A protein kinase A–dependent molecular switch in synapsins regulates neurite outgrowth

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Cyclic AMP (cAMP) promotes neurite outgrowth in a variety of neuronal cell lines through the activation of protein kinase A (PKA). We show here, using both *Xenopus laevis* embryonic neuronal culture and intact *X. laevis* embryos, that the nerve growth–promoting action of cAMP/PKA is mediated in part by the phosphorylation of synapsins at a single amino acid residue. Expression of a mutated form of synapsin that prevents phosphorylation at this site, or introduction of phospho-specific antibodies directed against this site, decreased basal and dibutyryl cAMP–stimulated neurite outgrowth. Expression of a mutation mimicking constitutive phosphorylation at this site increased neurite outgrowth, both under basal conditions and in the presence of a PKA inhibitor. These results provide a potential molecular approach for stimulating neuron regeneration, after injury and in neurodegenerative diseases.

In neurons, cAMP mediates diverse functions, including neurite outgrowth[1], neuronal differentiation[2] and survival[3,4]. The cAMP pathway also modulates axon guidance by diffusible factors, including neurotrophins[5], netrin-1[6] and inhibitory factors associated with myelin[7]. In addition, a cAMP-mediated pathway has been implicated in axon regeneration[8]. Almost all of the effects of cAMP are due to the activation of PKA[9]. Despite the well-known effects of cAMP signaling, the specific substrates involved in neurite outgrowth have remained elusive.

The most abundant substrates for PKA in adult neurons are synapsins, a family of neuronal phosphoproteins identified almost three decades ago[10] that regulate two distinct cellular functions: neuronal development and synaptic transmission. Synapsins are major components of mature nerve terminals, where they are highly enriched on small synaptic vesicles, and play an essential role in synaptic transmission[11]. They are also involved in neuronal development, although their level of expression is considerably lower in developing neurons than in mature neurons[12]. Overexpression of synapsins leads to presynaptic differentiation in cell lines[13,14] and accelerated synaptogenesis in embryonic cell cultures[15,16], whereas reduction of synapsins leads to delayed neuronal differentiation, axonal outgrowth and synaptogenesis[17–19]. All three synapsin genes (I, II and III) appear to be important for neurite outgrowth *in vitro*[19]. The molecular basis for these developmental actions is largely unknown. Because synapsins are excellent substrates for PKA[10] and play an important role in neuronal development, we hypothesized that they might be involved in mediating cAMP-dependent neurite outgrowth. We show here that PKA-dependent regulation of neurite outgrowth is mediated in large part through phosphorylation of synapsins at a single site, which acts as a molecular switch to regulate neurite outgrowth.

A single phosphorylation site for PKA, termed phosphorylation site 1 (P-site 1), is conserved at the amino-terminal region of all vertebrate synapsin isoforms examined to date[20,21]. P-site 1 in vertebrate synapsin I also serves as a substrate for Ca^{2+}/calmodulin-dependent protein kinase I[22]; however, there have been no reports indicating a role for this kinase in neurite outgrowth. P-site 1 is contained within a short region of conserved amino acids of 29 or 30 residues known as domain A (**Fig. 1a**). Domain A is among the most conserved regions of the synapsin molecule, with 90% of the residues identical in organisms as diverse as lamprey, *X. laevis* and mammals[20].

The use of *X. laevis* embryonic neurons in culture and of intact *X. laevis* embryos provided a useful system in which to dissect the molecular mechanisms of synapsin-dependent neurite

