

## Long-term increases in neurotransmitter release from neuronal cells expressing a constitutively active adenylate cyclase from a herpes simplex virus type 1 vector

(adenylate cyclase/cAMP/neuronal physiology)

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**ABSTRACT** Signal-transduction pathways mediate a wide range of short-term changes in the physiology of neuronal systems from invertebrates to mammals. However, examples of long-term changes in neuronal physiology mediated by these pathways have been limited to invertebrate systems. In this report, long-term changes in the physiology of mammalian neurons were studied by using genetic intervention to cause a long-lasting activation of the cAMP pathway. The catalytic domain of yeast adenylate cyclase (*cyr*), encoding a constitutive enzyme activity, was expressed in neuronal cells infected with a defective herpes simplex virus vector (pHSVcyr). In PC-12 cells infected with pHSVcyr, increases were seen in cAMP levels, protein kinase A activity, protein phosphorylation, phosphorylation of the tyrosine hydroxylase protein kinase A site (Ser<sup>40</sup>), and catecholamine release. Infection of sympathetic neurons with pHSVcyr increased cAMP levels, protein phosphorylation, and catecholamine release. Yeast adenylate cyclase immunoreactivity and elevated cAMP levels were localized to the cell bodies of sympathetic neurons. The increase in neurotransmitter release was both Ca<sup>2+</sup>- and activity-dependent and persisted for at least 1 week after infection of the sympathetic neurons, suggesting that sustained physiological activation of the cAMP pathway may mediate long-term changes in the neuronal physiology of mammalian systems.

Signal-transduction pathways are known to mediate short-term changes in mammalian neuronal function by modulating the activities of specific neurotransmitter (NT) receptors, voltage-gated ion channels, and components of the NT-release machinery (1, 2). In invertebrates, these same pathways mediate both short-term and long-term changes in nervous system function (3, 4). Thus, signal-transduction pathways may also mediate the changes in mammalian neuronal physiology that are responsible for long-term alterations in the strengths of synaptic connections.

The cAMP pathway mediates both short-term and long-term changes in invertebrate nervous system function associated with certain behavioral adaptations (4). Short-term changes include presynaptic facilitation of the gill withdrawal reflex in *Aplysia*, wherein a stimulus activates the cAMP pathway, which enhances NT release (3). The role of the cAMP pathway in mediating long-term changes in invertebrate nervous system function is illustrated by a mutation in a cAMP phosphodiesterase, *dunce*, that affects associative learning in *Drosophila* (4). The capability of the cAMP pathway to mediate long-term changes in invertebrate neuronal physiology suggests that this same pathway might function similarly in mammalian neuronal physiology.

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Signal-transduction pathways, including the cAMP pathway, mediate a wide range of short-term changes in mammalian neuronal physiology (1, 2). Substantial evidence supports the involvement of these pathways in mediating or modulating NT release (5, 6). In particular, the cAMP pathway can affect short-term changes in NT release from many neuronal cell types, including PC-12 cells and sympathetic, striatal, and cortical neurons (7-9). Most effects of the cAMP pathway are mediated by protein kinase A (PKA)-dependent phosphorylation, which can modulate the activity of specific NT receptors, voltage-gated ion channels, cytoskeletal proteins, NT-synthesizing enzymes, synaptic vesicle proteins, and other neuronal proteins (1, 2).

The evidence that signal-transduction pathways effect long-lasting changes in mammalian neuronal function is less direct. Transcription of specific neuronal genes can be regulated by the cAMP and other signal-transduction pathways (10). Physiological activity (action potentials) can elicit a complex transcriptional response mediated by *c-fos* and *c-jun* (10). The stable maintenance of long-term potentiation, a strengthening of synaptic connections due to stimulation of certain afferent pathways, appears to require activation of Ca<sup>2+</sup>-regulated protein kinase(s) (11). However, delineation of the molecular events in these pathways has been hindered by the short-term nature and the limited cellular and biochemical specificity of the pharmacological agents used.

