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Increased Site-Specific Phosphorylation of Tyrosine Hydroxylase Accompanies Stimulation of Enzymatic Activity Induced by Cessation of Dopamine Neuronal Activity

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ABSTRACT

Activation of striatal dopamine (DA) neurons by neuroleptic treatment or by electrical stimulation of the nigrostriatal pathway increases the activity of tyrosine hydroxylase (TH). The increase is mediated by phosphorylation of the enzyme. However, abolition of DA neuronal activity [by γ-butyrolactone (GBL) treatment or transection of the nigrostriatal pathway] also increases TH activity. Quantitative blot immunolabeling experiments using site- and phosphorylation state-specific antibodies to TH demonstrated that GBL treatment (750 mg/kg, 35 min) significantly increased phosphorylation at Ser19 (+40%) and Ser40 (+217%) without altering Ser31 phosphorylation. Concomitantly, GBL treatment [along with the 3,4-dihydroxyphenylalanine (dopa) decarboxylase inhibitor NSD-1015, 100 mg/kg, 30 min] increased in vivo striatal dopa accumulation and in vitro TH activity 3-fold. Likewise, cerebral hemitransection of the nigrostriatal pathway significantly increased phosphorylation of TH at Ser19 (+89%) and Ser40 (+158%) but not at Ser31; dopa levels were increased accordingly (+191%). Kinetic analysis of TH activity established that GBL treatment and hemitransection primarily decreased the K_m for the cofactor tetrahydrobiopterin (3-fold). The effects of GBL and hemitransection were abolished or attenuated by pretreatment with the DA agonist *R*-(−)-*N*-*n*-propylnorapomorphine (NPA; 30 μg/kg, 40 min), presumably via stimulation of inhibitory presynaptic DA autoreceptors. NPA dose-response curves for reversal of GBL-induced dopa accumulation and Ser40 phosphorylation were identical; however, only the highest dose of NPA reversed the small and variable increase in Ser19 phosphorylation. Thus, TH activity seems to be regulated by phosphorylation in both hyper- and hypoactive striatal DA neurons; in the latter case, activation seems to be caused by selective phosphorylation of Ser40.

It is now well established that short-term regulation of tyrosine hydroxylase (TH) is accomplished by dynamic changes in the phosphorylation state of the enzyme (Kaufman, 1995; Kumer and Vrana, 1996). Four phosphorylation sites in TH have been identified (Ser8, Ser19, Ser31, and Ser40), all in the amino-terminal regulatory region (Campbell et al., 1986; Haycock, 1990). With the exception of Ser8, the phosphorylation of each site seems to be physiologically regulated (Haycock, 1990; Haycock and Haycock, 1991), and at least seven different protein kinases have been identified that phosphorylate the enzyme in vitro (Kumer and Vrana, 1996). Until recently, assessment of site-specific phosphorylation of TH utilized a complex protocol involving ^{32}P incorporation into purified TH or in situ PC12 or adrenal chromaffin cells, followed by isolation or immunopurification of radiolabeled TH, tryptic digestion, and sequence analysis of purified phosphopeptides (Campbell et al., 1986; Haycock, 1990). With the development of site- and phosphorylation state-specific polyclonal antibodies to TH (Goldstein et al., 1995; Haycock et al., 1998), assessment of alterations in phosphorylation at specific TH serine residues in vivo has become more practicable. Applications include mapping the distribution of phosphorylated TH species in immunohistochemical studies (Xu et al., 1998) and evaluating the effects of dopamine (DA) receptor antagonist treatment (Harada et al., 1996a).

Electrical stimulation of the medial forebrain bundle (containing the dopaminergic afferents to the forebrain) is known to elicit activation of striatal TH (Murrin et al., 1976; Murrin and Roth, 1987). This activation is associated with an increase in phosphorylation of striatal TH at Ser19, Ser31, and

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ABBREVIATIONS: TH, tyrosine hydroxylase; DA, dopamine; GBL, γ-butyrolactone; NPA, *R*-(−)-*N*-*n*-propylnorapomorphine; NSD-1015, *m*-hydroxybenzylhydrazine; BH_4, tetrahydrobiopterin; HPLC, high-performance liquid chromatography; RT, room temperature; PP2A, protein phosphatase 2A.

Ser40 but not at Ser8 (Haycock and Haycock, 1991). Similarly, acute treatment with DA receptor antagonists, such as haloperidol, increases the firing rate of dopamine neurons (Bunney and Grace, 1978; Chiodo and Bunney, 1983) and activates TH (Zivkovic et al., 1975a,b; Lerner et al., 1977; Lazar et al., 1982). Recent studies demonstrate that the latter treatments increase the phosphorylation of TH at the same three sites (Harada et al., 1996a; Salvatore et al., 1998). However, it is well known that cessation of impulse traffic in dopaminergic neurons, whether attributable to treatment with γ-butyrolactone (GBL) (Walters and Roth, 1976; Roth, 1979) or to lesion (Walters et al., 1973) or transection (Kehr et al., 1977) of ascending dopaminergic fibers, also increases in vivo tyrosine hydroxylation. This has been interpreted to result from the relief of inhibitory nerve-terminal DA autoreceptor regulation of TH activity consequent to the absence of DA release, because treatment with DA agonists reverses the activation of TH (Roth, 1979; Meller et al., 1987). That phosphorylation of TH is the mechanism underlying its activation in the latter studies is supported by kinetic studies (Morgenroth et al., 1976) but challenged by others (Zivkovic et al., 1975a), which indicated that the K_m for pterin cofactor was reduced, analogous to changes after haloperidol treatment (Lerner et al., 1977; Lazar et al., 1982).

