Research Reports

Phosphorylation of neuromodulin in rat striatum after acute and repeated, intermittent amphetamine

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Repeated, intermittent treatment of rats with amphetamine results in a sensitization of locomotor and stereotyped behaviors that is accompanied by an enhancement in stimulus-induced dopamine release Increased phosphorylation of the neural specific calmodulin-binding protein, neuromodulin (GAP-43, B-50, F1) has been demonstrated in other forms of synaptic plasticity and plays a role in neurotransmitter release To determine whether neuromodulin phosphorylation was altered during amphetamine sensitization, the in vivo phosphorylated state of neuromodulin was examined in rat striatum in a post hoc phosphorylation assay Female, Holtzman rats received saline or 2 5 mg/kg amphetamine twice weekly for 5 weeks One week after the last dose of amphetamine, rats were challenged with either 1 mg/kg or 2 5 mg/kg amphetamine or saline and the rats were sacrificed 30 min later Purified synaptic plasma membranes were prepared in the presence of EGTA and okadaic acid to inhibit dephosphorylation, and were subsequently phosphorylated in the presence of purified protein kinase C and [γ-^{32}P]ATP The protein kinase C-mediated post hoc phosphorylation of neuromodulin was significantly reduced in groups that received either acute or repeated amphetamine suggesting that neuromodulin in those groups contained more endogenous phosphate The acute, challenge dose of amphetamine increased neuromodulin phosphorylation in the saline-treated controls but not in the repeated amphetamine-pretreated group Anti-neuromodulin immunoblots showed no change in neuromodulin levels in any group There was no significant change in protein kinase C activity in any treatment group To further investigate the effect of acute amphetamine, the ability of amphetamine to alter neuromodulin phosphorylation in ^{32}P$_i$-preincubated Percoll-purified rat striatal synaptosomes was examined Amphetamine (10 μM) significantly increased phosphorylation of a 53 kDa band that migrated with authentic neuromodulin in the synaptosomes by 22% while 500 nM 12-O-tetradecanoylphorbol 13-acetate (TPA) increased neuromodulin phosphorylation by 45% These data suggest that one injection of amphetamine can increase neuromodulin phosphorylation in rat striatum and that this increase is maintained for at least 1 week following a repeated, sensitizing regimen of amphetamine Since sensitization can be induced with one dose of amphetamine, it is possible that enhanced neuromodulin phosphorylation could contribute to neurochemical events leading to enhanced release of dopamine and/or behavioral sensitization

INTRODUCTION

In humans, amphetamine (AMPH) abuse can lead to a psychosis that clinically resembles paranoid schizophrenia and responds fairly rapidly to antipsychotic dopamine receptor blocking drugs Upon discontinuation of drug use, individuals remain hypersensitive to the psychotic effects of the drug for several years[2,22,51,52,54] In animals, repeated, intermittent, administration of AMPH results in a sensitization of a number of behaviors, including locomotor and stereotyped behaviors (for review see ref 48), that involve activation of dopaminergic systems Behavioral sensitization to AMPH in rats can develop after only one injection, but is more robust after multiple, intermittent, injections and will develop following a number of different injection regimens[46,48] After withdrawal from the drug, the sensitization intensifies with time and can persist for up to one year[40] Although considerable evidence suggests than an action of AMPH at dopamine cell bodies in the midbrain is necessary for induction of sensitization[13,23,56] the expression of AMPH sensitization resides in dopaminergic terminal areas, such as striatum and nucleus accumbens[28,39,46,48,58] An enhanced stimulus-induced release of dopamine in both striatum and nucleus accumbens has been reported after intermittent treatment with AMPH that exhibits characteristics similar to behavioral sensitization in-

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tensification with one dose, but heightened intensity upon multiple doses, increased activity and persistence after withdrawal[39,46,48,58] Enhanced AMPH-induced dopamine release after repeated AMPH is expressed in both the rat striatum and nucleus accumbens and could play a role in the expression of sensitization[46,48] Behavioral sensitization to AMPH exhibits characteristics similar to other forms of synaptic plasticity, such as long-term potentiation, in that it develops after intermittent stimuli, is long-lasting, involves an increase in neurotransmitter release and its induction is blocked by N-methyl-D-aspartate antagonists[25] Additional similarities in models of synaptic plasticity and AMPH-induced behavioral sensitization are morphological changes in pre- and postsynaptic elements[4,7,33,57] and alterations in activities involving Ca^{2+} and/or the endogenous Ca^{2+}-binding protein, calmodulin (CaM)[19,24,26,42,43,60] We and others have shown an increased CaM content and altered subcellular localization of CaM in striatum and limbic forebrain of rats repeatedly treated with AMPH[19,41,43,50]

Heightened phosphorylation in brain of a growth-associated, neural-specific, CaM-binding protein termed neuromodulin (as well as B-50, GAP-43, p57, F1, pp46,[8]) has been reported in several models of synaptic plasticity, such as long-term potentiation[32,37] and memory formation for passive avoidance in chicks[49] Neuromodulin is located presynaptically and plays a role in neuronal growth and neurotransmitter release[8] Neuromodulin phosphorylation has been correlated with neurotransmitter release from both synaptosome and slice preparations[10,18] Due to its low charge-to-mass ratio neuromodulin exhibits apparent molecular masses of between 43 and 67 kDa on SDS-polyacrylamide gels, rather than its exact molecular mass of 24 kDa[8] Protein kinase C (PKC)-mediated phosphorylation of neuromodulin or increased Ca^{2+} leads to dissociation of CaM from neuromodulin[31] It has been postulated that neuromodulin sequesters CaM in the unstimulated cell and releases it upon an increase in intracellular Ca^{2+} and phosphorylation by PKC[31] Since PKC-dependent phosphorylation of neuromodulin has been correlated with synaptic plasticity and may a play a role in neurotransmitter release and CaM localization in a cell, the phosphorylation of endogenous neuromodulin in response to acute or repeated AMPH treatment was investigated To study neuromodulin phosphorylation, the post hoc phosphorylation of neuromodulin in response to exogenous partially-purified PKC was assessed in purified rat striatal synaptic plasma membranes If enhanced neuromodulin phosphorylation is involved in behavioral sensitization and/or the increased dopamine release in response to AMPH, one

would predict that neuromodulin phosphorylation would be greater after repeated treatment with AMPH and would persist following withdrawal of the drug Enhanced phosphorylation of neuromodulin could also be detected after acute AMPH, since sensitization has been reported after a single dose of AMPH[48] We found that neuromodulin phosphorylation was enhanced in striatal synaptic plasma membranes from rats treated repeatedly with AMPH or in control rats that had received only one acute injection of AMPH Preliminary studies show that AMPH could increase phosphorylation of a 53 kDa band migrating with authentic neuromodulin in purified synaptosomes

