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Activity-dependent regulation of tyrosine hydroxylase expression in the enteric nervous system

Running title: Neurochemical plasticity in the enteric nervous system

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Footnote: * J. Chevalier and P. Derkinderen contributed equally to this study

Abstract

The regulation of neuromediator expression by neuronal activity in the enteric nervous system (ENS) is currently unknown. Using primary cultures of ENS derived from rat embryonic intestine, we have characterized the regulation of tyrosine hydroxylase (TH), a key enzyme involved in the synthesis of dopamine. Depolarization induced either by 40mM KCl, veratridine or electrical field stimulation produced a robust and significant increase in the proportion of TH immunoreactive (TH-IR) neurons (total neuronal population was identified with PGP9.5 or Hu) compared to control. This increase in the proportion of TH-IR neurons was significantly reduced by the sodium channel blocker tetrodotoxin (0.5 μ M), demonstrating that neuronal activity was critically involved in the effects of these depolarizing stimuli. KCl also increased the proportion of VIP-IR but not nNOS-IR enteric neurons. The KCl-induced increase in TH expression was partly reduced in the presence of the nicotinic receptor antagonist hexamethonium (100 μ M), of norepinephrine (1 μ M) and of the α 2 adrenoreceptor agonist clonidine (1 μ M). Combining pharmacological and calcium imaging studies, we have further shown that L-type calcium channels were involved in the increase of TH expression induced by KCl. Finally, using specific inhibitors, we have shown that both protein kinases A and C as well as the extracellular signal-regulated kinases were required for the increase in the proportion of TH-IR neurons induced by KCl. These results are the first demonstration that TH phenotype of enteric neurons can be regulated by neuronal activity. They could also set the basis for the study of the pathways and mechanisms involved in the neurochemical plasticity observed both during ENS development and in inflammatory enteric neuropathies.

Introduction

The enteric nervous system (ENS) is a complex network of neurons and glial cells located within the gastrointestinal tract, also named the "brain of the gut", which can function independently of the central nervous system (CNS). This system controls gastrointestinal (GI) motility, exocrine and endocrine secretions as well as microcirculation (Goyal & Hirano, 1996). Overall, more than 20 candidate neurotransmitters regulating GI functions have now been identified in enteric neurons, including acetylcholine, vasoactive intestinal polypeptide, and nitric oxide (Schemann & Neunlist, 2004).

Although the GI tract contains dopamine (Eaker *et al.*, 1988), it has been difficult to determine whether dopamine is present either in the enteric neurons themselves or in the sympathetic innervation where dopamine is the precursor of norepinephrine. However, recent reports demonstrated that dopamine is an enteric neurotransmitter in the human (Anlauf *et al.*, 2003) as well as in the mouse ENS (Li *et al.*, 2004; Li *et al.*, 2006). Tyrosine hydroxylase immunoreactive (TH-IR) neurons have been identified in the stomach of adult ferrets and guinea pigs as well as in the whole digestive tract of adult mice (Schemann *et al.*, 1995; Sann *et al.*, 1998; Li *et al.*, 2004). In adult humans, a recent report showed that TH-IR neurons constituted around 20% of all enteric neurons in the upper gastrointestinal tract (Anlauf *et al.*, 2003). These neurons were considered to be dopaminergic since they were not stained with antibodies against dopamine β hydroxylase (DBH) (Anlauf *et al.*, 2003). Although it has been suggested that endogenous dopamine inhibits colonic motility (Walker *et al.*, 2000) and modulate secretion (Zhang *et al.*, 2007), the precise function of enteric dopaminergic neurons remains unclear.

In the CNS, an up-regulation of TH expression can be induced by various stimuli such as depolarization and nicotine. Furthermore, several reports have demonstrated that the extracellular signal-regulated kinases (ERKs) pathway plays a critical role in the increase of TH expression (Guo *et al.*, 1998; Shah *et al.*, 2006). In contrast, little is known about the regulation of TH expression in the ENS. It has been shown recently that sympathetic denervation upregulates TH expression by increasing both the abundance of transcripts encoding TH and the numbers of TH-IR neurons in the bowel of mice (Li *et al.*, 2004). Some GI pathologies are also associated with a change in TH expression. In particular, an increase in TH-IR neurons is observed in the myenteric plexus of patients with Crohn's disease (Belai *et al.*, 1997) while a loss of dopaminergic neurons has been described in the myenteric plexus of patients with Parkinson's disease (Singaram *et al.*, 1995).

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The present study was therefore aimed at characterizing the regulation of TH phenotype in the enteric neurons. Primary cultures of ENS derived from rat embryonic intestine were used to study the regulation of TH expression by neuronal activity. In the first part of the study, addition of extracellular KCl, veratridine and electrical field stimulation (EFS) were used to study the regulation of TH expression in our primary culture of enteric neurons. In a second set of experiments, the signaling pathways involved in the regulation of TH expression by neuronal activity were studied by using KCl-induced depolarization as a stimulus (Kilbourne *et al.*, 1992; Brosenitsch *et al.*, 1998; Cigola *et al.*, 1998). In our study, we showed that KCl, veratridine and EFS increased the proportion of TH-IR enteric neurons and that these effects were dependent on neuronal activity. The increase of TH-IR neurons induced by KCl was mediated in part via nicotinic and noradrenergic pathways and required calcium (Ca^{2+}) influx through L-type Ca^{2+} channels. Eventually, we have shown that protein kinase A (PKA), protein kinase C (PKC) and ERKs were involved in this activity-dependent regulation of TH expression.

Material and methods

Cell culture.

Pregnant Sprague-Dawley rats were purchased (CERJ, Le Genest St Isle, France and Janvier-Breeding Center, Belgium) and manipulated in compliance with the French and Belgian institutional guidelines. These procedures were approved by the local institutional animal research committee (Agreement E. 44011; Inserm, Nantes, France) and by the Ethical Committee for Animal Experiments of the KU Leuven (Agreement P06077), Belgium. Every effort was made to minimize animal suffering and the number of animals used.

Pregnant rats were killed by an overdose of CO₂ followed by severing the carotid arteries. The embryos (E15; 35-45 per isolation from 3 pregnant rats) were removed and killed by decapitation. Then, the small intestine of embryos were removed and finely diced in HBSS (Sigma, Saint Quentin Fallavier, France). Tissue fragments were collected in 5mL of medium (DMEM-F12 (1:1) medium) and digested at 37°C for 15min in 0.1% trypsin (Sigma). The trypsin reaction was stopped by adding 10mL of medium containing 10% fetal calf serum and then treated by DNase I 0.01% (Sigma) for 10min at 37°C. After triturating with a 10mL pipette, cells were centrifuged at 750rpm for 10min. Cells were counted and then seeded at a density of $2.4 \cdot 10^5$ cells/cm² on 24-well plates previously coated for 6h with a solution of gelatin (0.5%; Sigma) in sterile PBS. After 24h, the medium was replaced with a serum-free medium (DMEM-F12 (1:1) containing 1% of N-2 supplement (Life Technologies, Cergy Pontoise, France). Cells were maintained in culture for 14 days. Half of the medium was replaced every other day.

Electrical activation of enteric neurons and pharmacological experiments

To study the effect of neuronal activity upon the neurochemical phenotype, enteric neurons were electrically stimulated in 24-well plates fitted with a pair of platinum electrodes connected to an electrical stimulator (Dual Impedance Research Stimulator, Harvard Apparatus Limited, Edenbridge, Kent.). The electrical field stimulation (EFS) was achieved with trains of constant current pulses with the following parameters : pulse duration: 200µs; amplitude: 8V; frequency: 15Hz, applied during 8 hours. Electrode polarity was changed every 15min and half of the medium was changed at the end of the stimulation protocol. Following EFS, cultures were left unstimulated for an additional 15 hours.

Immunohistochemistry and identification of neuronal cell populations

After fixation (in 0.1 M PBS containing 4% paraformaldehyde for 1h at room temperature), cells were washed 3 times in PBS. Cells were permeabilized for 30min in PBS/NaN₃ containing 0.5% Triton X-100 and 4% horse serum before being incubated with rabbit anti-Tyrosine Hydroxylase (anti-TH; 1:1000; Pel-Freez, Arkansas, USA), with mouse anti-dopamine β hydroxylase (anti-DBH; 1:200; Fitzgerald Industries International, MA, USA), with mouse anti-vasoactive intestinal peptide (anti-VIP; 1:800; US biological, MA, USA), with anti-neuronal nitric oxide synthase (anti-nNOS; 1:2000; Alexis, CA, USA) or anti-caspase 3 active (1:2000; Sigma, Saint Quentin Fallavier, France) diluted in PBS/NaN₃, 0.5% Triton X-100 and 4% horse serum for 1h30 at room temperature. After incubation with primary antisera, cells were washed 3 times with PBS and incubated for 30min with the following secondary antibodies coupled to fluorophores: donkey anti-rabbit or anti-mouse IgG conjugated to carboxymethylindocyanine (1:500; Jackson Laboratories, purchased from Immunotech, Marseille, France). In the following step, the cells were labeled with rabbit anti-PGP9.5 (1:2000; Ultraclone Limited, Isle of Wight, UK) or with mouse anti-Hu (1:200; Molecular Probes, Oregon, USA) (general neuronal markers) for 1h30. Following incubation with primary antisera, cells were washed with PBS and incubated for 30min with donkey anti-rabbit IgG or donkey anti-mouse conjugated to FluoProbes[®]488 (1:200; Interchim, Montluçon, France).

