Localization of Preganglionic Neurons That Innervate Choroidal Neurons of Pterygopalatine Ganglion

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PURPOSE. The pterygopalatine ganglion (PPG) receives preganglionic input from the superior salivatory nucleus (SSN) of the facial motor complex and is the main source of parasympathetic input to the choroid in mammals. The present study was undertaken to determine in rats the location and neurotransmitters of SSN neurons innervating those PPG neurons that target the choroid and to determine the location and neurotransmitters of the PPG choroidal neurons themselves.

METHODS. Retrograde labeling from rat choroid using a fluorescent tracer, in combination with immunofluorescence labeling for nitric oxide synthase (NOS), vasoactive intestinal polypeptide (VIP), and choline acetyltransferase (ChAT), was used to characterize the location and neurotransmitters of choroidal PPG neurons. To identify SSN neurons that innervate the choroidal PPG neurons, the Bartha strain of the retrograde transneuronal tracer pseudorabies virus (PRV-Ba) was injected into rat choroid, and immunolabeling for NOS or ChAT was used to characterize their neurochemistry.

RESULTS. Fluorescent retrograde labeling showed that PPG neurons projecting to the choroid contained NOS, VIP, and ChAT and were widely distributed in PPG and its preganglionic root, the greater petrosal nerve. SSN neurons were ChAT⁺, and a subset of them was found to contain NOS. PRV-Ba transneuronal retrograde labeling revealed that choroidal preganglionic neurons were localized to the rostral medioventral part of the ipsilateral SSN. The choroidal SSN neurons were ChAT⁺ and appeared largely to correspond to the NOS⁺ neurons of the SSN.

CONCLUSIONS. These results show that preganglionic neurons in rats that are presumed to regulate choroidal blood flow through the PPG reside within the rostral medioventral SSN, and that NOS is a marker for these SSN neurons. (Invest Ophthalmol Vis Sci. 2003;44:3713-3724) DOI:10.1167/iovs.02-1207

The choroid is the blood supply to the outer retina (including photoreceptors), and disturbances in choroidal blood flow (ChBF) can lead to impaired retinal function and damage to photoreceptors.¹⁻³ The choroid in both birds and mammals is innervated by parasympathetic, sympathetic, and sensory nerve fibers that adaptively regulate ChBF according to retinal needs.¹⁻⁴ Such adaptive control may be important for maintaining the health of retinal photoreceptors and maintaining normal visual functioning.¹⁻¹²⁻¹⁴ Numerous studies have shown that the pterygopalatine ganglion (PPG) is the major source of parasympathetic input to the choroid in mammals.⁶⁻⁹,¹⁶⁻¹⁸ The PPG efferent fibers to the choroid contain the vasodilators VIP and nitric oxide (NO).³⁻⁵,¹⁷⁻¹⁹ These fibers also appear to be, at least in part, cholinergic.²¹⁻²⁴

The PPG receives its preganglionic input from the superior salivatory nucleus (SSN) of the hindbrain through the greater petrosal branch of the facial nerve.²⁵⁻²⁹ The SSN itself is located dorsolateral to the facial motor nucleus. The SSN neurons, which are somewhat intermingled among and surrounded by noradrenergic neurons of the A5 cell group, are cholinergic, and, in rabbits and humans, some have been reported to contain nitric oxide synthase (NOS) as well.³⁰⁻³² The SSN also provides preganglionic input through the chorda tympani nerve to the submandibular gland.²⁵⁻²⁹,²⁸⁻³⁵ which sends postganglionic fibers to the submandibular and sublingual glands and thereby regulates blood flow and salivary secretion within these glands. The PPG, in addition to its innervation of choroidal blood vessels, innervates orbital blood vessels, the meibomian glands, the lacrimal gland, the hard-erian gland, blood vessels of the nasal mucosa and palate, and cerebral blood vessels.³⁴⁻³⁹

Thus, functionally diverse types of preganglionic neurons may be present within the SSN. Although the location within the SSN of the preganglionic neurons controlling the meibomian glands³⁰ and the lacrimal gland³⁹ has been described, the location within the SSN of the preganglionic neurons controlling choroid is unknown. In the present study, we sought to determine the location within the SSN in rats of those neurons that innervate the PPG neurons innervating the choroid (prechoroidal neurons). This study also sought to determine whether these SSN neurons contain NOS and can be distinguished by morphology or location from the nearby A5 adrenergic neurons. To this end, a transneuronal retrograde tracer, the Bartha strain of pseudorabies virus (PRV-Ba), was injected into the choroid of adult rats to identify prechoroidal neurons of SSN, and immunolabeling was used to further characterize these neurons. Our results show that the prechoroidal neurons of rat SSN reside within a characteristic location within the SSN and largely coincide with NOS⁺ preganglionic neurons within the SSN. In addition, retrograde labeling from the choroid using fluorescent tracer (FG; Fluorogold; Flurochrome, Englewood, CO), in combination with immunofluorescence, was used to show that PPG neurons projecting to choroid contain NOS, VIP, and ChAT and are widely distributed in the PPG and its preganglionic root, the greater petrosal nerve. The results of the present studies will aid in elucidating the central sources of input to these SSN neurons and thereby help clarify the signals driving parasympathetic control of ChBF.

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MATERIALS AND METHODS

Subjects and Approach

The results reported are based on studies in 40 adult Sprague-Dawley rats (220–550 g; Harlan Inc., Indianapolis, IN). All experiments performed were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with National Institutes of Health and institutional guidelines. To identify SSN neurons involved in the control of ChBF, we injected a retrograde transneuronal tracer, the Bartha strain of pseudorabies virus (PRV-Ba), into the choroid of rats.Brains from these injected rats, or from normal animals, were fixed by transcardial perfusion and processed by immunohistochemistry to visualize PRV-Ba. NOS, ChAT, tyrosine hydroxylase (TH) or pairs of the aforementioned (as well as by histochemistry to visualize reduced nicotinamide adenine dinucleotide phosphate diaphorase [NADPHd]), for the purpose of characterizing the location and neurochemistry of neurons in the rat SSN that innervate PPG neurons that target choroid. To characterize the PPG neurons themselves involved in the control of ChBF, a retrograde tracer, FG (FluoroGold, Fluorochrome) was injected into the choroid of additional rats. These animals were fixed by transcardial perfusion. The eyes, the greater petrosal nerves, and the PPG from the injected side were sectioned and FG in the PPG was visualized by fluorescence microscopy. FG sections with FG+ neurons were processed by immunofluorescence to visualize NOS, ChAT, or vasoactive intestinal polypeptide (VIP), to characterize the neurochemistry of PPG neurons innervating the choroid.

