Potent regulation of microglia-derived oxidative stress and dopaminergic neuron survival: substance P vs. dynorphin

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ABSTRACT **Unregulated microglial activation has been implicated as a pivotal factor contributing to Parkinson's disease. Using mesencephalic neuron-glia cultures, we address the novel possibility that peptides endogenous to the substantia nigra (SN), substance P and dynorphin (10^{-13}–10^{-14} M), are opposing mediators of microglial activation and consequent DA neurotoxicity. Here, we identify that substance P (10^{-13}–10^{-14} M) is selectively toxic to DA neurons in a microglia-dependent manner. Mechanistically, substance P (10^{-13}–10^{-14} M) activated microglial NADPH oxidase to produce extracellular superoxide and intracellular reactive oxygen species (ROS). Neuron-glia cultures from mice lacking a functional NADPH oxidase complex ($PHOX^{-/-}$) were insensitive to substance P (10^{-13}–10^{-14} M) -induced loss of DA neuron function. Mixed glia cultures from ($PHOX^{-/-}$) mice failed to show a significant increase in intracellular ROS in response to substance P compared with control cultures ($PHOX^{+/+}$). Further, dynorphin (10^{-14} M) inhibited substance P (10^{-13} M) -induced loss of [^{3}H] DA uptake. Here we demonstrate a tightly regulated mechanism governing microglia-derived oxidative stress, where the neuropeptide balance of dynorphin and substance P is critical to DA neuron survival.—Block, M. L., Li, G., Qin, L., Wu, X., Pei, Z., Wang, T., Wilson, B., Yang, J., Hong, J. S. Potent regulation of microglia-derived oxidative stress and dopaminergic neuron survival: substance P vs. dynorphin. *FASEB J.* 20, 251–258 (2006)**

Key Words: microglia · oxidative stress · DA neurotoxicity · superoxide · NADPH oxidase · femtomolar

PARKINSON'S DISEASE (PD) is characterized by the progressive and selective degeneration of dopaminergic (DA) neurons in the substantia nigra (SN). Increasing evidence has identified microglia as a predominant source of inflammation and oxidative stress contributing to DA neurodegeneration (1–3). In the disease state, microglia become overactivated and uncontrolled, resulting in either the initiation of DA neurotoxicity, or the amplification of DA cell death through reactive microgliosis (4). Currently, the homeostatic mechanisms regulating microglial function are unknown. In the following study, we sought to determine the influence of resident SN peptides, substance P (SP) and dynorphin (Dyn), on microglial activation and DA neuron survival. Specifically, we hypothesize that while neuromodulation occurs at micromolar concentrations, physiologically relevant concentrations for the regulation of microglia occur at 10^{-13}–10^{-14} M, perhaps after the peptides have diffused away from the synapse.

Substance P is both a classic proinflammatory peptide (5, 6) and neuromodulator colocalized in high concentrations with dynorphin in the SN (7–9). Substance P has also been associated with microglial inflammatory responses, such as the amplification of prostaglandin E_2 (PGE_2) (10), enhancement of tumor necrosis factor α (TNFα) (11), and the induction of microglial chemotaxis (12). The potent effects of substance P (10^{-13} M) on immune cells (13, 14) have been documented, but the mechanisms remain poorly defined.

Sub-picomolar concentrations of several peptides have been reported to regulate a diverse list of biological processes, such as chemotaxis (15, 16), antibody production (17–19), natural killer cell activity (20), phagocytosis (21, 22), production of proinflammatory factors (23, 24), and neuroprotection (24–27). In the current study, we are the first to address whether 10^{-13}–10^{-14} M of an endogenous neuropeptide (substance P) can have a deleterious effect on neuronal survival through activation of innate immunity in the brain (microglia). Further, we investigate the role of dynorphin on substance P neurotoxicity and provide evidence of a finely tuned neuropeptide homeostatic mechanism in the SN at the concentrations of 10^{-13}–10^{-14} M, offering novel insight into the potential etiology of sporadic PD.

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MATERIALS AND METHODS

Animals

Timed-pregnant (gestational day 14) adult female Fisher 344 rats were purchased from Charles River Laboratories (Raleigh, NC, USA). Eight-wk-old (25–30 g) male and female B6.129S6-*Cybb*^{tm1Din} ($PHOX^{-/-}$) and C57BL/6J ($PHOX^{+/+}$) mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA) and maintained in a strict pathogen free environment. The $PHOX^{-/-}$ mice lack the functional catalytic subunit of the NADPH oxidase complex, gp91. NADPH oxidase is an inducible electron transport system in phagocytic cells that is responsible for the generation of the respiratory burst. $PHOX^{-/-}$ mice are unable to generate extracellular superoxide in response to LPS or other immunological stimulus. Breeding of the mice was designed to achieve accurate timed-pregnancy ± 0.5 days. Because the $PHOX^{-/-}$ mutation is maintained in the C57BL/6J background, the C57BL/6J ($PHOX^{+/+}$) mice were used as control animals. Housing, breeding and experimental use of the animals were performed in strict accordance with the National Institutes of Health guidelines.

