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Norepinephrine and isoproterenol increase the phosphorylation of synapsin I and synapsin II in dentate slices of young but not aged Fisher 344 rats

(long-term potentiation/calcium/calmodulin kinase II/cAMP-dependent protein kinase/aging/basal phosphorylation)

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ABSTRACT **A number of recent reports have suggested that norepinephrine (NE) produces a form of synaptic enhancement that resembles long-term potentiation (LTP). LTP, thought to be an electrophysiological correlate of memory, in part involves an augmentation of transmitter release. Although the effects of NE have not been unequivocally linked to LTP, it is clear that NE can produce increased transmitter release in the dentate gyrus of the hippocampus. The purpose of this study was to determine whether NE was capable of enhancing the phosphorylation of synapsin I and synapsin II, two homologous phosphoproteins thought to be involved in modulation of neurotransmitter release. NE (10 μM) and isoproterenol (250 nM) produced an increase in the phosphorylation of synapsin I and synapsin II in dentate slices from young rats. Phosphorylation site analysis of synapsin I, performed by limited proteolysis, indicated that NE and isoproterenol increased the phosphorylation of synapsin I at sites modified by Ca^{2+}/calmodulin-dependent protein kinase II as well as cAMP-dependent protein kinase. These data demonstrate that NE stimulates the phosphorylation of synapsin I at its Ca^{2+}/calmodulin-dependent protein kinase II site, which is a site that has been shown to regulate the effect of synapsin I on neurotransmitter release. We have also examined the effects of NE and isoproterenol on synapsin phosphorylation in dentate slices prepared from aged animals. Such animals have previously been shown to exhibit deficits in NE sensitivity as well as significant impairment in their ability to exhibit LTP. Neither NE nor isoproterenol stimulated synapsin phosphorylation in slices prepared from aged animals. Interestingly, the basal level of phosphorylation of the synapsin proteins was higher in slices prepared from aged animals. This higher basal level of phosphorylation may underlie the failure of aged animals to exhibit NE-stimulated increases in phosphorylation of the synapsin proteins. We hypothesize that the β-adrenergic agonist-stimulated phosphorylation of synapsin I and synapsin II in young rats plays a role in the increase in transmitter release produced by NE in the dentate. Thus, the failure of the aged rats to show such phosphorylation may underlie, in part, their failure to exhibit normal responsiveness to NE. Moreover, these deficits in synapsin phosphorylation may also play some role in the deficits in plasticity seen in aged rats.**

A great deal of clinical evidence has indicated that the hippocampal formation in humans is critically involved in the memory of recent events (1–3). In addition, the hippocampus has been shown to exhibit a dramatic form of synaptic enhancement known as long-term potentiation (LTP). LTP was first described by Bliss and Lomo (4) as a long-lasting increase in the strength of synaptic circuits that can be elicited by a burst of high-frequency stimulation in the rat hippocampus. LTP has been widely regarded as a cellular substrate for learning and memory. Interestingly, aged (24 mo) rats that have been reported to have learning and memory deficits also appear to have a significant impairment in their ability to develop LTP (5). A number of recent reports have shown that synaptic potentiation that resembles LTP can be elicited by norepinephrine (NE) (6–10). The potentiation produced by NE is most clearly seen in the dentate gyrus and appears to be due, at least in part, to modulation of transmitter release (10, 11). It is still not clear, however, whether the potentiation produced by NE is identical to LTP.

NE is widely thought to produce its effects in its target cells via effects on second messengers, particularly cAMP. A substantial body of evidence suggests that second messenger-induced activation of protein kinases constitutes a primary molecular mechanism underlying neuronal response to extracellular signals (see refs. 12–16 for pertinent reviews). Our focus in this report is on the possibility that phosphorylation may underlie the ability of NE to enhance neurotransmitter release in the dentate gyrus. We also intend to test whether aged animals exhibit defects in NE-stimulated phosphorylation.