FG Injection

Seven rats received bilateral FG injections into the choroid. The rats were anesthetized with an intraperitoneal injection of a ketamine-xylazine mix of 87 and 15 mg/kg, and both the right and left choroid were injected with 1 μL of 2% to 4% FG. The FG was injected into the superior-temporal sector of the choroid. The animals were allowed to survive approximately 90 hours after the injections. All FG injections were performed with a syringe (Hamilton, Reno, NV) connected to a 30-gauge needle. The needle tip punctured the conjunctiva and then the sclera posterior to the ciliary complex before entering the choroidal space. Tracer was slowly injected into the choroid over 3 to 5 minutes. The conjunctival puncture site was carefully monitored through a surgical microscope for efflux of tracer. In all cases, there was minimal or no evident efflux, and any efflux was blotted with a sterile cotton swab. The conjunctival sac was rinsed with sterile normal saline at the conclusion of the injection procedure to prevent further spread of tracer to extraocular tissues. Inadvertent spread of FG from our intrachoroidal injections to the lacrimal glands can be ruled out in all cases, because the main lacrimal gland in rats is extrabulbar, and the secondary lacrimal gland—the infraorbital gland—is located below the eyeball, well removed from the site at which we injected into the choroid.41 Similarly, the meibomian glands of the eyelids were remote from our intrachoroidal penetration site,42 and thus accidental spread of tracer to them could be ruled out as well.

PRV-Ba Injections

Viruses such as PRV-Ba can be uniquely valuable as pathway tracing agents for delineating central circuits, because (unlike a conventional retrograde tracer such as FG) they are transported retrogradely transneuronally (i.e., across synapses) and provide robust labeling in recipient neurons due to virus replication.43 Nonetheless, use of viruses as neuronal tracers is not without potential pitfalls, which include virus-induced neuronal degeneration with longer postinoculation survival times, failure of certain sets of neurons to transport the virus, and variability between cases in the extent of transneuronal labeling. Use of the smallest effective doses of virus and an attenuated strain such as the PRV-Ba can mitigate neuronal injury caused by the virus.44 Use of several animals at each of several postinoculation survival times and comparison with the results obtained with conventional tracers can help overcome case-to-case variability and possible labeling failures.45 The experimental design of the present studies using PRV-Ba, therefore, incorporated these considerations. Twenty-three rats received PRV-Ba injections into the choroid. These rats were anesthetized with an intraperitoneal injection of a ketamine-xylazine mix of 87 and 15 mg/kg, and the right superior-temporal choroid was injected with 1.0 to 2.0 μL of PRV-Ba (3 × 10^7 plaque forming units/mL). The same injection approach was used as for the above described intrachoroidal injections of FG. In 7 of the 23 cases, a strain of PRV-Ba bearing a LacZ construct (which codes for the enzyme β-galactosidase) was used. The animals were allowed to survive between 52 and 88 hours after virus injection. All PRV-Ba injections were performed with a syringe (Hamilton) with a 30-gauge needle. The rats receiving an intrachoroidal injection of PRV-Ba had already received bilateral resections of the superior cervical ganglia (SCG) to prevent retrograde transneuronal labeling along sympathetic circuitry.46 Bilateral resections were performed rather than unilateral because of evidence that the superior cervical ganglion has a contralateral orbital projection.47,48 The SCG lies immediately dorsal to the bifurcation of the common carotid artery. A single ventral midline neck incision allowed access to both the right and left SCG. Careful blunt and sharp dissection was used to localize the common carotid artery and then the cervical portion of the sympathetic trunk. The cervical portion of the sympathetic trunk and SCG were freed from the carotid artery and excised in toto. Because PRV-Ba does not typically demonstrate transganglionic transport through sensory ganglia,49,50 there was no reason to transect the ophthalmic nerve to prevent central transport of PRV through trigeminal circuitry. The absence of PRV-Ba-labeled neuronal perikarya or terminals within the trigeminal nuclear complex in our studies confirmed that there was no transganglionic transport of virus through the trigeminal nerve in our rats. Thus, our bilateral SCG-ectomies in conjunction with the absence of central PRV-Ba transport through the trigeminal nerve served to prevent virus labeling in the central nervous system (CNS) in sympathetic preganglionic neurons, in the hindbrain (trigeminal nerve targets, and (in the case of longer survival times) in the higher order neurons projecting to them.40

Histochemical Studies and Colchicine Treatment

Ten rats were used exclusively in immunohistochemical and/or histochemical studies of the SSN region. None of these rats received ocular or orbital virus injections or had the superior cervical ganglia resected. Two of them, however, were treated with the axonal transport blocker colchicine to enhance visualization of neurotransmitter-related substances, notably NOS, in the SSN neuronal perikarya, as described previously.43 These rats were anesthetized with ketamine (0.66 mL/kg) and xylazine (0.33 mL/kg) and secured in a stereotaxic body. Body temperature was maintained at 38°C, and colchicine (45 μg/1 μL; Sigma-Aldrich, St. Louis, MO) was injected into the fourth ventricle. Coordinates for the injection site were from Paxinos and Watson.44 Four microliters of colchicine was injected with a 10-μL syringe (Hamilton) at a rate of 1 μL every 10 minutes. Animals were allowed to survive for 30 to 36 hours after the colchicine injection. All 10 animals were then processed for histologic analysis.

Histologic Tissue Preparation

Normal rats (n = 8), colchicine-treated rats (n = 2), rats that had received PRV-Ba injections (n = 25), and rats that had received FG injections (n = 7) were anesthetized with an intraperitoneal injection of 0.1 mL/100g of a ketamine-xylazine mixture (87 and 13 mg/kg). In all rats, 0.4 mL of heparinized saline was injected into the heart, and then they were transcardially perfused with 0.9% saline followed by either 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.2–7.4) or paraformaldehyde in 0.1 M lysine-0.1 M sodium periodate in 0.1 M PB (pH 7.2–7.4). Brains were postfixed for 1 to 4 hours at room temperature in the same fixative used for perfusion and then cryoprotected at 4°C for at least 24 hours in a 20% sucrose, 10% glycerol, and 0.02% sodium azide in 0.1 M PB solution. Once the brains were cryoprotected, they were frozen with dry ice and sectioned on a
sliding microtome at 40 μm. Sections were collected as six parallel series, and one series was mounted immediately during sectioning on gelatin-coated slides, allowed to dry, and stained with cresyl violet. The cresyl violet series for each case made it possible to identify unambiguously the rostral-to-caudal order of all sections from that case. The remaining free-floating sections were stored at 4°C in a 0.02% sodium azide and 0.02% imidazole in 0.1 M PB solution until they were labeled for PRV-Ba, β-galactosidase (β-gal), ChAT, NOS, and/or TH by immunohistochemistry, or for NADPHd by histochemistry.