Reagents

Substance P and dynorphin were purchased from American peptide Company (Sunnyvale, CA, USA); Lipopolysaccharide (LPS) (strain O111:B4) was purchased from Calbiochem (San Diego, CA, USA). Cell culture ingredients were obtained from Life Technologies (Grand Island, NY, USA). [^{3}H] Dopamine (DA, 28 Ci/mmol) and [2,3-H^3] GABA (81 Ci/mmol) were purchased from NEN Life Science (Boston, MA, USA). The polyclonal antibody against tyrosine hydroxylase (TH) was a kind gift from Dr. John Reinhard of Glaxo Wellcome (Research Triangle Park, NC, USA). The neuron-specific nuclear protein (Neu-N) monoclonal antibody and the monoclonal antibody raised against the CR3 compliment receptor (OX42) were obtained from PharMingen (San Diego, CA, USA). The biotinylated horse anti-mouse and goat anti-rabbit secondary antibodies were purchased from Vector Laboratories (Burlingame, CA, USA). Catalase was obtained from Calbiochem. 2′, 7′-Dichlorofluorescin diacetate (DCFH-DA) was obtained from Calbiochem (San Diego, CA, USA). WST-1 was purchased from Dojindo Laboratories (Gaithersburg, MD, USA). TNFα enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems Inc. (Minneapolis, MN, USA). PGE_2 ELISA kits were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). All other reagents came from Sigma Aldrich Chemical Co. (St. Louis, MO, USA).

Mesencephalic neuron-glia cultures

Rat and mouse ventral mesencephalic neuron-glia cultures were prepared using a described protocol (28). Briefly, midbrain tissues were dissected from day 14 Fisher 344 rat embryos or day 14 mouse embryos ($PHOX^{+/+}$ or $PHOX^{-/-}$). Cells were dissociated via gentle mechanical trituration in minimum essential medium (MEM) and immediately seeded (5×10^5/well) in poly D-lysine (20 μg/mL) precoated 24-well plates. Cells were seeded in maintenance media and treated with the treatment media described previously (28). Three days after seeding, the cells were replenished with 500 μL of fresh maintenance media. Cultures were exposed 7 days after seeding.

Microglia-enriched cultures

Primary enriched-microglia cultures were prepared from the whole brains of 1-day-old Fisher 344 rat pups, using the described previously procedure (28). Briefly, after removing menengies and blood vessels, the brain tissue was gently triturated and seeded (5×10^7) in 150 cm^3 flasks. One week after seeding, the media was replaced. Two weeks after seeding, when the cells had reached a confluent monolayer of glial cells, microglia were shaken off and either replated at 1×10^5 in a 96-well plate precoated with poly D-Lysine, or reseeded on top of a neuron-enriched culture in a 24-well plate at 5×10^4 (10%) or 1×10^5 (20%) for a microglia add-back culture. Cells were treated 24 h after seeding the enriched microglia.

Neuron-enriched cultures

Mesencephalic neuron-glia cultures were seeded (5×10^5/well) in 24 well plates precoated with poly D-lysine. Twenty-four hours postseeding, 5–10 μM cytosine β-D-arabinofuranoside was added to the culture. After 2 days, the cytosine β-D-arabinofuranoside was removed and replaced with fresh media. Neuron-enriched cultures are 98% pure, as indicated by ICC staining with OX-42 and GFAP antibodies. Neuron-enriched cultures were treated 7 days post-seeding.

For microglia add-back cultures, the microglia were plated on top of the neuron-enriched culture at 6 days postseeding, resulting in the addition of either 10% (500 μL of 1×10^5) or 20% (500 μL of 2×10^5) microglia. Cells were treated 7 days after the initial seeding of the neuron-enriched cultures.