Cell counting procedure

The number of TH-IR cells and PGP9.5-IR or Hu-IR cells was counted in at least 20 ganglia (649±19 neurons per preparation, n=12) per well and per condition. The data was expressed in percentage of TH-IR neurons normalized to the total PGP9.5-IR or Hu-IR neurons.

Quantitative PCR analysis

Total RNA extraction from cells was performed with RNAeasy Minikit (Qiagen S.A., Courtaboeuf, France) according to the manufacturer's instructions. For reverse transcription, 2µg RNA was combined with 0.5µg of random hexamers (Amersham, Piscataway, NJ), transcription buffer (50mM Tris HCl, pH 8.3, 75mM KCl, 3mM MgCl₂, 10mM DTT), dNTPs (10mM each), RNasin (20 units; Promega, Madison, WI), and RNaseH Maloney murine leukemia virus reverse transcriptase (200 units; Promega) in a total volume of 25µl. Incubation was performed at 42°C for 60min. The following primers were used: TH forward:

5'-GTG AAC CAA TTC CCC ATG -3', TH reverse: 5'-GGT CGC AGC TGG AAG C-3' and hypoxanthine-guanine phosphoribosyltransferase (HPRT) forward: 5'-CCT TGG TCA AGC AGT ACA GCC-3' and HPRT reverse: 5'-TTC GCT GAT GAC ACA AAC ATG A-3'. Real-time PCR procedure was used with Custom TaqMan® Gene Expression Assays (PE Applied Biosystems, Foster City, CA) and with Gene expression assay (PE Applied Biosystems) for the HPRT. TaqMan® Probes were chosen to avoid alternative splicing in the target gene. All samples were analysed in duplicate in a total volume of 25µL with 1µL of cDNA, 1.25µL probes and 12.75µL TaqMan® Universal PCR Master Mix 2X (PE Applied Biosystems) and processed in the ABI PRISM® 7900HT Sequence Detection System using universal cycling conditions (40 cycles of 95°C for 15 seconds; 60°C for 1 minute). The mRNA level of expression was determined using the formula of the comparative cycle threshold (Ct): $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{\text{gene}} - Ct_{\text{HPRT}})_{\text{sample}} - (Ct_{\text{gene}} - Ct_{\text{HPRT}})_{\text{calibrated}}$ as previously described (Livak & Schmittgen, 2001).

Intracellular [Ca²⁺]_i measurements

For intracellular Ca²⁺ measurements ([Ca²⁺]_i), primary cultures of ENS were seeded on 18 and 13mm coverslips. After 13 days of culture, experiments were performed at room temperature in a modified Krebs solution containing (in mM) 148 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, with pH adjusted to 7.38 with NaOH (5 M). The high-KCl buffer used for stimulating and identifying neurons contained 75mM KCl and Na⁺ was reduced to 78mM. The [Ca²⁺]_i changes were assayed by Fluo-4 fluorescence imaging. In brief, cultures seeded on coverslips were loaded for 30 minutes with 10µM Fluo-4 AM (F14217, Invitrogen, Merelbeke, Belgium) in modified Krebs solution and subsequently rinsed with fresh modified Krebs solution. After loading with the dye, the coverslips with cells were transferred to a recording chamber and mounted on a Zeiss Axiovert 200M microscope equipped with a monochromator (Poly V) and a cooled CCD camera (Imago QE) both from TILL Photonics (Gräfelfing, Germany). Fluo-4 was excited at 488 nm, and its fluorescence emission was collected at 525/50nm. Recordings were made using a 20X objective under constant perfusion (1 ml / minute) with modified Krebs solution. The high-KCl medium (75mM) used to identify neurons and the 10mM KCl modified Krebs solution (containing Bay-K8644 (1µM) or not) was applied onto the neurons for 5s.

Images were collected using TiLLVision software (TILL Photonics, Gräfelfing, Germany) and stored on a personal computer. Further analysis was done using custom written macros in IGOR PRO (Wavemetrics, Lake Oswego, OR, USA). Regions of interest (ROIs)

were drawn over each cell, fluorescence intensity was normalized to the basal fluorescence at the onset of the recording for each ROI, and peaks were analyzed. A peak was considered if the signal rose above baseline + 5 times the intrinsic noise level. The percentage of responsive cells (%RC) and the maximum $[Ca^{2+}]_i$ peak amplitude were determined.

Pharmacological experiments

To study the effect of neuronal activity using depolarizing agents, cells were incubated with KCl 40mM (Sigma) or 30 μ M veratridine (Sigma). An equimolar concentration of NaCl (40mM) was used to test for any osmotic effect. Tetrodotoxin (0.5 μ M, Sigma), was added 30min prior to the addition of 40mM KCl or 30 μ M veratridine or EFS. Hexamethonium (100 μ M), norepinephrine (1 μ M), clonidine (1 μ M) and nifedipine (1 μ M) were added 30min prior to the addition of 40mM KCl (all drugs from Sigma). Bay-K8644 (1 μ M, Sigma) was added in presence of 10mM KCl, as a low concentration of KCl was necessary to unravel the effects of Bay-K8644 in enteric neurons similarly to what was reported in primary sensory neurons (Brosenitsch *et al.*, 1998). To study the signaling pathway involved in the effects of KCl on TH expression, the following drugs (all purchased from Calbiochem) were added 30min prior to the addition of 40mM KCl: PD-98059, a MAP/ERK (MEK) inhibitor (50 μ M) (Alessi *et al.*, 1995), SB-203580, a p38 mitogen activated protein kinase (p38 MAPK) inhibitor (10 μ M) (Cuenda *et al.*, 1995), H89, a PKA inhibitor (2 μ M) (Chijiwa *et al.*, 1990), SQ 22536, an inhibitor of adenylyl-cyclase (100 μ M) (Haslam *et al.*, 1978), GF109203X a PKC inhibitor (1 μ M) (Toullec *et al.*, 1991) and PP2, a Src family kinase inhibitor (1 μ M) (Hanke *et al.*, 1996).

Statistical analysis

All data are given as mean \pm standard error of the mean (SEM). Comparisons of means between groups were performed by unpaired t-tests or by analysis of variance followed by a Bonferroni *t*-test. When data were not normally distributed, a Mann-Whitney test was performed. Differences were considered statistically significant if $p < 0.05$. *n* indicates the number of experiments.

Results

Primary culture of rat enteric nervous system

Dissociated cells of the embryonic gut were seeded at a density of $2.4 \cdot 10^5$ cells/cm². Following 14 days of culture, enteric neurons are organized in ganglia connected to each other by interganglionic fiber strands. Ganglia contained 29 ± 2 neurons identified with PGP9.5 (Figure 1A) and an identical number of neurons identified with Hu (30 ± 1 ; $n=4$; $p=1$) (Figure 1B). In addition, GFAP positive enteric glial cells were also present in enteric ganglia and along interganglionic fiber strands (Figure 1C). Analysis of neurochemically identified population revealed that $16 \pm 0\%$ of PGP9.5-IR neurons were VIP-IR (Figure 1D) and $68 \pm 6\%$ were nNOS-IR (Figure 1E). In contrast, only 1% of neurons were TH-IR (Figure 1F).

KCl modulates the neurochemical coding in primary cultures of enteric neurons

Treatment of primary culture of rat ENS with 40 mM KCl induced a significant and time-dependent increase in the proportion of TH-IR neurons. The KCl-induced increase in TH-IR neurons was observed as early as 24h of treatment ($14 \pm 0\%$ vs $0 \pm 0\%$ of PGP9.5 IR neurons, in the presence or in the absence of KCl respectively, $n=12$; $p<0.001$) and reached about 20% after 72h (22 ± 1 vs $0 \pm 0\%$ of PGP9.5 IR neurons, respectively, $n=8$; $p<0.001$) (Figure 2A-E). No cell death as assessed by active caspase 3 IR was observed even following 72h of treatment with KCl (data not shown). Addition of NaCl of equimolar concentration (40mM) did not induce TH-IR neurons (Figure 2E). In addition, these TH-IR neurons were not dopamine β hydroxylase (DBH)-IR (Figure 2F-H), suggesting that they are not noradrenergic. The KCl-induced increase in TH-IR was associated with a significant increase by 2.4 fold of transcripts encoding TH compared to control ($n=8$; $p<0.001$) (Figure 2I).