**Peroxidase-Antiperoxidase Single-Labeling Immunohistochemistry**

Immunohistochemical single labeling was performed as described previously. The primary antibodies used were goat anti-PRV-Ba diluted 1:15,000 to 1:50,000, rabbit anti-β-gal diluted 1:50,000 (Rockland Immunochemicals, Gilbertsville, PA), rabbit anti-chicken ChAT diluted 1:1,000 (generously provided by M. Epstein and Carl D. Johnson, University of Wisconsin), goat anti-ChAT diluted 1:250 (Chemicon International, Inc., Temecula, CA), rabbit anti-NOS diluted 1:1,000 to 1:2,000 (Alexis Biochemicals, San Diego, CA), rabbit anti-TH diluted 1:400 to 1:1,000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and mouse anti-TH diluted 1:1,000 (DiaSorin Inc., Stillwater, MN). The diluent for all antisera or antibodies was 0.1 M phosphate buffer, 0.3% Triton X-100, and 0.01% sodium azide solution (PB/Tx/Az) + 5% normal horse serum. The anti-PRV-Ba or anti-β-gal was used to detect transneuronal retrograde labeling with the virus. The anti-ChAT was used to identify PPG preganglionic neurons in the SSN, the anti-NOS was used to determine whether SSN neurons contain this enzyme (which produces nitric oxide), and the anti-TH was used to identify A5 neurons. The specificity of these primary antisera has been demonstrated previously. For the immunolabeling studies of brain, free-floating sections were rinsed and pretreated in 1% NaOH with 0.5% H2O2 in 0.1 M PB for 15 minutes followed by 1% nonfat dry milk for 15 to 30 minutes. The NaOH enhances antigenicity (especially for ChAT), the H2O2 inactivates endogenous peroxidases, and the 1% nonfat dry milk reduces nonspecific background immunostaining. The sections were incubated in primary antisera for 48 to 72 hours at 4°C in plastic 5 mL vials. Sections were then rinsed in 0.1 M PB and incubated for 1 hour at room temperature in a bridging secondary antisera directed against IgG of the host in which the primary antibody was raised (1:50, secondary antibodies; Jackson Immunoresearch Laboratories, Inc., West Grove, PA). The sections were then rinsed in 0.1 M PB and incubated for 1 hour at room temperature in rabbit, goat, or mouse peroxidase-antiperoxidase (PAP, all at 1:200, rabbit and mouse PAP from Sternberger Monoclonals, Lutherville, MD, and goat PAP from Jackson Immunoresearch Laboratories). The sections were then rinsed in 0.1 M PB (pH 7.2–7.4), and the labeling visualized using diaminobenzidine tetrahydrochloride (DAB) in a 0.2 M sodium cacodylate buffer (pH 7.2–7.4), as in our previous work. The sections were subsequently rinsed, mounted on gelatin-coated slides, air dried, dehydrated and coverslipped with nonaqueous mounting medium (Permount; Fisher Scientific, Pittsburgh, PA). The sections were examined with a microscope (BH2; Olympus, Lake Success, NY) with standard transmitted light or differential interference contrast optics. Camera lucida drawings were made of sections through the levels of SSN and A5 in representative cases to characterize the location and extent of the perikaryal labeling for PRV-Ba, ChAT, TH, and/or NOS, as well as for NADPHd (NADPHd labeling methods described later).

**Peroxidase-Antiperoxidase: Two-color DAB Double-Label Immunohistochemistry**

Immunohistochemical two-color DAB double labeling was performed to map the relative locations of the TH+ noradrenergic neurons of the A5 cell group and the cholinergic neurons of SSN in the same sections. This labeling method was performed as described previously. In brief, tissue from normal rats was incubated in a primary antibody cocktail of rabbit anti-chicken ChAT (1:1000) and mouse anti-TH (1:1000) diluted with PB/Tx/Az+5% normal horse serum for 48 to 72 hours at 4°C. Tissue was rinsed in 0.1 M PB and then incubated at room temperature for 1 hour in a donkey anti-mouse secondary antibody (1:50; Jackson Immunoresearch Laboratories). Sections were then rinsed in 0.1 M PB and incubated in mouse PAP for 1 hour at room temperature (1:200; Sternberger Monoclonals). The tissue was next rinsed in 0.1 M PB (pH 7.2–7.4), and the TH labeling visualized using DAB in 0.1 M PB with 0.04% nickel ammonium sulfate (pH 7.2–7.4), resulting in brown-black TH+ cells, as described in our prior studies. The tissue was then rinsed extensively, incubated in donkey anti-rabbit (1:50; Jackson Immunoresearch Laboratories) for 1 hour at room temperature, rinsed three times for five minutes each in 0.1 M PB, and incubated in rabbit PAP (1:200; Sternberger Monoclonals) for 1 hour at room temperature. The sections were subsequently rinsed in 0.1 M PB (pH 7.2–7.4), and the ChAT labeling visualized using DAB in a 0.2 M sodium cacodylate buffer (pH 7.2–7.4), resulting in brown DAB labeling of ChAT+ neurons, as described previously. The sections were subsequently rinsed in 0.1 M PB, mounted on gelatin-coated slides, air-dried, dehydrated, and coverslipped in nonaqueous medium (Permout; Fisher Scientific). The sections were then examined with a microscope (BH2; Olympus) with standard transmitted light or differential interference contrast optics. Camera lucida drawings were made through the levels of SSN/A5 of labeled neurons in sections double-labeled for ChAT and TH by the two-color DAB immunolabeling method.