We have developed a genetic approach to determine the ability of signal-transduction pathways to effect long-lasting changes in mammalian neuronal function. In this approach, the catalytic domains of signal-transduction enzymes are expressed in neurons. These unregulated enzymes cause long-lasting and direct activation of particular signal-transduction pathways. Alterations in specific aspects of the neuronal physiology in these genetically altered cells are then evaluated (for review, see ref. 12). The present study used this approach to cause a long-lasting activation of the cAMP pathway in mammalian neurons. The catalytic domain of yeast adenylate cyclase (*cyr*) (13) was expressed in neurons by using a defective herpes simplex virus type 1 (HSV-1) vector (pHSVcyr) (12, 14, 15). Expression of the recombinant *cyr* gene in either PC-12 cells or sympathetic neurons resulted in long-lasting increases in cAMP levels, protein phosphorylation, and NT release. Thus, activation of the cAMP pathway by pHSVcyr causes long-lasting changes in neuronal function *in vitro*, strongly suggesting that sustained

Abbreviations: Bt<sub>2</sub>cAMP, dibutyryl cAMP; *cyr*, yeast adenylate cyclase; DA, dopamine; HSV-1, herpes simplex virus type 1; IR, immunoreactivity; Nf, neurofilament; NT, neurotransmitter; pfu, plaque-forming units; PKA, protein kinase A; TTX, tetrodotoxin; TH, tyrosine hydroxylase.

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physiological activation of the cAMP pathway *in vivo* may also cause long-term changes in neuronal physiology, with consequent functional alterations in neuronal circuitry.

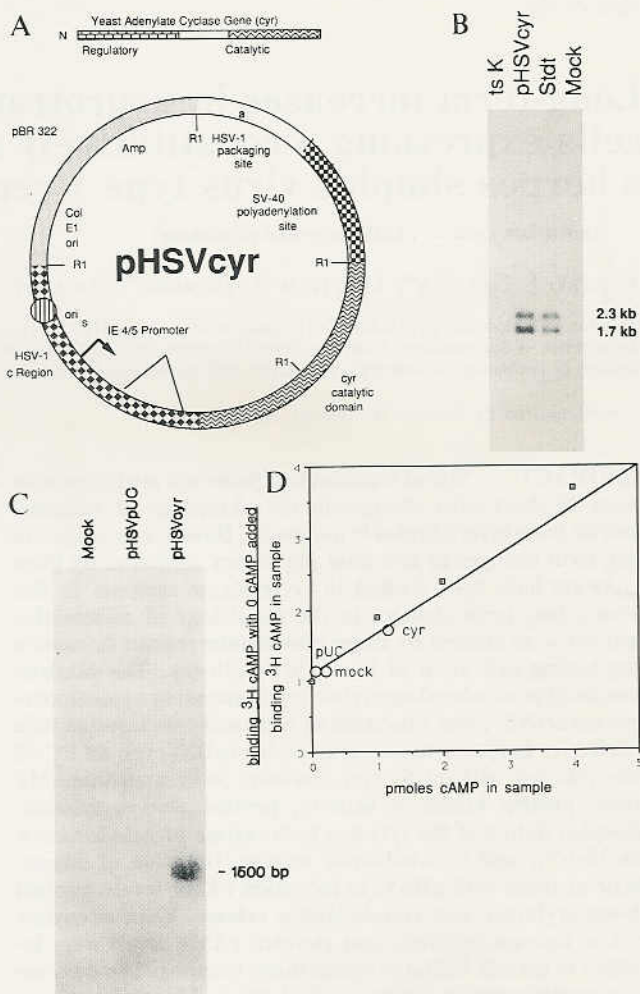
## MATERIALS AND METHODS

**Vectors and Cell Culture.** The *LacZ* gene in pHSVlac (14) was replaced with the *cyr* catalytic domain (5465–6968 bp, ref. 13, Fig. 1A, pHSVcyr) or with the pUC19 polylinker (pHSVpUC). The *cyr* catalytic domain was fused to an oligonucleotide encoding a 10-aa peptide (flag, ref. 16, pHSV-Flag-cyr), for detection by an anti-flag antibody; flag has been fused to many proteins without noticeably affecting biological activity (16). Vectors were packaged into HSV-1 particles (14, 15). Initially, we used HSV-1 strain 17 ts K (14), a temperature-sensitive mutant in the immediate-early 3 gene, as helper virus; later packaging procedures used a deletion mutant in the immediate-early 3 gene, D30EBA (15). The titers of the virus stocks were as follows: for pHSV-cyr— $6 \times 10^6$  plaque-forming units (pfu) of ts K per ml,  $5 \times 10^6$  infectious particles of pHSVcyr per ml or  $6 \times 10^6$  pfu of D30EBA per ml,  $6 \times 10^6$  infectious particles of pHSVcyr per ml; for pHSVflag-cyr— $5 \times 10^6$  pfu of D30EBA per ml,  $6 \times 10^6$  infectious particles of pHSVflag-cyr per ml. PC-12 and PC-18 cells (refs. 7 and 17;  $2 \times 10^5$  cells per 0.5 ml) were infected (7.5  $\mu$ l of virus unless specified; 12  $\mu$ l for phosphorylation studies). Cultures of dissociated superior cervical ganglia (14) were prepared from 4-day-old rats and treated with cytosine arabinoside (40  $\mu$ M, days 5–6); 1–3 weeks later, cultures ( $2 \times 10^5$  cells per 0.5 ml, 20% neurons) were infected (7.5  $\mu$ l).