In the present studies, quantitative blot immunolabeling with site- and phosphorylation state-specific antibodies was used in conjunction with assays of both in vivo and in vitro TH activity to assess changes in TH phosphorylation and activity after abolition of DA neuronal activity by either GBL treatment or cerebral hemitransection. Increases in phosphorylation at Ser19 and Ser40 occurred with both treatment paradigms, and the increases in phosphorylation and TH activity were reversed by pretreatment with a DA agonist. However, the increase in Ser19 phosphorylation was relatively small and variable. Moreover, whereas DA agonist reversal of GBL-induced dopa accumulation and Ser40 phosphorylation displayed the same dose dependence, there was no such correlation for reversal of Ser19 phosphorylation. Thus, the activation of TH in hypoactive DA neurons seems to be solely attributable to phosphorylation of Ser40.

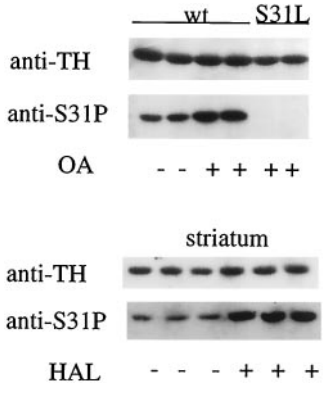

Fig. 1. Specificity and utility of anti-Ser31p antibody. Top, immunolabeling of protein extracts (30 μg/lane) from AtT-20 cells expressing recombinant wild-type (wt) or mutant TH (Ser31 replaced by leucine; S31L) (Wu et al., 1992), before and after exposure to okadaic acid (OA, 1 μM, 60 min). Bottom, labeling of rat striatal protein extracts (40 μg/lane) from vehicle and haloperidol- (HAL, 2 mg/kg, 60 min) treated rats.

Experimental Procedures

Materials. GBL was purchased from Aldrich Chemical (Milwaukee, WI). *R*-(−)-*N*-*n*-propylnorapomorphine (NPA) was obtained from Research Biochemicals Inc. (Natick, MA), *m*-hydroxybenzylhydrazine (NSD-1015) from Sigma Chemical Co. (St. Louis, MO), bicinchoninic acid protein assay reagent from Pierce Chemical Company (Rockford, IL), methoxyflurane (Metofane) from Mallinckrodt Veterinary (Mudelein, IL), and ^{125}I-protein A from New Englnand Nuclear Life Sciences (Boston, MA). Tetrahydrobiopterin (BH_4) was purchased from B. Schircks Laboratories (Jona, Switzerland). All other reagents were of the highest purity available commercially and were usually obtained from Sigma.

Animals and Drug Treatments. Male Sprague-Dawley rats weighing 175 to 250 g (Taconic Farms, Germantown, NY) were used in all experiments. Groups of rats were injected with NPA (0.3–30 μg/kg, s.c.) or its vehicle 5 min before GBL (750 mg/kg, i.p.); NSD-1015 (100 mg/kg, i.p.) was administered 5 min after GBL; and animals were sacrificed 30 min later. The corpus striatum was dissected in the cold and TH was extracted by sonication in 20 mM Tris·HCl, pH 7.2, containing 0.1% Triton X-100, 1 μM okadaic acid, 25 mM NaF, 25 μg/ml leupeptin, 25 μg/ml pepstatin A, and 0.1 mM phenylmethylsulfonyl fluoride. After centrifugation (56,000*g*, 20 min, 4°C) the supernatant was assayed for protein content using the Pierce bicinchoninic acid protein assay and stored in aliquots at −80°C.

Cerebral Hemitransection. The technique was essentially that described by Bedard et al. (1972). Rats were anesthetized with methoxyflurane and a dental drill was used to make an opening in the skull, 3.2 mm posterior to bregma, from the midline laterally. The dura was opened and a stainless steel knife was inserted to the base of the skull near the midline and moved laterally, completely hemitransecting the brain at the level of the caudal hypothalamus. NPA (30 μg/kg, s.c.) was injected 5 to 10 min before the transection, and NSD-1015 (100 mg/kg, i.p.) was injected immediately after. Rats were sacrificed 30 min after NSD-1015 treatment, the brain was removed, and the transection was extended to the unoperated side. The striatum was dissected from the rostral section and processed as described above.

TH Antibodies. Four primary antibodies were used in these experiments. The preparation and characterization of three of the antibodies has been described previously: 1) anti-TH, a pan-specific rabbit antiserum to total TH (Markey et al., 1980); 2) anti-Ser40p, an antipeptide, double-affinity-purified antibody to TH phosphorylated at Ser40 (Goldstein et al., 1995); and 3) anti-Ser19p, an antipeptide, double-affinity-purified antibody to TH phosphorylated at Ser19 (Haycock et al., 1998). The fourth antibody, anti-Ser31p, an antipeptide, double-affinity-purified antibody to TH phosphorylated at

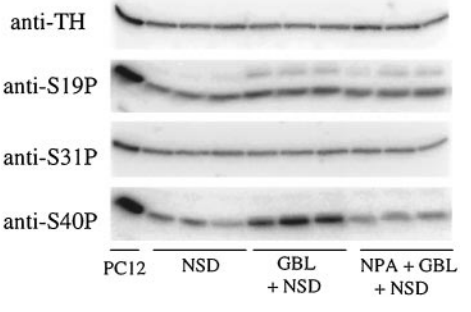

Fig. 2. Blot immunolabeling pattern of striatal TH to site- and phosphorylation state-specific antibodies as a function of drug treatment. Drug treatment times and doses are as described in *Experimental Procedures*. Equal amounts of protein (40 μg) were loaded in each lane. The anti-Ser19p antibody also weakly recognized an unknown protein of higher molecular mass; this band was not seen in PC12 extracts or in striatal extracts visualized with a fluorescence method that did not use ^{125}I-protein A. Interestingly, this band seems to covary with treatment. The blots shown are from one of three separate experiments. PC12, TH extract of PC12 cells.