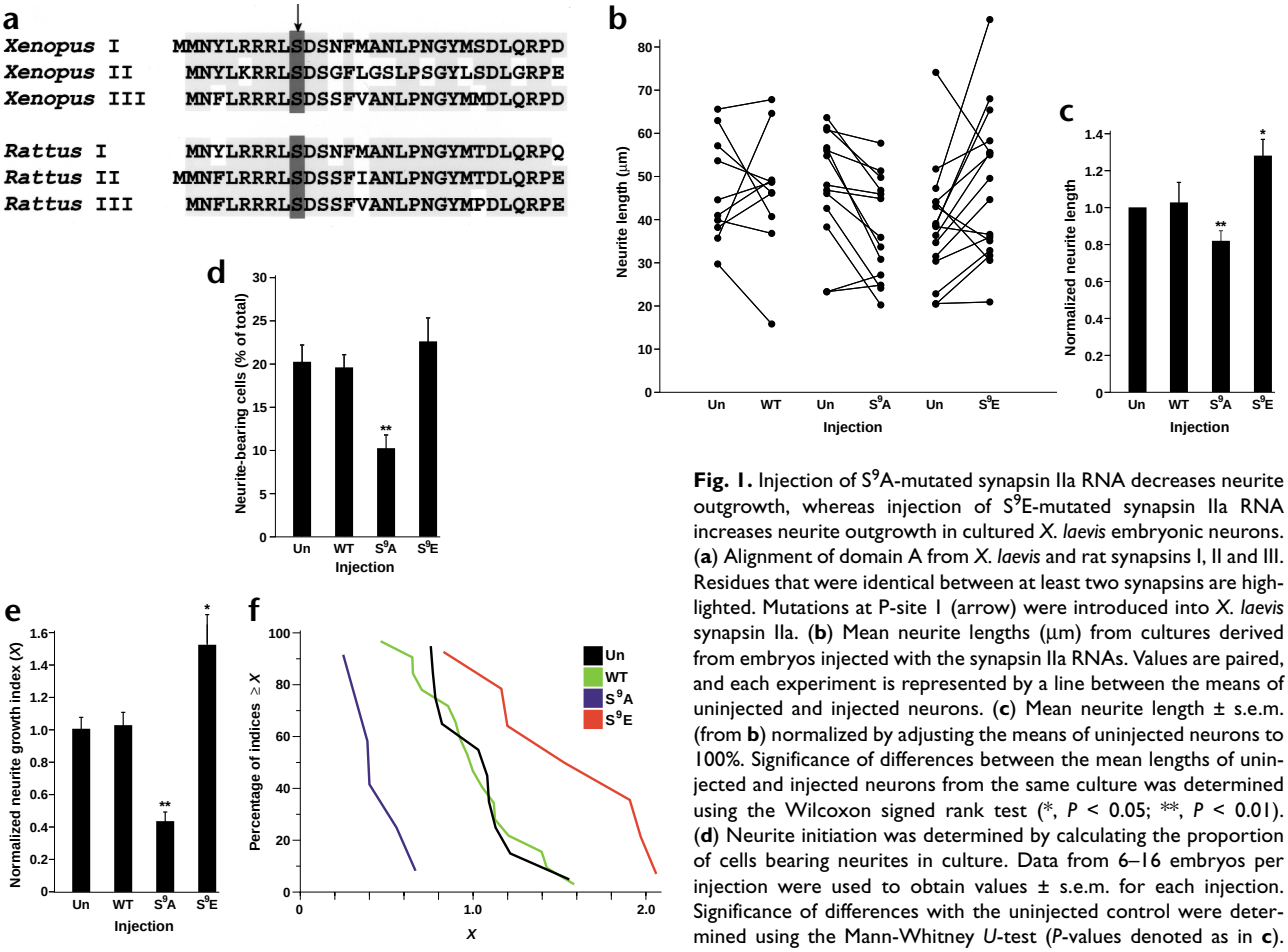

Fig. 1. Injection of S[9]A-mutated synapsin IIa RNA decreases neurite outgrowth, whereas injection of S[9]E-mutated synapsin IIa RNA increases neurite outgrowth in cultured *X. laevis* embryonic neurons. (**a**) Alignment of domain A from *X. laevis* and rat synapsins I, II and III. Residues that were identical between at least two synapsins are highlighted. Mutations at P-site 1 (arrow) were introduced into *X. laevis* synapsin IIa. (**b**) Mean neurite lengths (μm) from cultures derived from embryos injected with the synapsin IIa RNAs. Values are paired, and each experiment is represented by a line between the means of uninjected and injected neurons. (**c**) Mean neurite length ± s.e.m. (from **b**) normalized by adjusting the means of uninjected neurons to 100%. Significance of differences between the mean lengths of uninjected and injected neurons from the same culture was determined using the Wilcoxon signed rank test (*, $P < 0.05$; **, $P < 0.01$). (**d**) Neurite initiation was determined by calculating the proportion of cells bearing neurites in culture. Data from 6–16 embryos per injection were used to obtain values ± s.e.m. for each injection. Significance of differences with the uninjected control were determined using the Mann-Whitney *U*-test (*P*-values denoted as in **c**). (**e**) A single normalized parameter *X*, the neurite growth index, defined as the product of neurite initiation and neurite length[25], was used to depict the overall effect on neurite outgrowth (shown as mean ± s.e.m.; significance of differences with the uninjected control was determined using the Mann-Whitney *U*-test, *P*-values are denoted as in **c**). (**f**) Distribution curve depicting range of normalized neurite growth indices for each of the indicated injections.

outgrowth. We have identified three *X. laevis* synapsin orthologues corresponding to the three known mammalian synapsin genes (I, II and III)[20]. *X. laevis* synapsins are also multiply spliced to give rise to several isoforms that are similar to their mammalian counterparts[23,24]. We show here that the well-known ability of cAMP to regulate neurite outgrowth is mediated in part by PKA-dependent phosphorylation of synapsins at a single residue in the N-terminal domain.

Results

We first tested whether P-site 1 was involved in regulating neurite outgrowth in cultured embryonic *X. laevis* spinal neurons. Mutations were introduced into P-site 1 in *X. laevis* synapsin IIa, and the mutated and wild-type synapsins were overexpressed in *X. laevis* neurons. The mutation S[9]A precludes phosphorylation at Ser 9, whereas the mutation S[9]E was used as a means to mimic constitutive phosphorylation at P-site 1. A mixture of RNAs encoding green fluorescent protein (GFP) and synapsin was injected into one cell of two-cell-stage embryos. GFP was a reliable marker for neurons expressing exogenous synapsin in dissociated neural tube cultures 24–48 hours after injection, as >95% of cells expressing GFP also stained for the presence of synapsin. GFP-negative (uninjected) neurons, therefore, served as a control for GFP-positive (injected) neurons in the same *X. laevis* culture. Endogenous synapsins are first detected in 2-day-old embryos, and are present at low levels in these cultures at the times examined (data not shown).