The specificity of the effects of KCl upon other neuromediators was further studied by assessing the proportion of two other chemical markers : VIP and NOS. Treatment by KCl induced a significant increase in the proportion of VIP-IR neurons ($42 \pm 5\%$ vs $16 \pm 0\%$ of PGP9.5-IR neurons, in the presence or in the absence of KCl respectively, $n=4$; $p=0.003$) but not of NOS-IR neurons as compared to control ($68 \pm 2\%$ vs $68 \pm 6\%$ of PGP9.5 IR neurons, in the presence or in the absence of KCl respectively, $n=4$; $p=1$) (Figure 2J). These changes were also paralleled at the transcriptional levels (data not shown).

Neuronal activity mediates changes in TH expression.

In order to determine whether neuronal activity was involved in the regulation of TH expression, we first studied the effects of the sodium channel blocker tetrodotoxin (TTX) on the KCl-induced increase in TH expression. The KCl-induced increase in the proportion of TH-IR neurons was significantly reduced by 16% ($n=5$; $p=0.036$) in the presence of $0.5\mu\text{M}$ TTX, (Figure 3A). Next, we showed that electrical field stimulation (EFS) of enteric neurons for 8h induced a significant increase in TH-IR neurons as compared to control ($26\pm 3\%$ vs $4\pm 1\%$ of Hu IR neurons, respectively $n=3$; $p=0.003$) (Figure 3B). This effect was completely prevented in the presence of TTX (Figure 3B). Further reinforcing the role of neuronal activity in the control of TH expression, we showed that treatment of primary culture of ENS with $30\mu\text{M}$ veratridine, a potent activator of sodium channels, induced a significant increase in TH expression in enteric neurons as compared to control (17 ± 4 vs $1\pm 1\%$ of Hu IR neurons, respectively, $n=4$; $p=0.022$) (Figure 3C). Again, this effect was blocked by TTX (Figure 3C). Throughout the rest of the study, experiments were conducted using KCl as a model of activity-dependent stimuli.

Cholinergic and adrenergic pathways modulate the proportion of TH-IR neurons.

KCl-induced changes in TH expression were significantly reduced down to 70% ($n=9$; $p<0.001$) by hexamethonium ($100\mu\text{M}$), an antagonist of nicotinic receptors (Figure 4A). In order to “physiologically” decrease cholinergic activity, we used norepinephrine, which is known to inhibit acetylcholine release from excitatory cholinergic nerve terminals via $\alpha 2$ adrenoceptors (Scheibner *et al.*, 2002). Treatment of ENS with KCl in presence of norepinephrine ($1\mu\text{M}$), significantly reduced the proportion of TH-IR neurons compared to control cultures ($n=4$; $p=0.005$) (Figure 4B). A similar decrease in the proportion of TH-IR neurons was observed when clonidine ($1\mu\text{M}$), a specific $\alpha 2$ adrenoceptor agonist was used ($n=4$; $p=0.005$) (Figure 4B).

KCl-induced increase in the proportion of TH-IR neurons involves L-type Ca^{2+} channels

Previous studies performed in olfactory and sensory neurons showed that the KCl- induced increase of TH expression resulted at least in part from an increase in intracellular Ca^{2+} through L-type Ca^{2+} channels (Brosenitsch *et al.*, 1998; Cigola *et al.*, 1998). The involvement of L-type Ca^{2+} channels in KCl-induced increase in the proportion of TH-IR neurons was therefore first studied in enteric neurons with nifedipine, a specific antagonist of these

channels. Treatment of primary culture of ENS with nifedipine (1 μ M), completely prevented the KCl-induced increase in the proportion of TH-IR neurons (Figure 5A). Conversely, incubation of primary cultures of ENS with the selective L-type Ca²⁺ channels agonist Bay K-8644 (in presence of 10mM KCl, as previously described (Cigola *et al.*, 1998)) significantly increased the proportion of TH-IR neurons as compared to control (10mM KCl) (7.7 \pm 1% vs 0 \pm 0%, n=6; p=0.002) (Figure 5B). Addition of 10mM KCl *per se* did not modify the proportion of TH-IR neurons (Figure 5B). Using Ca²⁺ imaging studies, we have further shown that microapplication of 1 μ M BayK-8644 in presence of 10mM KCl to enteric neurons induced a significant increase in the transient Ca²⁺ rise as compared to application of 10mM KCl alone (n=93 neurons, p<0.0001) (Figure 5D-E). In addition, the percentage of KCl-responsive cells showed a slight increase in the presence of BayK-8644 (97.3% vs. 84.5% in 10mM KCl alone). After performing the experiments, neurons were identified by exposure to 75mM KCl (Figure 5C).

ERKs but not p38 are involved in KCl-induced increase in the proportion TH-IR neurons

The involvement of ERKs pathway in the regulation of KCl-induced increase in TH-IR in enteric neurons was studied with pharmacological tools. Pretreatment of enteric neurons with PD98059 (MEK inhibitor; 50 μ M) significantly reduced by 85% the KCl-induced increase in the proportion of TH-IR neurons (n=5; p<0.001) (Figure 6A-D and G). In contrast, pretreatment of enteric neurons with SB203580 (p38 inhibitor; 10 μ M) did not significantly modify the KCl-induced increase in TH expression in enteric neurons (n=5; p=0.11) (Figure 6A- B, E- F and G).

Protein kinase C and the cAMP pathway are involved in the KCl-induced increase in the proportion of TH-IR neurons

In neuronal cells, Ca²⁺ influx can activate ERK through several signaling pathways. This can be achieved through activation of the proline rich tyrosine kinase 2 (Pyk2)/Src complex, PKC, PKA and CaM kinase (Derkinderen *et al.*, 1999). The role of these signaling pathways linking Ca²⁺ influx, ERK and TH expression was studied using a pharmacological approach. First, two inhibitors of the cAMP pathway SQ22536 (adenylate cyclase inhibitor; 100 μ M), and H89 (PKA inhibitor; 2 μ M) significantly reduced the KCl-induced increase in the proportion of TH-IR neurons by 43% and 50%, respectively (n=5; p=0.008 and p<0.001, respectively) (Figure 7A). Treatment with 20 μ M forskolin, an activator of adenylyl cyclase induced a

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significant increase in the number of TH-IR neurons as compared to control (6 ± 1 vs $0\pm 0\%$ of PGP9.5-IR neurons, respectively, $n=5$; $p=0.001$), further reinforcing the role of the cAMP pathway in the regulation of TH expression (Figure 7B). Pretreatment of neurons with GF109203X ($1\mu\text{M}$), a specific inhibitor of PKC, significantly reduced the KCl-induced increase of TH-IR by 42% ($n=4$, $p=0.029$) (Figure 7C). Concomitant addition of both H89 and GF109203X induced a significant additive inhibition of the KCl-induced increase of TH-IR neurons by 74% (Figure 7D). In contrast, PP2 (inhibitor of the Src family kinase; $1\mu\text{M}$) did not inhibit KCl effects on TH-IR in enteric neurons ($n=4$; data not shown).

Discussion

Our study showed that activity dependent stimuli can modulate TH expression in enteric neurons in part *via* cholinergic pathways and activation of L-type Ca^{2+} channels. Furthermore, TH regulation was shown to involve both PKC and PKA dependent pathways leading to ERK activation. To the best of our knowledge, this is the first study characterizing the signaling pathways involved in the regulation of a neuromediator in the ENS. In addition, it is also one of the rare studies linking activity dependent signaling pathways regulating TH expression in primary culture of neurons (Figure 8).

Activity-dependent regulation of neuromediators has been well documented in the CNS (West *et al.*, 2001). In particular, depolarization was able to increase the proportion of dopaminergic/TH-IR neurons in primary cultures of mouse olfactory bulb and of rat brain (Brosenitsch *et al.*, 1998; Cigola *et al.*, 1998). Nicotine has also been demonstrated to increase TH expression both in the CNS and in sympathetic ganglia (Otten & Thoenen, 1976; Sun *et al.*, 2003). Consistent with these observations, we have shown that nicotinic cholinergic pathways can modulate TH expression in the ENS. Although, acetylcholine (ACh) is the main fast excitatory neurotransmitter in the ENS, its effect on regulation of gene expression remains largely unknown and needs further studies. In our model, we showed that a physiological inhibition of cholinergic activity mimicked by norepinephrine was able to antagonize the KCl-induced increase in the proportion of TH-IR, *via* activation of $\alpha 2$ receptors. These results could set the basis for the observation that *in vivo* sympathetic denervation increases the TH-expression in myenteric and submucosal neurons in mice (Li *et al.*, 2004). Furthermore, the observation that cholinergic activity increases the proportion of TH-IR neurons in the ENS, suggests a paradigm for a physiological regulation of TH expression in the ENS. An increase in the activity of the ENS could indeed lead to an up-regulation of dopamine synthesis aimed at reducing this neuronal activity. This hypothesis is reinforced by the observation that dopamine inhibits the evoked release of ^3H -ACh from enteric neurons (Kusunoki *et al.*, 1985; Takahashi *et al.*, 1991). A similar homeostatic regulation of neurotransmitters has been shown to occur in the CNS. Indeed, activity has been shown to participate to the homeostatic regulation of transmitter specification (Spitzer *et al.*, 2005). In particular, an increase in neuronal activity downregulates the expression of excitatory mediators such as ACh while it increases the expression of inhibitory mediators such as GABA or dopamine, through a regulation of TH expression (Spitzer *et al.*, 2005).

