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Tyrosine Hydroxylase Expression in Primary Cultures of Olfactory Bulb: Role of L-Type Calcium Channels

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Sensory activity mediates regulation of tyrosine hydroxylase (TH), the first enzyme in the dopamine biosynthetic pathway, in the rodent olfactory bulb. The current studies established for the first time primary cultures of neonatal mouse olfactory bulb expressing TH and tested whether L-type calcium channels mediate the activity-dependent regulation of the dopamine phenotype. After 1 d *in vitro* (DIV), a small population of TH-immunostained neurons that lacked extensive processes could be demonstrated. After an additional 2 DIV in serum-free medium, the number of TH neurons had doubled, and they exhibited long interdigitating processes. Membrane depolarization for 48 hr with 50 mM KCl produced a further 2.4-fold increase in the number of TH-immunoreactive neurons compared with control cultures. Increased TH neuron number required at least 36 hr of exposure to KCl. Forskolin, which increases intracellular cAMP levels, induced a 1.5- to 1.6-fold increase in the number of TH-immunostained neurons. Combined treatment with KCl and forskolin was not additive. Nifedipine, an L-type calcium channel blocker, completely prevented the depolarization-mediated increase in TH expression but did not block the response to forskolin. Treatment with Bay K8644, an L-type calcium channel agonist, also significantly increased the number of TH-expressing neurons. Depolarization also induced alterations in neuritic outgrowth, resulting in a stellate versus an elongate morphology that, in contrast, was not prevented by nifedipine. These results are the first demonstration that *in vitro*, as *in vivo*, depolarization increases TH expression in olfactory bulb and that L-type calcium channels mediate this activity-dependent regulation of the dopamine phenotype.

Key words: tyrosine hydroxylase; olfactory bulb; calcium; depolarization; primary cultures; dopamine; cAMP; L-type calcium channel; Bay K8644

Tyrosine hydroxylase (TH) is expressed in periglomerular dopamine neurons intrinsic to the olfactory bulb (Halasz et al., 1977; Baker et al., 1983). These neurons, hypothesized to modulate mitral cell excitability, receive excitatory glutamatergic input from olfactory receptor cells (Trombley and Westbrook, 1990; Berkowicz et al., 1994). TH expression developed concurrently with patterned synaptic activity between receptor and mitral cells (Gesteland et al., 1982; Baker and Farbman, 1993). In adults, reduced afferent activity produced by either primary afferent denervation or sensory deprivation resulted in profound decreases in TH expression (Nadi et al., 1981; Baker et al., 1983, 1993; Stone et al., 1990, 1991; Cho et al., 1996; Cummings et al., 1997). The mechanisms by which synaptic afferent activity either initiate or maintain olfactory bulb TH expression during development and in the adult have yet to be delineated.

Both basal and inducible TH gene regulation, previously investigated in PC12 cells and cultures of adrenal medullary chromaffin cells, occurred through several sites in the TH promoter, including the cAMP response element (CRE) (Kilbourne and Sabban, 1990; Fung et al., 1992; Kilbourne et al., 1992; Yoon and Chikaraishi, 1992; Kim et al., 1993; Lazaroff et al., 1995; Sabban, 1997). The CRE also acts as a calcium responsive element (CaRE). Treatments that increased intracellular calcium levels, veratridine, calcium ionophores, and depolarization also modulated TH expression (Kilbourne and Sabban, 1990; Kilbourne et al., 1992; Sabban, 1997). Depolarization-mediated induction of TH gene expression also occurred via the AP1 site in the TH promoter (Nagamoto-Combs et al., 1997). Recent studies also demonstrated an important role for L-type calcium channels in depolarization-induced expression of immediate early genes such as c-fos (Murphy et al., 1991), which bind to the AP1 site (Sheng and Greenberg, 1990; Sheng et al., 1990). Significantly, in olfactory bulb dopamine neurons, decreased expression of fos family genes paralleled the downregulation of TH expression, suggesting an important role for calcium in TH gene regulation (Guthrie et al., 1993; Guthrie and Gall, 1995a,b; Jin et al., 1996).

Neonatal olfactory bulb cultures were chosen to study the relationship between depolarization and TH expression for several reasons. First, both *in vivo* and *in vitro* studies showed cell type-specific regulation of the dopaminergic phenotype that differed between olfactory bulb and other brain dopaminergic systems (Weiser et al., 1993; Lazaroff et al., 1995; Tinti et al., 1996). Second, although all previous studies of olfactory bulb TH (Denis-Donini, 1989; McMillian et al., 1994) used primary cultures obtained from embryonic tissues, significant numbers of TH-expressing cells cannot be demonstrated until gestational day 18 (Baker and Farbman, 1993). Thus, to study authentic and not ectopic phenotypic expression, postnatal neurons must be cultured. Last, because most periglomerular neurons have birth dates between 1 and 4 days postnatal (Hinds, 1968: Bayer, 1983), robust TH expression could be expected in cultures of neonatal olfactory bulb.

Thus, the current studies used primary cultures derived from neonatal olfactory bulbs to delineate whether depolarization and cAMP mediate TH gene regulation in the olfactory bulb. Also-

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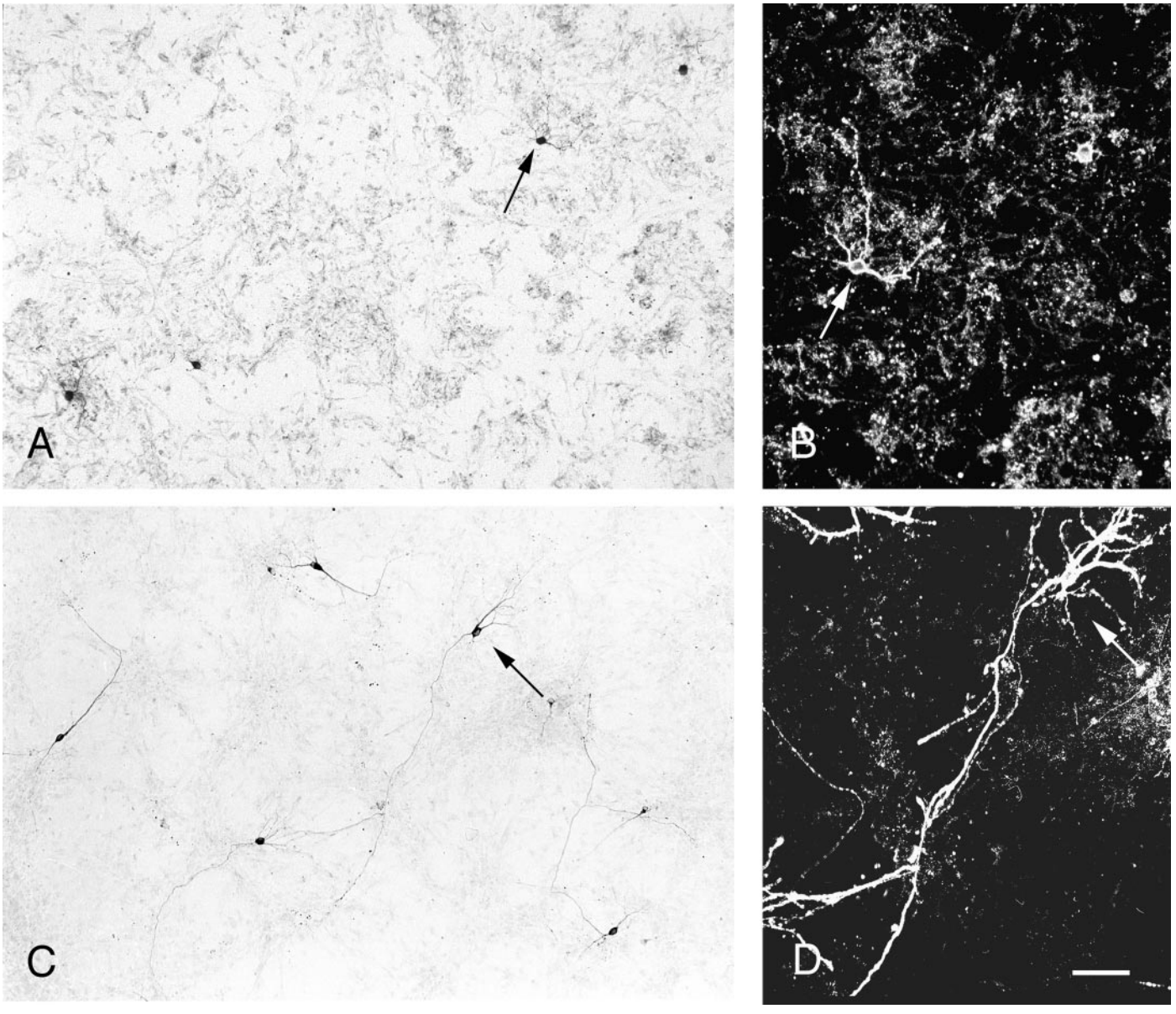