Two parameters of neurite outgrowth were initially measured: neurite length and neurite initiation. *X. laevis* spinal cultures consist of a heterogeneous population of neurons derived from different regions of the developing neural tube. Therefore, neurite lengths varied among neurons from the same culture and from culture to culture. Neurons injected with S[9]A-mutated synapsin IIa RNA showed significantly decreased neurite lengths compared to their uninjected counterparts (**Fig. 1b**). In contrast, neurons injected with S[9]E-mutated synapsin IIa RNA had increased neurite lengths, whereas neurons injected with wild-type synapsin IIa neurons showed no change (**Fig. 1b**,**c**). Similar results were observed when neurite lengths from uninjected and injected neurons were pooled for all experiments (data not shown). The proportion of neurite-bearing cells, a measure of neurite initiation, was relatively constant from experiment to experiment for a specific injection (**Fig 1d**). Neurons injected with S[9]A-mutated synapsin IIa RNA had significantly decreased neurite initiation, whereas neurons injected with wild-type or S[9]E-mutated synapsin IIa RNA did not show a significant change. To obtain a single overall measure of neurite outgrowth, the neurite growth index, defined as the product of neurite initiation and neurite length for each culture[25], was used here and in subsequent experiments. The neurite growth index was markedly decreased in neurons injected with

synapsin IIa RNA bearing the mutation S^9A (**Fig. 1e–f**); thus, S^9A acts as a dominant negative mutation of P-site 1. In contrast, the neurite growth index was significantly increased in neurons injected with S^9E-mutated synapsin IIa RNA. Thus, constitutive dephosphorylation of P-site 1 leads to decreased neurite outgrowth, whereas constitutive phosphorylation leads to increased outgrowth (**Fig. 1e–f**). Injection of wild-type synapsin IIa RNA did not result in a significant change in neurite outgrowth of progeny neurons derived from the injected blastomeres (**Fig. 1e–f**), suggesting that the phosphorylation state, rather than the quantity of synapsin IIa, was a factor in regulating neurite outgrowth.

To extend our observations, we examined the effects of phosphorylation *in vivo* by measuring nerve growth in intact *X. laevis* embryos. Embryos injected unilaterally with exogenous synapsin RNA at the two-cell stage were subjected to whole-mount immunocytochemistry[26] and analyzed by laser scanning confocal microscopy for the growth of spinal nerves. Optical sections were made through the embryo and nerve growth on the two sides of the embryo compared. Spinal nerves were clearly identifiable after stage 30, and staining was performed at stage 31–32, which corresponds to the developmental stage of the *X. laevis* cultures we had assessed previously (**Fig. 1**). Spinal nerves were revealed by staining with a monoclonal antibody against the synaptic vesicular protein SV2 (ref. 27), and the pattern of immunoreactivity was similar to that found for synapsin in stage 32 embryos (**Fig. 2a**). Embryos injected with wild-type or S^9E-mutated synapsin IIa RNA (**Fig. 2b**) did not show any gross morphological changes as compared to uninjected controls. However, embryos injected with S^9A-mutated synapsin IIa RNA frequently exhibited a shorter antero-posterior length (**Fig. 2b**) and were sometimes curved inward on the injected side. Injection of S^9A-mutated synapsin IIa RNA resulted in shorter or thinner cranial nerves (**Fig. 2d**) and shorter spinal nerves in the thorax and abdominal area (**Fig. 2e–f**). In contrast, injection of S^9E-mutated synapsin IIa RNA resulted in more pronounced nerve development on the injected side (**Fig. 2d–f**). These changes were also revealed by using cross-sectional projections of nerves after serial reconstruction (data not shown), and represented marked differences from nerve growth observed in embryos injected with wild-type synapsin IIa (**Fig. 2e–f**), uninjected (**Fig. 2f**) or injected with GFP RNA alone (data not shown). Furthermore, the changes were observed in nerves derived from the head, thorax and abdominal regions, indicating that much of the nervous system was affected by the injections. These *in vivo* results are consistent with the observations made in cell culture.