Modulation of enteric phenotype by sympathetic pathways is probably not restricted to TH as sympathetic denervation has been shown to increase the expression of neuronal nitric oxide synthase in rat jejunal myenteric neurons, an effect mediated by the activation of α_2 adrenoreceptors (Nishizaki *et al.*, 2003).

Interestingly, transient TH-IR neurons have been described in the mouse gut during development. These transient TH-IR neurons are the first to exhibit an adult-like phenotype (Gershon *et al.*, 1993; Young *et al.*, 1999; Young *et al.*, 2002). Thus, it could be tempting to speculate that the increase in the number of TH-IR neurons induced by neuronal activity in our experiments could be a correlate of the transient catecholaminergic phenotype observed during development. However, while TH-IR neurons increase, no change in the proportion of nNOS-IR is reported in this study. Since TH-IR transient neurons have been shown to be the progenitors of nNOS neurons, it is therefore unlikely that TH-IR neurons are the transient TH-IR neurons. Furthermore, KCl also induced a robust increase in the proportion of VIP-IR neurons. As VIP expression has been shown to peak when TH-IR fades during development (Pham *et al.*, 1991), we further believe that the concomitant increase induced by KCl in TH and VIP-IR neurons is probably not associated with a dedifferentiation.

In our study, activity-dependent regulation of TH expression was fully blocked by an inhibitor of L-type Ca^{2+} channels and mimicked by an agonist of these channels demonstrating a key role for L-type Ca^{2+} channels in the regulation of TH expression in the ENS. Such a critical role of L-type Ca^{2+} channels in the regulation of TH expression has been documented in both mouse olfactory neurons and in primary sensory neurons (Brosenitsch *et al.*, 1998; Cigola *et al.*, 1998). Ca^{2+} channels are present in enteric neurons but their role in ENS physiology has been poorly studied. Isolated myenteric ganglia of newborn rats and neurons from adult rats and guinea pigs express different types of voltage-dependent Ca^{2+} channels, from which the L- and the N-type seem to be the most important (Kirchgessner & Liu, 1999; Schaufele & Diener, 2005). Our results provide further evidences that L-type Ca^{2+} channels are not only expressed by enteric neurons but that they are also involved in ENS physiology and neuromediator plasticity.

Using a specific inhibitor of MEK, we showed that ERKs are involved in the regulation of TH expression in enteric neurons. Several studies have provided evidence implicating ERKs as a critical player in synaptic and neuronal plasticity, through their role in the regulation of gene expression (Curtis & Finkbeiner, 1999). Such a critical role of ERK in TH expression has already been documented in neuronal cells treated with uracil nucleotides, short chain fatty acids and fibroblast growth factor (Milosevic *et al.*, 2006; Shah *et al.*, 2006).

ERK could thus be a final common pathway to the regulation of TH expression. Depolarization has been shown to increase the expression of TH in primary sensory neurons and PC12 cells (Kilbourne *et al.*, 1992; Brosenitsch & Katz, 2001). Interestingly, in these neuronal cells, the effects of KCl on TH expression were insensitive to specific inhibitors of PKA and PKC (Kilbourne *et al.*, 1992; Brosenitsch & Katz, 2001). This contrasts with our results obtained in primary enteric neurons in which both PKC and PKA are involved in the regulation of TH expression by depolarization. It has been suggested that a PKA-dependent signaling pathway links neuronal Ca^{2+} influx to ERKs via the small G-protein, Rap1, and the neuronal Raf isoform, B-Raf (Grewal *et al.*, 2000; Baldassa *et al.*, 2003). Depending on the neuronal cell type (PC12 cells or primary cultures of rat hippocampal neurons), depolarization can activate either PKA-dependent or PKA-independent pathways to ERKs. Our results obtained in enteric neurons show that depolarization may be able to activate both PKA-dependent and -independent pathways equally, as already observed in hippocampal neurons (Grewal *et al.*, 2000). They also suggest that both Rap1 and B-Raf could be present in enteric neurons.

Whether activity dependent regulation of neuromediators also occurs in the ENS is currently unknown. However, in various GI pathologies both in infants and in the adults, plasticity in the neurochemical phenotype of the ENS occurs. These changes have been observed in inflammatory bowel disease such as ulcerative colitis (Neunlist *et al.*, 2003), Crohn's disease (Schneider *et al.*, 2001) or even in atresia (Khen *et al.*, 2004). Consistent with a putative involvement of activity and calcium signaling in these alterations is the observation that inflammatory mediators present in these pathologies such as IL-1 β , TNF- α modulate both neuronal excitability and $[\text{Ca}^{2+}]_i$ (Xia *et al.*, 1999; Kelles *et al.*, 2000; Rehn *et al.*, 2004). Therefore, depending on the inflammatory phenotype (Crohn's disease or Ulcerative colitis), the neuronal calcium response could differ and differentially regulates neuromediators gene expression. Besides a role in pathology, activity dependent control of neuronal phenotype could also be involved in the development of GI functions, in particular motility. Indeed, recent data in mice have shown that the establishment of neural control of motility is paralleled by an increase in the cholinergic phenotype of the ENS (Roberts *et al.*, 2007). One can speculate that activity dependent signaling due to the muscle distension or mucosal stroking (induced by luminal gut content) could be directly involved in maturation of the ENS neurochemical phenotype.

In conclusion, these results are the first demonstration that the TH phenotype of enteric neurons can be regulated by neuronal activity. They could also set the basis for the study of

the pathways and mechanisms involved in the neurochemical plasticity observed both during ENS development and in inflammatory enteric neuropathies.

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Figure Legends

Figure 1. Characterization of primary culture of ENS. After 14 days in culture, the presence of enteric neurons in primary culture of ENS was assessed by PGP9.5 (A) and Hu immunostaining (B). Enteric glial cells were identified by immunostaining with glial fibrillary acid protein antibodies (C). The neurochemical phenotype was determined by immunostaining using antibodies specific for VIP (D), nNOS (E) and TH (F). Scales bar represents 25 μ m.

Figure 2. KCl induces a significant increase in TH, VIP but not nNOS expression in enteric neurons. After 14 days in culture, enteric neurons identified with PGP9.5 (A) were not immunoreactive (IR) for TH (B). Treatment with 40 mM KCl induced a robust increase in TH-IR (D) in PGP9.5-IR enteric neurons (C). The proportion of TH-IR neurons normalized to the total neuronal population identified with PGP9.5 was significantly increased following KCl treatment for 24 and 72h (E; n=12; p<0.001 and n=8, p<0.001, respectively, t-test). In contrast, incubation of equimolar concentration of NaCl (40mM) did not modify the proportion of TH-IR neurons (E; n=8, p<0.001, t-test). TH-IR neurons (F) were not dopamine β -hydroxylase (DBH)-IR (G), although the antibody labeled neurons in adult rat ileum (H; arrow). KCl treatment increased TH mRNA transcript expression normalized to HPRT as compared to control (I; n=8; p<0.001, t-test). Treatment with KCl for 72h significantly increases the proportion of VIP-IR neurons (J; n=4; p=0.003, t-test) but not of nNOS-IR neurons (J; n=4; p=1, t-test). Scale bar represents 25 μ m for A-D and H and 50 μ m for F-G). Data are presented as the mean \pm SEM.

Figure 3. Neuronal activity is involved in the regulation of TH expression. The increase in the proportion of TH-IR neurons induced by KCl (normalized to the number of PGP9.5-IR neurons) was significantly reduced by 0.5 μ M tetrodotoxin (TTX+KCl) (A; n=5; p=0.036, t-test). EFS (8h of electrical stimulation) significantly increased the proportion of TH-IR neurons normalized to the number of Hu-IR neurons (+EFS) as compared to control (-EFS) (B; n=3; p<0.001, One Way ANOVA or Tukey test). This effect was blocked following EFS in the presence of 0.5 μ M tetrodotoxin (TTX+EFS) (B). Treatment of primary culture of enteric neurons with 30 μ M veratridine (24h) (+Vera) significantly increased the proportion of TH-IR neurons (normalized to the number of Hu-IR neurons) as compared to control (-Vera)

(C; n=4; p=0.022, t-test). These effects were also blocked in the presence of 0.5 μ M tetrodotoxin (TTX+Vera) (C).

