptic strength. *Learn. Memory* 3: 170–181, 1996.