Figure 1. Time-dependent development of TH expression in culture. *A*, A bright-field photomicrograph illustrates olfactory bulb cultures after 24 hr (time 0) in serum-containing medium. TH-immunoreactive cells are dispersed throughout the culture but have few relatively short processes that do not fasciculate. *B*, Dark-field photomicrograph emphasizes the paucity of processes. *C*, After an additional 48 hr in serum-free control medium (see Materials and Methods), the increased number of TH-immunostained neurons displayed many long interdigitating processes, illustrated in *D*. *Arrows* in *A* and *C* indicate the same cells shown at higher magnification in *B* and *D*. Scale bar (in *D*): *A*, *C*, 100 μm; *B*, *D*, 65 μm.

investigated was a role for L-type calcium channels in activity-dependent regulation of TH expression.

MATERIALS AND METHODS

Animals. C57Bl/6J females and CBA/J males, originally purchased from The Jackson Laboratory (Bar Harbor, ME), were bred in house to obtain mouse pups of appropriate age. Mice were housed in an approved animal facility under constant temperature, cycled lighting (12 hr light/dark cycle), and access to food and water *ad libitum*. All procedures were performed under protocols approved by the Cornell University Institutional Animal Care and Use Committee and conformed to National Institutes of Health guidelines.

Cell culture. Cell culture was performed according to previously published methods with slight modification (Iacovitti et al., 1992). Olfactory bulbs were collected from a single litter of 2-d-old mouse pups (6–10 per isolation), cleaned of investing membranes, minced, and dissociated at 37°C for 30 min in trypsin–EDTA (0.1% in HBSS) (Life Technologies, Grand Island, NY). After trituration with a fire-polished Pasteur pipette, the cells were rinsed with medium (DMEM high-glucose medium) containing 10% fetal calf serum (Life Technologies), 10 μM L-glutamine (Sigma, St. Louis, MO), 0.6% glucose, and 1% penicillin–streptomycin (Sigma). To avoid edge effects (Takeshima et al., 1996), 100 μl containing $0.2–0.3 \times 10^6$ cells were plated in the center of each well in a four-chamber slide (Nunc, Naperville, IL), previously coated overnight with a solution of polyornithine (1 mg/ml; Sigma) in 0.15 M borate buffer. After 30 min to allow the cells to attach, 400 μl of medium was added to each well, and the slides were placed in a humidified 5% CO_2–95% air incubator at 37°C. After 24 hr, 0 hr with respect to treatments, the medium was replaced with a serum-free neurobasal medium (Life Technologies) containing 1% N-2 supplement (Life Technologies), 0.5 mM L-glutamine (Sigma), 25 μM L-glutamic acid (Sigma), and the different treatments (see below). Defined serum-free medium was used to obviate the possibility that the observed responses were mediated by the actions

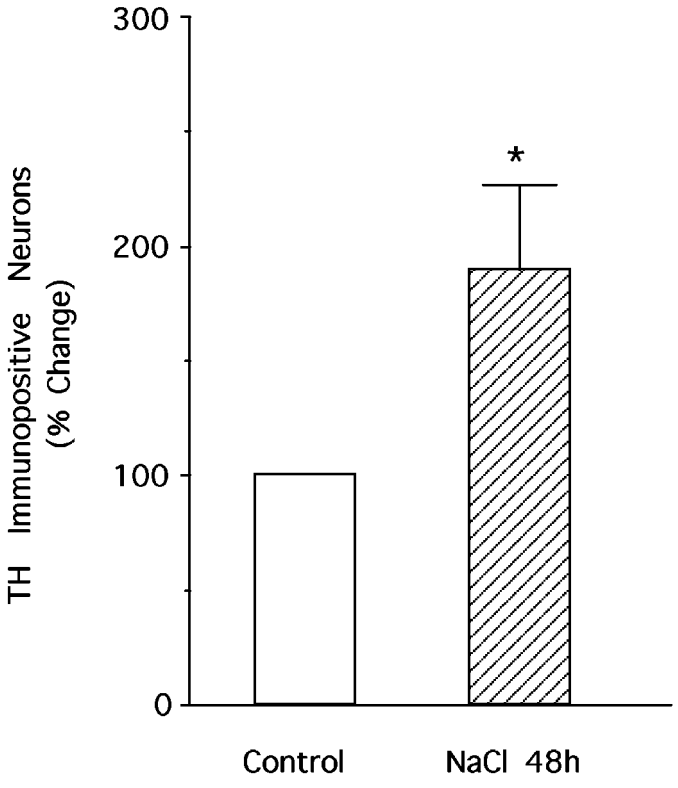

Figure 2. Significant ($p < 0.001$) increase in the number of TH-immunostained neurons after 48 hr in control (NaCl-containing) serum-free medium. Control cultures (time 0) contained 4.6 ± 0.65 TH-immunopositive cells/mm^2. Data were obtained from three experiments performed in triplicate. Comparisons were made by a paired Student's *t* test.

Table 1. Quantitative analysis of cell types in olfactory bulb cultures

Cell type	NaCl[a]	KCl
Neurons	1475 ± 157	1329 ± 324
Glia	452 ± 47	569 ± 81
Total nuclei	2344 ± 164	2422 ± 91
TH-immunopositive	8.75 ± 1.16	18.96 ± 2.12*

For each analysis condition, data were averaged from at least two wells obtained from at least three experiments (cultures prepared from different isolations). *$p < 0.005$.

[a]Data are presented as mean density per square millimeter ± SEM.

of unknown serum components. The cells were fixed for 20 min with 4% buffered formaldehyde either immediately after or 48 hr after replacement of the medium.

Treatments. To mimic *in vivo* afferent stimulation, parallel cultures were either depolarized with 50 mM KCl or, as a control, an equimolar concentration of NaCl. Treatments were started simultaneously with the replacement of the serum-containing medium with the serum-free medium and interrupted 48 hr later by fixation of the cells. In some cultures, depolarization was interrupted after either 4, 8, 16, 24, or 36 hr by replacing the KCl-containing medium with NaCl-containing medium. Medium was replaced at the same time points in control cultures. To determine the response to increased intracellular levels of cAMP, some cultures were stimulated with 10 μM forskolin in DMSO (Tinti et al., 1996). An equal concentration of DMSO was used as a control. To investigate the role of calcium in the induction of TH, nifedipine (Sigma), an L-type calcium channel blocker (10 μM in DMSO), was added to the serum-free medium 30 min before either KCl or forskolin treatments and to the respective control cultures (Miller, 1987). Cultures also were treated for 48 hr with the L-type calcium channel agonist Bay K8644 (1 μM in DMSO; Sigma) either alone or in the presence of 15 mM KCl (Brosenitsch et al., 1998), added with the serum-free medium (Nowycky et al., 1985). Because Brosenitsch et al. (1998) reported that low-level depolarization, which did not alter TH expression, potentiated the effects of Bay K8644 in primary sensory neurons, similar treatments were used in the current studies. All cultures were harvested after 48 hr in serum-free medium unless otherwise stated.