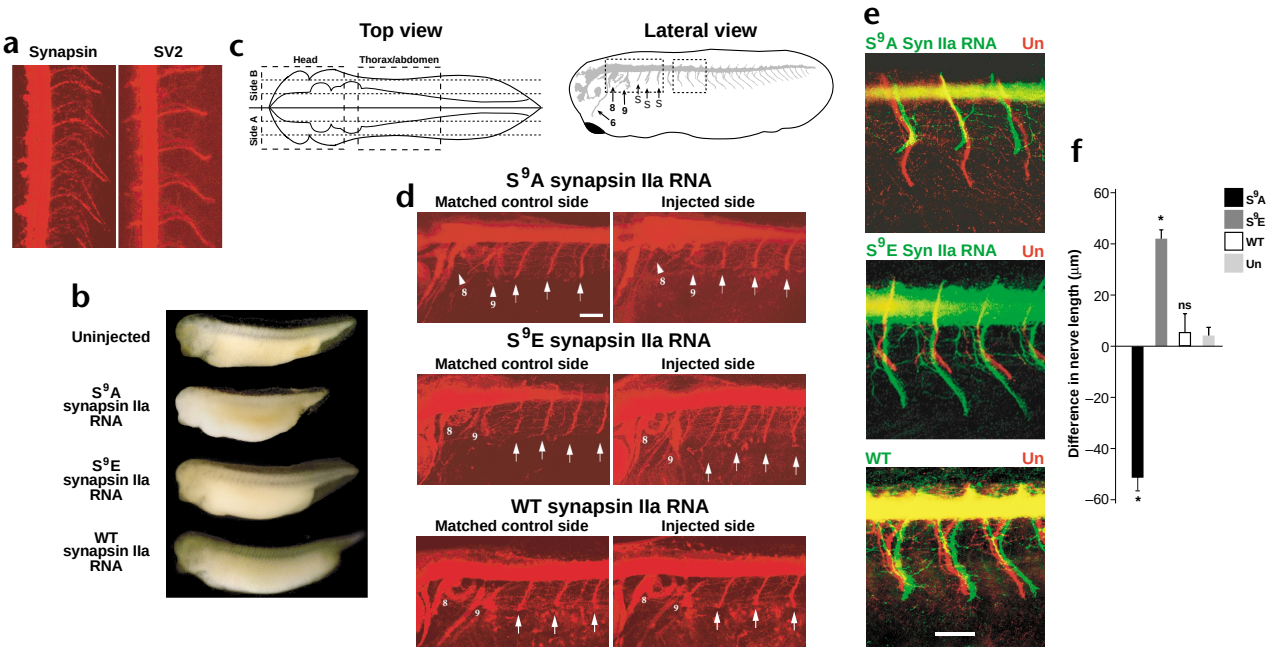

Fig. 2. Injection of S^9A-mutated synapsin IIa RNA decreases nerve growth, whereas injection of S^9E-mutated synapsin IIa RNA increases nerve growth in intact *X. laevis* embryos. Whole-mount immunocytochemistry was performed on uninjected and injected stage 31–32 embryos[26]. (**a**) Thoracic spinal nerves stained with anti-synapsin antibody G304 or with anti-SV2 monoclonal antibody. (**b**) Gross morphology of typical, bleached embryos injected with wild-type (WT) or mutated synapsin IIa RNA (S^9A or S^9E) or uninjected. (**c**) Diagram indicating the area scanned for lateral visualization of cranial and spinal nerves. Top view, optical sectioning from one side to the other; lateral view, region scanned. To ensure that embryos were flat during the scan, images from each side were checked for alignment using cranial nerves and retinas as landmarks. (**d**) Images of cranial and cervical spinal nerves derived from embryos injected on one side at the two-cell stage with wild-type or mutated *X. laevis* synapsin IIa RNA (boxed region in **c**). Each image is the summation of a Z-stack of optical slices spaced 4 μm apart from each side of the embryo (as depicted in **2c**). Cervical spinal nerves (arrows) and cranial nerves 8 and 9 are shown for each side of embryos injected unilaterally with wild-type (WT) synapsin IIa RNA or synapsin IIa RNA bearing the mutation S^9A or S^9E. Bar, 100 μm (applies to all frames). (**e**) Images of spinal nerves from the thoracic and abdominal area derived from embryos injected on one side at the two-cell stage with wild-type or mutated *X. laevis* synapsin IIa RNA. Each image is the summation of a Z-stack of optical slices spaced 4 μm apart from each side of the embryo. Green (pseudocolor), spinal nerves from injected side; red (pseudocolor), nerves from uninjected side (Un). Bar, 100 μm (applies to all frames). (**f**) Differences in nerve lengths (μm), as visualized from the two-dimensional lateral projection only. In embryos injected with wild-type (WT) synapsin IIa RNA (9 embryos) or synapsin IIa RNA bearing the mutation S^9A (7 embryos) or S^9E (10 embryos), nerve length differences (injected minus uninjected side, or left minus right side for controls) contrasted markedly from uninjected (Un) controls (14 embryos). For each embryo, three or four nerves were measured on each side. *P*-values (Student's *t*-test) represent comparisons with length difference of uninjected nerves (*, $P < 0.0001$).