Synapsin I (synapsin Ia, 85 kDa; synapsin Ib, 80 kDa; collectively referred to as synapsin I) and synapsin II [formerly protein III (17); synapsin IIa, 74 kDa; synapsin IIb, 55 kDa; collectively referred to as synapsin II] are synaptic vesicle-associated phosphoproteins that have been hypothesized to play a role in neurotransmitter release. Direct evidence for such a role for synapsin I has come from studies of the effects of microinjection of synapsin I into the squid giant synapse (18). These authors demonstrated that phosphorylation of synapsin I by Ca^{2+}/calmodulin kinase II (CAM kinase II) controlled the effect of synapsin I on transmitter release. In addition, McGuinness *et al.* (19) have recently shown that synapsin I can regulate vesicle movement in extruded squid axoplasm and, again, this effect was regulated by phosphorylation by CAM kinase II. No similar evidence for synapsin II has yet appeared. However, given the significant homology between synapsin II and synapsin I (17, 20, 21), some similar role for synapsin II seems likely.

Mobley and Greengard (22) have shown that NE stimulates the phosphorylation of synapsin I in rat frontal cortex, apparently through the activation of β-adrenergic receptors. However, the effects of NE on synapsin I were confined to the site phosphorylated by cAMP-dependent protein kinase (PKA); no NE effects were observed on the synapsin I site that is phosphorylated by CAM kinase II (22). Thus, in the cerebral cortex, NE does not phosphorylate synapsin I on the

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Abbreviations: NE, norepinephrine; ISO, isoproterenol; PKA, cAMP-dependent protein kinase; CAM kinase II, calcium/calmodulin kinase II; LTP, long-term potentiation.

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site that has been shown to regulate neurotransmitter release. As mentioned above, NE does produce an increase in transmitter release in the dentate gyrus of the hippocampus. However, the phosphorylation state of the synapsins during such NE stimulation has not been examined.

The objectives of this study were two fold. First, we were interested in determining whether phosphorylation of synapsin I and synapsin II might underlie the potentiating effects of NE in the dentate gyrus. Accordingly, we have tested whether concentrations of NE that produce increased transmitter release in the dentate gyrus would also lead to increased phosphorylation of synapsin I and synapsin II. We were particularly interested in NE-stimulated phosphorylation of synapsin I by CAM kinase II, since this kinase has been shown to regulate the effect of synapsin I on synaptic transmission (18). Second, we wanted to examine the possibility that defects in the phosphorylation of synapsin I and synapsin II might underlie some of the deficits described above for aged rats. Therefore, we have also examined the effects of NE on the phosphorylation of synapsin I and synapsin II in aged rats.

MATERIALS AND METHODS

Front Phosphorylation in Dentate Slices from Rat Hippocampus. Synapsin I is phosphorylated at distinct sites by PKA and by CAM kinase II. Previous studies (22–28) of synapsin I phosphorylation in intact nerve cell preparations have relied on an indirect "back-phosphorylation" assay. The back-phosphorylation assay of synapsin I uses only the catalytic subunit of PKA. When high levels of the catalytic subunit of PKA are included in the back-phosphorylation assay, it is possible to assay effects on the CAM kinase II site. However, under these conditions, the stoichiometry of the phosphorylation of this site is quite low (28). It is essential to assay the CAM kinase II phosphorylation of synapsin I since it is phosphorylation by this kinase that is thought to regulate the effect of synapsin I on neurotransmitter release (18), its binding to synaptic vesicles (29), and vesicle motility (19). Under the experimental conditions used to date, it has not been possible to demonstrate an effect of phosphorylation of synapsin I by PKA on these processes. Consequently, we chose to use a direct "front-phosphorylation" assay to determine whether synapsin I was phosphorylated on the CAM kinase II site with noradrenergic stimulation. This assay utilizes preincubation of dentate slices with $^{32}PO_4$ to label the ATP pools in the slice. Rodnight *et al.* (12) have recently demonstrated the utility of such direct labeling procedures for studies of neuronal protein phosphorylation *in vivo* and *in vitro*. DeGraan *et al.* (30) and Hemmings *et al.* (31) have recently reported the use of a similar procedure for studies of the phosphoproteins B-50 and ARPP-21, respectively.