Uptake assays (DA and GABA)

Cells were incubated in Krebs-Ringer buffer (16 mM NaH_2PO_4,1.2 mM $MgSO_4$, 1.3 mM EDTA, 4.7 nM KCL, for 15 min at 37°C with either 5 μM [^{3}H] GABA or 1 μM [^{3}H] DA. Nonspecific uptake was blocked for GABA with 10 μM NO-711 and 1 mM β alanine. Nonspecific uptake was blocked for DA with 10 μM mazindole. After incubation, cells were washed three times with 1 mL/well of ice-cold Krebs-Ringer buffer. Cells were then lysed with 0.5 mL/well of 1 N NaOH and mixed with 15 mL of scintillation fluid. Radioactivity was measured on a scintillation counter, where specific [^{3}H] GABA or [^{3}H] DA uptake was calculated by subtracting the mazindole or the NO-711 and β alanine counts from the wells without the uptake inhibitors.

Immunostaining

Dopamine neurons were detected with the polyclonal antibody against tyrosine hydroxylase (TH). Briefly, cells were fixed for 20 min at room temperature in 3.7% formaldehyde diluted in phosphate-buffered saline (PBS). After washing twice with PBS, the cultures were treated with 1% hydrogen peroxide for 10 min. The cultures were again washed three times with PBS, then incubated for 40 min with blocking solution (PBS containing 1% bovine serum albumin (BSA), 0.4% Triton X-100, and 4% goat serum. The cultures were incubated overnight at 4°C with the primary antibody diluted in DAKO antibody diluent and the cells were washed three times for 10 min each in PBS. The cultures were next incubated for 1 h with PBS containing 0.3% Triton X-100 and the appropriate biotinylated goat anti-rabbit secondary antibody (1:227). After washing three times with PBS, the cultures were incubated for 1 h with the Vectastain ABC reagents diluted in PBS containing 0.3% Triton X-100. Cells were then washed twice with PBS, the bound complex was visualized by

incubating cultures with 3,3′-diaminobenzidine. Color development was halted by removing the reagents and washing the cultures twice with fresh PBS. To quantify cell numbers, nine representative areas per well in the 24-well plate were counted under the microscope at 100× magnification by two individuals. The average of these scores was reported.

Superoxide assay

Extracellular superoxide (O_2^-) production from microglia was determined by measuring the superoxide dismutase (SOD) inhibitable reduction of 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4,-disulfophenyl)-2H-tetrazolium, monosodium salt, WST-1 (29–31). Briefly, 200 μL of primary enriched-microglia were seeded (1×10^5/well) in 96-well plates. The cells were then incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Immediately before treatment, cells were washed twice with Hanks balanced salt solution (HBSS). To each well, 100 μL of HBSS with or without SOD (600 U/mL), 50 μL of vehicle or LPS, and 50 μL of WST-1 (1 mM) in HBSS were added. The cultures were incubated for 30 min at 37°C and 5% CO_2 and 95% air. The absorbance at 450 nm was read with a Spectra Max Plus microtiter plate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Cell free experiments with and without substance P were conducted to determine that SP did not alter absorbance by itself. The amount of SOD-inhibitable superoxide was calculated and expressed as percent of vehicle-treated control cultures.

Intracellular reactive oxygen species assay

The production of intracellular reactive oxygen species (ROS) was measured by DCFH oxidation. The DCFH-DA reagent passively enters cell where it is de-acetylated by esterase to nonfluorescent DCFH. Inside the cell, DCFH reacts with ROS to form DCF, the fluorescent product. For this assay, 10 mM DCFH-DA was dissolved in methanol and was diluted 500-fold in HBSS to give a 20 μM concentration of DCFH-DA. Enriched-microglia cultures seeded (5×10^4) in 96-well plates were then exposed to DCFH-DA for 1 h, followed by treatment with HBSS containing several concentrations of LPS or substance P for 2 h. After incubation, the fluorescence was read at the 485 nm excitation and 530 nm emission on a fluorescence plate reader. Cell free experiments with and without SP were conducted to determine that SP did not alter fluorescence by itself. To calculate the amount of intracellular ROS produced, the mean control treatment was subtracted from the mean treatment group.

TNFα assay and PGE_2 assay

The production of TNFα was measured with a commercial ELISA kit from R&D Systems. The PGE_2 release was measured with a commercial ELISA kit from Cayman Chemical Company.

Nitrite assay

As an indicator of nitric oxide production, the amount of nitrite accumulated in culture supernatant was determined with a colorimetric assay using Griess reagent [1% sulfanilamide, 2.5% H_3PO_4, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride]. Briefly, 50 μL of Griess reagent and 50 μL of culture supernatant were incubated in the dark at room temperature for 10 min. After incubation, the absorbance at 540 nm was determined with the Spectra Max Plus microplate spectrophotometer. The sample nitrite concentration was determined from a sodium nitrite standard curve.