**Immunofluorescence Double Labeling**

Immunofluorescence double labeling was performed to determine whether PRV-Ba-labeled neurons in the SSN were cholinergic (and thus preganglionic) and whether the PRV-Ba–labeled neurons of SSN also contain NOS. Animals with intrachoroidal injection of PRV-Ba were used for this analysis. Immunofluorescence double labeling was also used to determine whether the NOS-immunolabeled neurons of SSN contain ChAT, as a means of further assessing whether the NOS+ neurons might be preganglionic. Colchicine-treated sections and sections with intrachoroidal injection of PRV-Ba were used for this analysis. The immunofluorescence double-labeling method was performed as described previously. In brief, tissue was pretreated by incubation in 1% NaOH with 0.5% H2O2 in 0.1 M PB for 15 minutes followed by 1% nonfat dry milk for 15 to 30 minutes. The NaOH enhances antigenicity (especially for ChAT), the H2O2 inactivates endogenous peroxidases, and the 1% nonfat dry milk reduces nonspecific background immunostaining. The sections were incubated in primary antisera for 48 to 72 hours at 4°C in plastic 5 mL vials. Sections were then rinsed in 0.1 M PB and incubated for 1 hour at room temperature. The immuno

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Inc.). The sections were then rinsed, mounted on gelatin-coated slides, air dried, and covered slipped with several drops of glycerol carbonate buffer (pH 10.5) or several drops of anti-fade coverslip-mounting medium (ProLong; Molecular Probes, Inc.).

In some PRV-Ba cases, the brains were sectioned translucently at 20 μm using a cryostat (Leica, Deerfield, IL), and sections were collected on slides. For immunofluorescence double labeling of this tissue, the slide-mounted sections were circled with a hydrophobic slide-marking pen (PAP pen; Electron Microscopy Sciences, Fort Washington, PA) and dried on a slide warmer. Sections were then rinsed in 0.02 M phosphate-buffered saline (PBS) with 0.1% sodium azide. Endogenous peroxidases were inactivated by a 20-minute incubation in 10% methanol and 3% hydrogen peroxide in PBS. Sections were next rinsed in PBS and pretreated with 2% nonfat milk and 0.3% Triton X-100 for 1 hour at room temperature and incubated overnight in droplets of a primary antibody cocktail containing goat anti-PRV-Ba (diluted 1:100,000 with PBS plus 0.1% Triton + 5% normal donkey serum) and either rabbit anti-nNOS (1:500; Santa Cruz Biotechnology, Inc.), rabbit anti-nNOS (1:100; Alexis Biochemicals), or rabbit anti-chicken ChAT (1:1000). Secondary antibodies—namely, donkey anti-goat IgG conjugated to Cy2 and donkey anti-rabbit IgG conjugated to Cy5—were used at 1:250 dilutions. Tissue was incubated in secondary antisera for 4 hours, rinsed, dehydrated, cleared, and coverslipped with 1,4-diazethyl-2-phenylxanithine (DPX; Sigma-Aldrich).

The sections double-labeled by immunofluorescence were viewed with an epi-illumination fluorescence microscope (Olympus), as described previously, or with a confocal laser scanning microscope (CLSM; MRC-1000; Bio-Rad, Richmond, CA), as described previously. For CLSM examination, a 20× objective (Euplan; Olympus) was used, and sections were scanned with a krypton-argon laser, with specific excitation wavelength settings for TRITC (568 nm) and dichlorotriazinylamino fluorescein (DTAF; 488 nm for green fluorescence visualization), or for far red (647 nm for Cy5) and DTAF (for Cy2). Images of the labeling for individual fluorophores were captured sequentially for examination and analysis.

Immunofluorescence Combined with FG Retrograde Labeling

In animals that had intrachoroidal injections of FG, the injected eyes and attached nerves were removed from the orbit and sectioned on a cryostat (Hacker-Bright Instruments, Fairfield, NJ) at 20 μm in the horizontal plane. Sections were then collected on slides (Superfrost Plus; Fisher Scientific). The eyes were similarly sectioned to assess the FG injection sites. The sections were then viewed with an epi-illumination fluorescence microscope (Olympus), as described previously. In some cases in which FG labeling had been identified in PPG neurons, sections through the PPG with FG+ neurons were processed for immunofluorescence. The sections were incubated overnight in a humid chamber at 4°C in either rabbit anti-nNOS (1:100; Alexis Biochemicals), rabbit anti-VIP (1:100; Dianorin), or goat anti-ChAT (1:250; Chemicon International, Inc.). After incubation in the primary antibodies, the sections were rinsed in 0.1 M PB and incubated for 1 to 2 hours at room temperature in TRITC-conjugated donkey anti-rabbit IgG or in the case of the tissue incubated in either anti-nNOS or anti-VIP (1:100; Jackson ImmunoResearch Laboratories) or in green fluorophore (Alexa 488)-conjugated donkey anti-goat IgG in the case of tissue incubated in anti-ChAT (1:500; Molecular Probes, Inc.).

NADPHd Histochemistry

A standard NADPHd histochemical procedure was used to localize NADPHd activity in eye tissue sectioned in the frontal plane and in free-floating sections through the pons of rats. The eyes were removed from the orbit and sectioned on a cryostat (Hacker-Bright) at 20 μm and the sections collected on slides (Superfrost Plus; Fisher Scientific) and stored at −20°C until histochemically processed for NADPHd. For NADPHd histochemistry, free-floating brain sections and slide-mounted eye sections were allowed to warm to room temperature, rinsed in 0.1 M PB three times for 5 minutes each, and placed in the incubation medium containing 0.1 M Tris-HCl (pH 8.0), 1 mM β-NADPH, 0.2 mM nitroblue tetrazolium (NBT) and 0.2% Triton X-100. Both free-floating and slide-mounted sections were incubated for 4 to 20 minutes at 37°C. Progress of the reaction was assessed by microscopy examination of the tissue. Free-floating sections were mounted on gelatin-coated slides when labeling was complete. All sections were dried, dehydrated, cleared, coverslipped, and examined with a transmitted-light microscope (BH2: Olympus). To determine the specificity of NADPHd staining, sections of brain tissue through the SSN were stained with a solution that contained all the ingredients for the NADPHd histochemical procedure, except the NADPH itself. Adherent sections from the same animal were stained in parallel for NADPHd with a solution containing all ingredients. This control procedure yielded no SSN labeling.