Young and aged male Fisher 344 (F344) rats were obtained from the National Institute on Aging contract colonies maintained by Harlan Laboratories (Haslett, MI). Rats were decapitated and their brains were quickly removed. Hippocampal slices (400 μm) were prepared as described (32). Slices were placed in 60-mm Petri dishes containing buffer A (125 mM NaCl/3.3 mM KCl/2.4 mM $MgSO_4$/2.5 mM $CaCl_2$/25.7 mM $NaHCO_3$/5.4 mM glucose), which was pregassed with 95% O_2/5% CO_2. Slices consisting primarily of dentate gyrus were prepared by making three knife cuts in the hippocampal slice. The first cut was made just dorsal to the hippocampal fissure and parallel to the CA1 cell layer. The second cut was made medial to the granule cell layer to remove any remaining retrohippocampal structures. The third cut was made at the lateral edge of the hippocampal fissure to remove the CA3 region. The dentate slices were then transferred to 50-ml beakers containing 6 ml of buffer A and $^{32}PO_4$ at 1 mCi/ml (1 Ci = 37 GBq). The oscillation of the shaking incubator was set to 60 per min and constant 95% O_2/5% CO_2 pressure (setting 10 on the gas flow gauge of the Dubnoff incubator) was provided in the enclosed incubator. Incubation temperature was maintained at 33.5°C ± 0.20°C. After 90 min, the slices were rapidly transferred to 50-ml beakers containing 6 ml of buffer A and any desired drug treatments. After 10 min of treatment in the incubator, the slices were sonicated in 1% (wt/vol) SDS to provide a rapid "stop" for the various treatment conditions and to stabilize the phosphorylation state of the proteins. An aliquot of each sample was taken for total protein determination by the method of Lowry (33) with bovine serum albumin used as a standard. In addition, an aliquot of each sample was subjected directly to SDS/polyacrylamide gel electrophoresis on 10% gels, and autoradiograms were generated to examine changes in total protein phosphorylation. The remaining sample was subjected to immunoprecipitation.

Because of the high levels of radioactivity required for these experiments, we were not able to use our typical hippocampal slice chambers for these experiments. Nevertheless, we felt it was essential to identify incubation conditions using the Dubnoff incubator that retained slice viability as assessed by electrophysiological criteria. Accordingly, in control experiments, slices that had been subjected to the manipulations described above (without $^{32}PO_4$) were transferred to a typical hippocampal slice chamber and the viability of the slices was assessed. Provided that the conditions described above were carefully adhered to, slices exhibited normal electrophysiological characteristics including evoked responses (M. Taylor, K.D.P., M.D.B., and T. V. Dunwiddie, unpublished observations).

Back-Phosphorylation. The back-phosphorylation assay followed the procedure of Forn and Greengard (23) as modified by Walaas *et al.* (34). The levels of the synapsin proteins present in the samples assayed by back-phosphorylation were determined by immunoblot assay as described below.

Immunoprecipitation of Synapsin I and Synapsin II. In all the immunoprecipitation and immunoblot experiments described below, a rabbit polyclonal antibody that was raised against synapsin II was used. This antibody exhibits essentially 100% cross-reactivity toward synapsin I. The antibody was purified from the rabbit serum by affinity purification on a synapsin I affinity column. Immunoprecipitation was performed as described (35).

One-Dimensional Phosphopeptide Maps. Synapsin I bands were removed from liquid scintillation mixture, rinsed in ether, agitated for 60 min in SDS/PAGE sample buffer, and resubjected to SDS/PAGE in the presence of *Staphylococcus aureus* V8 protease (5 μg per lane) according to the method of Cleveland *et al.* (36). Bands containing phosphorylated fragments of synapsin I were excised using the autoradiograms as guides, and $^{32}PO_4$ incorporation was quantified by liquid scintillation counting.

Immunoblot Assay of the Levels of Synapsin I and Synapsin II. Levels of synapsin I and synapsin II were assayed by immunoblot as described (17).