Figure 4. Cholinergic and noradrenergic pathways regulate the KCl-induced increase in the proportion of TH-immunoreactive neurons. The increase in the proportion of TH-IR neurons induced by KCl was significantly reduced by hexamethonium (Hex, 100 μ M) as compared to control (Vehicle) (A; n=9; p<0.001, t-test). The KCl-induced increase in the proportion of TH-IR neurons was also significantly reduced by norepinephrine (Nor, 1 μ M) and clonidine (Clo, 1 μ M) as compared to control (Vehicle) (B; n=4; p<0.001 and n=4 ; p=0.005, respectively, t-test). Data are presented as the mean \pm SEM.

Figure 5. L-type calcium channels are involved in the KCl-induced increase in the proportion of TH-immunoreactive neurons. The increase in the proportion of TH-IR neurons induced by KCl was completely prevented by nifedipine (Nif, 1 μ M) (A; n=6, p=0.002, Mann-Whitney Rank Sum Test). Treatment with Bay-K8644 (Bay, 1 μ M) in presence of 10mM KCl significantly increased the proportion of TH-IR neurons as compared to control (Vehicle) (B; n=6, p=0.002, Mann-Whitney Rank Sum Test). Intracellular calcium measurements were performed on cells loaded with Fluo-4 AM and identification of neurons was performed by application of 75mM KCl (C) and Ca²⁺ signals were calculated from individual neurons (e.g. as indicated with a black square) Scale bar represents 50 μ m. Microejection of 10mM KCl (5s ejection duration) onto neurons induced an transient relative fluorescence rises (F₁/F₀) of the Ca²⁺ indicator Fluo-4 over time (D). Microejection of 10mM KCl in the presence of Bay-K8644 (dark gray line, 1 μ M) induced a larger transient fluorescence rises as with 10mM KCl (D). The traces in this example are from the neuron marked (black square in C). The amplitude of the transient relative fluorescence rise induced by 10mM KCl is significantly larger in presence of Bay-K8644 (E; p<0.0001; n=93 neurons). Scale bar represents 50 μ m. Data are presented as the mean \pm SEM.

Figure 6. ERK-but not p38-dependent pathways mediate the KCl-induced increase in the proportion of TH-immunoreactive neurons. Photomicrographs illustrating that in presence of KCl a proportion of PGP9.5-IR neurons (A) were TH-IR (B). In presence of KCl and the inhibitor of ERK pathways (PD98059; 50 μ M), no PGP9.5-IR neurons (C) were TH-IR (D). In contrast, in presence of KCl and the inhibitor of p38 pathways (SB203580; 10 μ M) a proportion of PGP9.5-IR neurons (E) were still TH-IR (F). Quantification of the results

demonstrated that PD98059 (PD), but not SB203580 (SB) significantly reduced the KCl-induced increase in the proportion of TH-IR neurons (G; $p < 0.001$, $n = 5$, t-test). Scale bar represents $25\mu\text{m}$. Data are presented as the mean \pm SEM.

Figure 7. Both, cAMP- and PKC- dependent pathways mediate the KCl-induced increase in TH-immunoreactive neurons. The increase in the proportion of TH-IR neurons induced by KCl was significantly reduced by an inhibitor of adenylate cyclase SQ 22536 (SQ, $100\mu\text{M}$); $n = 5$; $p = 0.008$, t-test) and by a an inhibitor of PKA, H89 (A) (H89 ($2\mu\text{M}$); $n = 5$; $p = < 0.001$, t-test). Forskolin, an activator of adenylate cyclase induced a significant increase in the proportion of TH-IR neurons as compared to control (B) (Forsko, $20\mu\text{M}$); $n = 5$; $p = 0.001$, t-test). The increase in the proportion of TH-IR neurons induced by KCl was also significantly reduced the inhibitor of PKC, GF109203X (C) (GF ($1\mu\text{M}$); $n = 4$; $p = 0.029$, t-test). In presence of both PKC and PKA inhibitors, the increase in the proportion of TH-IR neurons induced by KCl was further significantly reduced as compared to the condition with the inhibitors alone (D) ($n = 5$; $p = < 0.001$, t-test). Data are presented as the mean \pm SEM.

Figure 8. Signaling pathways involved in the activity dependent regulation of TH-expression in enteric neurons. Neuronal activation by depolarization results in opening of L-type calcium channels leading to an increase in calcium entry. This increase in intracellular calcium induces an activation of ERKs through and activation of both PKA and PKC, resulting in an increase in TH expression.

Figure 1

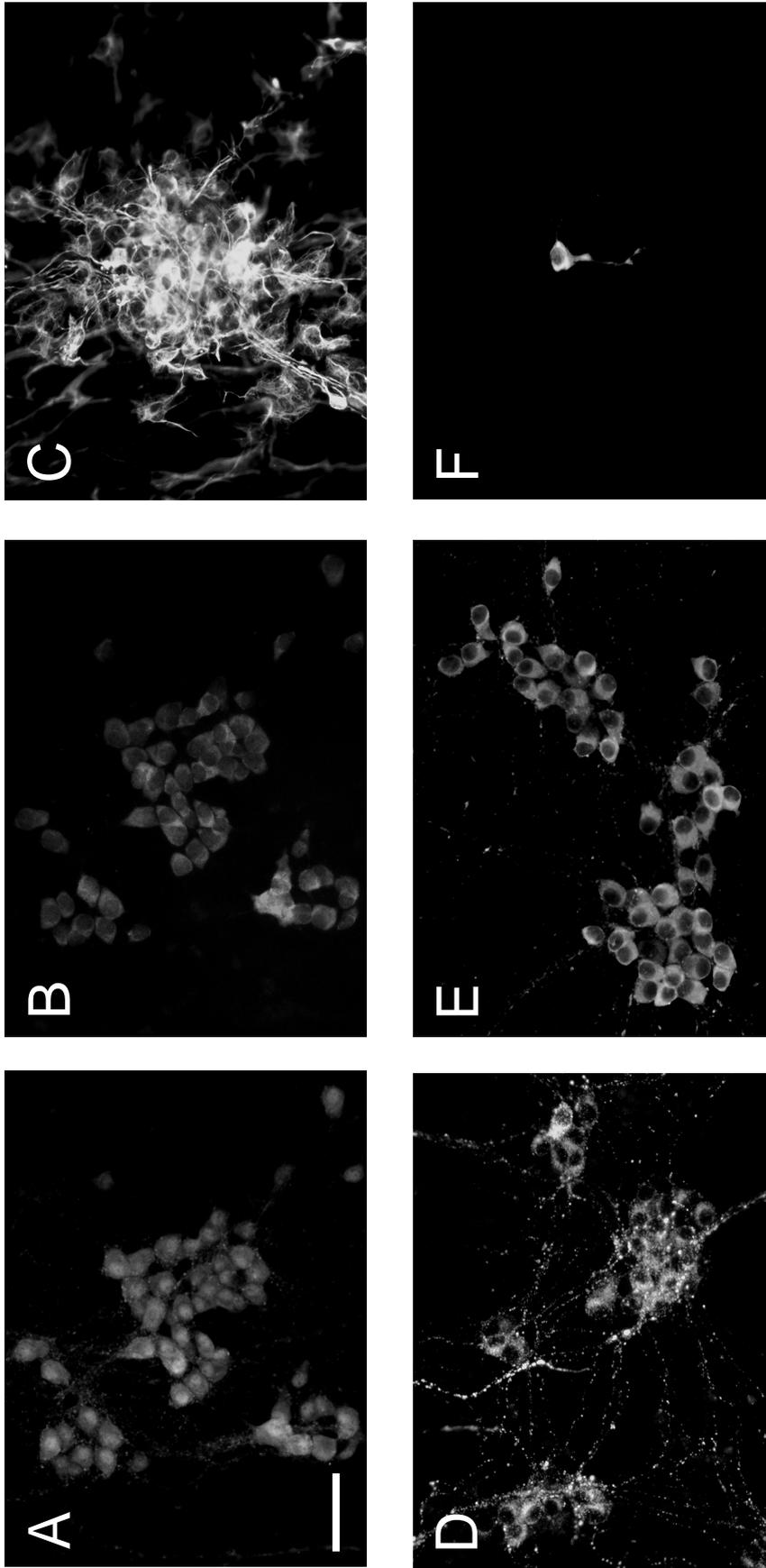
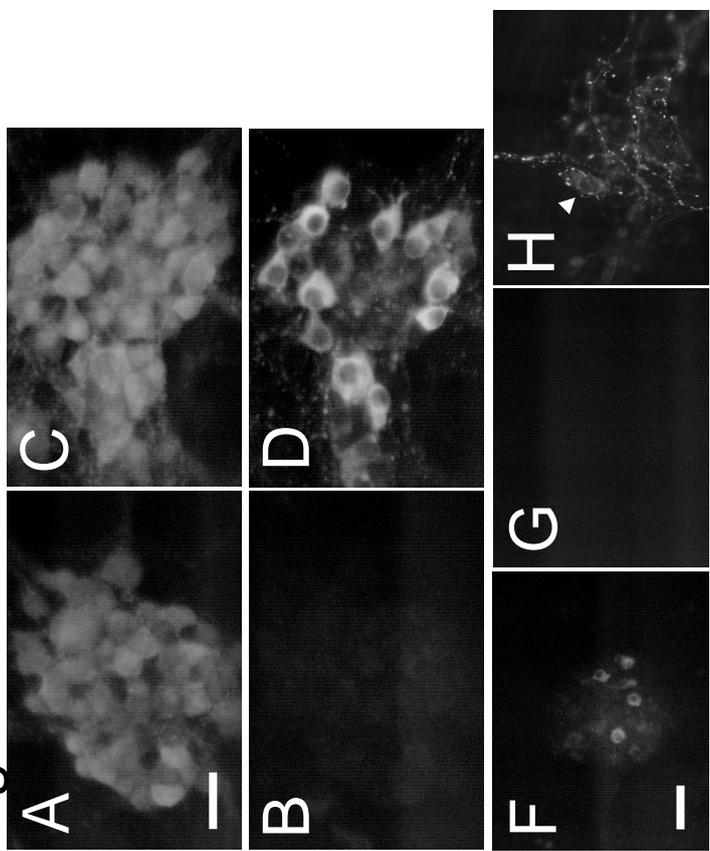
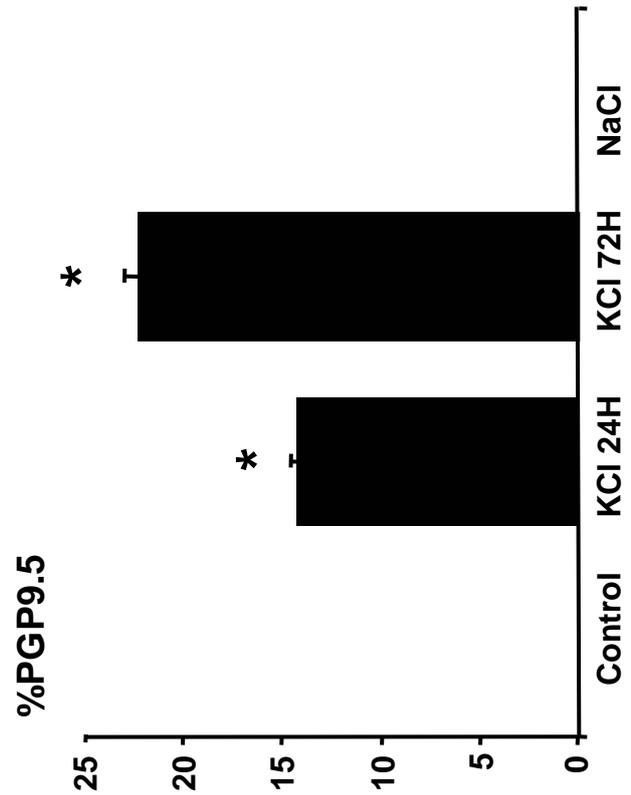