**DNA and RNA Analysis, Immunocytochemistry, and cAMP RIA.** Vector DNA in viruses (15), expression of *cyr* RNA (18), and immunocytochemistry (14) were determined as described. Primary antibodies were mouse anti-flag (M-5, 1:50 dilution; Immunex), rabbit anti-*cyr* (ref. 19; U1, 1:50 dilution), rabbit anti-cAMP (1:50 dilution; Chemicon), and mouse anti-rat neurofilament (Nf) (SML-33, 1:800 dilution; Sternberger-Monoclonal); secondary antibodies (Cappel) were fluorescein isothiocyanate-conjugated goat F(ab')<sub>2</sub> anti-mouse F(ab')<sub>2</sub> (1:200 dilution) and rhodamine isothiocyanate-conjugated goat F(ab')<sub>2</sub> anti-rabbit F(ab')<sub>2</sub> (1:250 dilution). cAMP was assayed with a [<sup>3</sup>H]cAMP RIA kit (Amersham).

**Phosphorylation Assays.** <sup>32</sup>P incorporation into protein was determined as described (20). PKA activity in cell extracts was determined by measuring <sup>32</sup>P incorporation into a peptide substrate (LRRASLG) in the presence or absence of 10  $\mu$ M cAMP (21). The extraction buffer maintains the relative dissociation of catalytic and regulatory PKA subunits in intact cells; the fraction of PKA active *in vivo* is measured as the PKA activity ratio [(PKA activity, no cAMP added)/(PKA activity, cAMP added)] (21). The phosphorylation state of Ser<sup>40</sup> in tyrosine hydroxylase (TH) (22) was determined in aliquots of these protein extracts by adding purified PKA catalytic subunit (1  $\mu$ M) and [ $\gamma$ -<sup>32</sup>P]ATP. Increased phosphorylation of Ser<sup>40</sup> in intact cells decreases the number of sites available for phosphorylation *in vitro* (1). <sup>32</sup>P incorporation into TH, restricted to Ser<sup>40</sup> by these conditions (23), was determined from the 60-kDa TH band after SDS/PAGE and normalized to the amount of TH protein (22).

**NT-Release Assays.** Cells were incubated (37°C, 15 min) in 1 ml (PC-12) or 0.2 ml (neurons) of release buffer (ref. 24; 135 mM NaCl/3 mM KCl/1 mM MgCl<sub>2</sub>/1.2 mM CaCl<sub>2</sub>/2 mM NaPO<sub>4</sub>, pH 7.4/10 mM glucose); the buffer was cooled, and 0.1 vol of 2 M HClO<sub>4</sub> and 1% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> were added. Cells were lysed (0.2 ml of 4 M HClO<sub>3</sub>; 0°C, 5 min), and the lysates were buffered [100  $\mu$ l of 1 M NaPO<sub>4</sub> (pH 7.0), 80  $\mu$ l of saturated NaOH, and 40  $\mu$ l of 1% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>]. Catecholamines were separated by HPLC and analyzed by a serial array of 16 electrode sensors (25). NT release is measured over a 15-min period; this assay measures steady-state changes in release.