RESULTS

Labeling in PPG after Intrachoroidal FG Injection

Labeling was observed in the ipsilateral PPG for five of the eyes that received an intrachoroidal FG injection. The FG-labeled neurons in each case were found within the proximal part of the ipsilateral PPG and scattered along the greater petrosal nerve itself, in which the preganglionic fibers to PPG travel. The FG labeling at the intrachoroidal injection sites showed that these intrachoroidal injections were largely restricted to choroid and typically involved no more than approximately 25% to 50% of the choroidal circumference (Fig. 1). Whereas slight leakage of FG into the musculature surrounding the temporal side of the eye was observed in all five of the eyes yielding PPG neuron labeling, similar extrachoroidal labeling was observed in six eyes in which the attempted intrachoroidal FG injections failed to yield PPG labeling and showed no evident labeling of the choroid at the injection site. Similarly, in two of the cases with neuronal labeling in the PPG, FG spread to a small laterally directed portion of the hardierian gland (constituting no more than 1%-2% of its volume) was observed. The pattern of PPG labeling was, however, the same as in the successful intrachoroidal cases without spread to the hardierian gland. Moreover, one of the failed choroidal injections showed slight spread to a small temporal portion of the hardierian gland, but yielded no PPG labeling. Thus, it seems unlikely that FG spread outside the choroid contributed significantly to the PPG labeling. Similarly, whereas some FG spread into temporal retina was evident in four of the cases with PPG labeling, no anterograde labeling was observed in the optic nerve, tract, or central retinal target areas with retinal spread, suggesting that FG had not leaked significantly into the vitreous from the choroidal injection site. Moreover, similar leakage into the retina was evident in the failed intrachoroidal injected eyes without PPG retrograde labeling, thus further indicating that such leakage was not the basis of the retrograde PPG labeling in the four eyes with effective intrachoroidal injections. Based on the maximum number of retrogradely labeled neurons observed in the PPG and greater petrosal nerve after intrachoroidal FG injection, our results suggest that at least 200 to 400 PPG neurons innervate the choroid in the rat. Our immunolabeling studies on the FG-labeled sections through PPG confirmed that PPG neurons in general and those innervating choroid in particular contain NOS (Figs. 2A, 2D), VIP (Figs. 2B, 2E), and ChAT (Figs. 2C, 2F) in rats. Moreover, we confirmed that the choroid is rich in nerve fibers containing the NOS marker NADPHd (Fig. 1C), as reported previously by

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Both the SSN and A5 are parts of the central motor nucleus and the SSN constitute the A5 noradrenergic system. 

Neurochemistry of SSN Neurons: ChAT versus TH

The distributions of ChAT+ neurons and TH+ neurons in and around the SSN were somewhat overlapping but nonetheless distinct (Fig. 3). The ChAT+ neurons in this region included the somatomotor neurons of the facial nucleus as well as neurons of the SSN. The somatomotor facial nucleus neurons are found within a circumscribed oval-shaped field that begins just caudal to the superior olive and the exit of the facial nerve itself, and it extends to just rostral to the level of the inferior olive. The somatomotor neurons are relatively large (20–30 μm) and labeled intensely for ChAT. The neurons of the SSN, by contrast, were smaller (10–15 μm) and more lightly labeled for ChAT, and they occupied a field at the dorsolateral margin of the somatomotor facial nucleus, which extended from the lateral aspect of the somatomotor facial nucleus toward the periventricular gray (Figs. 3, 4). A small rostral part of this field was observed to extend over the dorsolateral margin of the caudalmost part of the superior olive, flanking the medial aspect of the facial nerve as it exits the brain stem.

The TH+ neurons within the vicinity of the facial somatomotor nucleus and the SSN constitute the A5 noradrenergic cell group.29,59,60 Both the SSN and A5 are parts of central circuitry controlling the peripheral vasculature. For this reason and because of their proximity, it was important to distinguish the two neuronal populations. The TH+ perikarya were smaller (15–20 μm) and more spindle-shaped than the cholinergic neurons of the facial somatomotor nucleus, but slightly larger than the cholinergic neurons of the SSN. In addition, the TH+ neurons of A5 were most abundant at the level of the superior olive and facial nerve root (Fig. 3). Although the rostral part of the cholinergic SSN neuron field overlapped the caudal part of the TH+ neuron field, at this point of overlap the bulk of the TH+ neurons were located more medially and ventrally than the bulk of the SSN neurons. At more caudal levels, the few TH+ neurons observed tended to be ventral to the SSN neurons. Thus, although the TH+ neurons of A5 and the cholinergic ChAT+ neurons of the SSN overlap slightly in their distributions, the two cell types occupy distinct territories and differ in their size and shape. This was evident in both adjacent sections that had been single-labeled for TH and ChAT, respectively, and in individual sections in which two-color DAB double labeling (nickel intensified brown-black DAB for TH and brown DAB for ChAT) had been used to distinguish the two neuron types (Fig. 5).

Labeling in the SSN after Intrachoroidal PRV-Ba Injection

Retrograde transneuronal PRV-Ba labeling was observed in the SSN after intrachoroidal injection of the virus, after various postinjection survival periods (52–88 hours). With longer survival times, more neurons in and around the SSN were labeled, whereas with short survival times the labeling was more confined to a limited area within the SSN. For example, in six successful cases with less than 70 hours’ survival, an average of 16.8 PRV-Ba+ neurons was observed in the SSN at the 10.5 mm level (Fig. 3), and these were confined to a small cluster at the rostral ventromedial aspect of the SSN. By contrast, in five successful cases with more than 70 hours’ survival, an average of 33.8 PRV-Ba+ neurons was observed in the SSN at the 10.5-mm level (Fig. 3), and these were more widely distributed in the SSN. In addition, with survival times beyond 70 hours, perikaryal labeling for PRV-Ba became evident within the A5 region. Given its late appearance, the PRV-Ba-labeled neurons within the A5 after intrachoroidal injection of the virus may be higher-order labeling from the SSN cluster.