Ser31, was prepared by methods similar to those used in the preparation of the other site- and phosphorylation state-specific antibodies, and its detailed characterization will be described elsewhere. Evidence for the specificity of the antibody and its ability to visualize changes in Ser31 phosphorylation are shown in Fig. 1. Immunolabeling experiments (Fig. 1, top) demonstrated that the antibody recognized recombinant wild-type TH, and labeling was increased after phosphatase inhibition with okadaic acid; in contrast, mutant TH, in which Ser31 was replaced by leucine (Wu et al., 1992), was not labeled by the antibody even after phosphatase inhibition. In addition, anti-Ser31p immunolabeling of rat striatal TH was increased by haloperidol treatment (Fig. 1, bottom).

Blot Immunolabeling. Aliquots of striatal extracts were diluted with sample buffer (50 mM Tris·HCl, pH 6.8, containing 2% SDS, 5% β-mercaptoethanol, 12% glycerol, and 0.001% bromphenol blue), boiled for 4 min, and cooled. Aliquots containing equal amounts of protein were applied to each lane of a 1.5-mm-thick 7.5% gel, and proteins were separated by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (1970) as described previously (Goldstein et al., 1995; Harada et al., 1996b). A positive control (PC12 cell extract), protein molecular mass markers, and at least four serial dilutions (over a 4–5-fold range) of a striatal extract from a GBL-treated (or cerebral hemitransected) rat were also loaded onto each gel (to generate standard curves). After electrophoresis, proteins were electrophoretically transferred overnight at 4°C to nitrocellulose sheets, blocked with 5% nonfat milk in phosphate-buffered saline, pH 7.2, for 1 h at room temperature (RT), washed repeatedly, and incubated with primary antibodies (0.3–2 μg/ml) for 1 h. The membranes were rinsed twice with phosphate-buffered saline, washed three times, and incubated with ^{125}I-protein A (200,000–400,000 cpm/ml) for 1 h at RT. After extensive washing, the membranes were air-dried and exposed to a phosphor-imaging plate for 1 to 3 days. Relative quantification of immunoreactivity was accomplished with ImmunoQuant software on a Molecular Dynamics PhosphorImager SI (Molecular Dynamics, Sunnyvale, CA). Lane-specific backgrounds were subtracted for each band, values were normalized for small variations in protein loading by probing an additional blot (always run concurrently) with the anti-TH antibody for total TH, and immunoreactivity was quantified by interpolation from the standard curves (which were linear over the range used).

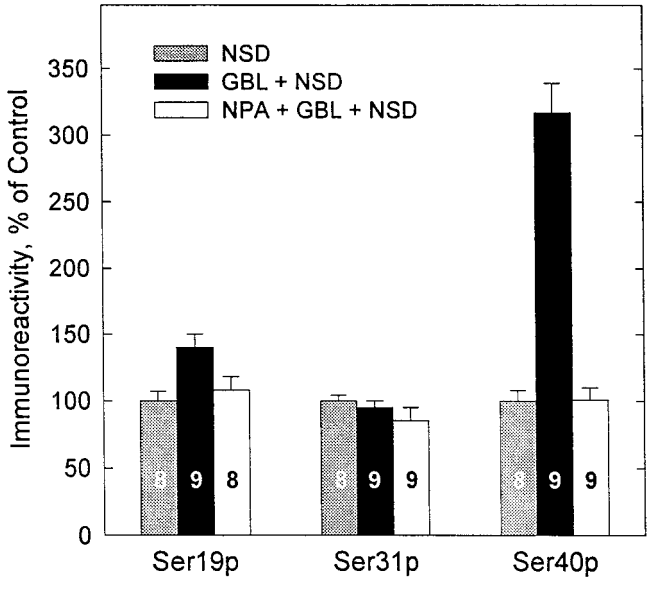

Fig. 3. Relative changes in serine site- and phosphorylation state-specific immunolabeling of striatal TH after drug treatment. The data were combined from three separate experiments; each value is the mean ± S.E. of the total number of rats shown in the columns. One-way analysis of variance: Ser19p, $F_{2,22} = 5.32$, $p = .013$; multiple comparisons, GBL + NSD versus NSD, $p < .02$; NPA + GBL + NSD versus NSD, not significant. Ser31p, $F_{2,23} = 1.14$, not significant. Ser40p, $F_{2,23} = 68.95$, $p < .001$; multiple comparisons, GBL + NSD versus NSD, $p < .001$; NPA + GBL + NSD versus NSD, not significant.

dopa Assay. dopa levels were determined by high-performance liquid chromatography (HPLC) with electrochemical detection as described previously (Meller et al., 1987) with minor modifications. Aliquots of striatal extracts were acidified with perchloric acid (0.4 N final concentration), chilled in ice for 20 min, and centrifuged at 12,000*g* for 15 min at 4°C. 3,4-Dihydroxybenzoic acid (internal standard; 20–100 ng) and 0.6 mM $NaHSO_3$ were added to the supernatants, and aliquots were transferred to tubes containing acid-washed alumina and 0.9 ml of a solution of 0.6 M Tris·HCl buffer, pH 8.6, 0.6 mM $NaHSO_3$ and 0.04 mM EDTA. After mixing for 20 min, the alumina was recovered by centrifugation and the supernatant was discarded. The alumina was washed three times with a solution containing 6 mM Tris·HCl and 1 mM $NaHSO_3$, pH 8.6, and catechols were extracted by shaking with 0.1 to 0.4 ml of 0.2 N perchloric acid for 10 min at RT. The mixture was centrifuged and aliquots of the supernatant were assayed for dopa with an ESA (Chelmsford, MA) HPLC system consisting of an ESA HR-80 column and a model 5100A coulometric electrochemical detector equipped with a model 5011 dual-electrode, high-sensitivity analytical cell. The working electrode potentials for the coulometric amperometric analytical cell were +0.07V and −0.25V for analysis of dopa. The mobile phase was ESA CAT-A-PHASE, pH 2.56, containing 5% methanol and 0.003% sodium octyl sulfate at a flow rate of 1 ml/min.