**FIG. 1.** pHSVcyr (A), analysis of pHSVcyr DNA in HSV-1 particles (B), *cyr* RNA expression (C), and cAMP levels in infected PC-12 cells (D). (A) The *cyr* gene, top line, contains regulatory (block segment) and catalytic (wavy line segment) domains (13). The pHSVcyr transcription unit contains the HSV-1 immediate-early 4/5 promoter (arrow), the intervening sequence following the promoter (triangle), the *cyr* catalytic domain (wavy line segment), and the simian virus 40 (SV-40) early region polyadenylation site (checkboard segment). A HSV-1 origin of DNA replication (*ori<sub>s</sub>*, circle with vertical lines) and the packaging site (a sequence, clear segment) support packaging into HSV-1 particles (14). R1, *Eco*RI. (B) pHSVcyr DNA in HSV-1 particles. DNA was isolated from pHSVcyr virus, ts K, uninfected cells (Mock), and *Escherichia coli* harboring pHSVcyr (Std). DNA was digested with *Eco*RI and subjected to Southern analysis (15). The probe contained the pBR (A, gray segment) and HSV-1 c (diamond segment) regions. pHSVcyr contains four *Eco*RI fragments: (i) 2.3-kb pBR region, (ii) 1.7-kb HSV-1 c region (1065 bp) and 5' portion of *cyr* (nt 5465–6129), (iii) 3' portion of *cyr*, and (iv) HSV-1 a region (iii and iv, not homologous to probe). The probe recognizes the 15.1-kb HSV-1 *Eco*RI H fragment; this large fragment was inefficiently transferred to the membrane but was detected on longer exposures (data not shown). (C) *cyr* RNA 1 day after infection of CV1 cells (multiplicity of infection, 0.25). RNA was isolated, *cyr* cDNA was synthesized and amplified using the PCR, and the products were subjected to Southern analysis using a *cyr* probe. (D) pHSVcyr increases cAMP levels in PC-12 cells. One day after infection, cAMP was measured by RIA. Squares represent the standard curve, and open circles are pHSVcyr (*cyr*), pHSVpUC (pUC), and mock. Each experimental condition was done in triplicate; values differed by <10%; the experiment was done three times.

## RESULTS

**pHSVcyr Is Packaged into HSV-1 Particles That Express *cyr* RNA.** DNA isolated from HSV-1 particles was subjected to



Southern analysis (15). The results showed that pHSVcyr (Fig. 1B) and control vectors (data not shown) were properly packaged into HSV-1 particles. Expression of cyr RNA was analyzed (18) in pHSVcyr-infected CV1 fibroblast cells by reverse transcription-PCR, and the expected 1.5-kb band was observed (Fig. 1C). In pHSVcyr-infected PC-12 cells, cyr RNA was detected by *in situ* hybridization (data not shown).

**In PC-12 Cells, pHSVcyr Expresses cyr Protein That Increases cAMP Levels.** One day after pHSVflag-cyr infection, flag-immunoreactivity (IR) was detected in the same cells that exhibited elevated cAMP-IR (Fig. 2 A-C); >99% of the cells that contained flag-IR also contained elevated cAMP-IR. Of the cells that lacked flag-IR, >99% lacked elevated cAMP-IR. pHSVlac-infected cultures lacked cells with flag-IR and displayed only low basal levels of cAMP-IR (Fig. 2 D-F). In pHSVflag-cyr-infected cultures, replacing the primary antibodies with preimmune sera abolished flag-IR and cAMP-IR (Fig. 2 G-I). In pHSVcyr-infected cultures, cyr-IR was detected with anti-cyr (20% positive cells), and parallel cultures contained elevated cAMP-IR (20% positive cells; data not shown). Because the antibodies against cyr and cAMP were raised in rabbits, it was not possible to colocalize cyr-IR and cAMP-IR.

One day after pHSVcyr infection, an overall 10-fold increase in cAMP was measured by RIA (Fig. 1D). Because pHSVcyr infected  $\approx 20\%$  of the cells (demonstrated by *in situ* hybridization and by cyr-IR), pHSVcyr increased cAMP  $\approx 45$ -fold per infected cell. Similarly, in yeast, the cyr catalytic domain directs a 20-fold increase in cAMP levels (13). Process outgrowth from PC-12 cells was not evident either 1 day after infection with pHSVcyr or after treatment with dibutyl cAMP (Bt<sub>2</sub>cAMP) for 1 day.

**pHSVcyr Directs Increases in PKA Activity and Phosphorylation of the PKA Site in TH.** The fraction of active PKA was determined in PC-12 and PC-18 cells. PC-18 cells, a derivative of PC-12 cells (17), had a lower fraction of active PKA than did PC-12 cells (Table 1), enabling a more sensitive assay for changes in PKA activity. Infection of PC-18 cells with pHSVcyr, but not with pHSVlac, directed a 28% increase in the fraction of active PKA relative to that in mock-infected cells (Table 1). Because pHSVcyr infected  $\approx 15\%$  of the cells, the percentage of active PKA increased from 29% in mock-infected cells to  $\approx 80\%$  in pHSVcyr-infected cells. Similar effects were produced by pHSVcyr in PC-12 cells; however,