MATERIALS AND METHODS

AMPH treatment

Female Holtzman rats were treated with saline (SAL) or 2 5 mg/kg AMPH i p twice weekly for 5 weeks One week after the repeated treatment a challenge dose of SAL or AMPH was administered 30 min before sacrifice such that 4 groups were formed SAL-SAL, SAL-AMPH, AMPH-SAL and AMPH-AMPH In Study 1 the challenge dose of AMPH was 1 mg/kg and in Study 2 the challenge dose was 2 5 mg/kg The lower challenge dose in Study 1 was chosen because the differences in sensitized behavior and AMPH-induced dopamine release are accentuated after a lower challenge dose of AMPH (ref 47, T E Robinson, personal communication) This treatment regimen was shown to result in robust increases in rotational activity[45]

Preparation of synaptic plasma membranes

Striata were dissected within 60 s of sacrifice and homogenized in 0 32 M sucrose, 1 mM EGTA 2 mM Tris, pH 7 4, 10 μM leupeptin, 10 μM pepstatin and 1 mM phenylmethylsulfonyl fluoride (PMSF) In Study 2 the medium included 1 μM okadaic acid Synaptic plasma membranes (SPM) were prepared as described by Dokas et al[12] Briefly a P2 pellet prepared from an individual striatum (approximately 50–60 mg wet weight) was lysed, centrifuged at 10 000 × g for 20 min washed and applied to a sucrose gradient consisting of 4 0 ml of 0 4 M sucrose overlaid on 8 ml of 1 0 M sucrose The gradient was centrifuged at 100,000 × g for 80 min in a Beckman SW27 rotor SPM's were collected by aspiration from the interface of the 0 4 M and 1 0 M layers, diluted 2- to 3-fold in 10 mM Tris-HCl, pH 7 5 and 1 mM EGTA and centrifuged in the SW 27 rotor at 100,000 × g for 30 min The final pellet was resuspended in 10 mM Tris buffer pH 7 5 Approximately 7 μg of SPM protein was obtained per mg wet weight tissue

Post hoc phosphorylation assay

PKC was purified from rat brain as described by Kitano et al[27] SPMs were phosphorylated in an assay containing 10 mM sodium acetate, pH 6 5, 10 mM magnesium acetate, 7 5 μM [γ-^{32}P]ATP (2 mCi/assay), 0 6 μg purified PKC, 0 1 mM $CaCl_2$ in a total volume of 75 μl as described by Dokas et al[12] After 30 s of incubation, the reaction was terminated by the addition one-third volume of an SDS-containing stop solution containing 12 5 mM Tris-HCl, pH 6 8 4% SDS, 10% glycerol, 0 008% bromphenol blue and 40% mercaptoethanol In Study 1, SPMs were preincubated in assay buffer (10 mM sodium acetate, pH 6 5, 10 mM magnesium acetate and 0 1 mM $CaCl_2$) for 5 min at 30° There was no preincubation in Study 2 Samples were immediately subjected to SDS–PAGE on gels containing either 10% or 8 75% polyacrylamide as indicated with subsequent autoradiography Results were analyzed by either cutting the neuromodulin band and counting the radioactivity or by scanning

the films with a Hoefer GS365W scanning densitometer The total peak areas were quantified by Gaussian integration using the Hoefer GS365W electrophoresis data system Statistical significance was determined by one way analysis of variance (ANOVA) with post test Bonferroni t-test analysis calculated using GraphPad Instat

Immunoblotting

SPM samples subjected to SDS–PAGE were electrophoretically transferred to Immobilon-P membranes for 2 h at 1 A at 4°C in a Transphor Transfer Unit (Hoefer Scientific) Blots were incubated in 10 mM Tris-HCl, pH 7 4, 150 mM NaCl with 0 1% Tween 20 and 1% (w/v) bovine serum albumin (blocking buffer) for 1–2 h at 4°C The Immobilon membrane was incubated overnight with affinity-purified antineuromodulin produced in rabbit (obtained from Dr Daniel Storm, Department of Pharmacology, University of Washington) diluted 1 1000 in blocking buffer ^{125}I-labeled donkey (1 mCi) anti-rabbit IgG or ^{125}I-protein A (1 mCi) was used for quantification and autoradiography

PKC assay

PKC activity of the SPMs was measured using a synthetic substrate peptide, myelin basic protein$_{4-14}$ (MBP$_{4-14}$), as described by Yasuda et al[59] Phosphorylation of MBP$_{4-14}$ by endogenous PKC in the SPM fractions was carried out in plastic tubes in a reaction mixture (50 μl) containing 20 μM Tris-HCl, pH 7 5, 5 mM magnesium acetate, 0 1 mM CaCl$_2$, 20 mM ATP (0 3 μCi/assay), 15 mM MBP$_{4-14}$, 0 5 mg phosphatidyl serine, 50 ng of diolein and 2 μg of SPM protein for 6 min at 30°C The reaction was stopped by spotting a 40 μl aliquot of the mixture onto a piece of P-81 paper which was immediately placed in 75 mM H$_2$PO$_4$ and washed 4 times as described by Yasuda et al[59] Parallel reactions were conducted in the presence of 1 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) and in the absence of MBP$_{4-14}$ Phosphorylation of the specific PKC substrate MBP$_{4-14}$ was generally 4–5 times that of the non-peptide containing blank PKC activity was also measured in the 10,000× g S2 supernatant fraction prepared from striata as described above using 200 μg/ml histone H1 as substrate and 30 μg of S2 protein[27] The PKC activity of the S2 fraction was determined to be the total activity (CaCl$_2$ plus lipids) minus that activity measured in the presence of CaCl$_2$ All radioactivity was determined using β-scintillation counting in a Beckman LS8100