Statistical analysis

The data are expressed as the mean ± SE and statistical significance was assessed with an ANOVA followed by Bonferroni's t test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Substance P is toxic to DA neurons at femtomolar concentrations

To discern the effect of substance P on DA neurotoxicity, mesencephalic neuron-glia cultures were exposed to substance P (10^{-7}–10^{-17} M) and the ability of cells to take up [^{3}H] DA was measured. Here, we show that substance P has a bimodal dose response, with peak inhibition of DA cell function occurring at 10^{-7} M and 10^{-13} M to 10^{-14} M, whereas 10^{-10} M and 10^{-17} M of substance P failed to demonstrate [^{3}H] DA uptake inhibition (**Fig. 1*A***). Immunohistochemical (ICC) analysis was performed on substance P (10^{-13} M), LPS, and control treated mesencephalic neuron-glia cultures, where cells were stained with an anti-tyrosine hydroxylase (TH) antibody and the numbers of TH-immunoreactive cells per well were counted. Here, we also show that substance P (10^{-13} M) results in the loss of TH-immunoreactive neurons (Fig. 1*B*). Substance P (10^{-13} M) also drastically altered DA neuron morphology, where cells exposed to substance P (10^{-13} M) showed loss of dendrites and axon disintegration in addition to the loss of DA neurons compared with control wells (Fig. 1*C*).

Substance P is selectively toxic to DA neurons at femtomolar concentrations

In an effort to establish whether substance P toxicity was selective for DA neurons, neuron-glia cultures were exposed to substance P and assessed for their ability to take up GABA, the predominant inhibitory neurotransmitter in the brain. Figure 1*D* indicates that while substance P (10^{-13} M and 10^{-14} M) results in an ~ 35–40% loss of [^{3}H] DA uptake, this peptide has no effect on [^{3}H] GABA uptake, supporting that substance P (10^{-13}–10^{-14} M) is selectively toxic to DA neurons.

Microglia are critical for SP-mediated neurotoxicity

The role of microglia in substance P-induced DA neurotoxicity was determined by comparing the ability of substance P to alter DA uptake in neuron-glia cultures, compared with neuron-enriched cultures, which contain <2% glia (microglia or astrocytes). Figure 1*E* demonstrates that the substance P (10^{-14} M) -induced loss of DA neuron function requires the presence of glia. Further, SP (10^{-14} M) neurotoxicity