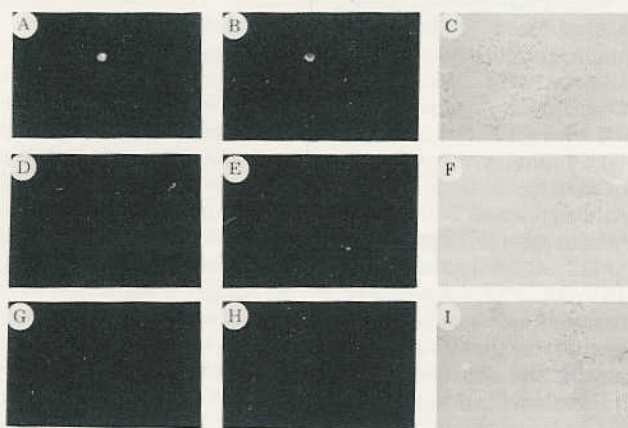


FIG. 2. In PC-12 cells, pHSVcyr directs cyr expression and increased cAMP. Cultures were infected with pHSVflag-cyr (multiplicity of infection 0.05) or were mock infected, and 1 day later immunocytochemistry was done (14). (A-C) pHSVflag-cyr: flag-IR (A); cAMP-IR (B); and phase-contrast (C). (D-F) Mock: flag-IR (D); cAMP-IR (E); and phase-contrast (F). (G-I) pHSVflag-cyr, pre-immune serum in place of primary antibodies: fluorescein fluorescence (G); rhodamine fluorescence (H); and phase-contrast (I). (Width of each photomicrograph is 230  $\mu\text{m}$ .)

the relative magnitude was lower than in PC-18 cells due to the higher percentage of active PKA in untreated PC-12 cells.

In initial studies of the effects of pHSVcyr upon protein phosphorylation, <sup>32</sup>P incorporation into trichloroacetic acid-precipitable protein (after incubation with <sup>32</sup>P<sub>i</sub>) was higher in pHSVcyr-infected cells and in Bt<sub>2</sub>cAMP-treated cells ( $\approx 100\%$  increases) relative to either pHSVlac or mock-infected cells. Analysis of these protein extracts by SDS/PAGE and autoradiography showed that pHSVcyr increased <sup>32</sup>P incorporation into specific bands that were also increased by Bt<sub>2</sub>cAMP (data not shown). As a more direct measure of PKA-dependent phosphorylation, we analyzed the phosphorylation state of Ser<sup>40</sup> in TH, a specific substrate for PKA in intact PC-12 cells (22). In this assay (1), increased phosphorylation *in vivo* reduces the number of dephospho-sites available for subsequent phosphorylation *in vitro*. Thus, the data are presented as percentage decreases in dephospho-Ser<sup>40</sup>. pHSVcyr, but not pHSVlac, directed a 20% decrease in phosphorylation of TH Ser<sup>40</sup> *in vitro* (Table 1).

**pHSVcyr Increases NT Release.** Pharmacological activation of the cAMP pathway causes a short-term increase in NT release from PC-12 cells (7), suggesting that pHSVcyr might also induce changes in NT release. One day after infection, dopamine (DA) release was quantitated by HPLC. pHSVcyr, but not pHSVpUC, caused a 60% increase in DA release compared with mock-infected cultures (Table 1). Similar results were seen with pHSVflag-cyr (data not shown). The increase in NT release required Ca<sup>2+</sup> but not physiological activity (action potentials), as indicated by the lack of effect of tetrodotoxin (TTX), a sodium channel inhibitor. This result is not surprising because PC-12 cells exhibit minimal numbers of processes or sodium channels unless treated with specific agents, such as nerve growth factor. Addition of Bt<sub>2</sub>cAMP or high K<sup>+</sup> to the release buffer also increased DA release. In that infection with pHSVcyr or pHSVlac did not alter cellular levels of DA (Table 1), the effects of pHSVcyr upon DA release are not attributable simply to differences in

Table 1. Effects of pHSVcyr on PKA activity, phosphorylation of the TH PKA site, and NT release in PC cells

PC line	Treatment	PKA, % mock	TH Ser <sup>40</sup> phos, % change	DA <sup>†</sup>
18	pHSVcyr	128 $\pm$ 8		
	pHSVlac	101 $\pm$ 4		
	Mock	100		
12	pHSVcyr	109 $\pm$ 3	20 $\pm$ 7	
	pHSVlac	102 $\pm$ 2	5 $\pm$ 6	
	Mock	100	0	
12	pHSVcyr			320
	pHSVpUC			202
	Mock			200
	pHSVcyr + TTX			318
	pHSVcyr - Ca <sup>2+</sup>			197
	Mock - Ca <sup>2+</sup>			204
	Mock + Bt <sub>2</sub> cAMP			244
Mock + high K <sup>+</sup>			3650	