Phosphorylation of Percoll-purified synaptosomes

Percoll-purified striatal synaptosomes from 4 rats were prepared as described by Dunkley et al[14] Fractions 3 and 4, enriched with synaptosomes, were combined, washed and resuspended to a protein concentration of 2–3 mg/ml in oxygenated Krebs Ringer buffer (118 mM NaCl, 4 7 mM KCl, 1 2 mM CaCl$_2$, 1 2 mM MgCl$_2$, 24 9 mM NaHCO$_3$, 10 mM glucose, oxygenated for 1 hr and brought to pH 7 4 at 37°C) Synaptosomes were prelabeled with ^{32}P$_i$ (0 5 mCi/ml) for 45 min at 37°C in Krebs–Ringer buffer The synaptosomes were then incubated with 10 μM AMPH, 500 nM 12-O-tetradecanoylphorbol 13-acetate (TPA) or buffer for 10 min In some experiments synaptosomes were incubated with Krebs–Ringer buffer or high K$^+$ buffer (62 mM KCl and 85 7 mM NaCl) for 30 s The reaction was stopped with one-fourth volume of an SDS-buffer solution containing 200 mM Tris-HCl, pH 6 8, 8% SDS, 28% glycerol, 0 1% bromphenol blue, 77 mM mercaptoethanol and 6 mM EGTA and was subjected to SDS–PAGE on gels containing 8 75% polyacrylamide with subsequent autoradiography Results were analyzed by either by scanning the peaks using a Hoefer GS365W scanning densitometer as described above or cutting the neuromodulin band and counting the radioactivity The peak areas were quantified by Gaussian integration using the Hoefer GS365W electrophoresis data system Peak areas were always compared with those of controls run on the same gel Statistical significance was determined by one way analysis of variance (ANOVA) with posttest Bonferroni t-test analysis calculated using GraphPad Instat and by a two-tail Student's t-test or Wilcoxon signed-rank test

Materials

AMPH was purchased from The University of Michigan Laboratory of Animal Medicine Leupeptin, pepstatin, phenylmethylsulfonyl fluoride, TPA, histone H1, phosphatidylserine, diolein, high molecular weight standards, BSA and Tween were obtained from Sigma Chemical Co (St Louis, MO) Okadaic acid and MBP$_{4-14}$ were obtained from UBI Biochemicals (Lake Placid NY) P-81 paper was obtained from Whatman (Maidstone, UK), Percoll from Pharmacia (Upsalla, Sweden) and Immobilon from Millipore (Bedford, MA) ^{125}I-labeled donkey anti-rabbit IgG and ^{125}I-protein A were purchased from Amersham (Arlington Heights, IL) [γ-^{32}P]ATP (spec act > 4000 Ci/mmol) was from ICN (Irvine, CA) Affinity-purified rabbit antineuromodulin was the generous gift of Dr Daniel Storm, Department of Pharmacology, University of Washington Purified rat brain neuromodulin was generously donated by Dr Linda Dokas, Department of Biochemistry, Medical College of Ohio

RESULTS

Effect of EGTA on phosphorylation of neuromodulin

SPM preparations were used to assess phosphorylation of neuromodulin because neuromodulin is a prominent phosphorylated protein in SPM and is readily detected (Fig 1) The apparent molecular weight of neuromodulin on SDS–PAGE with 10% polyacrylamide was 48 kDa In later experiments performed on 8 75% polyacrylamide gels, which we found gave greater separation of the proteins, neuromodulin appeared to have a molecular weight of 51–53 kDa Dephosphorylation of neuromodulin during preparation of the SPMs, however, could confound the results The ability of EGTA to reduce the endogenous dephosphorylation of
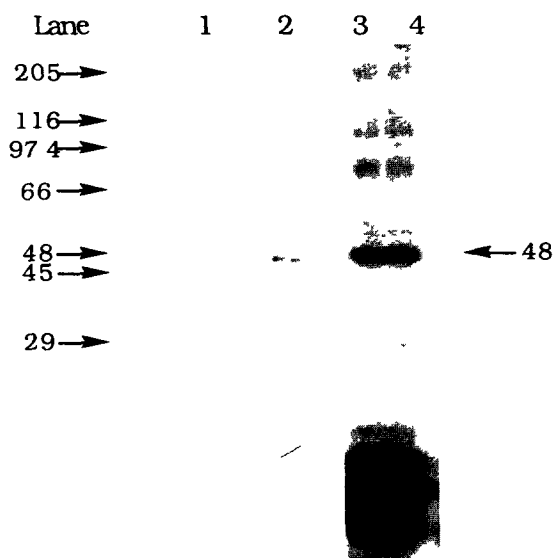

Fig 1 Localization of neuromodulin in striatal synaptic plasma membranes Rat striatal SPMs were prepared and phosphorylated in the presence of partially purified PKC and [γ-^{32}P]ATP as described in Methods and subjected to SDS–PAGE on gels containing 10% polyacrylamide Lane 1 high molecular weight standards (myosin, 205 kDa, β-galactosidase, 116 kDa, phosphorylase B, 97 4 kDa, bovine plasma albumin, 66 kDa, egg albumin, 45 kDa, carbonic anhydrase, 29 kDa), Lane 2 purified rat brain neuromodulin (10 μg), Lanes 3 and 4 27 μg of phosphorylated SPM Neuromodulin is one of the most prominent phosphorylated bands in the SPM preparation