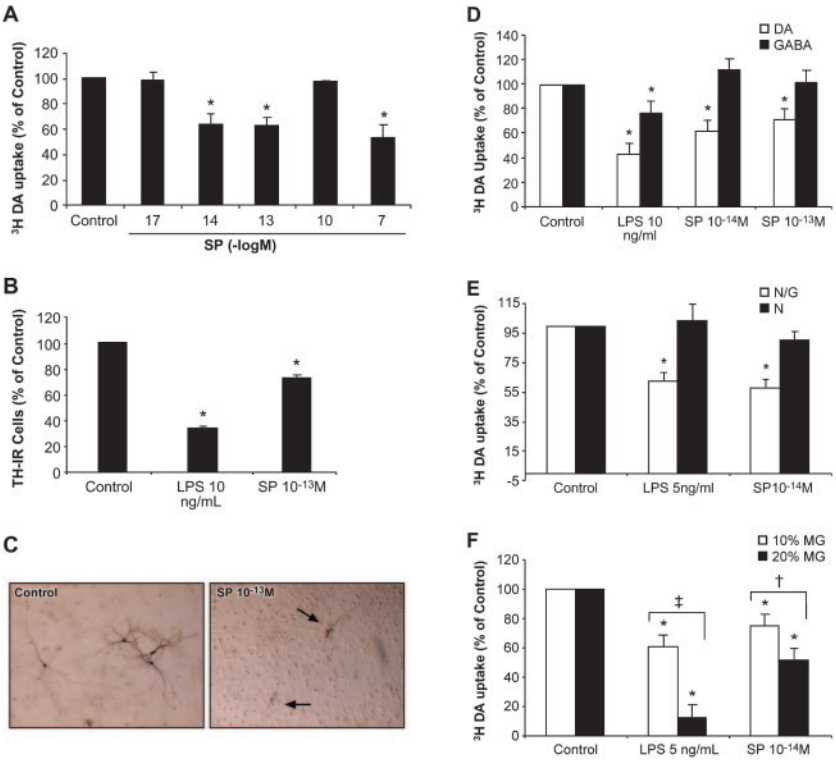

Figure 1. Substance P is selectively toxic to DA neurons in the presence of microglia. Cultures were treated with vehicle, lipopolysaccharide (LPS) or SP. LPS was used as a positive control for microglia activation. *A)* DA neurotoxicity was measured at 9 days post-treatment using the [^{3}H] DA uptake assay. The data are expressed as the % of the control cultures and are the mean ± SE from 5 independent experiments, each performed with triplicate samples. $*P < 0.05$ compared with control. *B)* SP-induced DA neuronal death was determined at 9 days post-treatment by counting the number of TH-positive neurons. The data are expressed as the % of the control cultures and are the mean ± SE from 3 independent experiments, each performed with triplicate samples. $*P < 0.05$ compared with control. *C)* Analysis of TH-immunoreactive neurons demonstrates the ability of SP to induce morphological damage to DA neurons. The images shown are representative of 3 independent experiments. *D)* Neurotoxicity was compared at 9 days post-treatment using the [^{3}H] DA and [^{3}H] GABA uptake assay. *E)* To determine the role of glia in SP-induced neurotoxicity, neuron-enriched cultures and neuron-glia cultures exposed to SP were compared for their ability to uptake [^{3}H] DA. *F)* Microglia (10% and 20%) were added back to neuron-enriched cultures to test the importance of microglia for SP-induced DA neurotoxicity. DA neurotoxicity was measured 8–9 days post-treatment using the [^{3}H] DA uptake assay. The data are expressed as the % of the control cultures and are the mean ± SE from 3 independent experiments, each performed with triplicate samples. $*P < 0.05$ compared with control.

can be reinstated in neuron-enriched cultures by the addition of microglia back to the culture, where increasing the number of microglia added back results in increased neurotoxicity (Fig. 1*F*). Together, these data support that substance P (10^{-13}–10^{-14} M) is selectively toxic to DA neurons through microglia.

Several proinflammatory parameters were measured in an attempt to discern how substance P influences microglia to become toxic to DA neurons. Supernatant from substance P-treated neuron-glia cultures and enriched microglia cultures were assessed for the presence of the soluble proinflammatory factors. Substance P treatment failed to induce TNFα and PGE_2 production (data not shown). However, enriched-microglia cultures showed an increase in intracellular ROS (**Fig. 2*A***) and extracellular superoxide (Fig. 2*B*) in response to substance P (10^{-13}–10^{-14} M). Taken together, Fig. 2 demonstrates that substance P (10^{-13}–10^{-14} M) activates microglia, resulting in the production of extracellular and intracellular ROS, implying that the mechanism through which substance P is toxic to DA neurons is through microglia generated oxidative insult.

Femtomolar concentrations of SP are toxic through NADPH oxidase

NADPH oxidase is the enzyme complex responsible for the respiratory burst in phagocytes. Recently, we have demonstrated that activation of this enzyme in microglia induces both the production of extracellular superoxide and an increase in intracellular ROS, resulting in the amplification of the production of proinflammatory factors (32). Pretreatment for 30 min with femtomolar concentrations of substance P (10^{-14} M) enhances the production of TNFα in LPS-treated neuron-glia cultures (**Fig. 3**). Several reports have linked substance P to the activation of NADPH oxidase in other immune cells, such as the production of superoxide in neutrophils (33). To discern whether SP increases intracellular ROS through NADPH oxidase activation, mixed glia cultures (containing both 30% microglia and 70% astroglia) from PHOX $^{-/-}$ (Phagocyte oxidase, PHOX) mice were exposed to substance P. PHOX$^{-/-}$ mice are missing a functional gp91 protein, the catalytic subunit of the NADPH oxidase enzyme complex, and cannot produce the respiratory burst in response to immunological stimulus. **Figure 4*A*** demonstrates that while LPS, a traditional immunological stimulus for microglia, does produce intracellular ROS at reduced levels in PHOX$^{-/-}$ cultures, substance P failed to induce a significant increase in intracellular ROS in PHOX$^{-/-}$ cultures, supporting the hypothesis that substance P-induced intracellular ROS is mediated primarily through NADPH oxidase. Further, mesencephalic neuron-glia cultures from PHOX$^{-/-}$ mice are resistant to substance P (10^{-13} M and 10^{-14} M) -induced DA neurotoxicity compared with PHOX$^{+/+}$ mice, as deter-