The cluster of PRV-Ba-labeled neurons observed in the rostral ventromedial SSN with short survival times (<70 hours) appears attributable to transneuronal retrograde transport from the choroid through the PPG neurons innervating the choroid. For example, although there was evidence of some variable spread of the PRV-Ba from the injection site to nearby periorbital facial muscles (such as the orbicularis oculi) or into the extraocular muscles, as evidenced by labeling in the somatomotor neurons of the facial nucleus or the nuclei innervating the extraocular muscles (oculomotor, trochlear, and abducens), the size and location of the PRV-Ba-labeled neuronal cluster in the SSN was invariant at short survival times, regardless of the occurrence of spread to any of this somatic musculature. Moreover, there is no known route by which SSN labeling could arise transneuronally after retrograde labeling of others, which are known to arise from the PPG and to contain VIP.17,18

A series of images of fluorescent labeling showing that neurons of the PPG that project to the choroid contain NOS, VIP, and ChAT. (A) Neurons of the PPG that had been retrogradely labeled by intrachoroidal FG injection into the temporal sector of the choroid, whereas (D) shows NOS+ immunolabeling in this same field. NOS is present in most of the FG-labeled PPG neurons. (A, D, arrows) Some of the FG-labeled neurons that are also NOS+. (B) Neurons of the PPG that were retrogradely labeled by the same intrachoroidal injection of FG. (E) VIP+ immunolabeling in this same field. VIP is also present in most of the FG-labeled PPG neurons. (B, E, arrows) Some of the FG-labeled neurons that were also VIP+. (C) Neurons of the PPG that had been retrogradely labeled by the same intrachoroidal injection of FG as in (A) and (B). (F) ChAT+ immunolabeling in the same field. ChAT was present in most of the FG-labeled PPG neurons. (C, F, arrows) Some of the FG-labeled neurons that are also ChAT+. All images are at the same magnification.
these somatomotor neuron pools.\textsuperscript{27} Note that these cases were also not confounded by leakage of virus into the vitreous, because this would have resulted in labeling of neurons in the nucleus of Edinger-Westphal\textsuperscript{61} and several hypothalamic and pretectal retinorecipient groups, and no such labeling was observed. Thus, the prechoroidal neurons of the SSN (i.e., those innervating PPG neurons targeting ipsilateral temporal choroid) appear to be restricted to a ventromedial and somewhat rostral part of the SSN field, as the field is defined by ChAT immunolabeling (Figs. 3, 4, 5).

**Neurochemistry of PRV-Ba–labeled SSN Prechoroidal Neurons: ChAT**

Double labeling for ChAT and PRV-Ba was performed to determine whether the PRV-Ba\textsuperscript{+} neurons within the SSN cluster observed in the single-labeling studies of virus localization (P10.5 level in Fig. 3) in fact were cholinergic (Table 1). Examination of short survival cases (<70 hours postinjection survival) revealed that nearly all of the PRV-Ba\textsuperscript{+} neurons in the small cluster of PRV-Ba\textsuperscript{+} neurons in the SSN contained ChAT.

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**FIGURE 3.** Camera lucida drawings of the right side of the brain in a rostral (top row)-to-caudal (bottom row) series of transverse sections showing the distribution within the rat SSN region of cholinergic neurons immunolabeled for ChAT (illustrated by dots in the drawings in the left column), noradrenergic neurons immunolabeled for TH (illustrated by triangles in the middle column), and neurons transneuronally retrogradely labeled from the choroid with PRV-Ba (right column). Numbers at right: rostrocaudal level of each section in the respective row in stereotaxic coordinates,\textsuperscript{44} with the rostrocaudal position specified in terms of the distance behind (P for posterior) the skull suture Bregma. The ChAT and TH immunolabeled sections are from one normal rat, and the ChAT and TH labeling were mapped from the same individual sections that had been double-labeled using the two-color DAB method (brown DAB for ChAT and brown-black DAB for TH). The PRV-Ba–labeled sections are from a different rat, which received bilateral resections of the superior cervical ganglia and survived 63 hours after right intrachoroidal PRV-Ba injection. The first two columns show that the TH\textsuperscript{+} neurons of the A5 cell group and the cholinergic neurons of SSN had only slight overlap, with the SSN neurons largely lying caudomedial to the A5. The PRV-Ba\textsuperscript{+} neurons transneuronally retrogradely labeled from the choroid were restricted in their location to the rostral and ventromedial part of the SSN (at P10.5). g7, genu of the facial nerve; M5, trigeminal motor nucleus; mlf, medial longitudinal fasciculus; n6, abducens nucleus; n7, facial motor nucleus; N7, facial nerve root; nTTd, nucleus of the descending trigeminal tract; Pr5, principal trigeminal nucleus; py, pyramidal tract; SO, superior olivary nucleus; SSN, superior salivatory nucleus; TTd, descending trigeminal tract; A5, adrenergic cell group 5.
shown in the drawings in Figure 3. The PRV-Ba
same animal, which survived 63 hours after intrachoroidal PRV-Ba, as
yields a greater number of SSN neurons labeled for virus, the
cluster were virus-labeled. With longer survival times, which
level. Note that this region contained numerous
facial nucleus, in the more ventromedial part of the SSN
were restricted to a region at the dorsolateral edge of the somatomotor
nucleus (n7), showing the location of the neurons within the SSN that
appear to be the preganglionic neurons for the choroidal neurons of
PPG. (A, C) Sections from a normal rat at the P10.5 level (Fig. 3)
immunolabeled for ChAT. This pair of images shows that SSN and n7
neurons were ChAT+, and SSN was located at the dorsolateral edge of
n7. (B, D) PRV-Ba+ neurons in the SSN. These images are from the
same animal, which survived 63 hours after intrachoroidal PRV-Ba, as
shown in the drawings in Figure 3. The PRV-Ba+ prechoroidal neurons
were restricted to a region at the dorsolateral edge of the somatomotor
facial nucleus, in the more ventromedial part of the SSN field at this
level. Note that this region contained numerous fiber bundles (pale, round areas) that coursed longitudinally through the brain stem, with
the dendrites of SSN neurons surrounding these fiber bundles. All four
images are of the right side of the brain, with medial to the left and
dorsal toward the top. (A, B) Same magnification; (C, D) same magnification.

FIGURE 4. A series of images at two different magnifications of transverse sections of the SSN at the rostral level of the facial somatomotor
nerve bundles. All four cases with the shortest survival times examined by double
injection did not include all SSN neurons, because in the four
cases with the shortest survival times examined by double
labeling for ChAT and PRV-Ba (which provide the most reliable
delineation of the prechoroidal neurons of the SSN), only
27.5% of the ChAT+ neurons within the SSN at the level of the
cluster were virus-labeled. With longer survival times, which
yields a greater number of SSN neurons labeled for virus, the

(Fig. 5). For example, counts of 91 PRV-Ba+ neurons in the SSN
cluster in four cases with short survival (<70 hours) revealed
that 94.2% of these PRV-Ba+ contained ChAT. The observation
that the PRV-Ba+ neurons in the small cluster within the SSN
(at least at short survival times) are typically cholinergic supports
the view that they are parasympathetic preganglionic
neurons that project to the PPG neurons that innervate the
choroid. By contrast, within the A5 field, PRV-Ba+ neurons rarely were ChAT+, supporting the view that virus-labeled neurons within this area are not SSN neurons.