RESULTS

The pattern of protein phosphorylation in dentate slices of young and aged F344 rats after immunoprecipitation of synapsin I and synapsin II is shown in Fig. 1. Incubation of dentate slices from a young rat in 10 μM NE led to increased phosphorylation of synapsin I, synapsin IIa, and synapsin IIb. In slices from an aged rat, no NE-stimulated enhancement of phosphorylation was observed. This experiment was repeated on triplicate slices from nine pairs of animals, and the data are summarized in Fig. 2*A*. In slices from young rats, NE significantly enhanced phosphorylation of synapsin I, whereas this adrenergic agonist failed to enhance phosphor-

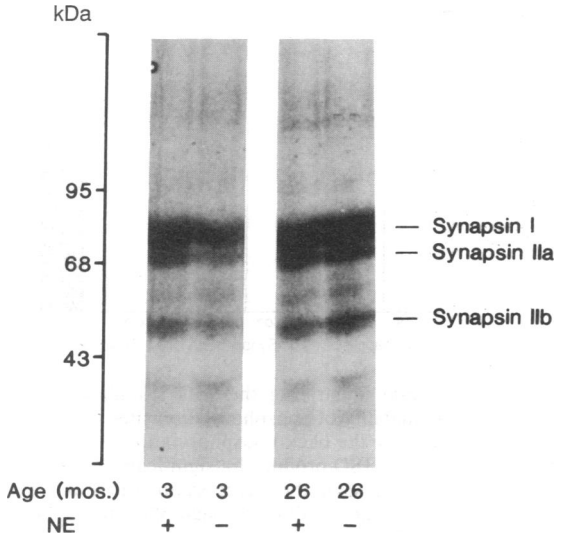

FIG. 1. Autoradiogram showing the effect of 10 μM NE on the front-phosphorylation of synapsin I, synapsin IIa, and synapsin IIb in dentate slices from young and aged (26 mo old) rats. Slices were incubated in $^{32}PO_4$ for 90 min, followed by incubation in the absence (−) or presence (+) of NE. Slices were then homogenized in 1% SDS. Synapsin I and synapsin II were immunoprecipitated, subjected to SDS/polyacrylamide gel electrophoresis, and exposed to x-ray film to generate an autoradiogram. In the dentate gyrus from a 3-mo-old rat, NE stimulated ^{32}P incorporation in the synapsin I, synapsin IIa, and synapsin IIb bands, whereas the agonist failed to stimulate phosphorylation of these proteins in the 26-mo-old rat. Note the high basal levels of ^{32}P incorporation in the aged control.

ylation of these proteins in aged rats. Similarly, the β-adrenergic agonist isoproterenol (ISO) significantly enhanced phosphorylation of these proteins in slices from young, but not aged, F344 rats (n = 7; Fig. 2*B*). As is apparent in Figs. 1 and 2, the aged animals appeared to exhibit higher basal levels of ^{32}P incorporation into the synapsin proteins.

Table 1 shows basal ^{32}P incorporation (expressed as cpm per mg of dentate slice protein) in synapsin I, synapsin IIa, and synapsin IIb. These data indicate that basal levels of phosphorylation of synapsin I, synapsin IIa, and synapsin IIb in slices from aged rats were significantly higher than those in slices from young rats. To determine whether the increase in basal phosphorylation in the aged slices was due to an increase in amount of synapsin I or synapsin II, immunoblot analyses of the levels of synapsin I and synapsin II in the hippocampi of young and old rats were also performed. The data shown in Fig. 3 indicate that there was no difference in the concentration of synapsin I, synapsin IIa, or synapsin IIb in hippocampi of young and aged rats. Quantitative analysis of the immunoblot data revealed that the young/old ratios for the levels of synapsin I, synapsin IIa, and synapsin IIb were 0.89, 1.08, and 0.96, respectively.

Phosphorylation site analysis of synapsin I using one-dimensional phosphopeptide maps was also performed. A phosphopeptide map of synapsin I obtained after treatment with *S. aureus* V8 protease is shown in Fig. 4. This autoradiogram shows an upper peptide fragment of ≈30 kDa, and a lower peptide fragment of ≈10 kDa. Previous studies have shown that the 30-kDa fragment contains the site phosphorylated by CAM kinase II and that the 10-kDa fragment contains the site phosphorylated by PKA (37). Incubation of dentate slices from a young animal in NE led to increased incorporation of ^{32}P at both the 10- and 30-kDa sites. Neither NE nor ISO treatment altered ^{32}P incorporation at either of these sites in slices from aged rats. Data from six experiments done in triplicate examining the effects of NE and ISO on the phosphorylation of these two fragments in young and aged