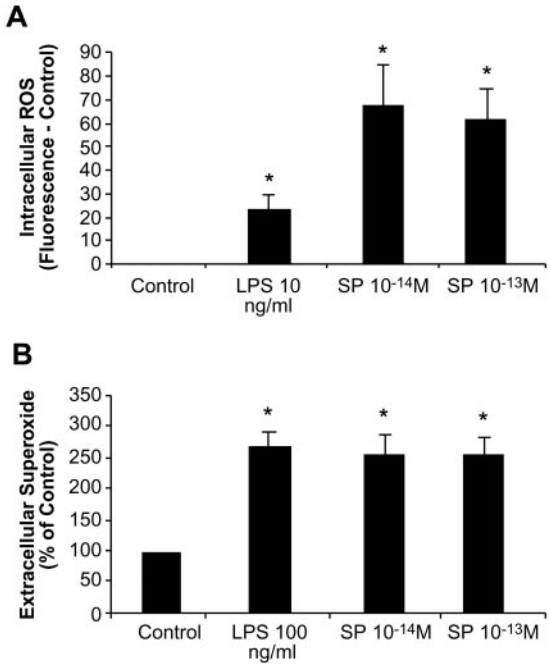

Figure 2. Substance P activates microglia and induces the production of ROS. Cultures were treated with vehicle, LPS or SP. LPS was used as a positive control for microglia activation. *A)* The production of intracellular ROS was measured in microglia-enriched cultures 3 h poststimulation by observing the fluorescent product of DCFH-DA oxidation. *B)* The production of extracellular superoxide was measured in microglia-enriched cultures by monitoring the superoxide dismutase (SOD) inhibitable reduction of tetrazolium salt, WST-1. The data are the mean ± SE and are the average of 3 separate experiments. *Significant difference ($P<0.05$) compared with control.

mined by [^{3}H] DA uptake (Fig. 4*B*). Thus, these results indicate that substance P (10^{-13}–10^{-14} M) induces microglial activation and consequent neurotoxicity through activation of NADPH oxidase.

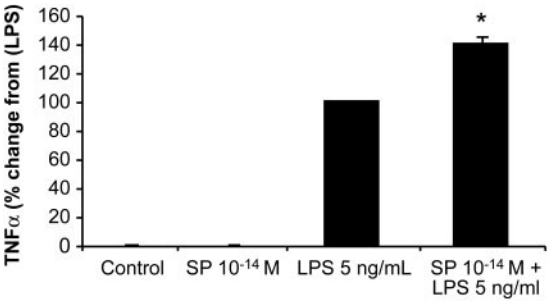

Figure 3. Femtomolar concentrations of substance P amplify LPS-induced TNFα production in neuron-glia cultures. Cultures were pretreated with substance P (SP 10^{-14} M) for 30 min before the addition of LPS. At 3 h post-LPS treatment, supernatant was collected and analyzed for TNFα production by ELISA. The data are expressed as the % of LPS response and are the mean ± SE. The data are the average of 4 separate experiments.

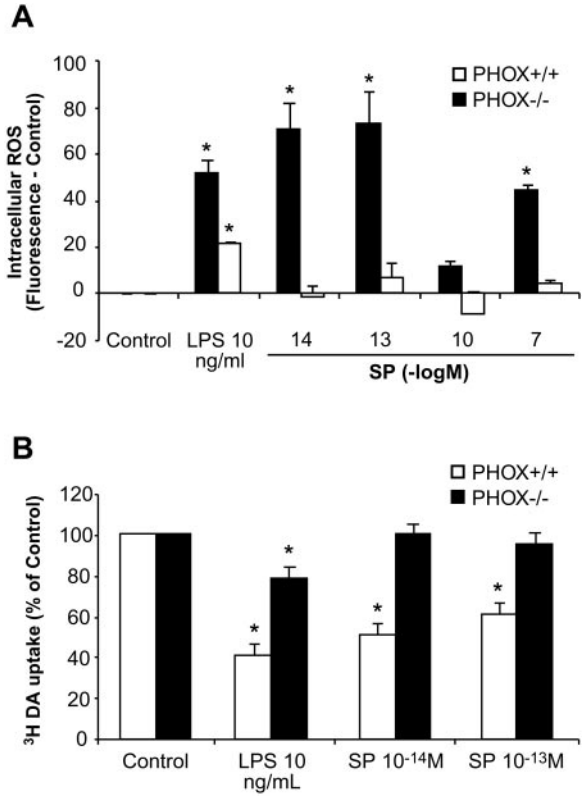

Figure 4. Substance P DA neurotoxicity is mediated through NADPH oxidase. *A)* PHOX$^{-/-}$ mice lack the functional catalytic subunit of the NADPH oxidase complex, gp91, and fail to generate the phagocytic respiratory burst. PHOX $^{-/-}$ mice failed to produce a significant increase in intracellular ROS in response to SP. The data are the mean ± SE and are the average of 5 separate experiments. *Significant difference ($P<0.05$) compared with control. *B)* Mesencephalic midbrain neuron-glia cultures from PHOX $^{-/-}$ mice were insensitive to SP-induced neurotoxicity compared with PHOX $^{+/+}$ mice. LPS was used as a positive control for microglia-mediated DA neurotoxicity. DA neurotoxicity was measured 8–9 days post-treatment using the [^{3}H] DA uptake assay. The data are the mean ± SE and are the average of 4 separate experiments. *Significant difference ($P<0.05$) compared with control.