Immunocytochemistry. Fixed cultures were rinsed two times for 10 min each in PBS, preincubated 30 min in 0.1 M PBS with 1% bovine serum albumin (BSA) and 0.2% Triton X-100, washed two times for 10 min each in 0.1 M PBS with 0.5% BSA, and incubated overnight in primary antisera to either TH (1:25,000), glial fibrillary acidic acid (GFAP) (1:12,000), or neuron-specific β-tubulin isotype III (1:10,000). Specificity of the antisera was established previously (Baker et al., 1983; Debus et al., 1983; Grill and Pixley, 1997). After two 10 min washes in 0.1 M PBS and 0.5% BSA, cells were incubated for 1 hr in secondary antibody of either biotinylated anti-rabbit or anti-mouse IgG. The wells were then washed twice in 0.1 M PBS–0.5% BSA and further incubated for 1 hr in the avidin–biotin complex (Elite Kit) purchased from Vector Laboratories (Burlingame, Ca). Antigens were visualized by incubation for 5 min in 0.05% 3,3′-diaminobenzidine HCl and 0.003% hydrogen peroxide. Some wells were counterstained with Mayer's hematoxylin, and all slides were dehydrated through graded alcohols, cleared in xylene, and coverslipped with Permount (Fisher Scientific, Fairlawn, NJ).

Cell counting procedures. The number of TH-immunolabeled cells was counted in at least 20 fields per well at 160×. In most preparations, more than five TH-immunoreactive cells were found in the same field of view under basal conditions. The area sampled in each well was ≥11 mm^2. Cells were counted only if they exhibited a clearly stained cell body. Similar counting procedures were used for evaluating the density of GFAP- and β-tubulin-immunoreactive cells. Total nuclear density in hematoxylin-stained cultures was estimated at 400× magnification in at least 20 fields.

To standardize the changes in the number of immunopositive TH cells, the data were expressed in terms of percent of increase relative to within culture controls instead of absolute numbers. For each treatment condition, data were averaged from at least four wells obtained from at least three experiments (cultures prepared from different isolations).

Measurement of neurite length. Neurite length was measured by visualizing the TH-immunopositive neurons in dark-field conditions at 100×. In each well, at least 50 TH-immunopositive neurons were analyzed for neurite length. The longest neurite on each TH-positive cell was measured as an estimate of the altered morphology. These judgments were validated between two examiners. Measurement of the neurite length was accomplished by transferring the dark-field image to a digital image on which a cursor was used to trace the longest neurite for each neuron (Ibas20; Zeiss, Thornwood, NY). Programs, which have been validated, transformed cursor lengths into micrometers (Volpe et al., 1995; Saji et al., 1996).

Statistical analysis. All values are presented as mean ± SEM. Sample sizes (*n*) are listed in the text or in the legend to each figure. ANOVA was used to test for multiple comparisons among independent groups of data. The presence of significant differences between groups was then determined by the Bonferroni method (Wallenstein et al., 1980). Comparisons between data with only two variables were performed by a paired Student's *t* test (Wallenstein et al., 1980). $p < 0.05$ was considered to be significant.

RESULTS

Tyrosine hydroxylase immunostaining in culture

After 24 hr in a serum-containing medium, TH-immunoreactive neurons were dispersed throughout the culture (Fig. 1*A*). The cells generally displayed strong immunoreactivity, were round in shape, and lacked elaborate processes (Fig. 1*B*). With an additional 48 hr *in vitro* in a serum-free control (NaCl-containing) medium, TH-immunoreactive cells exhibited variable morphology ranging from round to fusiform and were now characterized by the presence of long complex processes (Fig. 1*C*,*D*). To examine the consequences of time *in vitro* on the number of TH-containing neurons, immunostained cells were counted either 24 hr (time 0 with respect to treatments) after plating or after an

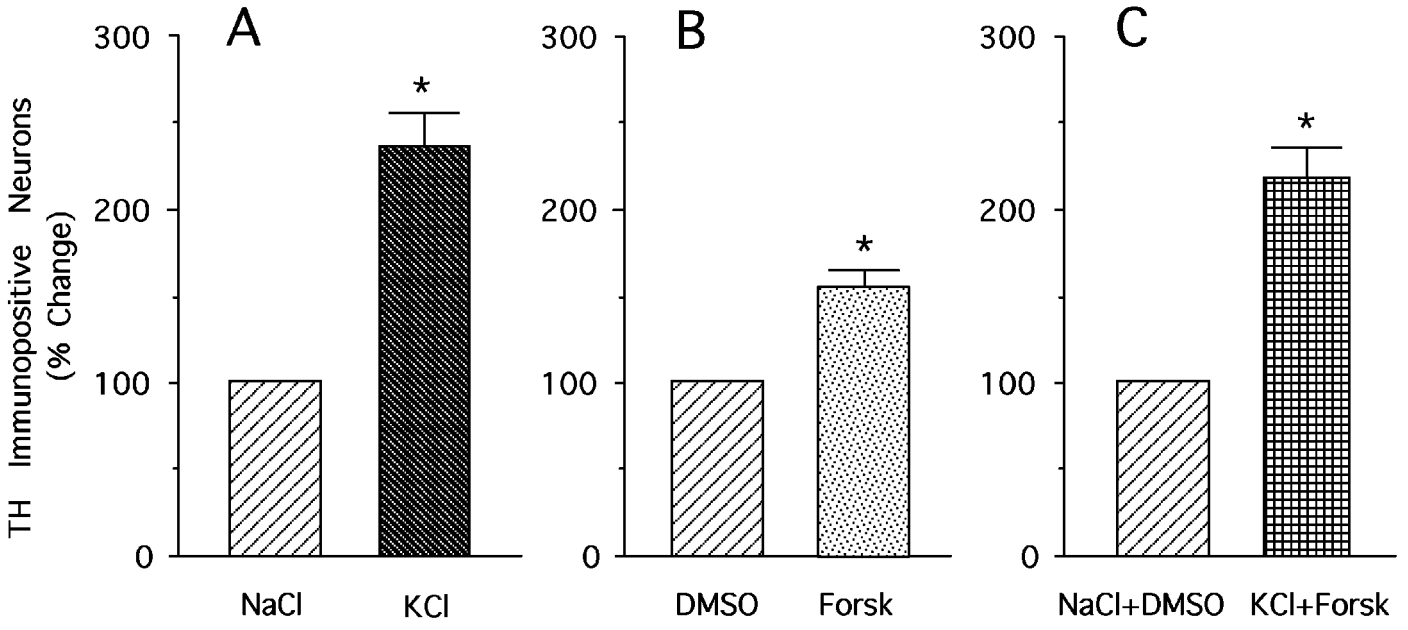

Figure 3. Depolarization- and forskolin-induced expression of TH. *A*, Membrane depolarization with 50 mM KCl for 48 hr produces a significant ($p < 0.001$) 2.4-fold increase in the number of TH-immunoreactive neurons compared with control NaCl-treated cultures. The latter contained 8.75 ± 1.2 TH-immunopositive cells/mm^2. Data were obtained from four experiments performed in at least triplicate. *B*, Forskolin (*Forsk*) produced a significant ($p < 0.001$) 1.6-fold induction in the number of TH-immunostained neurons compared with control DMSO-treated cultures. The mean number of TH-immunoreactive cells in DMSO control cultures was 10.35 ± 1.99 per square millimeter. Four experiments performed in at least triplicate were analyzed to produce these data. *C*, Combined treatment with both KCl and forskolin was not additive, producing no further induction in the number of TH-immunostained neurons. DMSO–NaCl-treated control cultures had 8.12 ± 0.46 TH-immunopositive cells/mm^2. Comparisons were made by a paired Student's *t* test.

additional 48 hr in the serum-free control medium. The number of TH-immunostained cells increased almost twofold at 48 hr ($p < 0.005$) compared with cells exposed only to serum-containing medium for 24 hr (time 0) (Fig. 2). Although a significant increase occurred with time in culture, the number of TH-immunoreactive cells at 48 hr represented only 0.37% of the total number of cells in each well (Table 1). The relative proportion of TH stained to the total number of neurons was similar to that reported previously in other primary cultures of dopamine neurons (McMillian et al., 1994).