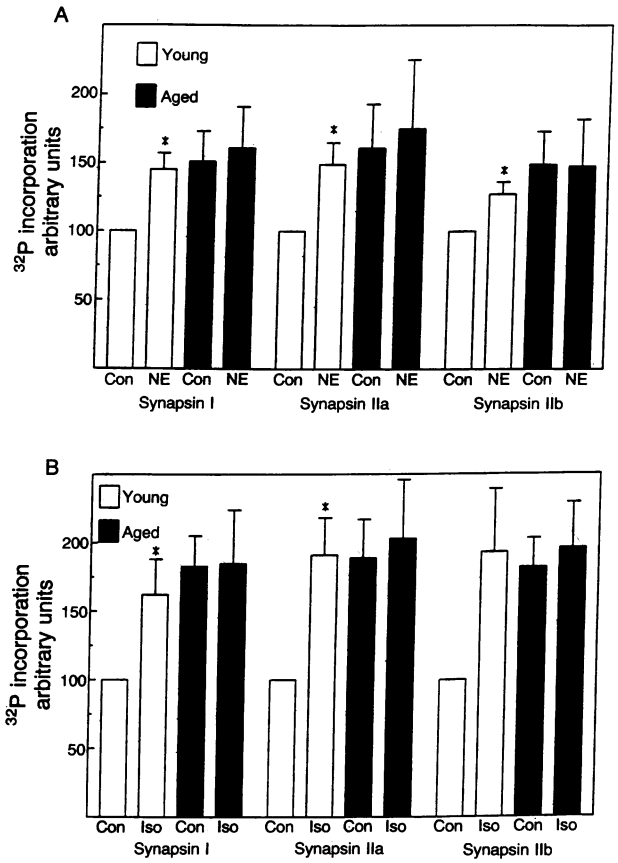

FIG. 2. Histograms illustrating the effects of NE (10 μM) and ISO (250 nM) on phosphorylation of synapsin I, synapsin IIa, and synapsin IIb. (*A*) NE significantly enhanced phosphorylation of synapsin I, synapsin IIa, and synapsin IIb over age-matched controls (Con) in 3-mo-old rats (n = 9). NE failed to significantly enhance the phosphorylation of these proteins over aged-matched controls in aged rats (n = 9; *, $P < 0.05$). (*B*) Likewise, ISO significantly enhanced phosphorylation of synapsin I and synapsin IIa in the young rats (n = 7; *, $P < 0.05$) and failed to significantly enhance phosphorylation of these proteins in aged rats (n = 7). Note that in both cases the basal levels of ^{32}P incorporation in aged controls are greater than in young controls. Treatment conditions were compared to control conditions by Student's t test. Values shown are means ± SEM.

slices revealed that NE and ISO significantly enhanced phosphorylation of both the 10- and 30-kDa fragments of synapsin I in young rats (data not shown).

It was not possible to maintain viability of the slices for sufficient time to obtain isotopic equilibrium. Consequently, it is possible that the effects of NE on phosphorylation of the synapsins might represent some nonspecific effect (e.g., an increase in the specific activity of the ATP). To control for such a possibility, samples of the dentate slice total homogenate were also analyzed on SDS/polyacrylamide gel. Incorporation of $^{32}PO_4$ into polypeptide bands of approximately

Table 1. Effect of aging on basal phosphorylation in slices of dentate gyrus (n = 16)

Protein	Basal ^{32}P incorporation	
	Young	Aged
Synapsin I	2646 ± 612	4104 ± 815*
Synapsin IIa	925 ± 135	1787 ± 422*
Synapsin IIb	744 ± 101	1271 ± 193

Results are expressed as cpm per mg of protein (mean ± SEM).
*Different from young control; $P < 0.05$.