Femtomolar concentrations of dynorphin protect against SP-induced neurotoxicity

To discern which of these two peptides (dynorphin or substance P) could prevail and exert the greatest influence on DA neuron survival, rat mesencephalic neuron glia cultures were pretreated with dynorphin (10^{-14} M) for 30 min, followed by substance P (10^{-13} M) treatment. **Figure 5** indicates that dynorphin (10^{-14} M) can inhibit substance P (10^{-13} M) -induced loss of DA neuron function at 9 days post-treatment, indicating that microglial activation and consequent DA neuron survival may be dependent on neuropeptide balance at femtomolar concentrations (10^{-13}–10^{-14} M).

DISCUSSION

The factors responsible for the overactivation of microglia and the consequent selective DA neurotoxicity in

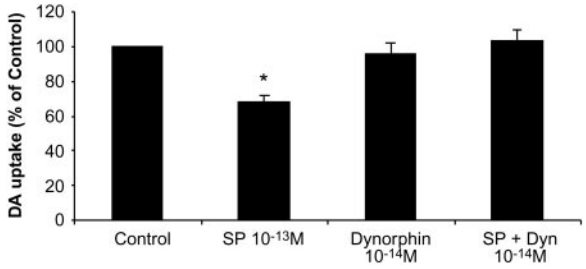

Figure 5. Dynorphin protects DA neurons from substance P-induced neurotoxicity. The effect of dynorphin on SP-induced DA neurotoxicity was compared in mesencephalic neuron-glia cultures. DA neurotoxicity was measured at 9 days post-treatment using the [^{3}H] DA uptake assay. The data are the mean ± SE and are the average of 3 separate experiments. *Significant difference ($P<0.05$) compared with control.

the SN of the PD patient are largely undefined. Here, we demonstrate that an endogenous peptide present in the SN, substance P, is toxic at 10^{-13}–10^{-14} M. Given the high concentrations of substance P localized in the SN, at first glance, the DA neurotoxicity of both micromolar (10^{-7} M) and femtomolar (10^{-13}–10^{-14} M) concentrations of SP defined by this work is alarming. However, in vivo, substance P is colocalized with several peptides and neuromodulators in the SN that may have an opportunity to intervene to assist the DA neuron survival, such as dynorphin. Previously, we identified dynorphin as both anti-inflammatory and neuroprotective at 10^{-13}–10^{-14} M (24). Of further interest, we recently identified the smallest biologically active fragment of dynorphin, glycine-glycine-phenylalanine (GGF), and reported that the mechanism through which dynorphin and GGF confer their anti-inflammatory and neuroprotective qualities was through the inhibition of microglial NADPH oxidase activation (34). Here, we demonstrate that 10^{-14} M dynorphin can inhibit substance P-induced DA neurotoxicity, supporting the critical importance of peptide balance in DA neuron survival (Fig. 5) and identifying a femtomolar-acting mechanism of control over microglial activation.

While the majority of the work presented here demonstrates the oxidative stress and neurotoxic consequences of unregulated microglia, it should be noted that not all activation of NADPH oxidase and increases in ROS are deleterious. In fact, slight elevation of ROS is protective to neurons (35), suggesting a potential benefit for the femtomolar (10^{-13}–10^{-14} M) activities of SP. Further, microglia continually inhibited by dynorphin and unable to respond to immunological stimuli also represents a deleterious situation. Thus, we propose that substance P serves as an opposing force in the homeostatic regulation of the microglial PHOX-ROS pathway, which governs inflammation, oxidative stress, and normal microglial functioning. The protective effect of dynorphin (Fig. 5) suggests that in the normal healthy brain, where both peptides are present in abundance in the SN, substance P is not likely toxic to DA neurons. Rather, we propose that perturbation of the neuropeptide system and the destruction of a harmonious peptide balance where the SP influence on microglia would become unregulated could either cause or potentiate neurodegeneration. In fact, this work suggests that only the micromolar and the femtomolar levels of substance P are toxic to DA neurons in vitro, leaving a nanomolar window of inactivation, which implies that the regulation of microglia is very tightly regulated.