KCl-induced increase in number of TH-immunopositive neurons

Continuous depolarization for 48 hr produced a 2.4-fold ($p < 0.001$) increase in the number of TH-immunostained neurons compared with control (NaCl-treated) cultures (Fig. 3*A*). The immunopositive neurons, as in control wells, were dispersed throughout the cultures; however, depolarization altered the morphology of their processes (see below).

Control and KCl-treated cultures also were examined to confirm the specificity of the depolarization-induced increase in the number of TH neurons. Both the density of neurons stained with anti-β-tubulin and glial cells stained with anti-GFAP showed no significant differences ($p > 0.05$) between KCl- and NaCl-treated cultures (Table 1). There was no alteration ($p > 0.05$) in the total number of cells between the two treatment conditions, evaluated by hematoxylin staining of nuclei (Table 1). Moreover, the magnitude of the increase in the number of TH-immunostained neurons was independent of both plating density and the number of TH neurons in control cultures (data not shown).

Time course of TH induction

To determine whether shorter-term exposure to KCl was sufficient to increase TH gene expression, cells were treated with KCl for either 4, 8, 16, 24, 36, or 48 hr. Depolarization was terminated by replacement of the KCl-containing medium with the NaCl-containing medium. All cultures were harvested 48 hr after the onset of treatment. The medium was replaced at the same time in the control cultures. No significant increase was seen at 16 and 24 hr of stimulation (Fig. 4). The minimal duration of depolarization that produced a significant increase (1.47-fold) in TH immunoreactivity was 36 hr ($p < 0.001$). The peak increase occurred at 48 hr ($p < 0.001$), which was the longest time investigated. The KCl-induced increase obtained at 48 hr did not differ between this and other experiments. Replacement of control medium with fresh NaCl-containing medium did not alter the number of TH-immunopositive neurons compared with cultures maintained continuously in the same control medium for 48 hr (data not shown).

Nifedipine treatment blocks KCl-induced increase in TH expression

Depolarization-induced increases in TH expression in other model systems result at least in part from an increase in intracellular calcium (Sabban, 1997). To examine the role of calcium in the induction of TH expression by depolarization in the olfactory system, parallel wells were treated for 48 hr with either KCl or NaCl alone or in the presence of nifedipine, a drug that inhibits calcium entry through L-type calcium channels. Nifedipine completely prevented the depolarization-induced 2.1-fold increase ($p < 0.05$) in the number of TH-immunostained neurons (Fig. 5*A*). Pretreatment with the calcium blocker did not alter the

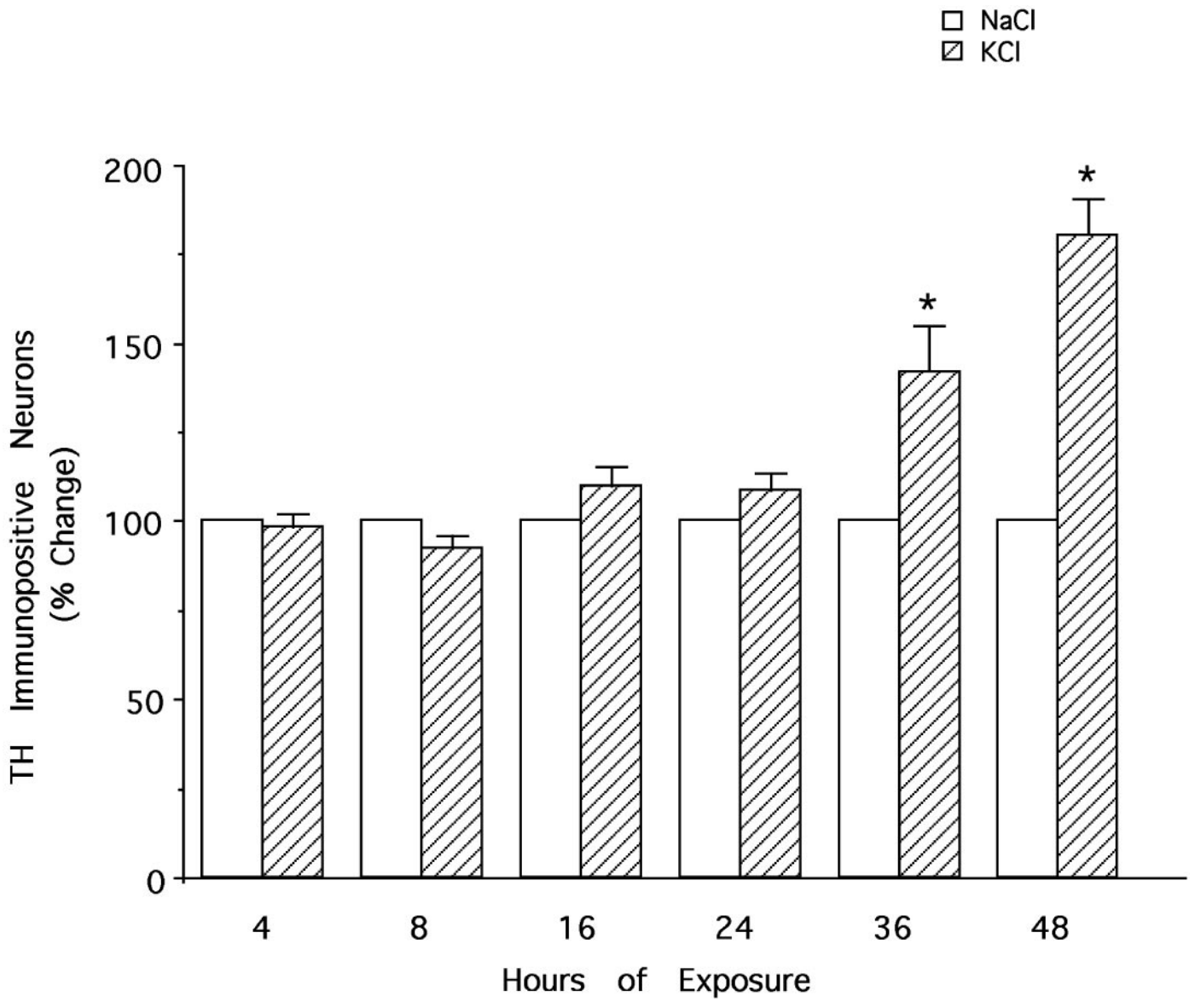

Figure 4. Time dependence of depolarization-induced TH expression. An increase in the number of TH-immunostained neurons occurred only when cultures were treated 36 and 48 hr with 50 mM KCl (*hatched bars*). Cultures were exposed to KCl for the time indicated and then incubated in control medium until fixation at 48 hr after the onset of stimulation. Values were calculated relative to their respective control culture (*open bars*). In control cultures, the NaCl-containing medium was replaced at the same time as in the KCl-treated cultures and maintained until 48 hr. Data were obtained from three experiments performed in duplicate.

number of immunostained neurons in NaCl-treated cultures (Fig. 5*A*).

Bay K8644 treatment mimics KCl-induced increase in TH expression

To establish whether a selective L-type calcium channel agonist alone was able to induce TH-expression, cultures were treated with 1 μM Bay K8644 added to the serum-free medium. Confirming the role of L-type calcium channels, this study showed that compared with the vehicle DMSO, Bay K8644 alone produced a significant increase (mean ± SE; 1.43 ± 0.09-fold; $p < 0.05$; $n = 18$) in the number of TH-immunoreactive neurons after 48 hr of treatment. In agreement with the findings of Brosenitsch et al., (1998) that slight depolarization augmented the effects of Bay K8644, the agonist was added in the presence of a concentration of KCl (15 mM), which alone did not increase the number of TH-immunostained neurons ($p > 0.05$), and produced a 2.1 ± 0.27-fold increase ($p < 0.05$; $n = 9$) in the number of TH-immunoreactive neurons compared with control cultures treated with DMSO and KCl. In this series of experiments, treatment with 50 mM KCl produced the expected 2.1 ± 0.3-fold increase ($p < 0.05$; $n = 14$) in the number of TH-immunoreactive neurons.