The fraction of active PKA in mock-infected cells was 0.29  $\pm$  0.03 (PC-18) and 0.46  $\pm$  0.05 (PC-12). Changes in TH Ser<sup>40</sup> phosphorylation reflect decreases in dephospho-Ser<sup>40</sup>. PKA and TH Ser<sup>40</sup> data were normalized to the mock-infected values from each experiment and are presented as means  $\pm$  SEMs of normalized values from three to five experiments in which treatments were done in duplicate or triplicate. Where indicated, NT-release buffer contained TTX (1  $\mu\text{M}$ ), no Ca<sup>2+</sup> (0.1 mM EGTA and no CaCl<sub>2</sub>), Bt<sub>2</sub>cAMP (2 mM), or high K<sup>+</sup> (56 mM KCl). Treatments were done in duplicate, and values differed by <10%; the experiment was done five times with similar results. phos, Phosphorylation.

\*Units of DA in the medium are pg per min per 10<sup>6</sup> cells.

†Units of cellular DA are pg  $\times 10^{-5}$  per 10<sup>6</sup> cells.



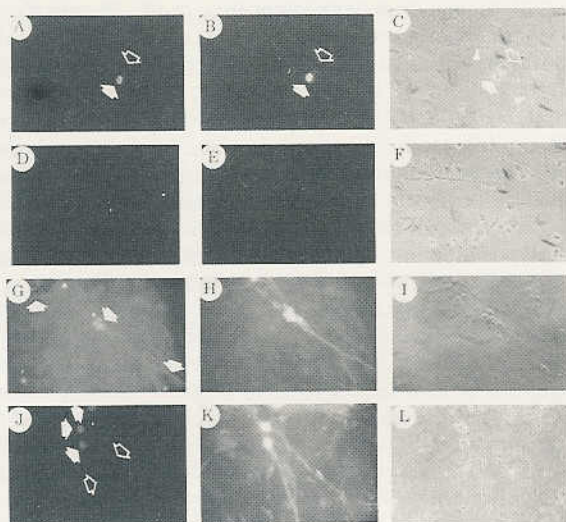


FIG. 3. pHSVcyr directs cyr expression and increased cAMP to neuronal cell bodies. One week after infection, immunocytochemistry was performed (14). (A–C) pHSVflag-cyr: flag-IR (A); cAMP-IR (B); and phase-contrast (C). Filled arrows, flag-IR or increased cAMP-IR; empty arrows, no flag-IR or basal cAMP-IR; arrowheads, neuronal processes partially masked by supporting cells. (D–F) Mock: flag-IR (D), cAMP-IR (E), and phase-contrast (F). (G–I) Forskolin (50  $\mu$ M, 30 min): cAMP-IR (G), Nf-IR (H), and phase-contrast (I). Filled arrows, increased cAMP-IR in neuronal cell bodies and processes. Nonneuronal cells (visible in I) contain increased cAMP-IR. (J–L) pHSVcyr: cAMP-IR (J), Nf-IR (K), and phase-contrast (L). Filled arrows, increased cAMP-IR in cell bodies; empty arrows, basal cAMP-IR in processes (visible in K and L). The photomicrographs in G and J were taken under similar conditions. (Width of each photomicrograph is 230  $\mu$ m.)

DA-synthesis rates or intracellular DA levels. The unaltered cellular levels of DA, combined with the  $Ca^{2+}$ -dependence of pHSVcyr-induced release, establish that pHSVcyr-induced release was not an unregulated efflux of DA due to saturation of the storage capacity of the cell. Thus, these results suggest that pHSVcyr-induced release is mediated by stimulus–secretion coupling processes.

**In pHSVcyr-Infected Neurons, cyr Protein and Increased cAMP Are Localized to the Cell Body.** This same genetic approach was applied to normal neurons. One week after pHSVflag-cyr infection of cultured sympathetic neurons, cells containing flag-IR also contained elevated cAMP-IR and appeared to be neurons by morphological criteria (Fig. 3 A–C). Greater than 99% of the cells containing flag-IR also contained elevated cAMP-IR; of the cells that lacked flag-IR, >98% also lacked elevated cAMP-IR. Flag-IR and elevated cAMP-IR were absent from pHSVpUC (data not shown) or mock-infected cultures (Fig. 3 D–F). pHSVflag-cyr-infected cultures assayed with preimmune serum lacked cells with flag-IR or elevated cAMP-IR. One week after pHSVcyr infection, some cells containing cyr-IR (data not shown) or cAMP-IR (Fig. 3 J–L) also exhibited Nf-IR. Parallel cultures infected with pHSVcyr (multiplicity of infection 0.2) exhibited similar numbers ( $\approx$ 20%) of neurons containing cyr-IR and cAMP-IR. pHSVcyr-infected cultures, assayed using anti-Nf and anti-cAMP preabsorbed with cAMP, contained Nf-positive cells but no cAMP-IR (data not shown). The data in Fig. 3 were obtained 1 week after infection; similar results were seen 1 day after infection (data not shown).