2A

2B

Lane   1   2   3   4   1   2

48→   ←48

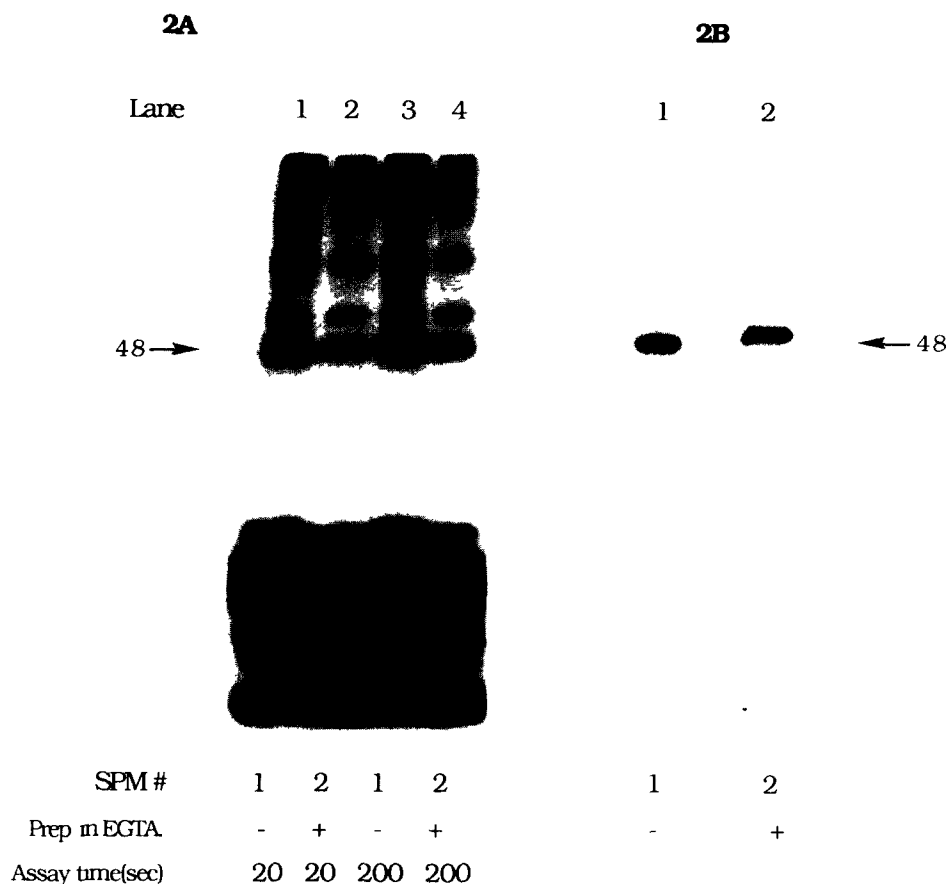| SPM #	1	2	1	2		1	2
Prep in EGTA	-	+	-	+		-	+
Assay time(sec)	20	20	200	200			

Fig 2 A in vitro phosphorylation by PKC of SPMs prepared in the absence (SPM #1) and presence (SPM #2) of EGTA 1 mM EGTA was included in the buffers in the preparation of SPM #2 Lane 1 SPM #1, 20 s incubation Lane 2 SPM #2, 20 s incubation Lane 3 SPM #1 200 s incubation Lane 4, SPM #2 200 s incubation B immunoblots for neuromodulin contained in 15 μg of SPM #1 (lane 1) and SPM #2 (lane 2) Samples of SPM #1 and #2 were subjected to immunoblotting using affinity-purified antibody to neuromodulin and [^{125}I]protein A for detection The cpm of [^{125}I]protein A for the samples are 2677 cpm for SPM #1 and 2916 cpm for SPM #2 showing that the detectable neuromodulin in the two preparations is the same

S-S   S-A   A-S   A-A   S-S   S-A   A-S   A-A   S-S   S-A   A-S   A-A
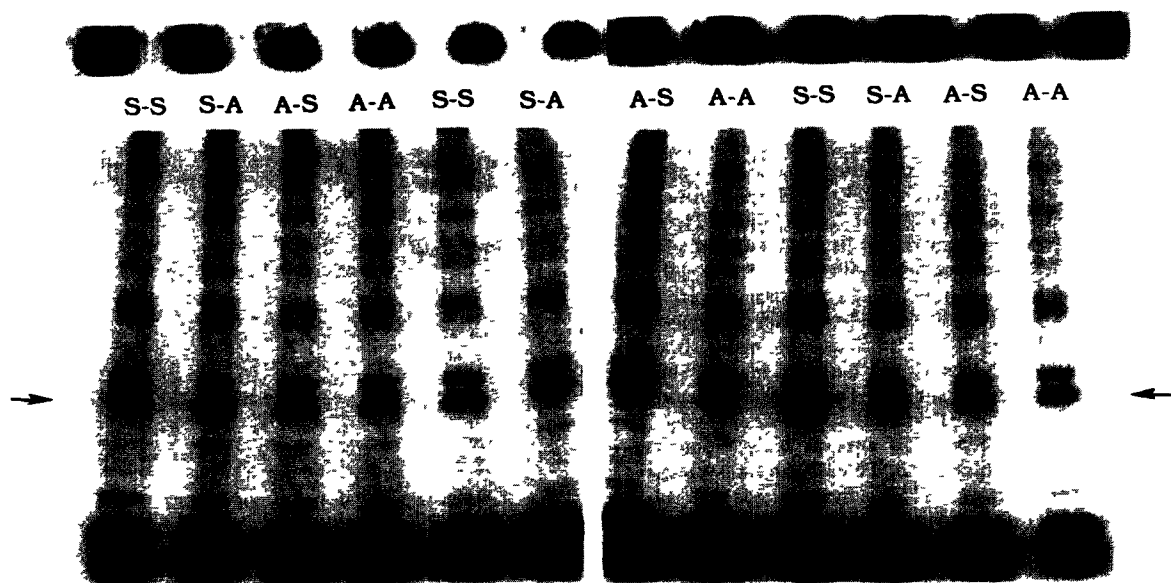
Fig 3 Post hoc phosphorylation of striatal SPMs from female rats treated with saline (S) or repeated intermittent AMPH (A) twice weekly for 5 weeks, as described in Materials and Methods and given a challenge dose of either saline or AMPH such that four groups were formed S-S S-A, A-S A-A Samples were phosphorylated in the presence of partially purified PKC and [γ-^{32}P]ATP and subjected to SDS–PAGE on gels containing 8 75% polyacrylamide as described in Methods Each lane contains 27 μg protein Shown are the results for 3 different sets of animals Immunoblots for neuromodulin in the SPMs (15 μg) using [^{125}I]donkey anti-rabbit for detection are shown above the SDS gels Only one band was present upon immunoblotting