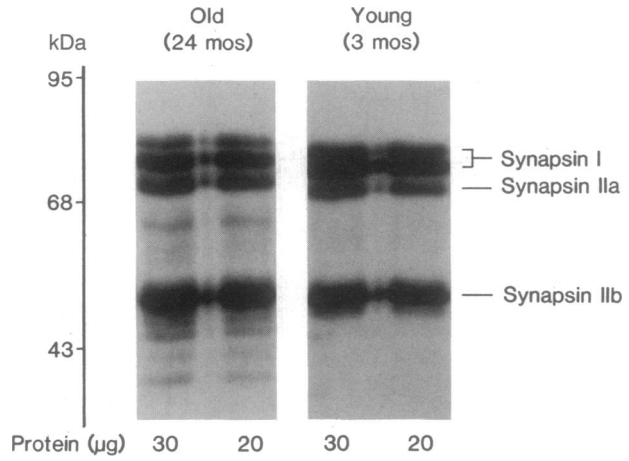

FIG. 3. Autoradiogram showing an immunoblot of various concentrations of young and aged rat hippocampal tissue. Various concentrations of rat hippocampal tissue from aged animals were subjected to SDS/PAGE and then the proteins in the gel were transferred to nitrocellulose sheets. The sheets were then incubated with antibody specific for the synapsin proteins followed by incubation in ^{125}I-labeled protein as described.

100, 65, and 45 kDa was analyzed by liquid scintillation counting. Neither NE nor ISO had any significant effect on the incorporation of $^{32}PO_4$ into these polypeptide bands in either the young or aged rats (data not shown). As an additional control to establish that the increases in synapsin phosphorylation were actually increases in phosphorylation state, we used the back-phosphorylation assay of Forn and Greengard (23). This assay revealed (Fig. 5) that ISO treatment of dentate slices resulted in significantly fewer sites for phosphorylation of synapsins I and II. As shown previously, this indicates that the effect of ISO was to increase the phosphorylation state of the synapsin proteins.

DISCUSSION

The results of the present study indicate that NE and ISO are both capable of enhancing the phosphorylation state of synapsin I, synapsin IIa, and synapsin IIb in dentate slices from young F344 rats. The specificity of this phosphorylation process is substantiated by the observation that NE and ISO did not change the phosphorylation state of polypeptide bands of undefined function (100, 65, and 45 kDa).

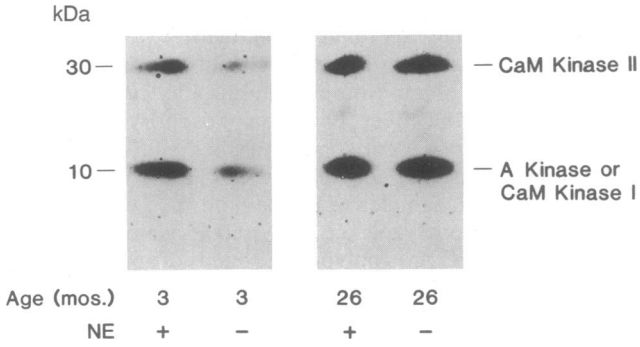

FIG. 4. Autoradiogram showing the effect of NE on phosphorylation of the PKA site (lower site, 10 kDa) and of the CAM kinase II site (upper site, 30 kDa) of synapsin I. Synapsin I bands were treated with *S. aureus* V8 protease and subjected to SDS/PAGE; gels were exposed to x-ray film to generate autoradiograms. This autoradiogram illustrates the NE (+) enhancement over control (−) of ^{32}P incorporation into both the 10- and 30-kDa fragments in dentate gyrus from a 3-mo-old rat. NE failed to stimulate phosphorylation of either of these fragments in an aged rat.

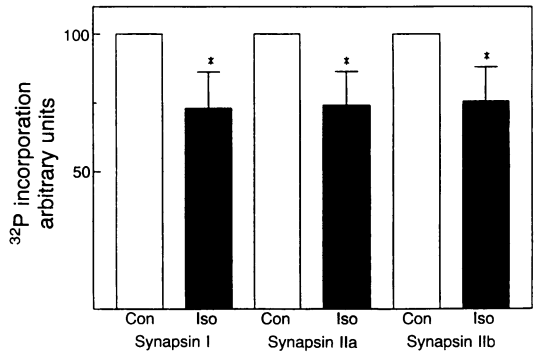

FIG. 5. Histogram illustrating the ISO stimulation-dependent decrease in the availability of phosphorylation sites on the synapsin proteins as revealed by the back-phosphorylation assay. In dentate slices from young rats, ISO produced a significant decrease ($n = 8$; *, $P < 0.05$; mean ± SEM) in the incorporation of ^{32}P into synapsin I, synapsin IIa, and synapsin IIb in the back-phosphorylation assay. Con, control.