The PRV-Ba+ neurons in the SSN labeled by intrachoroidal injection did not include all SSN neurons, because in the four
cases with the shortest survival times examined by double
labeling for ChAT and PRV-Ba (which provide the most reliable
delineation of the prechoroidal neurons of the SSN), only 27.5% of the ChAT+ neurons within the SSN at the level of the
cluster were virus-labeled. With longer survival times, which
yields a greater number of SSN neurons labeled for virus, the

FIGURE 5. CLSM images of a single field of view of the SSN from tissue
double labeled with immunofluorescence for PRV-Ba (A) and ChAT (B), from an animal that underwent bilateral resection of the superior
cervical ganglia and survived 65 hours after unilateral virus injection
into the choroid. Arrows: neurons within the SSN that were double
labeled for PRV-Ba and ChAT. Most of the PRV-Ba+ neurons shown were ChAT+, but not all ChAT+ neurons were PRV-Ba+. These results indicate that the PRV-Ba+ neurons within the SSN labeled transneuronally from the choroid were cholinergic preganglionic neurons, and they represented only a subset of SSN neurons. The field shown is of the right side of the brain, with medial to the left and dorsal toward the top. Both images are at the same magnification. The ChAT labeling was visualized with a secondary antibody conjugated to CY5 and pseudocolored red for ease of visualization with an
image-analysis program (Photoshop; Adobe Systems, Mountain
View, CA).

<table>
<thead>
<tr>
<th>Localization of ChAT or NOS in PRV-Ba+ SSN Neurons</th>
<th>Less Than 70-h Survival</th>
<th>More Than 70-h Survival</th>
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<tbody>
<tr>
<td>94.2% of PRV-Ba+ neurons contain ChAT (n = 4)</td>
<td>76.7% of PRV-Ba+ neurons contain ChAT (n = 3)</td>
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<tr>
<td>27.5% of ChAT+ neurons are PRV-Ba+ (n = 2)</td>
<td>30.5% of ChAT+ neurons are PRV-Ba+ (n = 2)</td>
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<tr>
<td>28.9% of PRV-Ba+ neurons contain NOS (n = 3)</td>
<td>40.0% of PRV-Ba+ neurons contain NOS (n = 1)</td>
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<tr>
<td>35.4% of NOS+ neurons are PRV-Ba+ (n = 3)</td>
<td>40.0% of NOS+ neurons are PRV-Ba+ (n = 1)</td>
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<tr>
<th>Co-localization of ChAT and NOS in SSN Neurons</th>
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<tr>
<td>36.4% of ChAT+ neurons contain NOS (n = 2)</td>
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<tr>
<td>79.1% of NOS+ neurons contain ChAT (n = 2)</td>
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Neurochemistry of SSN Neurons: NOS and NADPHd

Single-label NADPHd histochemistry and single-label NOS immunohistochemistry were used to assess the possibility that SSN neurons that project to the choroid contain NOS and therefore perhaps use NO as a transmitter. The SSN in normal and colchicine-treated rats contained a small but distinct population of neurons in both the tissue labeled by NADPHd histochemistry and that labeled by NOS immunolabeling (Fig. 6). The location of this cluster matched the PRV-Ba+ cluster observed within the SSN after intrachoroidal injection of the virus (Fig. 3; P10.5 level), and the labeling of perikarya for NOS was more prominent with colchicine-treatment. The NADPHd+/NOS+ neurons, like the PRV-Ba+ neurons from intrachoroidally injected animals, included only a small, spatially restricted subset of the SSN neurons defined by ChAT immunolabeling. Distinct NADPHd+ and NOS+ perikarya were not observed in the facial somatomotor nucleus in either normal or in colchicine-treated rats. Counts of 52 NOS-labeled neurons in two cases double-labeled by immunofluorescence for ChAT and NOS (Table 1) revealed that 79.1% of the NOS+ perikarya within the SSN were ChAT+ (Fig. 7), thereby suggesting most NOS+ neurons to be parasympathetic preganglionic SSN neurons. Because of the limited extent of the NOS+ field, however, only approximately 36.4% of the ChAT+ SSN neurons at the level of the cluster were NOS+.

Neurochemistry of PRV-Ba-Labeled SSN Prechoroidal Neurons: NOS

Immunofluorescence double labeling for NOS and PRV-Ba in sections from three animals with bilateral resection of the superior cervical ganglia that survived less than 70 hours after virus injection was used to determine whether the NOS+ neurons in the SSN were parasympathetic preganglionic neurons that project to prechoroidal PPG neurons (Table 1). The double labeling for NOS and PRV-Ba (Fig. 8) in these animals showed that 28.9% of the PRV-Ba+ were NOS+. For all NOS+ neurons observed in the SSN cluster, 33.4% were observed to be PRV-Ba+. These results confirm that many NOS+ neurons of the SSN are prechoroidal parasympathetic neurons. It is possible that those NOS+ neurons not labeled for PRV-Ba either are not prechoroidal SSN neurons or innervate PPG neurons projecting to parts of the choroid not injected with virus in this study (for example, the inferior–nasal choroid).

Discussion

The SSN contains preganglionic neurons projecting to two different cranial parasympathetic ganglia: the PPG through the greater petrosal nerve and the submandibular ganglion through the chorda tympani.25–29,35 The PPG and submandibular ganglion innervate different targets, with the PPG sending projections to orbital, nasal, and brain targets, and the submandibular ganglion innervating the submandibular and sublingual salivary glands. Consistent with the difference in peripheral targets, a number of neuroanatomical studies have shown that the preganglionic neurons innervating the PPG are segregated from those projecting to the submandibular ganglion, with the former located ventrally within the SSN and the latter dorsally.25–29,35 The PPG itself, however, innervates diverse cranial targets, including the lacrimal gland, meibomian glands, orbital conjunctiva, choroidal blood vessels, cerebral vasculature, and nasal and palatal mucosa.34–39 Prior studies in which PRV-Ba was injected into different peripheral PPG targets have suggested that the SSN populations responsible for innervation of the different PPG targets may differ in their location within the SSN. For example, SSN neurons controlling lacrimal gland may be centered slightly caudal to those responsible for meibomian gland control.29,40 Nonetheless, the degree to which the various functionally distinct types of SSN preganglionic neurons might be spatially segregated has not been fully determined. Similarly, although there is some evidence for segregation of function among PPG neurons innervating different targets,25–40,62 their spatial segregation is not yet fully resolved. The present results suggest that preganglionic neurons controlling PPG choroidal neurons in rat reside within a specific region of SSN, and that NOS within this region defines the location of and is a marker for prechoroidal SSN neurons. The neurons preganglionic to PPG neurons that innervate the choroid appear to be localized to a specific subregion of the SSN, because they are slightly more rostral or medial in location than those observed after injection of PRV-Ba into other peripheral