neuromodulin was assessed since neuromodulin is a substrate for the Ca^{2+}/CaM dependent phosphatase, calcineurin[30] Striata were homogenized in 0 32 M sucrose containing protease inhibitors without (SPM #1) and with 1 mM EGTA (SPM #2) (Fig 2) SPMs were prepared and phosphorylated in the presence of purified PKC and [γ-^{32}P]ATP in the post hoc phosphorylation assay In this assay, a greater incorporation of ^{32}P into the neuromodulin band would signify that the endogenous neuromodulin contained less phosphate As shown in Fig 2A, SPMs prepared in the presence of 1 mM EGTA (SPM #2) incorporated less ^{32}P in the presence of purified PKC than samples prepared without EGTA suggesting that the endogenous neuromodulin in those samples contained more phosphate The neuromodulin immunoblot in Fig 2B demonstrates that the amount of immuno-detectable neuromodulin per unit protein was the same in SPMs prepared in the presence (SPM #2) or absence of EGTA (SPM #1) The cpm of [^{125}I]protein A for the blots are 2677 cpm for 15 μg of SPM 1 (SPM #1) and 2916 cpm for 15 μg of SPM 2 (SPM #2) The recovery of SPM protein was not altered by the presence of EGTA in the preparation buffers Note that there was no significant dephosphorylation of neuromodulin in a 200 s assay (lanes 3 and 4) as compared to a 20 s assay (lanes 1 and 2) regardless of whether the SPMs were prepared in the presence or absence of EGTA

Post hoc phosphorylation of neuromodulin in striatal SPMs prepared from rats given repeated, intermittent AMPH or SAL

The phosphorylated state of endogenous neuromodulin was determined in striatal SPMs prepared from rats treated intermittently with SAL or AMPH using the post hoc phosphorylation assay In Study 1, there was a decreased ability of neuromodulin to be phosphorylated in the post hoc assay with purified PKC and [γ-^{32}P]ATP in SPMs prepared from rats receiving either acute treatment with 1 mg/kg AMPH or repeated treatment with 2 5 mg/kg AMPH Autoradiographs showing phosphorylation of SPMs from 3 different groups of rats are shown in Fig 3 To quantify the ^{32}P incorporated into neuromodulin, the bands corresponding to purified neuromodulin were cut and counted As shown in Table I, the ^{32}P incorporation into those bands was significantly decreased in the SAL-AMPH, AMPH-SAL and AMPH-AMPH groups There was no significant difference in the ^{32}P incorporation among any group that received AMPH The decrease in incorporation of ^{32}P in the post hoc assay suggests that both acute and repeated AMPH treatment increased the in vivo phosphorylation of neuro-

TABLE I

In vitro phosphorylation and immunoblot alues for neuromodulin in striatum from rats treated with acute or repeated AMPH

[a] Female Holtzman rats ($n = 6$) were treated with repeated saline (S) or amphetamine (A) and given a challenge dose of S or 1 mg/kg A before sacrifice as described for Study 1 in Materials and Methods The first initial represents the repeated treatment and the second represents the challenge treatment Bands in the SDS–PAGE corresponding to the R_f of purified NM were cut and counted after phosphorylation in the back-phosphorylation assay (^{32}P) as were bands containing ^{125}I-secondary antibody antibody from immunoblots For ^{32}P values ANOVA, $P < 0\,0001$ In post analysis Bonferroni t-tests * $P < 0\,01$ for A-S and A-A as compared to S-S and $P < 0\,05$ for S-A as compared to S-S ^{125}I-values were not significantly different, ANOVA, $P = 0\,56$

[b] Female Holtzman rats ($n = 5$) were treated with repeated amphetamine (A) or saline (S) and challenged with S or 2 5 mg/kg A as described for Study 2 in Materials and Methods Results were analyzed using densitometry as described in Materials and Methods For densitometry values $P < 0\,02$ by ANOVA In post analysis Bonferroni t-test ** $P < 0\,05$ for S-A and A-S as compared to S-S

Treatment group	Study 1 [a]		Study 2 [b]
	^{32}P (cpm)	^{125}I (cpm)	Densitometry values (Units × 10^{-3})
S-S	293 ± 5	8 613 ± 581	47 8 ± 1 5
S-A	231 ± 15 *	7 807 ± 680	26 8 ± 3 **
A-S	201 ± 16 *	8 773 ± 479	36 5 ± 2 7 **
A-A	199 ± 11 *	7 948 ± 537	41 6 ± 2 7

modulin Although the challenge dose of 1 mg/kg AMPH was able to increase neuromodulin phosphorylation in control SAL-treated rats, challenge with AMPH produced no further change in neuromodulin phosphorylation in rats repeatedly treated with AMPH To assess whether AMPH altered the amount of neuromodulin in the SPMs, immunoblots were performed using affinity-purified antibody to neuromodulin Neuromodulin immunoreactivity was detected with ^{125}I-labeled donkey anti-rabbit IgG The immunoblots for neuromodulin in the SPMs are shown above the SDS gels in Fig 3 Only one band was present upon immunoblotting The bands were cut and counted and the results are shown in Table I The amount of immuno-detectable neuromodulin was not altered by AMPH treatment further indicating that the decreased ^{32}P incorporation represents an alteration in the phosphorylated state of neuromodulin in vivo