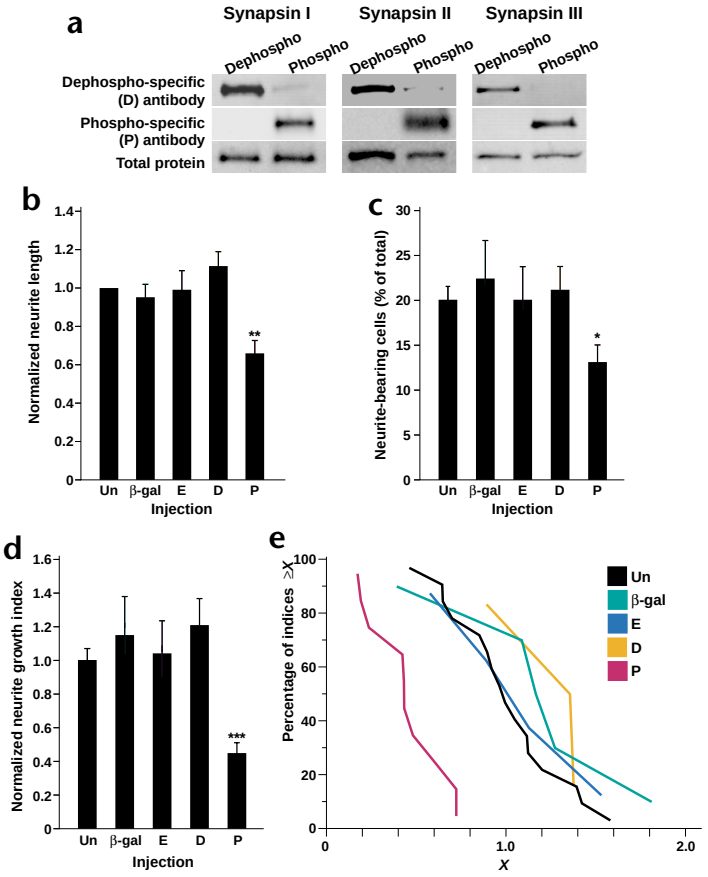

Fig. 3. Injection of phospho-specific P-site 1 antibodies decreases neurite outgrowth, whereas injection of dephospho-specific P-site 1 antibodies has no effect in cultured *X. laevis* embryonic neurons. (**a**) Phosphorylated or dephosphorylated recombinant fusion proteins containing P-site 1 of synapsins I, II and III were immunoblotted with the indicated antibodies. Relative levels of total protein were revealed by incubation with a marker that binds to polyhistidine, because the recombinant proteins contain a polyhistidine tag. Dephospho-specific antibodies recognize a small amount of protein in the lane with phosphorylated protein due to incomplete phosphorylation of the protein. (**b**) Normalized neurite length ± s.e.m. for uninjected neurons (Un) or neurons injected with anti-β-galactosidase (β-gal), domain E (E), P-site 1 dephospho-specific (D) and P-site 1 phospho-specific (P) antibodies. (**c**) Neurite initiation ± s.e.m. for each injection. (**d**) Neurite growth index ± s.e.m. for each injection. For (**b**–**d**), the Kruskal-Wallis test indicated strongly that at least one group of measurements differed significantly from the rest for neurite length, initiation or the growth index ($P < 0.001$). Significance of differences from the uninjected control (Mann-Whitney *U*-test) is indicated by asterisks: *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$. (**e**) Distribution curve comparing the normalized neurite growth index from neurons that were uninjected, injected with dephospho-specific antibody and injected with phospho-specific antibody.

The findings obtained using RNA injections were corroborated through injection of phosphorylation state–specific antibodies. Antibodies were generated against either phospho or dephospho peptides corresponding to P-site 1 of synapsins I, II and III[21,28]. The dephospho-specific antibodies detected the dephospho forms of all three synapsins and the phospho-specific antibodies detected the phospho forms of all three synapsins (**Fig. 3a**). Phospho- and dephospho-specific antibodies were co-injected with fluorescein isothiocyanate (FITC)–dextran, as a tracer, into one cell of a two-cell embryo, and neuronal cultures were made from stage 21–23 embryos. FITC–dextran was a reliable marker, as 98% of all neurons that contained fluorescein also stained for the presence of injected antibodies. The cultures were fixed 14 hours after plating and analyzed for neurite outgrowth.

Injection of phospho-specific antibodies resulted in a decrease of neurite length, neurite initiation and neurite growth index (**Fig. 3b–e**). Another antiserum, G-257 (ref. 28), which recognizes phosphorylated P-site 1 of all three synapsins, gave similar results (data not shown). In contrast, dephospho-specific antibodies had no significant effect (**Fig. 3b–e**). Because these antibodies are not subtype specific, this effect may not be restricted to a particular synapsin. Injection of an irrelevant antibody (anti-β-galactosidase), as control, had no effect on neurite outgrowth (**Fig. 3b–e**). In addition, anti-synapsin antibodies directed against a different region of the molecule, domain E, had no significant effect on neurite outgrowth (**Fig. 3b–e**). These findings indicate that phosphorylation of P-site 1 is required for stimulation of neurite outgrowth, whereas dephosphorylated P-site 1 does not play a role in neurite outgrowth. Taken together, these results are consistent with the RNA injection experiments and clearly demonstrate that the phosphorylation of a single site, P-site 1, can act as a molecular switch.

To test the possibility that synapsins are major effector molecules regulating PKA-dependent neurite outgrowth, we determined the degree to which synapsins account for the known effects of cAMP signaling on neurite outgrowth. Neurite outgrowth was measured in cultured neurons injected with phospho-specific antibodies or S^9A-mutated synapsin IIa RNA, to interfere with the phosphorylation of P-site 1, and then treated with a selective activator of PKA, dibutyryl cAMP. Dibutyryl cAMP increased neurite outgrowth of uninjected neurons in culture (**Fig. 4a–c**). Injection of phospho–P-site 1 antibodies abolished the neurite-promoting effects of dibutyryl cAMP (**Fig. 4a–c**). Injection of S^9A-mutated synapsin IIa RNA also significantly inhibited the promoting effects of cAMP on neurite outgrowth (**Fig. 4a–c**). In contrast, injection of wild-type synapsin IIa RNA, which possesses an intact P-site 1, had no significant effect on the neurite-promoting effects of dibutyryl cAMP (**Fig. 4a–c**). Thus, inhibition of P-site 1 by phospho-specific antibodies, or by S^9A-mutated synapsin IIa, either abolished or markedly decreased neurite outgrowth in response to PKA activation. These observations support a model in which PKA stimulates neurite outgrowth predominantly by phosphorylating P-site 1.