Figure 2



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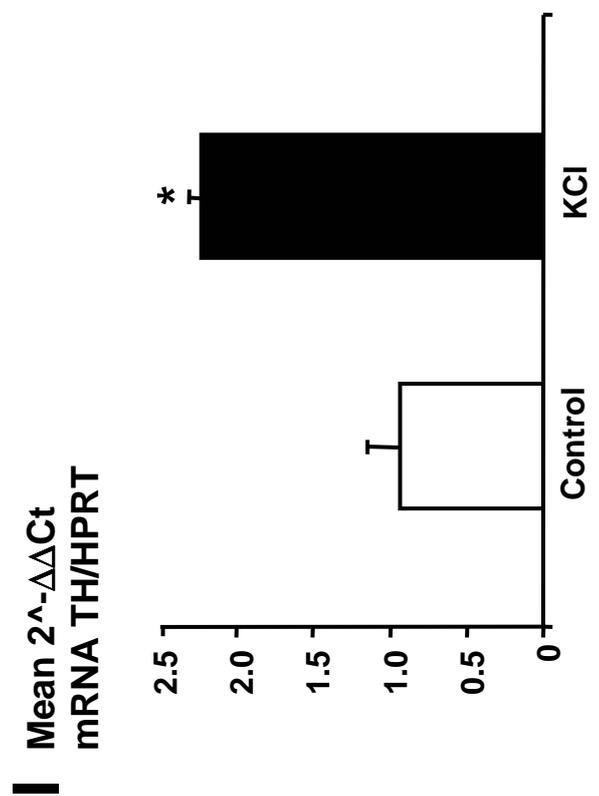
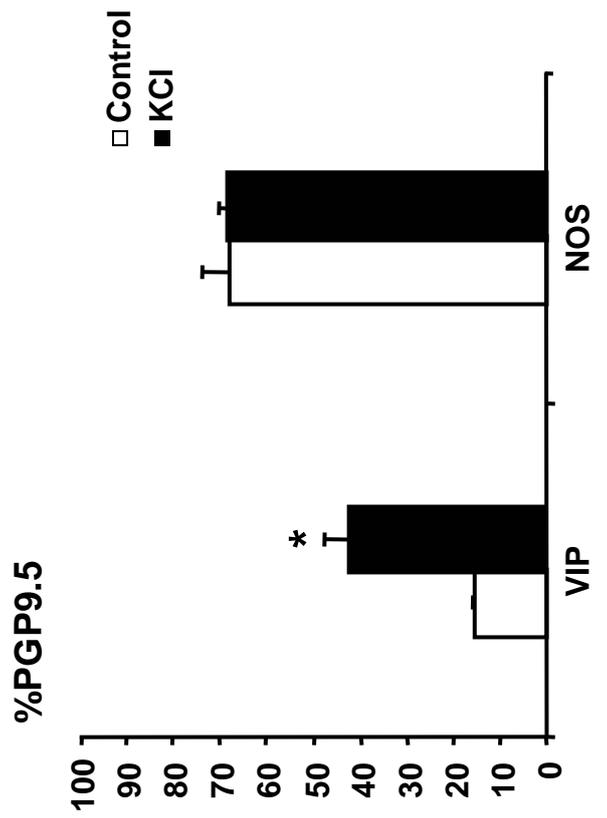