To further reduce dephosphorylation of neuromodulin during preparation of SPMs as a factor in our results, the experiment was repeated including 1 μM okadaic acid with 1 mM EGTA in SPM preparation buffers (Study 2) In addition, the challenge dose of acute AMPH was increased to 2 5 mg/kg to correspond with the dose of AMPH given repeatedly for 5 weeks The experiments were performed as described above but the results were analyzed by densitometry as described in Methods As shown in Table I, essentially the same results were achieved as those in Study 1

TABLE II

PKC activity in striatal fractions from rats treated with acute or repeated AMPH

Female Holtzman rats were treated with saline (S) or amphetamine (A) and challenged with S or 2 5 mg/kg A as described for Study 2 in Materials and Methods PKC activity was measured in striatal SPM or S2 cytosol as described in Materials and Methods n = 4

Treatment group	SPM (pmol / min / mg prot ± S E M)		S2 cytosol
	Total	Ca^{2+} dependent	
S-S	459± 85	264±90	939± 22
S-A	494± 79	261±57	821± 41
A-S	661±105	401±68	964±149
A-A	424± 74	248±74	1 103±157

The ability of neuromodulin to be phosphorylated in the post hoc assay was reduced in SPMs from control rats receiving a challenge dose of 2 5 mg/kg AMPH or from rats receiving repeated AMPH In this experiment, there was not a significant reduction in phosphorylation in the AMPH-AMPH group, but it demonstrated again that a challenge dose of AMPH does not elicit further neuromodulin phosphorylation in rats that have received repeated AMPH

To determine whether the altered neuromodulin phosphorylation was due to increased PKC activity, this activity was measured in SPM and soluble fractions from the four treatment groups PKC activity in the SPMs was determined using the specific PKC substrate MBP$_{4-14}$ since very little activity could be measured using histone H1 as a substrate The PKC activity in the SPMs was variable among the four groups but the results in Table II suggest that there was no significant difference in PKC activity among the groups Therefore neither repeated AMPH nor a challenge dose of AMPH appeared to alter the PKC activity To determine whether AMPH treatment would alter PKC activity in general, PKC activity in the soluble S2 fraction

TABLE III

Phosphorylation of neuromodulin by AMPH, TPA and high K$^+$ in percoll-purified rat striatal synaptosomes

Percoll-purified striatal synaptosomes were equilibrated with ^{32}P$_i$ and incubated with 10 μM AMPH, 500 nM TPA for 10 min or 62 mM KCl for 30 s as described in Materials and Methods Results were analyzed by scanning the neuromodulin band with densitometry as described in Materials and Methods [a] P < 0 007 as determined by a 2-tail paired t-test [b] P < 0 003 as determined by a 1-tail paired t-test [c] P < 0 008 as determined by a Wilcoxon signed rank test

Treatment	n	Densitometry values (Units × 10^{-3})	% Control
Buffer	9	6 ±0 8	
AMPH	9	7 3±0 8 [a]	125±8 [c]
Buffer	4	6 9±1	
TPA	4	9 7±1 [b]	145±11
Buffer	1	11 6	
High K$^+$	1	21	181

was measured As shown in Table III, there was no significant difference in activity among any of the groups

Phosphorylation of a 53 kDa protein in Percoll-purified rat striatal synaptosomes

The results of both Study 1 and Study 2 indicated that an acute in vivo dose of AMPH could increase the phosphorylation of neuromodulin in SPMs To determine whether this effect of AMPH could be measured
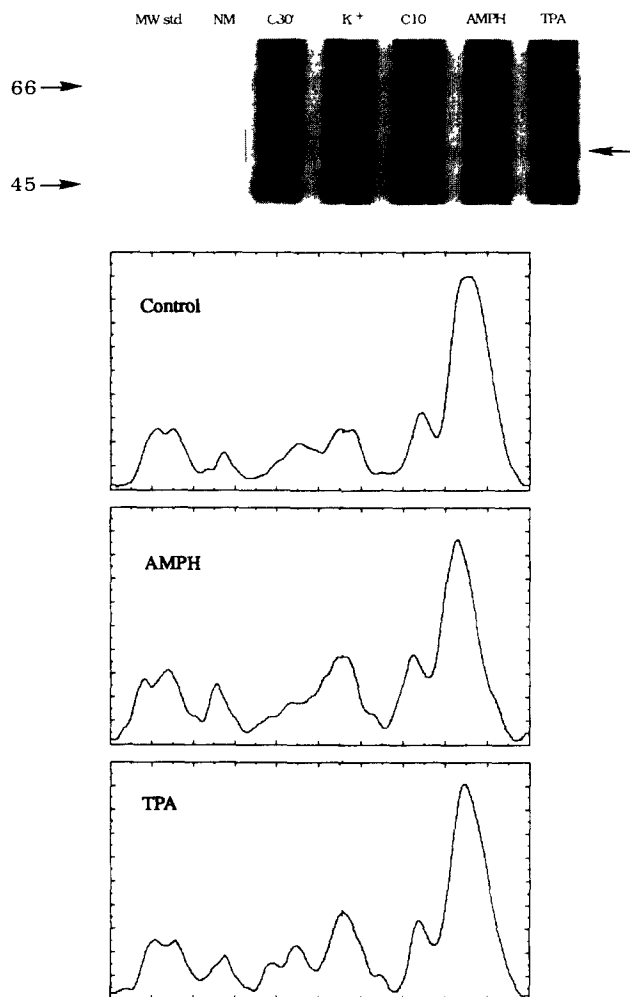