One-dimensional phosphopeptide maps of synapsin I yielded two fragments of 30 kDa and 10 kDa. Previous studies (38) have shown that the 30-kDa fragment is phosphorylated by CAM kinase II and the 10-kDa fragment is phosphorylated by PKA. NE- and ISO-stimulated phosphorylation of synapsin I was observed at both the CAM kinase II (30 kDa) and PKA (10 kDa) sites. The mechanism by which NE and ISO promote the CAM kinase II phosphorylation of synapsin I is not known, but this phosphorylation may play a significant role in the effects of NE in the dentate gyrus. However, as ISO was equally effective at eliciting these effects, it appears that activation of β-adrenoreceptors is sufficient to produce the increases in the phosphorylation of the synapsins. Phosphorylation of synapsin I by CAM kinase II has been shown to be important in binding the protein to synaptic vesicles (29). Llinas *et al.* (18) have recently obtained direct evidence that phosphorylation of synapsin I by CAM kinase II plays a role in regulating neurotransmitter release. Moreover, Nichols *et al.* (39) have recently shown that CAM kinase II can potentiate glutamate release from synaptosomes. Given that NE has been shown to potentiate transmitter release and increase excitatory postsynaptic potentials in the rat dentate gyrus (10, 11), it is possible that the NE-stimulated phosphorylation of synapsin I at its CAM kinase II site could underlie this increase in transmitter release. It should be emphasized that the data provided here are simply correlational and no direct evidence for such a link between phosphorylation of the synapsins and NE-stimulated potentiation has been obtained.

In aged (26 mo old) rats, the phosphorylation of synapsin I and synapsin II was not altered by treatment with NE or ISO. Similarly, the 100-, 65-, and 45-kDa polypeptides showed no change in phosphorylation with adrenergic agonist treatment. Phosphopeptide analyses indicated that neither the 10- nor 30-kDa peptides of synapsin I were significantly affected by agonist treatment in the aged dentate gyrus; thus, both the CAM kinase II-mediated and PKA-mediated phosphorylation mechanisms appear to be altered with aging. One could argue that the absence of phosphorylation in the aged dentate gyrus is due to morphological changes that accompany senescence, such as the marked decrease in number of afferent synapses within the terminal zone of the perforant pathway, degeneration of hippocampal fibers (40), and atrophy of granule cell dendrites (41). However, the results of the immunoblot data (Fig. 3) show that despite these morphological changes the concentration of synapsin I and synapsin II in the aged animals is comparable to that in the young.

It is important to note that basal phosphorylation of synapsin I, synapsin IIa, and synapsin IIb were significantly higher in the dentate gyrus of aged rats. In fact, the basal level of phosphorylation in aged rats is comparable to that seen for NE-stimulated phosphorylation in young rats. This raises the possibility that in aged animals the synapsin proteins may already be maximally phosphorylated. These high basal levels do not appear to represent a maximally stimulated condition, however, since drug treatments such as addition of isobutylmethylxanthine (final concentration, 5 μM), a cAMP phosphodiesterase inhibitor, to the NE incubation medium was capable of enhancing phosphorylation of synapsin I and synapsin II 30–40% (data not shown) in aged animals. It is possible that the increased basal state of phosphorylation of the synapsins in aged animals may represent an ongoing adaptation in nerve terminals to compensate for some underlying pathology that reduces neurotransmitter release from these terminals. The mechanism of this increase in basal phosphorylation remains to be investigated.

Taken together, the data presented here are consistent with the hypothesis that phosphorylation of synapsin I and synapsin II may underlie the increase in transmitter release produced by NE in the dentate gyrus of the hippocampus. Moreover, the failure of aged rats to exhibit NE-stimulated phosphorylation may be part of the deficit in NE sensitivity seen in aged animals. Although we have not directly studied age-related alterations in LTP in these same animals, the possibility that the phosphorylation of synapsin I and synapsin II is related to LTP and the possibility that the absence of this phosphorylation in the aged dentate gyrus is related to LTP deficits in aged animals deserve further attention.

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