Figure 3

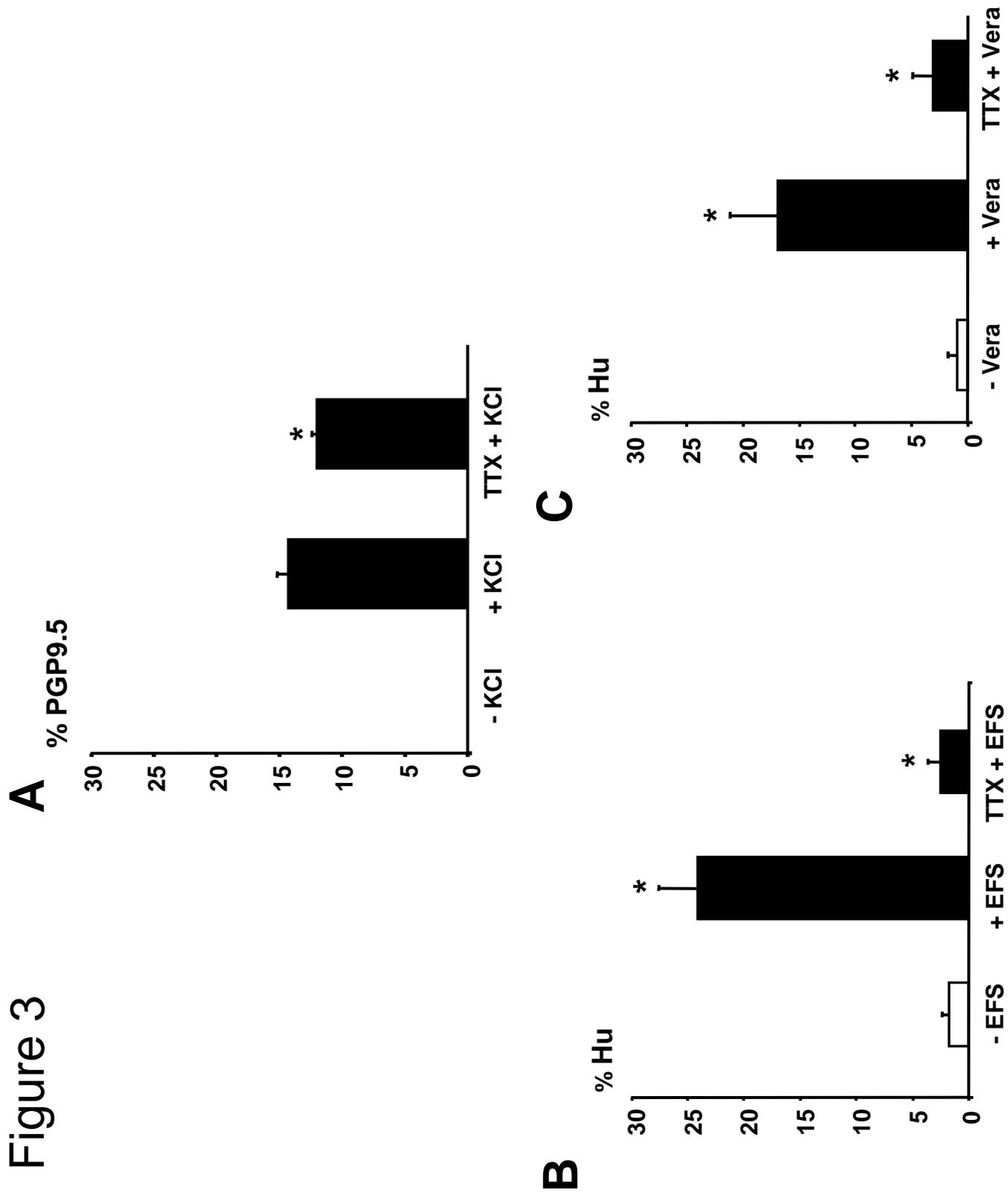


Figure 4

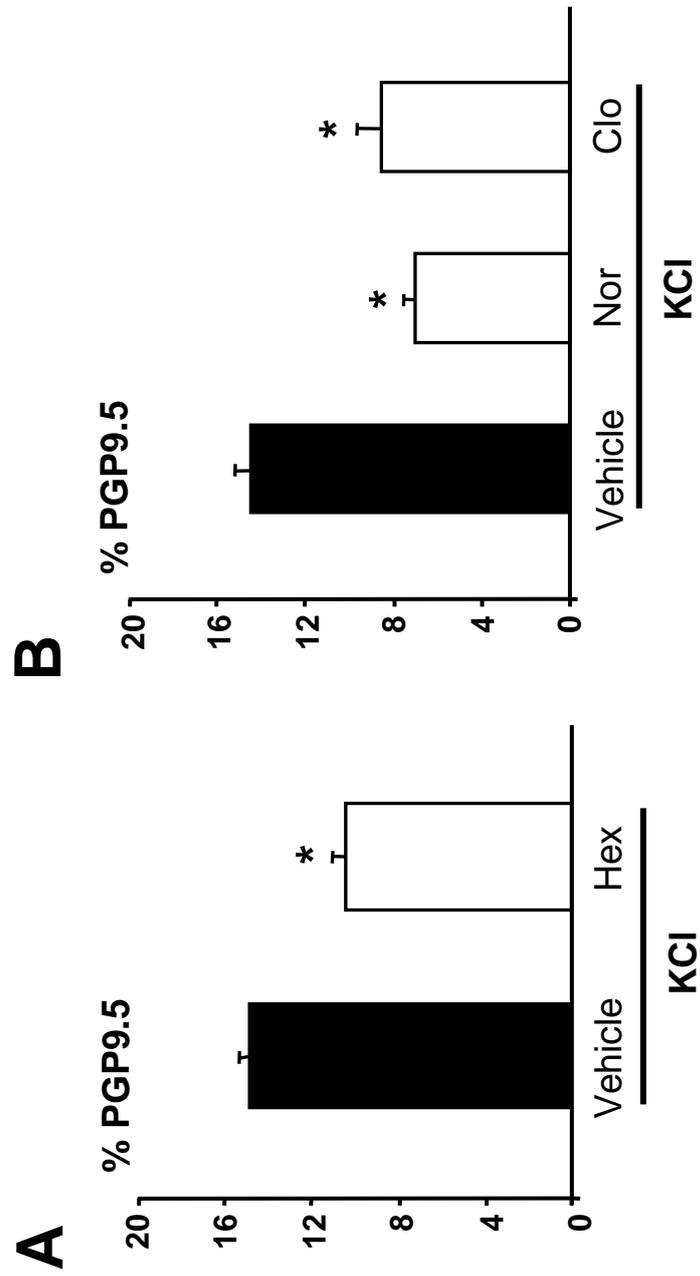


Figure 5

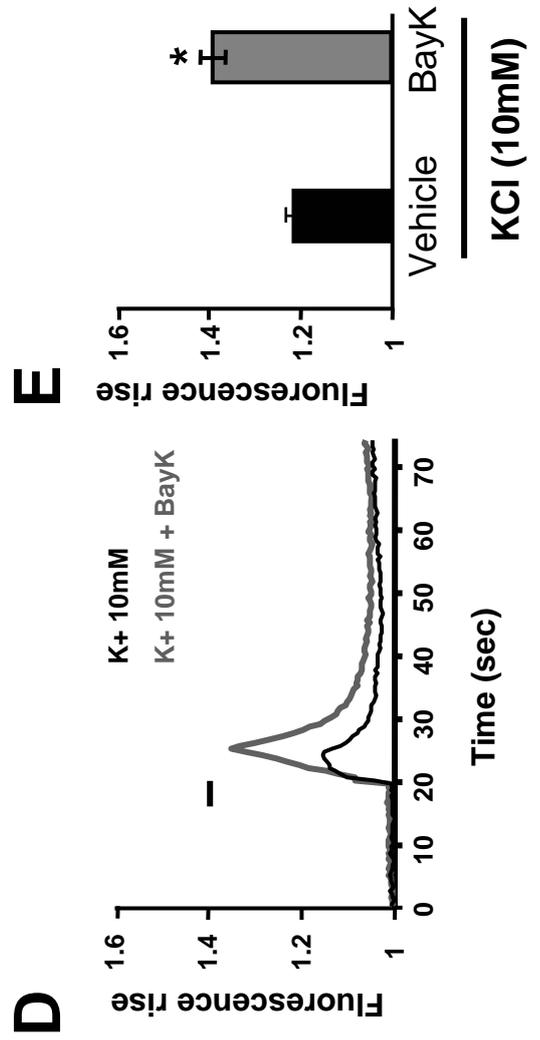
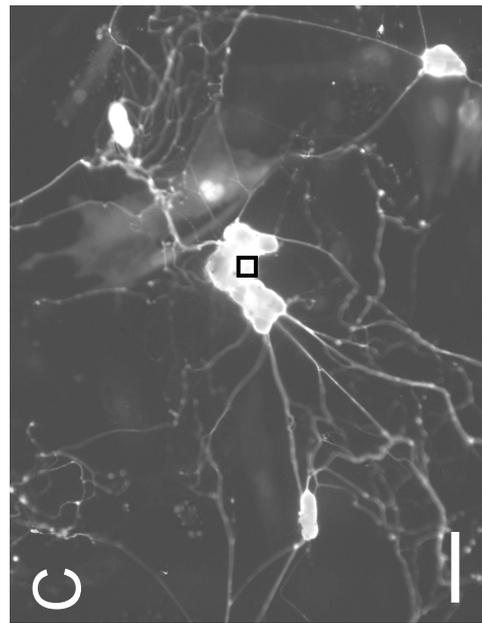
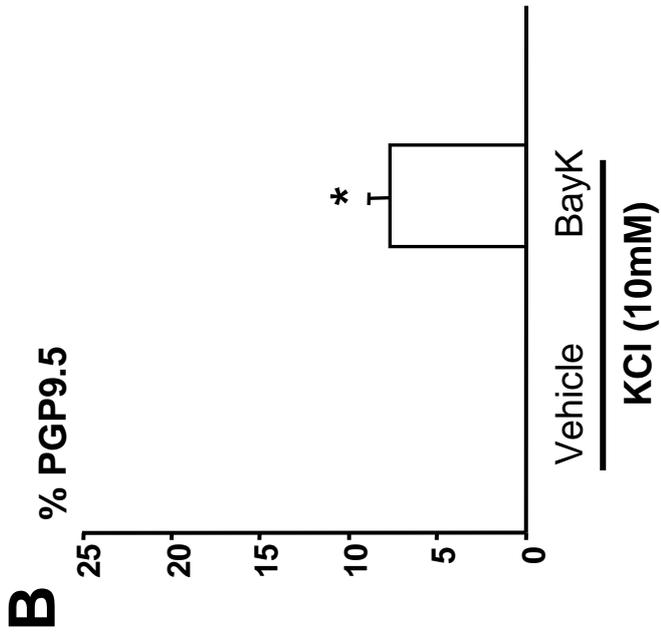
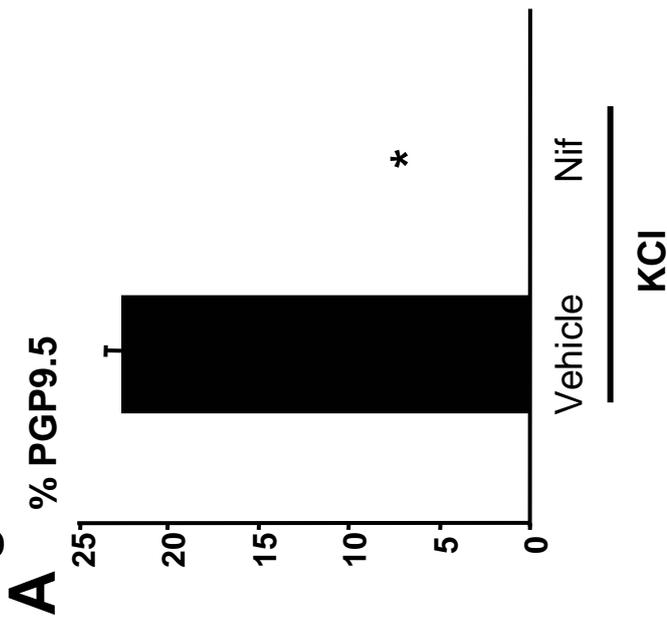