Forskolin-induced increase in number of TH-immunostained neurons

Forskolin, an adenylate cyclase agonist, at a maximally effective concentration (10 μM) produced a 1.6-fold increase in the number of TH-immunopositive neurons (Fig. 3*B*) compared with control (DMSO-treated) cultures ($p < 0.005$). To determine whether depolarization and cAMP stimulated TH expression through either a common rate-limiting mechanism or through different

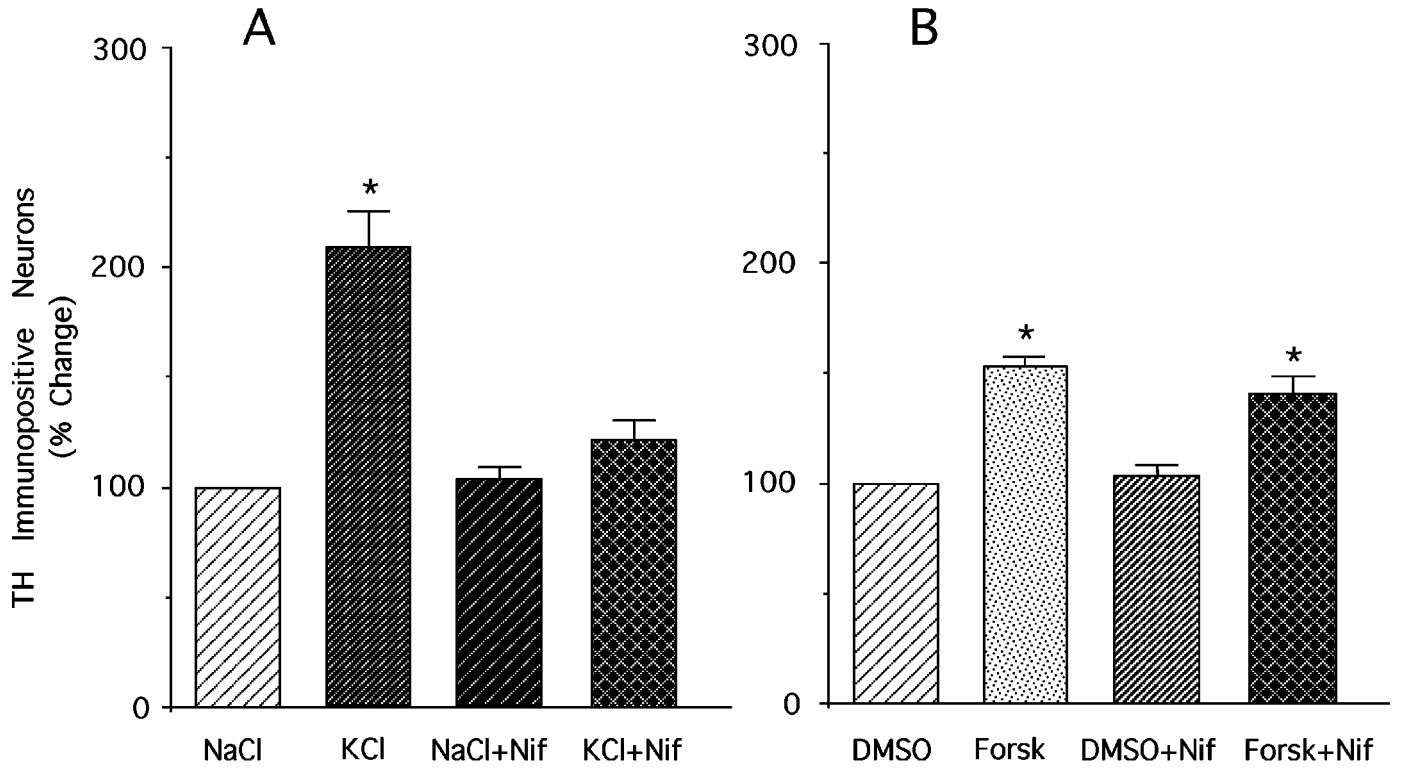

Figure 5. Nifedipine blocks KCl-induced, but not forskolin-induced, increase in TH expression. *A*, The 2.1-fold increase ($p < 0.001$) in the number of TH-immunopositive neurons was prevented by nifedipine pretreatment, resulting in the same number of TH-immunopositive cells in NaCl-treated and KCl plus nifedipine-treated cultures. Pretreatment with nifedipine did not alter the number of cells in control NaCl-treated cultures ($p > 0.05$). The mean number of immunopositive cells per square millimeter in NaCl-treated cultures was 10.49 ± 2.0. Data were obtained from four experiments performed in triplicate. *B*, The forskolin-induced increase in TH-immunopositive neurons continued in cultures pretreated with nifedipine ($p < 0.01$). Nifedipine does not alter the number of stained cells in control DMSO-treated cultures. The mean number of immunopositive cells per square millimeter in DMSO-treated cultures was 13.87 ± 2.1. Data are expressed relative to control in either NaCl-treated (*A*) or DMSO-treated (*B*) cultures. Statistical comparisons were made by ANOVA with a Bonferroni *post hoc* test to determine significant differences between treatments.

additive mechanisms, cultures were treated with both KCl and forskolin. Compared with a 2.4-fold increase induced by KCl alone and a 1.6-fold increase produced by forskolin alone, combined treatment produced a 2.2-fold increase in the number of positive cells (Fig. 3*C*). The magnitude of increase did not differ significantly ($p > 0.05$) from the effects obtained with either KCl or forskolin alone. These findings suggest that either depolarization and cAMP stimulate TH expression through the same pathway or that depolarization produces the maximal possible increase in TH expression, which is a ceiling effect.

Nifedipine does not alter forskolin-mediated TH induction

A role for calcium also has been hypothesized for TH gene induction by cAMP-dependent mechanisms (Stachowiak et al., 1994; Sabban, 1997). To further examine the role of L-type calcium channels in the regulation of TH, the effects of nifedipine were evaluated in the TH induction produced by forskolin. Nifedipine pretreatment did not change ($p > 0.05$) the number of TH-immunopositive neurons produced by forskolin stimulation (Fig. 5*B*). Addition of nifedipine to the control (DMSO-treated) cultures did not change the number of TH-immunopositive cells (Fig. 5*B*).

KCl- and forskolin-induced changes in cellular and neurite morphology

Treatment with KCl produced not only a change in the number of TH-expressing neurons but also in their morphology. In control cultures, TH-immunoreactive neurons usually displayed long, relatively unbranched neurites (Figs. 6*A*, 7*A*). After depolarization, the cells took on a stellate appearance, with more neurites and a more complex aspect (Figs. 6*B*, 7*B*). However, no major change in staining intensity of TH-immunopositive neurons was apparent after a 2 d treatment with KCl. In contrast to the nifedipine-mediated blockade of the KCl-induced increase in the number of neurons expressing TH, the calcium blocker did not prevent the change in neurite morphology (Figs. 6*D*, 7*D*). Nifedipine pretreatment also did not alter neurite morphology in control cultures (Figs. 6*C*, 7*C*).

Because of the close apposition of the cells in the KCl-treated cultures, it was difficult to distinguish reliably between all the neurites of individual cells and total neurite number, and length could not be determined. Because the neurites appeared shorter in the depolarized condition, the changes in neurite morphology were assessed by determining the length of the longest process independent of their number. At 48 hr after treatment, the longest neurites were significantly shorter in KCl-treated than NaCl-treated cultures (Fig. 8). Examination of cultures at other treatment intervals did not reveal any apparent differences in neurite length. The alteration in neurite length was not prevented by pretreatment with nifedipine (Fig. 8).