Forskolin, which activates adenylate cyclase throughout the cell, increased cAMP-IR both in neuronal cell bodies and in processes (Fig. 3 G–I), as well as in underlying glia. In contrast, flag-IR, cyr-IR, and elevated cAMP-IR were restricted to the cell body in neurons. This localization is not surprising because the cyr catalytic domain, when expressed

Table 2. NT release from neurons after pHSVcyr infection

Treatment (T)	Additions to release buffer	Medium CA*		Cellular DA, pg/10 <sup>6</sup> cells
		NE	DA	
pHSVcyr (1 D)		20	9.6 (0.12)	2000
(1 W)			11 (0.14)	2000
pHSVpUC (1 D)		8.0	4.8 (0.06)	2100
(1 W)			<4.8 (<0.06)	2400
Mock (1 D)		6.4	4.8 (0.06)	2500
(1 W)			<4.8 (<0.06)	2100
pHSVcyr (1 D)	TTX		<4.8 (<0.06)	
pHSVcyr (1 D)	– $Ca^{2+}$		<4.8 (<0.06)	
Mock (1 D)	Forskolin		15 (0.19)	
pHSVcyr (1 D)	High $K^{+}$		34	
pHSVpUC (1 D)	High $K^{+}$		35	
Mock (1 D)	High $K^{+}$	23	36	
pHSVcyr (1 D)	Veratridine		25	
pHSVpUC (1 D)	Veratridine		24	
Mock (1 D)	Veratridine		26	

Additions to release buffer are as in the legend for Table 1 or forskolin (50  $\mu$ M) or veratridine (100  $\mu$ M). Numbers in parentheses are concentrations of DA (pg/ $\mu$ l) in release buffer. Treatments were done in duplicate, and values differed by <10%; the experiment was done five times with similar results. CA, catecholamines; NE, norepinephrine. T, time; D, day; W, week.

\*Units are in pg/min per 10<sup>6</sup> cells.

in yeast, is not associated with the membrane but is found in the cytoplasm (the cyr catalytic domain lacks the membrane-directing signals present in the regulatory domain of the full-length cyr) (26); thus, the cyr catalytic domain is unlikely to contain signals directing it to neuronal processes. Similarly, expression of *E. coli*  $\beta$ -galactosidase from pHSVlac is predominantly localized to the cell body (14), and this  $\beta$ -galactosidase is a cytoplasmic protein in *E. coli*.

**pHSVcyr Increases Protein Phosphorylation and NT Release from Neurons.** <sup>32</sup>P incorporation into total protein was increased (50%) in neuronal cultures infected with pHSVcyr or treated with forskolin, compared with pHSVpUC or mock-infected cultures. Autoradiograms, after SDS/PAGE of protein extracts, showed that pHSVcyr directed an increase in <sup>32</sup>P incorporation into a set of bands similar to those affected by forskolin, whereas pHSVpUC did not change the pattern of bands compared with mock-infected cells (data not shown). PKA-specific phosphorylation experiments were hindered by the limited material provided by neuronal cultures, and subsequent studies focused upon the effects of pHSVcyr on NT release from these cells.

One day or 1 week after infection NT release was quantitated by HPLC. In initial studies, norepinephrine, the NT used by adult sympathetic neurons, was measured; in subsequent experiments we measured the more easily detectable DA, a significant NT in cultured sympathetic neurons from newborn rats (27). Similar results were obtained for either NT (Table 2). One day after infection with pHSVcyr, but not with pHSVpUC, NT release was approximately doubled. In addition, this increase in NT release was observed 1 week after infection. Similar results were obtained with pHSVflag-cyr (data not shown). As with PC-12 cells, the increased release depended on  $Ca^{2+}$ , implying that it was vesicular. In contrast to PC-12 cells, TTX inhibited release, suggesting that the increase in release required physiological activity (action potentials). The increase in release was probably from neurons; glia do not synthesize, accumulate, or secrete significant amounts of catecholamines (28). Forskolin caused a short-term increase in release, consistent with reports (8, 29).