To further test the hypothesis that P-site 1 is the major regulator of neurite outgrowth via PKA, neurite outgrowth was measured in cultured neurons injected with S^9E-mutated synapsin IIa RNA, which mimics constitutive phosphorylation, and then treated with a selective inhibitor of PKA, KT5720[29]. If this hypothesis is correct, constitutive phosphorylation of P-site 1 should be sufficient to overcome the neurite-inhibiting effects of PKA inactivation. As expected, KT5720 had actions that were opposite to those of dibutyryl cAMP, decreasing neurite outgrowth in uninjected neurons and in wild-type neurons injected with synapsin IIa RNA (**Fig. 4d–f**). In neurons injected with S^9E-mutated synapsin IIa RNA, neurite outgrowth was stimulated both in the absence and in the presence of KT5720 (**Fig. 4d–f**). Some inhibition by KT5720 was still present in neurons injected with S^9E-mutated synapsin IIa RNA, probably due

to endogenous synapsins. Thus, the phosphorylation state of P-site 1, a phosphorylation site that is conserved in all vertebrate synapsins, largely accounts for the effects of cAMP in promoting the early phase of neurite outgrowth.

Discussion

In several biological systems, synapsins have been shown to have various roles in neuronal development, including differentiation, neurite outgrowth and synaptogenesis. All of these studies used embryonic cell lines to examine the effects of overexpressing or abolishing the expression of synapsins, and the effects were most apparent at an early phase of neuronal development. The present study is the first to show that synapsins do have effects that are readily discernible in an intact animal. Because dissociated cell cultures may have alterations in the regulatory signals that normally allow neurons to develop, the demonstration that synapsins have potent effects on nerve growth in an intact animal indicates that synapsins are an integral regulatory component of normal neurite development.

Synapsins are avid substrates for multi-site phosphorylation by a number of protein kinases, including PKA[21], CamKII[21,30], MAP kinase[31,32] and Cdk5[33]. The sequence surrounding P-site 1, which is highly conserved, attracted our attention as a potential regulatory site that has co-evolved with other regulatory molecules. In contrast, the primary sequences surrounding the other known phosphorylation sites are less conserved or are absent among synapsins. The role of cAMP and PKA in neuronal differentiation has been well documented, and synapsins, the most abundant substrates for PKA, are attractive candidates as mediators of the actions of PKA. Here, we show that a single site in synapsins, P-site 1, accounts for much of the effects of cAMP and PKA in promoting neurite growth. Because P-site 1 is so well conserved, it is likely that the phosphorylation-dependent mechanisms by which P-site 1 operates have been maintained among vertebrates.

We used a variety of molecular approaches to show that P-site 1 of synapsins regulates neurite outgrowth. These included injection of mutated forms of synapsin IIa followed by examination of cultures or intact embryos, injection of phosphorylation state–specific antibodies and manipulation of PKA activity using pharmacological agents. We measured two closely related parameters of neurite outgrowth in cultured neurons: neurite length and neurite initiation. Synapsins, as well as a variety of other molecules, such as neurofilaments[34], GAP43[35] and tau[36], affect both neurite length and initiation in culture, suggesting that these two processes have overlapping molecular mechanisms. However, quantification of these processes in cell culture showed that they were not uniform: pharmacological agents that stimulate PKA preferentially promoted neurite length, whereas agents that inhib-