TH Enzyme Activity. Two different assay methods were used in different experiments, primarily as a matter of convenience. The radioisotopic assay was a modification of that described previously (Strait and Kuczenski, 1986; Bohmaker et al., 1989). A typical assay (final volume, 0.36 ml) contained 50 μl of 1 M Tris-maleate buffer, pH 7.0, 50 μl of 0.7 mM ferrous sulfate, 50 μl of water, or 0.7 mM 3-iodotyrosine (as blank), 50 μl of BH_4 (0.025–0.8 mM final concentration), 50 μl of a solution consisting of 14 mM phenylmethylsulfonyl fluoride, 7 mM pepstatin and 7 μg/ml leupeptin, and 50 μl of 42 mM dithiothreitol containing 2000 U of catalase and 50 μl of striatal extract (~300 μg of protein). The reaction was initiated by the addition of 10 μl of purified [^{3}H]tyrosine (specific activity, 0.1 Ci/mmol) to a final concentration of 13.9 μM. After incubation for 20 min at 37°C, the reaction was terminated by the addition of 100 μl of glacial acetic acid. The reaction product, [^{3}H]H_2O, was separated on a 2.5-cm combination column of anion and cation exchange resins by elution with water. Eluates were mixed with scintillation cocktail and counted at 50% efficiency.

The second, nonisotopic assay was a modification of that described by Hooper et al. (1997). Assay tubes (total volume, 0.275 ml) contained 0.143 mM Tris-maleate buffer, pH 7.0, 60 μM L-tyrosine, 1 mM NADPH, 0.66 U dihydropteridine reductase, 0.025 to 0.8 mM BH_4, 50 μM NSD-1015, 350 U catalase, and 0.2 M glycerol. Blanks containing 300 μM 3-iodotyrosine were included for each treatment group. Reactions were initiated by the addition of striatal enzyme extract (50–100 μg of protein) and were carried out for 15 min at

TABLE 1

GBL-induced increases in TH activity in vivo and in vitro and reversal by in vivo NPA treatment

Rats were treated with NPA (30 μg/kg), GBL (750 mg/kg), and NSD-1015 (100 mg/kg) at 40 min, 35 min, and 30 min before sacrifice, respectively. Aliquots of striatal extracts were analyzed for dopa content by HPLC and for TH activity in vitro at pH 7.0 and 0.1 mM BH_4. Similar results were obtained in two additional experiments. Data are mean ± S.E. of the number of animals shown in parentheses.

Treatment	In Vivo L-dopa	In Vitro ^3H_2O
	ng/mg protein/30 min	*pmol/mg protein/min*
Veh + Veh + NSD (3)	22.07 ± 3.40	2.03 ± 0.38
Veh + GBL + NSD (3)	59.10 ± 5.45[a]	5.87 ± 0.76[a]
NPA + GBL + NSD (3)	24.60 ± 1.00	2.16 ± 0.36

[a] $p < .01$ versus NSD-1015 alone (one-way analysis of variance).
Veh, vehicle.

37°C. Samples were treated with perchloric acid and dopa production was assayed by HPLC-EC as described above.

Kinetic analysis of TH activity as a function of BH_4 concentration was analyzed according to the method of Lineweaver-Burk as described previously (Wu et al., 1992). Where appropriate, estimates of kinetic parameters were analyzed as described by Segel (1975).

Statistics and Curve Fitting. Statistical comparison of treatment groups, using SigmaStat software (v. 2.03; SPSS, Inc., Chicago, IL), employed one- and two-way analyses of variance followed by multiple comparisons (Newman-Keuls) where appropriate. Dose-response curves were fit using the ALLFIT computer program (De Lean et al., 1978) as described previously (Meller et al., 1987; Bohmaker et al., 1989).

Results

Effects of GBL on Site-Specific Phosphorylation of TH. GBL treatment markedly increased TH Ser40 phosphorylation but did not modify the phosphorylation of TH Ser31 in rat striatum (Fig. 2). The phosphorylation of TH Ser19 was also increased but to an obviously smaller extent. Moreover, NPA pretreatment reversed or attenuated the effects of GBL. The pooled results from three separate experiments are shown in Fig. 3. GBL increased the phosphorylation of Ser40 by 217%, which was completely prevented by treatment with a dose of NPA (30 μg/kg) previously shown by Meller et al. (1987) to be maximally effective in reversing the effects of GBL on tyrosine hydroxylation. In contrast, the phosphorylation of Ser19 exhibited a much smaller but significant mean increase of 40% after GBL; this was largely (80%) reversed by this dose of NPA. The effect on Ser19 was inconsistent; three separate experiments showed mean increases of 6, 40, and 75% (see below for further discussion).