The specificity of the increase in NT release directed by pHSVcyr was investigated. Depolarizing agents (56 mM  $K^{+}$  and veratridine, which opens voltage-gated sodium channels) caused similar elevations in NT release from pHSVcyr and



control cultures (Table 2). Thus, pHSVcyr directed an increase in basal release (low  $K^+$ ) relative to control cells; in contrast, evoked release (high  $K^+$  or veratridine) was comparable in pHSVcyr and control cultures. As in PC-12 cells, intracellular DA levels were similar in pHSVcyr and control cultures (Table 2), minimizing the likelihood that elevated cellular DA levels could account for the observed increase in DA release directed by pHSVcyr.

Cultured sympathetic neurons can be induced to change from the catecholaminergic to the cholinergic NT system (30). pHSVcyr did not cause this change; 5 days after pHSVcyr infection the number of cells with TH-IR or choline acetyltransferase-IR was not changed (data not shown), and no acetylcholine ( $<10$  fmol/ $\mu$ l) was released.

## DISCUSSION

We have used genetic intervention to produce a long-lasting activation of the cAMP pathway in neuronal cells. In PC-12 cells, pHSVcyr directed efficient expression of cyr RNA and cyr protein, resulting in long-lasting increases in cAMP levels, PKA activity, protein phosphorylation, phosphorylation of the TH PKA site, and NT release. pHSVcyr-infected PC-12 cells did not undergo process extension; therefore, cyr protein and the increased cAMP were found throughout the cell and could act directly on the NT-release machinery. The increased NT release required  $Ca^{2+}$ , presumably for fusion of synaptic vesicles to the plasma membrane, but did not require physiological activity.

In pHSVcyr-infected sympathetic neurons, cyr protein was also efficiently expressed, resulting in long-term increases in cAMP levels, protein phosphorylation, and NT release. Forskolin directed an increase in cAMP that was detected throughout the cell, including cell processes. In contrast, cyr protein and the resulting increase in cAMP were restricted to the cell body, suggesting that pHSVcyr may not have directly affected the NT release machinery in the axon terminal. A possible indirect mechanism by which pHSVcyr could influence release is to induce or increase the frequency of action potentials in the relatively quiescent sympathetic neurons; for example, short-term ( $<10$  min) elevation of cAMP in hippocampal neurons can increase the frequency of action potentials in response to depolarizing stimuli, presumably due to PKA activation (31). A similar mechanism may operate in mammalian (32) and frog (33) sympathetic neurons. This mechanism is consistent with the observation that TTX prevented the pHSVcyr-induced increase in NT release from neurons, but not from PC-12 cells, and that depolarizing agents, which substitute for action potentials in effecting release, cause the same amount of NT release from pHSVcyr, pHSVpUC, and mock-infected cells. Such mechanisms may be mediated by specific voltage-gated ion channels or other PKA-regulated proteins.

This report describes a paradigm in which a stable increase in cAMP causes long-term changes in NT release. Stable activation of the cAMP pathway is probably of physiological significance. In *Aplysia*, facilitation of the gill withdrawal reflex is mediated by the cAMP pathway, which regulates NT release from a specific neuron (3, 4). In *Drosophila*, *dunce*, a mutation in a cAMP phosphodiesterase, affects long-term associative learning (4). In sympathetic neurons, activation of the cAMP pathway causes multiple short-term changes in neuronal function, including increased NT release (1, 2, 8). Several long-term changes in sympathetic neuron physiology, including one termed long-term facilitation (32), have been described (29, 32). Relatively stable changes in the cAMP pathway mediate long-term changes in the physiology of specific central nervous system neurons, such as the norepinephrine cells in the locus coeruleus (34). Our results raise the possibility that activation of the cAMP pathway may play a role in long-lasting changes in sympathetic neuron

physiology. Because this report focuses on establishing the paradigm, elucidating the significance of the long-term increases in NT release and the mechanism(s) that mediate these changes will be the focus of subsequent work.

HSV-1 vectors expressing catalytic domains of various signal-transduction enzymes may represent a general approach to analyzing the role of signal-transduction pathways in neuronal function (12). Delivery of pHSVcyr virus into the adult mammalian brain, with a consequent increase in NT release from infected neurons, will offer the opportunity to correlate molecular changes in neurons with altered behavioral responses in learning paradigms (12).

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