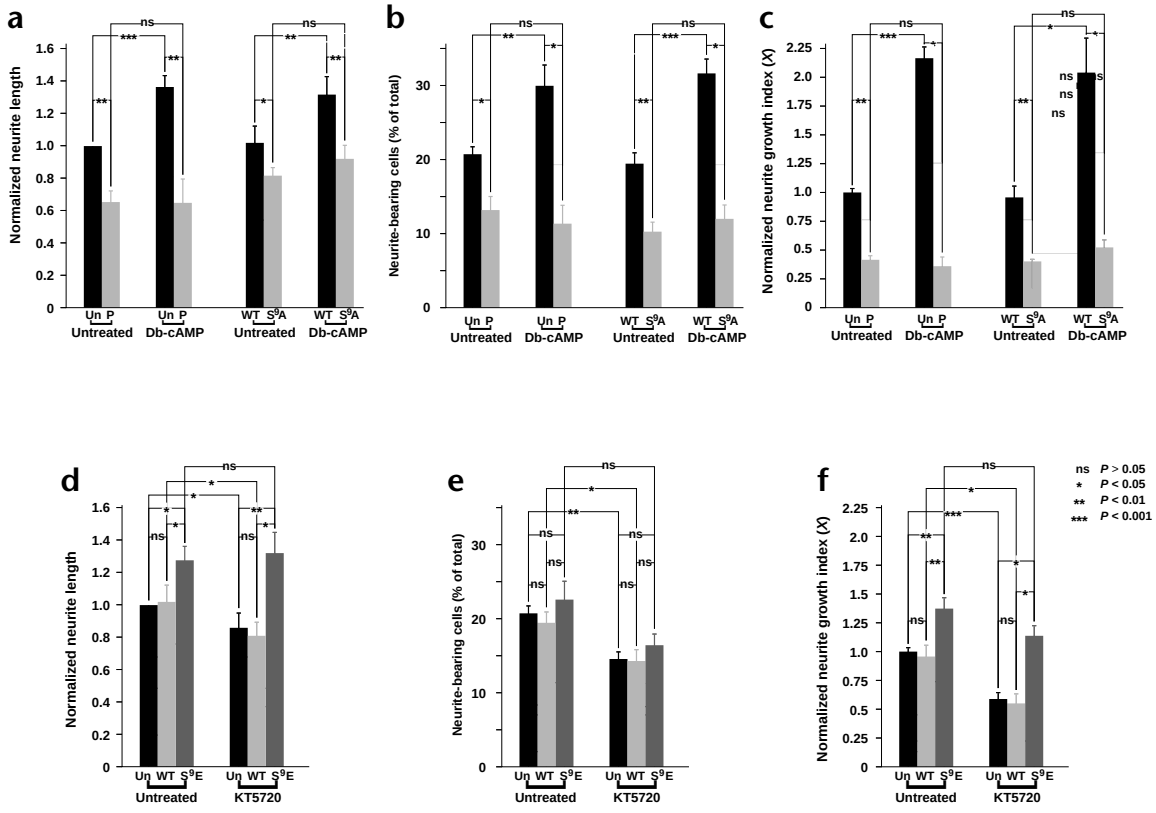

Fig. 4. PKA stimulates early neurite outgrowth predominantly by phosphorylating P-site 1. *X. laevis* neuronal cultures derived from injected embryos were grown in the presence of (**a**–**c**) a PKA activator (3 mM dibutyryl cAMP (Db-cAMP)) or (**d**–**f**) a PKA inhibitor (1 mM KT5720). Embryos were either uninjected (Un) or injected with phospho-specific antibodies (P), wild-type synapsin IIa RNA (WT) or mutated synapsin IIa RNA (S^9A or S^9E). Analyses were performed 14 h after plating. The normalized neurite length (**a**,**d**), neurite initiation (**b**,**e**) and neurite growth index (**c**,**f**) were determined (mean ± s.e.m; each measurement represents the mean derived of at least three different experiments). At least one group of measurements differed significantly from the rest for neurite length, initiation or growth index (Kruskal-Wallis test, $P < 0.0005$). For neurite length, the Wilcoxon signed rank test was used to determine significance for pairwise comparisons to the untreated, uninjected control (which was normalized to 1 with no standard error bars). For other comparisons of neurite length, the Wilcoxon rank-sum test was used. For comparisons of neurite initiation and the neurite growth index, significance was determined using the Mann-Whitney *U*-test as indicated.

it PKA preferentially inhibited neurite initiation (**Fig. 4**). This pattern of neurite outgrowth was also reflected by the injection of phospho-mutated synapsin IIa RNA (**Fig. 1**). We therefore calculated an additional parameter, the neurite growth index, that provides a single measure of neurite outgrowth per cell culture. We also provided quantification for nerve lengths in intact animals, where conditions for nerve growth are not subject to the variables created in cell culture. The various approaches yielded consistent results and, taken together, provide considerable evidence that phosphorylation of P-site 1 promotes neurite outgrowth through a PKA-dependent mechanism.

In addition to the signal transduction pathway mediated by PKA described here, several other signal transduction pathways regulate neurite outgrowth. For example, regulation of neurite outgrowth via PKC[37], calcium[37], MAP kinase[37] and Cdk5[38] has been described. The precise molecular mechanisms by which neurite outgrowth is regulated by each pathway remain to be elucidated. We have shown that synapsins appear to play a prominent role as a PKA-dependent switch during the early phase of neurite outgrowth, but it is likely that other molecules are involved as well.

The mechanism by which phosphorylation of synapsin at P-site 1 mediates the effects of cAMP is not known. Synapsins are distributed widely in the developing neuron and become concentrated at nerve terminals in mature neurons. Previous studies have shown that synapsins interact with actin filaments and synaptic vesicles[30]. In developing neurons, interaction of synapsin with actin filaments may regulate actin polymerization[39,40], a process intimately linked to neurite initiation. Alternatively, the phosphorylation state of P-site 1 may regulate the binding of synapsins to post-Golgi membrane precursor vesicles, which in turn may determine the rate of new membrane insertion during neurite outgrowth. In addition, synapsins are ATP-binding proteins[41,42], and may possess enzymatic activity[43] that in turn is regulated by P-site 1.

The present study points out the importance of synapsin P-site 1 in early neural development as a molecular switch operating through PKA-dependent protein phosphorylation. As several signal transduction pathways have been implicated in the regulation of neurite outgrowth, it is of interest that manipulation of a single pathway by alteration of a single amino acid was sufficient to cause overall changes in nerve growth in an intact animal. These results suggest that targeting specific molecules to affect changes in nerve growth is a feasible objective in designing pharmacological agents. It has recently been shown that the neuronal plasticity of the embryonic nervous system also exists, to a degree, in adult nervous systems[44]. Thus, an understanding of the mechanism by which P-site 1 acts may provide a molecular approach for stimulating neurons to regrow in neurodegenerative diseases and after injury.