Fig 4 A neuromodulin phosphorylation in Percoll-purified striatal synaptosomes After equilibration with ^{32}P$_i$, synaptosomes were incubated with either buffer or 62 mM KCl for 30 s or buffer 10 μM AMPH or 500 nM TPA for 10 min as described in Materials and Methods The reaction was stopped with SDS-stop buffer and subjected to SDS-PAGE on gels containing 8 75% actylamide with subsequent autoradiography Lane 1 molecular weight markers Lane 2, 5 μg purified rat brain neuromodulin Lane 3, buffer control, 30 s (C30") Lane 4 62 mM KCl, 30 s (K$^+$) Lane 5 buffer control, 10 min (C10") Lane 6 10 μM AMPH 10 min Lane 7, 500 nM TPA, 10 min Under these conditions, purified neuromodulin ran with an apparent molecular weight of 53 kDa B densitometric scans of phosphoproteins in lanes 5, 6 and 7, 10 min incubation times with buffer (top), 10 μM AMPH (middle) or 500 nM TPA (bottom) The neuromodulin peak is marked with a dotted line Results were analyzed by Gaussian integration with the GS365W densitometric program as described in Materials and Methods

more directly, preliminary experiments were undertaken to examine the ability of AMPH to increase neuromodulin phosphorylation in an isolated synaptosomal preparation Percoll-purified synaptosomes were equilibrated with $^{32}P_i$ and then incubated with buffer, 10 μM AMPH or 500 nM TPA In one experiment, the effect of depolarization with high K^+ on neuromodulin phosphorylation was assessed Phosphorylation of a 53 kDa band migrating with authentic neuromodulin was assessed by scanning densitometry of the films and, in some experiments, by cutting the band from the gel and counting radioactivity The results in Table III show that 10 μM AMPH had some effect in increasing phosphorylation of the 53 kDa band Addition of 10 μM AMPH to the synaptosomes significantly increased the phosphorylation of the 53 kDa neuromodulin band by 25%. TPA averaged a 45% increase in phosphorylation of the 53 kDa neuromodulin band and K^+ depolarization increased the phosphorylation by 81% Shown in Fig 4A is an autoradiograph demonstrating phosphorylation of the 53 kDa band by 62 mM KCl, 10 μM AMPH and 500 nM TPA A lane containing authentic neuromodulin is also shown The densitometry traces corresponding to the autoradiograph for the control, AMPH and TPA incubations are shown in Fig 4B Increased phosphorylation of the 53 kDa neuromodulin band is clearly shown in response to AMPH and TPA Integration values for the densitometry tracings are given in Table III and show a significant increase in phosphorylation of neuromodulin by both AMPH and TPA In 5 of the AMPH experiments, the ^{32}P-containing 53 kDa neuromodulin band was cut and counted Values for ^{32}P in CPM were buffer, 307 $\pm$ 52, AMPH, 385 $\pm$ 72, p < 0 05 as determined by a 2-tailed Student's t-test

DISCUSSION

A post hoc phosphorylation assay was devised to determine the effect of repeated AMPH, given in an intermittent regimen that results in behavioral sensitization, on the in vivo phosphorylated state of neuromodulin in rat striatum Our results suggest that one injection of AMPH can increase the phosphorylation of neuromodulin and the heightened phosphorylation remains at least one week after withdrawal from a regimen of repeated, intermittent, AMPH The phosphorylated state after repeated AMPH appears 'maximal' and stable such that a challenge dose of AMPH 1 week later elicits no further change in phosphorylation of neuromodulin Although dephosphorylation of neuromodulin during preparation of SPMs could confound these results, our experiments suggested that inclusion

of EGTA in the homogenization buffer reduced the dephosphorylation of neuromodulin during SPM preparation These results are consistent with those of Meiri and Burdick[35] who found that dephosphorylation of neuromodulin in growth cones was prevented by EGTA but not okadaic acid Since, however, neuromodulin has been shown to be dephosphorylated by protein phosphatases 1 and 2A[20] in synaptic membranes, the experiment was repeated including 1 μM okadaic acid which would inhibit both phosphatases The results of both studies were comparable An alternative explanation of the data is that EGTA treatment could increase binding of endogenous CaM to neuromodulin, which could block the post hoc phosphorylation of the protein However, this explanation is unlikely for two reasons First, striata from SAL- and AMPH-treated animals were both homogenized with EGTA in exactly the same way Second, the preincubation of membranes in a calcium-containing buffer in Study 1 should have dissociated endogenous CaM, permitting phosphorylation by PKC In Study 2, SPMs were not preincubated to reduce the possibility of phosphatase action on endogenous neuromodulin during the preincubation period Results from Study 1 and Study 2 were essential the same

The post hoc assays were conducted using an excess of partially purified PKC Recent studies have shown that neuromodulin in cultured neurons and in neonatal rat brain can be phosphorylated in vivo at 3 sites, only one of which, serine 41, is phosphorylated by PKC[55] Neuromodulin can be phosphorylated in vitro by casein kinase II[3] Phosphorylation at serine 41 is important in the regulation of local concentrations of calmodulin[31] and may play a role in modulating neurotransmitter release[18,44] The relatively modest changes in phosphorylation could be due to the fact that not all neurons in the striatum are affected by the acute or repeated AMPH, and the fact that only one-third of the possible phosphorylation sites in neuromodulin are affected by PKC Although the n was low and the results variable, measurement of PKC activity suggested that AMPH treatment did not increase PKC activity It is possible that there is a transient increase in PKC elicited by AMPH that was not detected by our assay, or that a change in phosphatase activity is responsible for the alteration of neuromodulin phosphorylation

Many studies have demonstrated changes in neuromodulin content, localization or phosphorylation associated with synaptic plasticity and nerve growth[8,18,38,53] Increases in neuromodulin phosphorylation have been shown to accompany long-term potentiation[17,32,37] and the one-trial passive avoidance learning task in chicks[49] The molecular mechanisms by which neuromodulin