Effects of GBL on Tyrosine Hydroxylation and In Vitro Enzymatic Activity. As has been shown previously, GBL treatment produced an approximately 3-fold increase in the accumulation of dopa in striatum (Table 1). NPA pretreatment (30 μg/kg) abolished the increase. Likewise, the activity of TH in vitro exhibited a similar 3-fold increase that was also reversed by NPA. Kinetic analysis of TH activity as a function of [BH_4] for the three treatment groups (Fig. 4) demonstrated that Lineweaver-Burk plots were not linear over the entire range of cofactor concentration; they exhibited a downward deflection at high [BH_4]. This has been noted previously using crude striatal extracts and has been interpreted to indicate the presence of a mixture of (activated) phosphorylated and (nonactivated) nonphosphorylated forms of the enzyme, with different kinetic properties (Lazar et al., 1982; Strait and Kuczenski, 1986; Bohmaker et al., 1989). The kinetic properties of the activated and nonactivated enzyme species were estimated by subjecting the low and high BH_4 concentration ranges to separate linear regressions (Segel, 1975; Lazar et al., 1982) (Fig. 4, A and B, respectively). The results demonstrate that GBL decreases the K_m for cofactor about 3-fold at low [BH_4], but does not alter it at high [BH_4]. We interpret this to reflect an enrichment of low K_m (activated) forms of the enzyme by GBL, and partial reversal to higher K_m (nonactivated) forms by NPA, consistent with the phosphorylation data (striatal extracts from rats treated with a submaximal 10 μg/kg dose of NPA

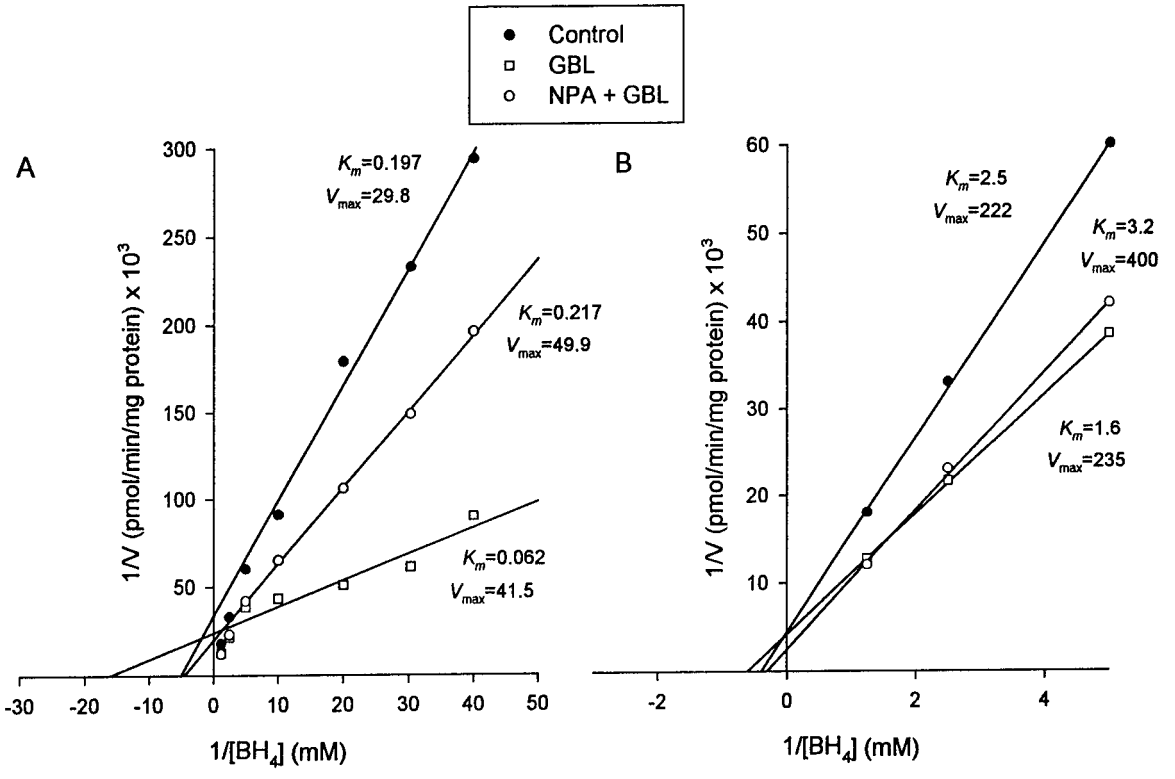

Fig. 4. Lineweaver-Burk analysis of striatal TH activity in vitro as a function of BH_4 concentration. TH activity was assayed (with 60 μM tyrosine) in pooled striatal extracts from three to four rats for each treatment group as described in *Experimental Procedures*. A, all of the data points are shown, but the linear regression line reflects only the lowest four BH_4 concentrations (0.025–0.1 mM). B, linear regression of the three highest BH_4 concentration points (0.2–0.8 mM) shown in A. The data were analyzed as described by Segel (1975). The experiment was repeated twice with similar results. K_m units shown are in mM; V_{max} units are in pmol/min/mg protein.

were used for this analysis; see below). Although the V_{max} of the low K_m form of the enzyme seemed to be increased (see legend to Fig. 4A), this effect was considerably smaller than the decrease in K_m. Several repetitions of this experiment yielded in each case a similar relationship between the treatment groups; the estimated nature of the analysis, however, resulted in considerable variance in the kinetic parameters, precluding a statistical comparison. Similar results were obtained when the analysis was conducted at a 4- to 5-fold lower tyrosine concentration (data not shown). The results are similar to those reported by Morgenroth et al. (1976); others, however, did not observe any changes in TH kinetics after treatment with the related substance γ-hydroxybutyrate (Zivkovic et al., 1975a).