Figure 6

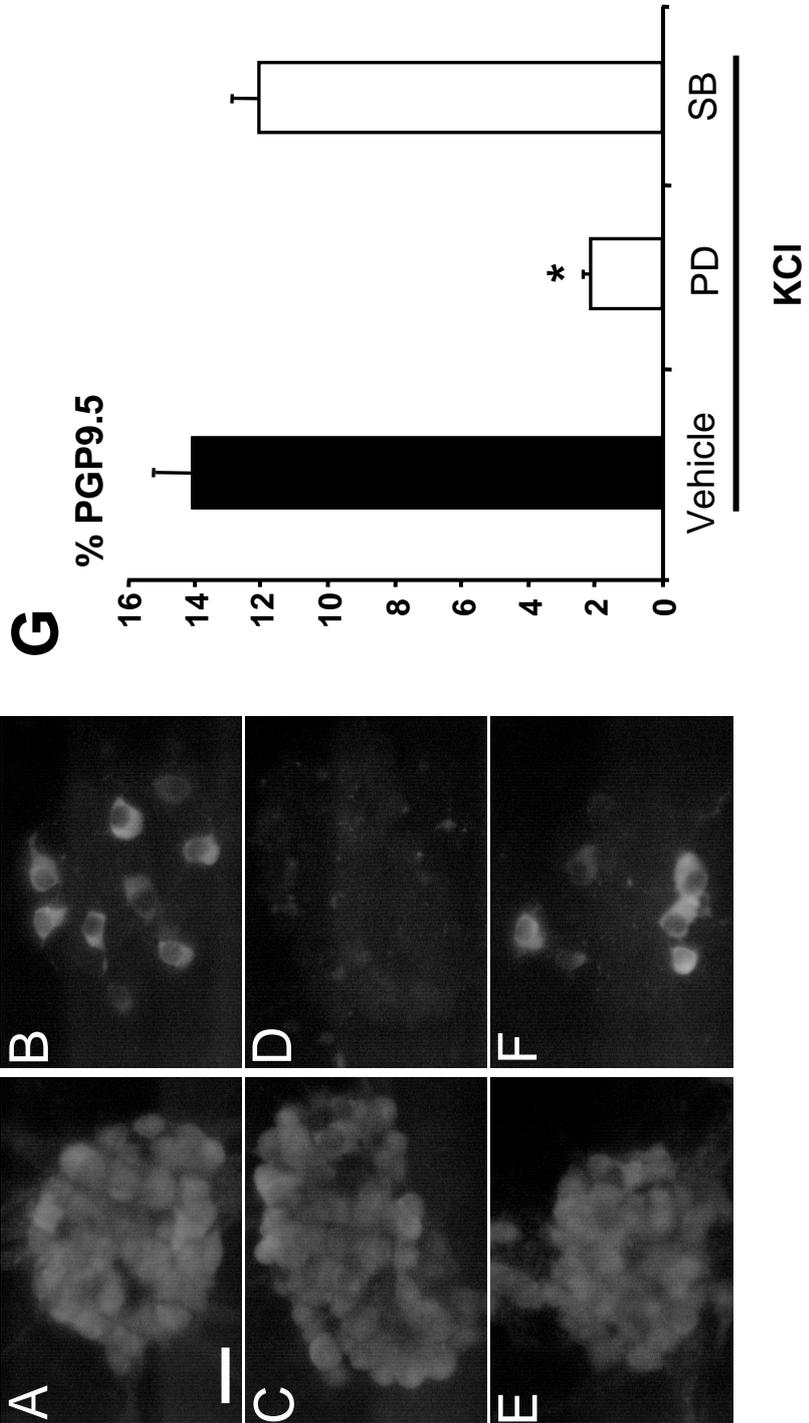


Figure 7

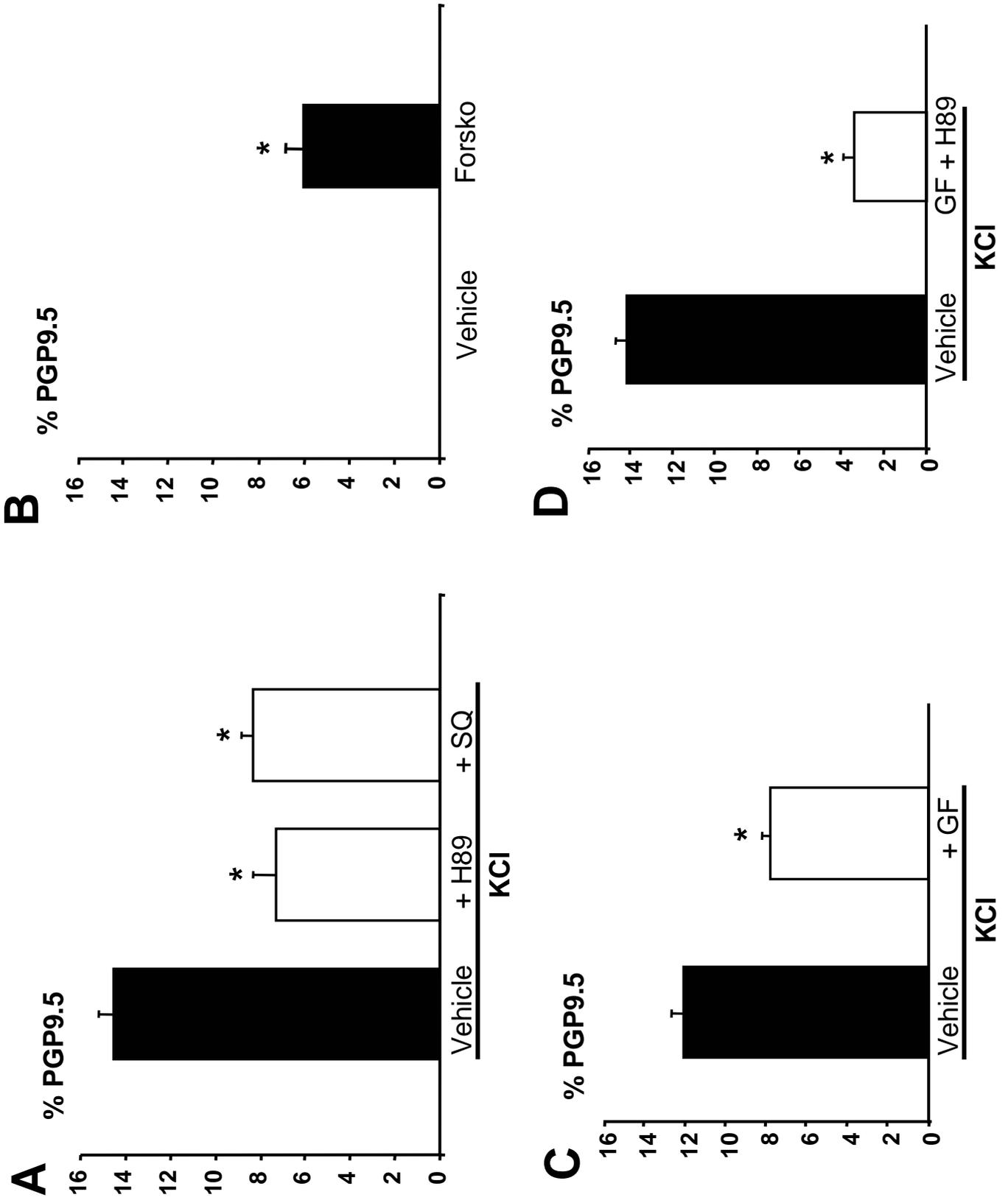


Figure 8