modifies synaptic function in the adult brain, however, are unclear Although neuromodulin is highly concentrated in areas such as the neocortex and hippocampus[8], neuromodulin mRNA is also expressed at high levels in catecholaminergic neurons in rat brainstem[5] This strongly suggests that it is involved in physiological processes, such as neurotransmitter release, signal transduction or other responses of functional plasticity, that regulate the synaptic function of the catecholamines There are several ways in which neuromodulin could alter synaptic function during AMPH sensitization One possible role is modulation of neurotransmitter release Enhanced stimulus-induced dopamine release has been demonstrated in both rat striatum and nucleus accumbens after repeated AMPH treatment Both AMPH- and Ca^{2+}-requiring K^+-stimulated release are increased[6] The increased dopamine releasability is expressed after a single injection of AMPH, is evident only after intermittent dosage regimens, persists for months after discontinuation of AMPH and is strengthened with greater time of withdrawal[46 48] These characteristics are expressed by many behaviors in AMPH-induced sensitization PKC-mediated phosphorylation of neuromodulin has been postulated to play a modulatory role in neurotransmitter release[18 44] Phosphorylation of neuromodulin can be stimulated by depolarization-dependent Ca^{2+} influx, receptor activation or phorbol esters[18 44] and has been correlated with neurotransmitter release from synaptosomes and hippocampal slices[18] In a study in streptolysin-O-permeabilized synaptosomes, however, anti-neuromodulin antibodies, which inhibit neuromodulin phosphorylation by PKC, inhibited depolarization-evoked neurotransmitter release but not phorbol ester-induced neurotransmitter release[11] This result must be interpreted with caution since neuromodulin may not be involved in mediation of release, but may have a role in priming or modulation of release[44] In addition, permeabilized synaptosomes, while retaining some function, may not maintain all functions of an untreated, healthier synaptosome The fact that an AMPH challenge had no further effect on neuromodulin phosphorylation in rats repeatedly treated with AMPH supports the concept that neuromodulin phosphorylation does not mediate the immediate release of dopamine or the immediate expression of behavioral sensitization An AMPH challenge given to rats sensitized to AMPH elicits enhanced dopamine release and an exaggerated behavioral response (see refs 47, 48) Although enhanced release of dopamine may play a role in mediating the behavioral sensitization to AMPH, it is unlikely to be the sole mechanism underlying this phenomenon[29]

Morphological changes in synapses also accompany expressions of synaptic plasticity[4 7 49 57] Uranova et al[57] found an increase in the area of presynaptic terminals and length of postsynaptic density in axo-dendritic and axo-spinous synapses in rat medial prefrontal cortex Similar studies were not performed in striatum Neuromodulin is primarily presynaptic[18] in neurons and associated with a membrane skeleton in cells[36] Although molecular events leading to morphological changes in synapses are not known, the known association of neuromodulin with reactive synaptogenesis[38] and nerve growth suggests it could have a role in this process

Neuromodulin could also affect signal transduction in the neuron through its interaction with Go or its regulation of local CaM concentrations[8] A change in PKC-mediated phosphorylation of neuromodulin may lead to increased available CaM in the neuron In vitro studies have shown that PKC-mediated phosphorylation of neuromodulin on serine 41 leads to a dissociation of CaM, since serine 41 borders the CaM-binding site[1] Although a direct correlation between phosphorylation of serine 41 in neuromodulin and CaM dissociation has not been demonstrated in vivo, CaM has been shown to bind to neuromodulin in membranes[9] We have shown that stimulation of PKC leads to a membrane-to-cytosol redistribution of calmodulin in human neuroblastoma SK-N-SH cells, which contain neuromodulin[34] An increase in CaM in the cell could lead to a rise in activation of various CaM-dependent enzymes An increase in neuromodulin phosphorylation could enhance neurotransmitter release through dissociation of CaM, which would activate CaM-dependent protein kinase II (CaM-kinase II) CaM-kinase II, which has been localized in synaptosomes[15], could increase the phosphorylation of the actin-binding vesicle-associated protein, synapsin I Increased CaM-Kinase II-mediated phosphorylation of synapsin I has been demonstrated to increase neurotransmitter release[21] Phosphorylation of synapsin I releases the vesicle from cytoskeletal constraints and may allow more vesicles to migrate toward the active zone in the neuron We have demonstrated an increase in CaM in Percoll-purified striatal synaptosomes prepared from rats treated with repeated, intermittent, AMPH (Gnegy and Farrell, data not shown)

Our data suggest that AMPH could directly elicit phosphorylation of a 53 kDa that migrated with authentic neuromodulin in a synaptosomal preparation Even at 10 μM AMPH, the effect was modest as compared to that of TPA and high K^+ The mechanism by which this might occur is unknown TPA directly activates PKC and the K^+-induced influx of Ca^{2+}

would increase CaM dissociation and stimulate phosphorylation by PKC AMPH, however, is taken up into the synaptosome through the catecholamine transporter and appears to release cytosolic and vesicular catecholamines through passive diffusion[16] PKC phosphorylation of neuromodulin is primarily by the β-isoform, which is activated by Ca^{2+} and diacylglycerol It is possible that AMPH could either alter ionic fluxes in the synaptosome to cause a change in Ca^{2+} or change enzyme activities such as phospholipases to increase production of diacylglycerol[44] Alternatively, AMPH could release a substance which could then act upon the synaptosome to elicit neuromodulin phosphorylation Specific saturable sequestration sites for AMPH in synaptosomes, especially at higher concentrations such as 10 μM, that may interact with N-acetylaspartate and abolish a transsynaptosomal pH gradient have been reported[61] There could be many, as yet unknown, actions of AMPH in a synaptosome Since neuromodulin is phosphorylated by a protein kinase other than PKC[55], this unknown enzyme could be activated by AMPH Further, although the 53 kDa band migrated with purified neuromodulin, we cannot state unequivocally that the band is neuromodulin at this time It could be another protein comigrating with neuromodulin We are presently conducting experiments to further characterize this effect of AMPH on the 53 kDa band and determine absolutely that it is neuromodulin that is being phosphorylated

In summary, the results of the post hoc phosphorylation assays suggests that both acute, in vivo, AMPH and repeated, intermittent, AMPH lead to an increase in neuromodulin in rat striatal synaptic plasma membranes Thus neuromodulin phosphorylation could contribute to the synaptic changes and plasticity that occur as a result of acute and repeated AMPH treatment and may play some role in behavioral sensitization that develops to AMPH These also demonstrates a similarity in neurochemical events in several models of synaptic plasticity, including long-term potentiation and other models of memory

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