In DMSO-treated cultures, TH-immunopositive neurons displayed a similar morphology, as observed in NaCl control cultures. Neurons had long, relatively less branched neurites (Fig. 9*A*,*C*). Forskolin produced both an increase in the number of

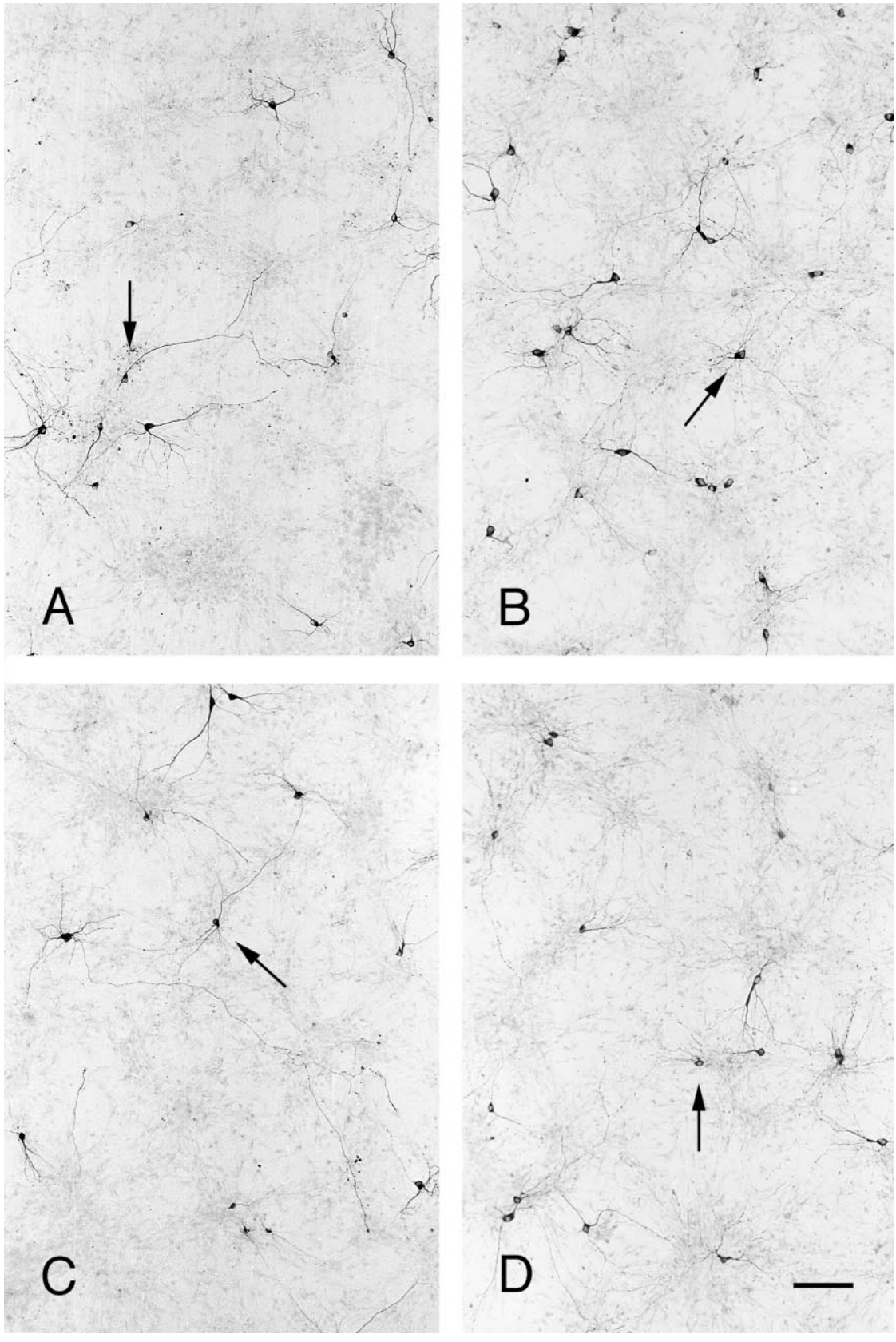

Figure 6. Bright-field micrographs illustrating the KCl-induced increase in number of TH-immunoreactive cells and its prevention with nifedipine. The number of TH-immunostained cells increased by more than twofold in KCl-treated compared with NaCl-treated cultures (*B* and *A*, respectively). *D*, Nifedipine prevented the increase in the number of TH-immunostained neurons in KCl-treated cultures without altering the number of immunopositive cells in NaCl-treated control cultures (*C*). See Figure 5 for quantitative comparisons. *Arrows* indicate the same cells shown at higher magnification in the corresponding panels in Figure 7. Scale bar, 100 μm.

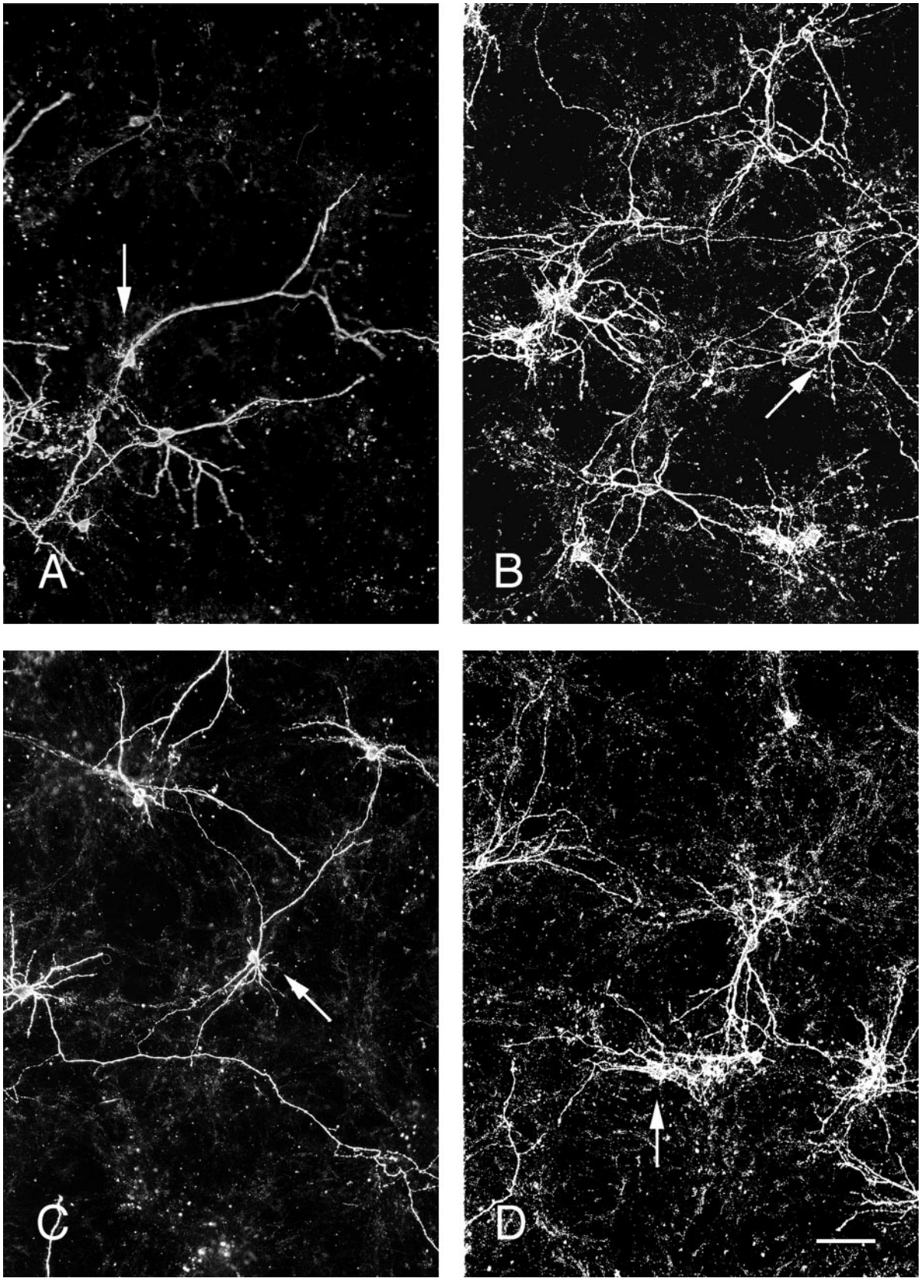

Figure 7. Dark-field photomicrographs illustrating the KCl-induced change in neurite morphology. *A*, Neurons in NaCl-treated cultures often displayed long, relatively unbranched TH-immunoreactive neurites. *B*, In contrast, depolarization produced cells with a stellate appearance resulting from the many processes of perikaryal origin and the paucity of long neurites. The morphological changes were assessed by measuring the length of the longest neurite (Fig. 8). *C*, Nifedipine did not alter neurite morphology in control cultures. *D*, The depolarization-induced change in cell morphology was not altered by nifedipine pretreatment, resulting in a continuing stellate appearance of the KCl-treated cells. Scale bar, 65 μm.