Methods

Neuronal cultures and measurement of neurite outgrowth. Adult female *X. laevis* were induced to ovulate and their eggs fertilized[45]. Staging was performed as described[26]. After the embryos developed to stage 21–23, the dorsal regions of the embryos were dissected in Steinberg's solution (4.6 mM Tris, pH 7.8, 58 mM NaCl, 0.67 mM KCl, 0.44 mM $Ca(NO_3)_2$, 1.3 mM $MgSO_4$) and incubated in 1 mg/ml collagenase (type 1A, Sigma, St. Louis, Missouri) dissolved in Steinberg's solution for 1 h at room temperature. Neural tubes were then dissected from the dorsal regions, dissociated and plated onto clean glass coverslips (Carolina Biological Supply, Burlington, North Carolina) in culture medium as described[45]. After 14 h, neuronal cultures were fixed in 4% paraformaldehyde in PBS for 15 min, washed in PBS and viewed in an upright epifluorescent Leitz (Leica Microsystems, Wetzlar, Germany) Aristoplan microscope. The cultures were highly enriched for neurons, as the large majority of cells (>80%) were immunoreactive for neurofilament protein 14 h after plating (data not shown). Approximately half the cells or neurons in each experiment are expected to be derived from the side of an embryo previously injected unilaterally at the two-cell stage. Injected neurons showed a range of fluorescence. The degree of fluorescence was quantified using the 'threshold' tool in Adobe Photoshop (http://www.adobe.com). Neurons were at least two standard deviations brighter than their uninjected counterparts before they were scored as injected. Neurite lengths >5 μm were used in the measurement of neurite length and neurite initiation. Phase-contrast and fluorescent digital images of neurons from a Leitz Aristoplan microscope were captured using the MDS120 (Microscopy Documentation System version 120, Kodak, Rochester, New York). Images were assembled and neurites traced using Adobe Photoshop. Measurements were made using NIH Image 1.61 (http://rsb.info.nih.gov/nih-image). Statistical analyses were performed using Statview (http://www.statview.com).

For the data presented here, neurite length was depicted as the average ± s.e.m. of the mean of injected neurite lengths divided by the mean of uninjected neurite lengths from several cultures. Thus, neurite length was normalized to uninjected counterparts, and one measurement was used per culture. Qualitatively similar data were obtained using individual neurite lengths pooled from all cultures. Neurite initiation was quantified by determining the percentage of neurofilament-positive cells that possessed neurites, and was determined for progeny cells derived from the uninjected or the injected blastomere. The neurite growth index was defined as the product of neurite length and neurite initiation, and was analogous to a previous definition[25]. The neurite growth index was calculated by multiplying the neurite initiation of each culture by the corresponding mean normalized neurite length for a particular injection. Neurite growth indices were normalized by adjusting the index obtained with uninjected cultures to 1. Significance between paired means of injected and uninjected, untreated neurite lengths was determined using the Wilcoxon signed rank test. Significance between unpaired means of neurite lengths was determined using the Wilcoxon rank-sum test. The Kruskal-Wallis test was used to determine if values for neurite initiation or the neurite growth index differed significantly from the rest of the group. The Mann-Whitney *U*-test was used to determine if there was a significant difference between two groups of neurite initiation or neurite growth index values.

Injections. Fertilized embryos at the two-cell stage were injected unilaterally using the Eppendorf microinjector 5242 (Eppendorf, Hamburg, Germany). For RNA injections, a 1:1 mixture of synapsin RNA (2–3 μg/μL) and GFP RNA (2–3 μg/μL) was injected into embryos. Mutated synapsin II RNAs (S^9A or S^9E) were derived from the synapsin IIa isoform. For antibody injections, a mixture of FITC–dextran (50 mg/ml final concentration; mol. wt. 10,000; Sigma) and antibodies (final concentration 1.0–1.5 μg/μl) was injected. RNA-injected progeny neurons were identified by GFP fluorescence and antibody-injected progeny neurons were identified by FITC fluorescence.

Whole-mount immunocytochemistry. Embryos at stage 31–32 were fixed, blocked and incubated with primary and secondary antibodies as described[26]. Embryos were placed in chamber slides, covered with coverslips and visualized using the Zeiss LSM510 laser scanning confocal microscope (10× objective). Cranial and spinal nerves were visualized using the monoclonal antibody (SV2) developed by K. M. Buckley[27], which was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa, Department of Biological Sciences (Iowa City). For whole-mount immunocytochemistry, the primary antibody consisted of a 1:1 dilution of anti-SV2 monoclonal antibody supernatant and a 1:100 dilution of rabbit anti-GFP antibodies (Santa Cruz Biotechnology, Santa Cruz, California). The secondary antibodies consisted of Cy5-conjugated donkey anti-mouse and Cy2-conjugated donkey anti-rabbit antibodies (1:200 dilution; Jackson ImmunoResearch, West Grove, Pennsylvania).

Phosphorylation state–specific antibodies. Antiserum G-143 was generated as described[28] against the dephospho form of the synapsin I/III

peptide YLRLSDSNF-amide. Antisera, designated RU442 and RU440, were generated against the dephospho and phospho forms, respectively, of the synapsin II peptide YLKRRLSDSGFLC. Synapsin II peptides were coupled to *Limulus* hemocyanin and injected into rabbits, and the resulting antisera were affinity purified using the same peptide coupled to SulfoLink resin (Pierce, Rockford, Illinois).

Phosphorylated or dephosphorylated recombinant fusion proteins containing domain A of synapsins I, II and III were used to test the specificity of these antibodies. G-143 recognized dephosphorylated domain A from synapsins I and III, whereas RU442 recognized only dephosphorylated domain A from synapsin II (data not shown). Therefore, a combination of G-143 and RU442 was used in all experiments that involved dephospho-specific antibodies. RU440 recognized phosphorylated domain A of all three synapsins, and was used in all experiments presented here that involved phospho-specific antibodies. Dephospho-specific and phospho-specific antibodies recognize the same set of proteins of the expected molecular weight for synapsins in *X. laevis* neural tissue (data not shown).

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Competing interests statement

The authors declare that they have no competing financial interests.

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