Cerebral Hemitransection. As discussed above, abolition of impulse traffic in nigrostriatal DA neurons by acute surgical transection of the ascending medial forebrain bundle elicits an increase in tyrosine hydroxylation comparable to that produced by reversible chemical deafferentation with GBL. Consequently, similar alterations in the pattern of site-specific phosphorylation state of TH would be expected. Hemitransection produced a significant ($p < .001$) 3-fold increase in dopa accumulation after dopa decarboxylase inhibition; after NPA pretreatment (30 μg/kg), the effect of transection was no longer significant (Fig. 5). Although NPA treatment reduced dopa accumulation by 20% on the intact side, the effect was not significant, presumably because a relatively low dose was used; higher doses of DA agonists have been shown to significantly reduce dopa levels even on the intact side (Kehr et al., 1977).

In complete analogy with the effects of GBL, cerebral hemitransection elicited a large and significant ($p < .001$) increase in the phosphorylation of Ser40, a smaller yet significant ($p < .01$) increase in that of Ser19, and no change in the phosphorylation of Ser31 (Fig. 6). NPA pretreatment (30 μg/kg) abolished the transection-induced increase in phosphorylation of Ser40. Although two-way analysis of variance in the case of Ser19p indicated that the interaction effect was not significant, our hypothesis predicted that NPA would attenuate the effect of transection; post hoc (Newman-Keuls) comparisons were therefore performed, which confirmed that the difference between the intact and transected sides was significant only in the vehicle-pretreated animals (Fig. 6). NPA also appeared to elicit a general reduction in phosphorylation, because the main effect of NPA was significant for all three serine sites (see legend to Fig. 6); however, a post hoc Newman-Keuls analysis (permissible only for Ser40p, which showed a significant interaction effect) indicated that NPA did not significantly lower Ser40 phosphorylation on the intact side.

Transection produced changes in kinetic parameters that were analogous to those obtained after GBL treatment (data not shown) and were qualitatively similar to those reported previously (Zivkovic et al., 1975b).

NPA Dose-Response Curves for Reversal of dopa Accumulation and TH Phosphorylation after GBL Treatment. As noted above, although GBL robustly and reliably increased the phosphorylation of Ser40 and dopa accumulation, the increase in phosphorylation of Ser19 was relatively small and quite variable; in some experiments, it was not significantly increased at the same time that a large and significant increase in dopa accumulation was clearly apparent. This suggested that the increase in Ser19 phosphorylation might be a fortuitous event unrelated to the activation of TH; however, the apparent reversal of the effect by pretreatment with NPA (30 μg/kg) suggested otherwise. In an attempt to reconcile these conflicting findings, a dose-response

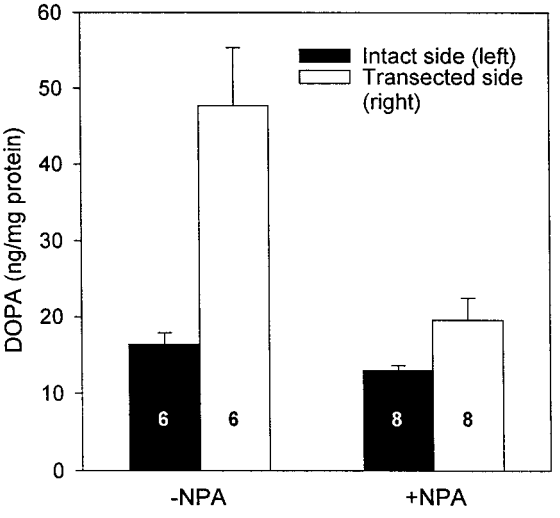

Fig. 5. Effects of cerebral hemitransection with and without NPA pretreatment on striatal L-dopa accumulation after decarboxylase inhibition. Groups of rats were treated with NPA (30 μg/kg, s.c.) or vehicle 5 to 10 min before hemitransection, followed immediately by NSD-1015 (100 mg/kg, i.p.) and sacrificed 30 min later. Values are mean ± S.E. of the number of rats shown in the columns. Two-way analysis of variance: main effect of transection, $F_{1,24} = 24.87$, $p < .001$; main effect of NPA, $F_{1,24} = 17.27$, $p < .001$; interaction, $F_{1,24} = 10.69$, $p < .01$. Multiple comparisons, intact versus transected (−NPA), $p < .001$; intact versus transected (+NPA), not significant; intact (−NPA) versus intact (+NPA), not significant.

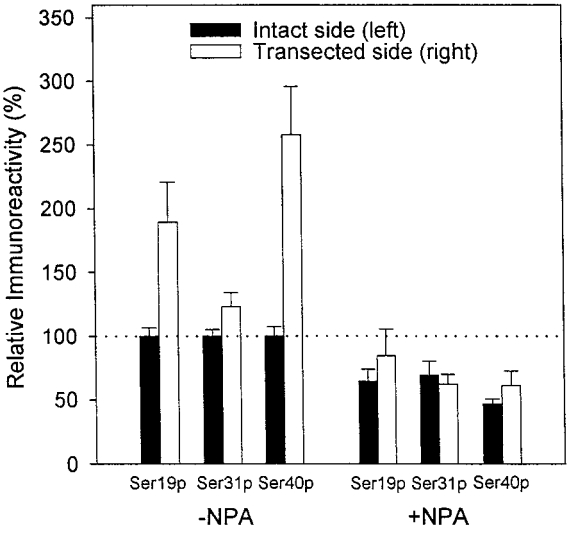

Fig. 6. Effects of cerebral hemitransection with and without NPA pretreatment on serine site- and phosphorylation state-specific immunolabeling of striatal TH. Values are mean ± S.E. of five to six rats per group. Two-way analysis of variance: Ser19p, main effect of transection, $F_{1,18} = 7.07$, $p < .02$; main effect of NPA, $F_{1,18} = 11.59$, $p < .01$; interaction, not significant. Multiple comparisons: intact versus transected (−NPA), $p < .01$; intact versus transected (+NPA), not significant. Ser31p, main effect of transection, not significant; main effect of NPA, $F_{1,18} = 24.70$, $p < .001$; interaction, not significant. Ser40p, main effect of transection, $F_{1,18} = 15.10$, $p < .001$; main effect of NPA, $F_{1,18} = 32.01$, $p < .001$; interaction, $F_{1,18} = 10.55$, $p < .01$. Multiple comparisons: intact versus transected (−NPA), $p < .001$; intact versus transected (+NPA), not significant.

analysis for NPA reversal of dopa accumulation and Ser40 and Ser19 phosphorylation was carried out. Figure 7 shows that the dose-response curves for reversal of dopa accumulation and Ser40 phosphorylation were indistinguishable. However, although GBL repeatedly (but variably) increased Ser19 phosphorylation, NPA at lower doses did not significantly reverse the increase in Ser19 phosphorylation (Fig. 8); only at the highest NPA dose used was the reversal significant.