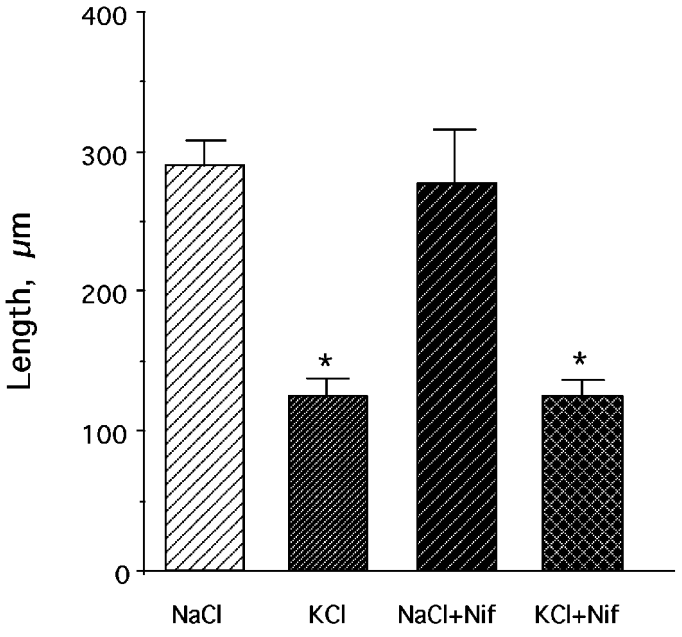

Figure 8. KCl-induced change in neurite length. The length of the longest TH-immunoreactive neurite was significantly ($p < 0.001$) reduced by treatment with depolarizing concentrations of KCl (Figs. 6, 7). Nifedipine pretreatment did not prevent the depolarization-induced change in neurite morphology. Data were analyzed by ANOVA using a Bonferroni *post hoc* test for between group comparisons. *Asterisks* indicates significant difference from respective control.

TH-immunoreactive cells and a stellate morphology compared with DMSO-treated cultures (Fig. 9*B*,*D*). The morphological alterations appeared comparable to those produced by depolarization and were not assessed quantitatively.

DISCUSSION

A number of *in vivo* studies demonstrated that expression of the dopaminergic phenotype in periglomerular neurons intrinsic to the olfactory bulb, as assessed by expression of TH, required odor-induced afferent activity (Brunjes et al., 1985; Brunjes and Frazier, 1986; Kosaka et al., 1987; Baker, 1990; Guthrie et al., 1990; Stone et al., 1990, 1991; Baker et al., 1993; Cummings et al., 1997). *In vitro* studies, primarily in cell lines, indicated that membrane depolarization, which mimics afferent stimulation, modulated transcription of a number of genes, including TH (Kilbourne and Sabban, 1990; Kilbourne et al., 1992; Stachowiak et al., 1994; Sabban, 1997). The goal of the current studies was to investigate the mechanisms underlying depolarization-dependent regulation of TH expression using primary cultures derived from neonatal mouse olfactory bulb.

Tyrosine hydroxylase expression *in vitro*

The current studies demonstrated that TH-immunoreactive neurons occurred 1 d after plating in serum-containing medium in primary cultures prepared from 2 d-old mouse pups. The presence of short, unbranched processes indicated the relatively undifferentiated state of these neurons. After an additional 48 hr in serum-free medium, the number of TH-immunostained neurons increased by almost twofold. Because the isolation of cells results in deafferentation of the periglomerular neurons, a process that downregulates TH expression *in vivo* (Nadi et al., 1981; Baker et al., 1983, 1984), the presence of TH neurons in the primary cultures after 1 d *in vitro* (DIV) requires explanation. One possibility is that degradation of TH protein may require several days, as demonstrated previously (Cho et al., 1996).

However, the presence of previously synthesized TH protein does not account for the increase in the number of TH-containing cells in unstimulated cultures after an additional 2 DIV. The developmental origin of TH neurons in olfactory bulb *in vivo* may be relevant. TH expression was found only after dopaminergic neurons, which derive from the anterior subventricular zone and migrate into the olfactory bulb (Betarbet et al., 1996), reached the periglomerular region in which they received afferent stimulation (McLean and Shipley, 1988; Baker and Farbman, 1993). Thus, the increased number of TH-immunostained neurons after 2 DIV could be a consequence of TH protein synthesis in migrating cells that received afferent stimulation before dissociation but before completion of the transduction program. The formation of extensive processes also suggested that an intrinsic neuronal maturation program occurred during this time. In addition, either low levels of synaptic activity (Murphy et al., 1991) or low concentrations of glutamic acid, the putative receptor cell afferent transmitter (Berkowicz et al., 1994), in the cultures could induce TH synthesis in an already committed population of cells.

KCl-induced increase in cells expressing TH

Treatment of the cultures with a depolarizing concentration of KCl produced a further 2.5-fold increase in the number of TH-expressing neurons. This is the first report of a direct effect of membrane depolarization on the number of TH neurons in primary olfactory bulb cultures. The only previous study, performed on cultures prepared from embryonic day 16 rat embryos before few, if any, TH neurons have been generated, required pretreatment with the opiate antagonist nalaxone before an effect of depolarization could be demonstrated (McMillian et al., 1994). Thus, both differences in culture conditions and embryonic age could account for the requirement for opiate antagonists to induce TH expression. Importantly, the TH induction was not a consequence of a generalized increase in the number of neurons, glia, or total cells.

At issue is the origin of the newly demonstrable TH-containing neurons. A likely source could be a population of cells committed to express TH *in vivo* (Denis-Donini, 1989; Betarbet et al., 1996) but have not yet received afferent stimulation, which is then mimicked *in vitro* by the KCl treatment. In addition, selective effects on proliferation of TH-containing neurons also could occur, because division of cells expressing a neuronal phenotype was found during their migration from the anterior subventricular zone to the olfactory bulb (Menezes et al., 1995). Last, prolonged neuronal survival specific to the TH-expressing cells also may contribute to the increase. However, although depolarization was shown to promote neuronal survival (Franklin et al., 1995), it also increased calcium entry into cells, a condition associated with cell death (Ellis et al., 1991; Cigola et al., 1997). Further studies are planned to evaluate a possible contribution of hyperplasia and apoptosis to the increase in TH-expressing cells. Surprisingly, short-term depolarization (<36 hr) did not induce an increase in the number of TH-immunostained cells, suggesting that prolonged stimulation is necessary to modulate the regulatory gene cascade required for induction of TH expression. This hypothesis is supported by a recent study showing that several days of odor stimulation are required to reverse the effects of sensory deprivation on olfactory bulb (Cummings et al., 1997).