The bimodal dose response is a common phenomenon seen in femtomolar-acting neuroprotective compounds (dynorphin, dynorphin fragments, naloxone, and dextromethorphan) tested in our laboratory (36–38). This bimodal response can be categorized by three unique sections of the dose curve. The physiological effects seen in the higher micromolar concentrations (10^{-7}–10^{-9} M) are often assumed to be the traditional receptor mediated effects, but this assumption may not be applicable for all cases. The femtomolar (10^{-13}–10^{-14} M) region of the curve is attributed to an atypical mechanism that is independent of traditional receptor-mediated events. The third component of the bimodal dose response is the middle portion of the curve (10^{-10}–10^{-12} M), where in the case of SP, the respiratory burst is absent. Presently, the mechanisms responsible for the lack of microglial response to SP at 10^{-10} to 10^{-12} M are unknown. One interpretation is that multiple sites of action exist for SP that result in NADPH oxidase activation or inhibition of the respiratory burst, depending on the dose. In the case of two sites of action (a micromolar and a femtomolar site), the inactivation of NADPH oxidase (and lack of neurotoxicity) seen in the picomolar range (Fig. 1*A*) may be due to an effect similar to "substrate inhibition," where the site of action for femtomolar concentrations is overwhelmed with increasing dose. Alternatively, a hypothetical third binding site in the picomolar range may mask or inhibit the SP-induced respiratory burst at femtomolar concentrations. Currently, there is a multitude of pathways through which microglial NADPH oxidase is activated in response to a diverse set of triggers (32, 39–43) where, in addition to SP, many receptors responsible for ligand recognition and generation of the respiratory burst are unknown. Chemotactic peptides may provide some insight, as fMLP is reported to induce the respiratory burst in neutrophils and monocytes and is chemotactic through two independent G-protein receptors (a high- and a low-affinity receptor) (44).

Other work from our laboratory has demonstrated that N-acetyl-L-trypotophan 3, 5-bis (trifluoromethyl)-benzyl ester, an antagonist for the substance P neurokinin-1 receptor, is unable to inhibit substance P-induced microglial activation or the associated neurotoxicity, indicating that substance P-induced inflammation-mediated DA neurodegeneration at femtomolar (10^{-13}–10^{-14} M) concentrations is independent of neurokinin-1 receptor (Block et al., unpublished results). At this time, the identity of the SP site(s) of action across this bimodal dose response is unknown, but ongoing work in our laboratory is aimed at identifying these complex mechanisms for SP and dynorphin. Micromolar concentrations are likely to occur primarily between

nerve terminals and are relevant for communication between neurons. However, we hypothesize that after diffusion and degradation, the peptides may encounter glial cells at femtomolar (10^{-13}–10^{-14} M) concentrations. It remains possible that the degradation of the peptide could be a critical component to the process.

Here we attempt to understand the mechanisms guarding the survival of DA neurons in the SN. DA neurons are ill equipped to endure oxidative stress, as evidenced by low intracellular glutathione, which renders DA neurons more vulnerable to oxidative stress and microglial activation relative to other cell types (45). The mesencephalon houses the SN and contains 4.5-fold as many microglia compared with other regions of the brain (46), making the strict regulation of microglial function in SN essential for DA neuron survival. Together, this work suggests that the neuropeptide balance could be critical to survival of DA neurons and supports our hypothesis of the peptide mediation of inflammatory homeostasis in the substantia nigra. The importance of this finding is emphasized by the fact that brains from postmortem PD patients often show an imbalance in SN peptides (24, 47, 48) and that chemical lesion of the SN has been reported to increase SP production (49). However, there are conflicting reports as to which neuropeptide will decrease or increase in PD patients, which may reflect compensatory mechanisms, differences in disease process, age of the patient, or responses to different drug therapy (48). An intriguing problem for future research will be to determine whether the neuropeptide imbalance found in the SN of postmortem PD patients is an initial trigger of inflammation and neuronal death or a further deleterious consequence of disease progression. Further research in our laboratory will focus on the relationship of dynorphin and substance P in DA neuron survival and microglia activation in vivo.

In summary, we report that SP (10^{-13}–10^{-14} M) is selectively toxic to DA neurons in the presence of microglia. The mechanism of SP neurotoxicity is through the activation of microglia, which induces NADPH oxidase activation and the production of ROS. We also demonstrate that the opiate peptide dynorphin protects DA neurons from substance P neurotoxicity, implying the regulated control of microglial NADPH at femtomolar concentrations (10^{-13}–10^{-14} M), offering novel insight into the potential causes and propagation of neurodegeneration in Parkinson's disease. FJ

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