Discussion

The present data demonstrate that the activation of TH that occurs after cessation of dopaminergic neurotransmission in the nigrostriatal pathway is accompanied by an increase in the phosphorylation of the enzyme. The use of site- and phosphorylation state-specific antibodies established that, of the three serine sites in the amino-terminal regulatory region of TH (Campbell et al., 1986; Haycock, 1990) whose phosphorylation is susceptible to physiological regulation (Haycock, 1990; Haycock and Haycock, 1991), an increase in phosphorylation occurred only at Ser40 and Ser19 (Figs. 2 and 3). Moreover, although the increase at Ser40 was robust and reproducible, that at Ser19 was less consistent. The effect was not paradigm-dependent, because abolition of DA neuronal activity either chemically (with GBL) or surgically (by cerebral hemitransection of afferent fibers) produced almost identical results (Figs. 3 and 6). However, although phosphorylation of TH seems to mediate the increased TH activity observed after either stimulation or inhibition of dopaminergic neurotransmission, both the pattern and extent of phosphorylation at specific serine sites differ substantially. Thus, in contrast to the results shown above after cessation of DA neuronal activity, stimulation of neuronal activity either electrically (Haycock and Haycock, 1991) or by treatment with DA receptor antagonists (Harada et al., 1996a; Salvatore et al., 1998) increased the phosphorylation of TH at all three serine sites 2- to 3-fold.

The latter observations made it difficult to ascertain the relative involvement of Ser19, Ser31, and Ser40 phosphorylation in the activation of TH in vivo. However, the more restricted changes observed after abolition of neuronal activity strongly support the idea that phosphorylation at Ser40 is the sole mediator of short-term regulation of TH in vivo, for several reasons. First, the most consistent effect of both GBL treatment and cerebral transection was a large and reproducible 3-fold increase in Ser40 phosphorylation, which was paralleled in each experiment by a similar large and reproducible increase in tyrosine hydroxylation. In contrast, the increase in Ser19 phosphorylation varied widely and consequently did not correlate with the increase in tyrosine hydroxylation. Indeed, in several individual experiments, Ser19 phosphorylation was not significantly altered at the same time that dopa levels were robustly elevated. Moreover, dose-dependent NPA reversal of the increase in dopa levels correlated precisely with reversal of Ser40, but not Ser19, phosphorylation (Figs. 7 and 8). Finally, Ser31 phosphorylation was unchanged; thus it is reasonable to presume that phosphorylation at this site did not contribute to the activation of TH. Although an early and transient increase in Ser31 phosphorylation, which disappeared by the time the animals were sacrificed (30 min), might potentially have influenced the results, a preliminary time course study found no changes in Ser31 phosphorylation at any time (0–30 min) after GBL treatment (data not shown).

Although the present data support the exclusion of Ser19 phosphorylation in the activation of TH elicited by cessation of DA neuronal firing, the inconsistency in the extent of phosphorylation at this site is rather curious. Two potential mechanisms that might account for the results (a rapid but transient increase or a greater post-mortem lability of

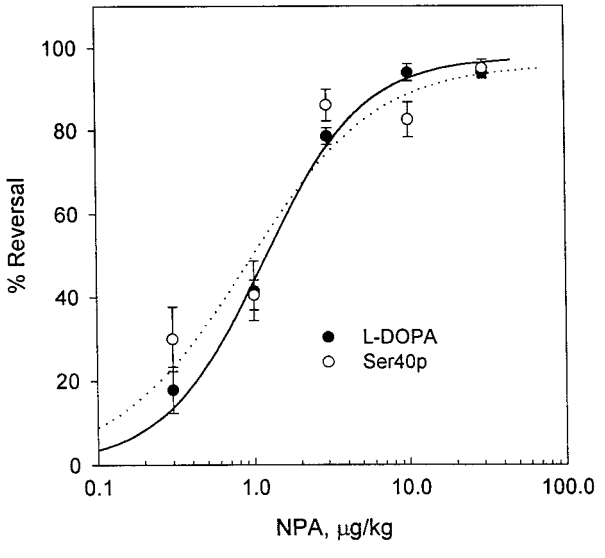

Fig. 7. Dose-response curves for NPA reversal of GBL-induced dopa accumulation or Ser40 phosphorylation. Both sets of data were obtained from the same animals and reflect 8 to 12 animals/group. The curves were fit by ALLFIT and the fit lines shown were obtained in the absence of any constraints. ALLFIT analysis indicated that all four parameters of the logistic equation used to fit the curves (minimum, maximum, slope factor, and ED_{50}) (De Lean et al., 1978; Meller et al., 1987) could be shared without a significant degradation in the fit (i.e., all of the data can be collapsed to describe a single curve).