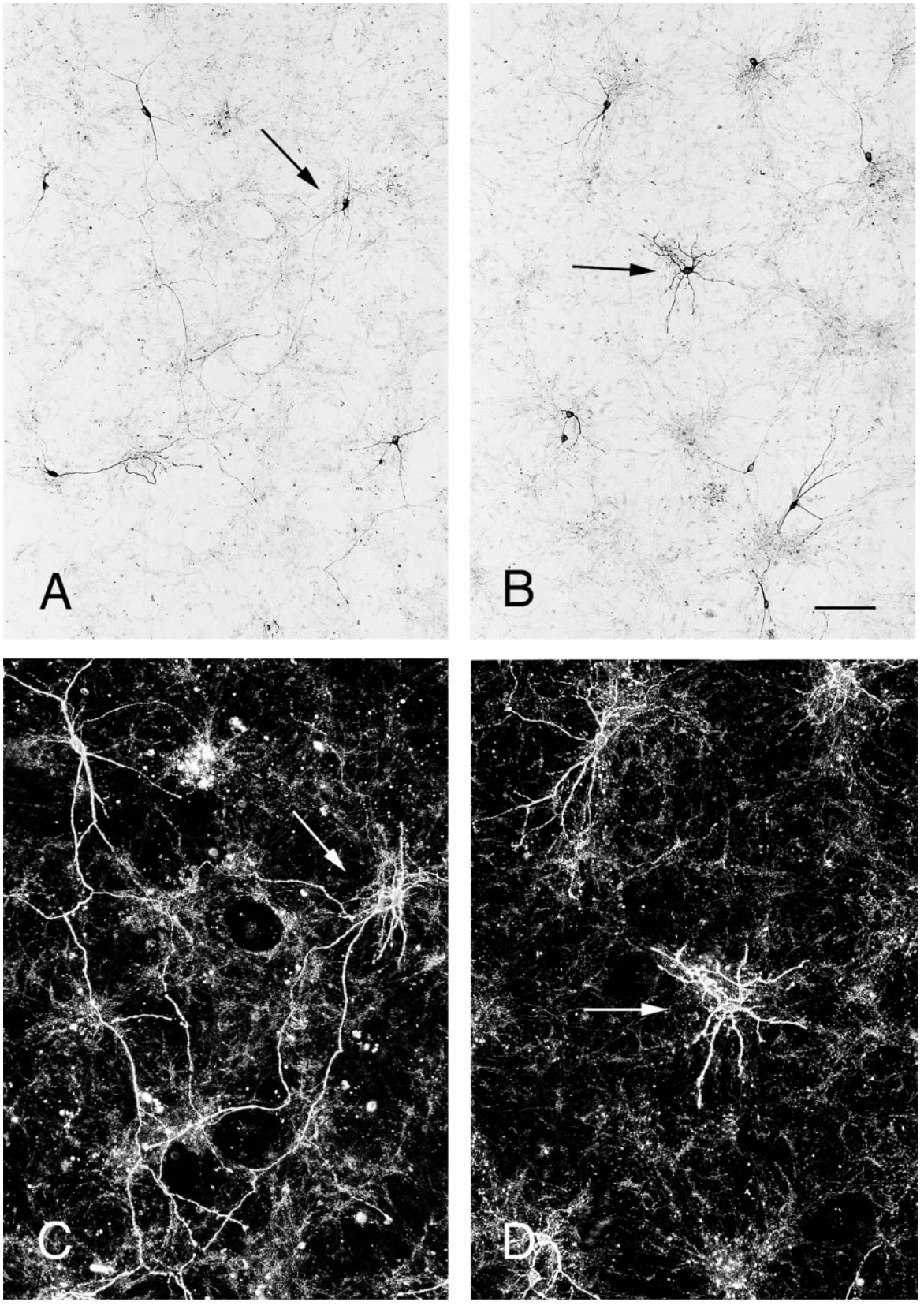

Figure 9. Forskolin-induced change in TH neuron number and neurite morphology. Forskolin (*B*) produced an increase in the number of TH-immunopositive neurons compared with DMSO-treated control cultures (*A*). Dark-field photomicrographs illustrate that in forskolin-treated cultures (*D*) compared with DMSO (*C*)-treated cultures, TH neurons displayed a stellate morphology. In the latter cultures, immunostained processes were the same length as those observed in NaCl control cultures. *Arrows* in *A* and *B* indicate the same cells shown at higher magnification in *C* and *D*. Scale bar (in *B*): *A*, *C*, 100 μm; *B*, *D*, 65 μm.

Nifedipine blocks KCl-induced increase in TH expression

The current studies showed that nifedipine, an L-type calcium channel blocker, prevented the depolarization-induced increase in the number of TH-immunostained neurons but had no effect on control cultures, indicating that the induction of TH expression was mediated by an increase in intracellular calcium through these channels. A further direct demonstration of the role of this channel in inducing TH-expression was the ability of the L-type calcium channel agonist Bay K8644 to mimic the effects obtained with KCl treatment. A recent study demonstrating KCl-mediated TH regulation in primary sensory neurons also supported the role of L-type calcium channels in depolarization-induced TH expression (Brosenitsch et al., 1998). As indicated above, increases in intracellular calcium were shown to act via the CRE site in the TH promoter, which then acts like a CaRE (Sabban, 1997). In both adrenal medullary cultures and cell lines, depolarization induced TH expression (Kilbourne and Sabban, 1990; Kilbourne et al., 1992; Dahmer, 1995; Nagamoto-Combs et al., 1997). Calcium-chelating agents prevented the depolarization-mediated increase in TH, and a calcium ionophore induced TH gene expression in PC12 cells. Previous studies also indicated that, in other systems including prenatally isolated olfactory bulb cultures, depolarization produced its effects via L-type calcium channels (McMillian et al., 1994). These channels were shown to play a minor role in mediating either spontaneous electrical activity or synaptically induced calcium currents in cortical neurons but an important role in producing synaptic activation of immediate early genes, such as c-fos (Murphy et al., 1991). Previous studies linked expression of early genes, including c-fos, to TH expression in the olfactory bulb (Guthrie and Gall, 1995a,b; Jin et al., 1996). Thus, these channels are key in coupling synaptic excitation to activation of transcription.

Forskolin induction of TH expression

The forskolin-induced 1.5-fold increase in TH-expressing neurons, in contrast to the response to depolarization, was not prevented by pretreatment with nifedipine. The lack of effect was unexpected, because combined treatment with forskolin and KCl was not more effective than KCl alone, suggesting a common mechanism. In addition, in other experimental systems, forskolin, by increasing cAMP levels, regulated both basal and inducible TH gene expression (Fader and Lewis, 1990; Tinti et al., 1996) and, like depolarization, acted via the CRE sequence present in the TH promoter (Fader and Lewis, 1990; Kim et al., 1994). The current data indicate that forskolin and depolarization induce TH-expression via different, possibly parallel but not additive, mechanisms.

Alterations in neurite extension

Depolarization and forskolin produced not only a change in the number of TH-containing neurons but in the morphology of their neurites. Depolarization dramatically reduced the length of the longest neurite, resulting in neurons with a stellate appearance. The response was not prevented by nifedipine pretreatment. These data are consistent with the effects of afferent stimulation on the morphology of TH-expressing cells *in vivo* during development. Migrating periglomerular cells, including those with ectopic expression of TH, displayed an unbranched leading neurite (Baker and Farbman, 1993; Luskin, 1993). Once in the periglomerular layer, the neurons expressed high levels of TH and developed highly branched dendritic processes (Baker and Farbman, 1993; Betarbet et al., 1996). The morphological changes observed in the TH-expressing neurons *in vitro* may reflect a depolarization-mediated maturation similar to that which occurs *in vivo*. In addition, growth and differentiation may occur via different molecular pathways, as shown in a recent study in which depolarization promoted neuronal survival but did not mediate neurite growth (Franklin et al., 1995). The ability of nifedipine to prevent TH gene expression, but not the morphological changes, supports this hypothesis. Furthermore, in cultured frog olfactory bulb neurons, nifedipine blocked the depolarization-induced increase in intracellular calcium concentration in perikaryal, but not neuritic, compartments, indicating a segregation of channels and therefore possibly the consequences of their activation (Bischofberger and Schild, 1995).

Summary

The current studies are the first to demonstrate survival and differentiation of TH neurons in cultures obtained from neonatal olfactory bulbs. Furthermore, they demonstrate that depolarization, which mimics afferent stimulation *in vivo*, acts via L-type calcium channels to produce an increase in the number of TH-expressing neurons. They also indicate that this model system can be used to delineate specific mechanisms regulating gene expression in olfactory bulb.

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