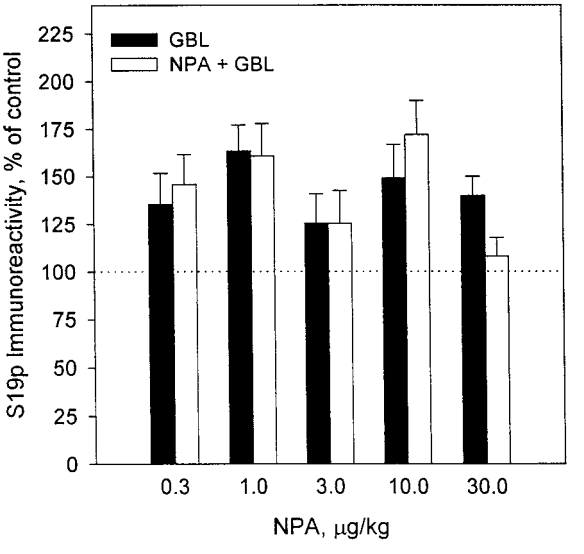

Fig. 8. Absence of a dose-dependent effect of NPA to reverse the GBL-induced increase in Ser19 phosphorylation. GBL significantly ($p < .05$) increased Ser19p immunoreactivity in every experiment except the one in which 3 μg/kg NPA was used. Only at 30 μg/kg was the effect of NPA significant (see Fig. 3). The data were generated from the same experiment as that shown in Fig. 7.

Ser19p) are not supported by preliminary experiments (data not shown).

The primary utility of GBL (as well as cerebral hemitransection) has been to provide an accessible model for studying terminal DA autoreceptor regulation of TH activity. GBL abolishes impulse traffic in nigrostriatal (and mesolimbic) DA neurons. Consequently, the effects of DA agonists are necessarily restricted to mediation by DA receptors on nerve terminals, because interaction with either postsynaptic striatal or somatodendritic nigral DA autoreceptors is irrelevant in the absence of impulse flow (Walters and Roth, 1976; Roth, 1979; Meller et al., 1987). The present results demonstrate that stimulation of inhibitory terminal DA autoreceptors not only blocks GBL-induced activation of tyrosine hydroxylation, but also site-specific phosphorylation of Ser40 in TH. The ED_{50} for both effects was 1 μg/kg, identical with the previously established value for reversal of dopa accumulation (Meller et al., 1987). However, only the highest dose of NPA used (30 μg/kg) reversed Ser19 phosphorylation. We have no ready explanation for this effect.

The present data strongly support the idea that the tonic activity of TH is dynamically regulated in a bidirectional manner. Moreover, a single mechanism, phosphorylation of the enzyme, although differing in specifics, seems to be used in this regulation. Thus, when DA neuronal activity is stimulated, protein kinase pathways are recruited that phosphorylate TH and increase DA synthesis. However, this activation is limited by the increased stimulation of presynaptic autoreceptors by DA released into the synaptic cleft. Because multiple serine sites are phosphorylated under these conditions (see above), it is possible that multiple protein kinases are activated that act in concert to phosphorylate TH. Moreover, because Ser40 has been shown to be phosphorylated in vitro by multiple protein kinases (Kumer and Vrana, 1996), activation of TH under conditions of increased neuronal activity may reflect the phosphorylation of Ser40 by these multiple kinases; the increased phosphorylation of Ser19 and Ser31 may be incidental effects of these increased kinase activities, which, by themselves, may not productively activate TH. Support for this hypothesis is provided by preliminary studies in this laboratory using striatal slices. It was found [as reported previously by Simon and Roth (1979) and El Mestikawy et al. (1983)] that forskolin and K^+ depolarization each activate TH, and their combination elicits a near-additive increase. However, it seems that this additive increase correlates with a further enhancement of Ser40, rather than Ser19 or Ser31, phosphorylation.

On the other hand, when DA neuronal activity is abolished, tonic inhibition of the phosphorylation of TH by autoreceptor stimulation is relieved because of the absence of synaptic DA, which leads to an increase in TH activity. However, the specific protein kinase pathways recruited upon stimulation or inhibition of DA neuronal activity clearly differ, because the pattern of TH phosphorylation is not the same. In this instance, the increased TH activity seems to reflect a selective increase in Ser40 phosphorylation. One or more potential mechanisms may be considered. Terminal DA autoreceptors may be coupled to inhibition of adenylate cyclase (El Mestikawy and Hamon, 1986); a GBL-induced diminution in synaptic DA would relieve this inhibition, elevate cAMP levels, and increase protein kinase A activity, leading to a selective and specific increase in Ser40 phosphorylation. Alternatively, inhibition of neuronal activity may decrease the activity of striatal cAMP phosphodiesterase(s) (Polli and Kincaid, 1994; Yamashita et al., 1997), yielding the same effect. Another possibility is that a decrease in the activity of striatal protein phosphatases ensues, leading to an increase in phosphorylated TH. Interestingly, it has been shown that protein phosphatase 2A (PP2A) is the major phosphatase responsible for dephosphorylation of TH in striatum (Haavik et al., 1989; Berresheim and Kuhn, 1994). TH phosphorylated by both protein kinase A (on Ser40) and Ca^{++}/calmodulin-dependent protein kinase II (on Ser19) (Kumer and Vrana, 1996) were substrates for PP2A; a decrease in activity of PP2A would therefore be consistent with the results obtained. (In this regard, it would be interesting to determine whether TH phosphorylated on Ser31 is a good substrate for this phosphatase). It must be noted, however, that neither of the latter two potential mechanisms readily accounts for DA agonist reversal, unless a heretofore unknown direct or indirect coupling of DA autoreceptors with these enzyme activities is postulated. Elucidation of the mechanisms operating in vivo that result in differential phosphorylation of TH and the relationship between site-specific phosphorylation and activation of the enzyme awaits further investigation.

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