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**FIGURE 6.** Images and drawings showing that nitrergic (i.e., NADPHd+/NOS+) neurons are located within the ventromedial part of the superior salivatory nucleus (SSN). (A, C) Transverse section from a normal rat at the level of the facial somatomotor nucleus (n7) and SSN, labeled histochemically for NADPHd-diaphorase to reveal nitrergic neurons. A small number of neurons within the confines of SSN label for NADPHd, some of which are indicated by arrows in (A). This population was located within ventromedial SSN at the rostral dorsolateral edge of n7. (B, D) Transverse section from a colchicine-treated rat at the level of n7 and SSN, immunolabeled for NOS. Arrows: some of the neurons within the confines of SSN labeled for NOS. This population of NOS+ neurons was located within the rostral, ventromedial part of SSN at the dorsolateral edge of n7, and they appeared to be the same population that labeled for NADPHd. The fields shown in (A) and (B) are on the right side of the brain, with medial to the left and dorsal toward the top. Abbreviations are as in Figure 3. (A, B) Same magnification; (C, D) drawings of the right side of the sections from which images (A) and (B), respectively, were taken.
PPG targets, such as the meibomian glands and the lacrimal gland. The possibility remains, nonetheless, that the same population of preganglionic SSN neurons may control more than one of the target structures of the PPG. For example, it may be the case that, given the similar metabolic needs of the brain and eye, the same SSN neurons mediate vasodilation of brain and choroidal vessels. Studies using two or more different transneuronal tracers are needed to determine whether the ventral SSN is divided into spatially distinct subpopulations of preganglionic neurons for each PPG target.

Although our studies thus cannot establish whether populations of PPG neurons that innervate the choroid in rats and the SSN neurons that innervate those PPG neurons control only ChBF, they establish a number of points regarding these neuronal populations. First, these studies have directly shown that the PPG neurons that innervate the choroid contain NOS, VIP, and ChAT. This is consistent with prior evidence that VIP and NOS fibers arising from the PPG innervate the choroid. Published data suggest that the same PPG neurons that innervate the choroid or additional PPG neurons may innervate orbital vessels that feed into the choroid and thereby exert a further influence on ChBF. The identification of prechoroidal neurons in the SSN is consistent with prior studies showing that facial nerve or SSN activation yields choroidal vasodilation, which appears to be mediated by NOS and VIP. Our

![Figure 7](https://iovs.arvojournals.org/pdfaccess.axd?url=/data/journals/iovs/932921/) Figure 7. CLSM images of a single field of view of the SSN from tissue double labeled by immunofluorescence for NOS (A) and ChAT (B), from a normal rat. Most of the NOS\(^+\) neurons also contained ChAT, suggesting they were cholinergic preganglionic neurons. Arrows: examples of these double-labeled neurons. Note that there were many more ChAT\(^+\) neurons than there were NOS neurons, suggesting that NOS\(^+\) neurons are a subset of cholinergic preganglionic SSN neurons. The field shown is of the right side of the brain, with medial to the left and dorsal toward the top. Both images are at the same magnification.

![Figure 8](https://iovs.arvojournals.org/pdfaccess.axd?url=/data/journals/iovs/932921/) Figure 8. CLSM images of a single field of view of the SSN from tissue double labeled by immunofluorescence for PRV-Ba (A) and NOS (B), from an animal that received bilateral resection of the superior cervical ganglia and survived for 65 hours after unilateral intrachoroidal virus injection. Many of the PRV-Ba\(^+\) neurons in the SSN were labeled for NOS (arrows) and many of the NOS\(^+\) neurons were labeled for PRV-Ba (arrows). These results indicate that NOS within the SSN is found in preganglionic neurons that innervate PPG neurons projecting to the choroid. The NOS labeling was visualized with a secondary antibody conjugated to CY5 and pseudocolored red with an image-analysis program (Photoshop; Adobe Systems, Mountain View, CA). The field shown is of the right side of the brain, with medial to the left and dorsal toward the top. Both images are at the same magnification.
findings on the localization of ChAT in PPG neurons that innervate the choroid also suggest a possible role of cholinergic PPG mechanisms in control of ChBF in rats, although the physiological evidence for such a mechanism is equivocal. 65,66 It is important to note that, in addition to the parasympathetic influence on the choroid mediated by the PPG, the choroid also is regulated by sensory fibers from the ophthalmic nerve and sympathetic fibers from the superior cervical ganglia. 4–5

In any event, the location of the prechoroidal SSN neurons revealed by the present study can be of use in determining the central circuitry that governs control of ChBF. Prior studies have shown that the paraventricular nucleus (PVN) of the hypothalamus and the nucleus of the solitary tract (NTS) are major sources of input to the SSN. 27,67,68 The PVN region of the diencephalon is known to be responsive to systemic blood pressure (BP) and to exert a vasodilatory influence on cerebral blood flow. 72,73 It is unknown, however, whether the NTS or the PVN region has an impact on ChBF, and it is not established that the NTS and the PVN projections to the SSN include among their targets the prechoroidal neurons of the SSN. The fact that we observed higher-order labeling in both the PVN and the NTS after intrachoroidal injection of PRV-Ba (Reiner A, LeDoux MS, Cuthbertson S, unpublished observations, 2000) is consistent with the possibility that these sites innervate prechoroidal neurons of the SSN. Because the present study shows that the prechoroidal neurons of the SSN can be identified by PRV-Ba transneuronal retrograde labeling or by NOS immunolabeling, it should be possible to combine this means of detecting prechoroidal SSN neurons with anterograde labeling from the PVN or the NTS to confirm that the PVN and/or the NTS innervate prechoroidal SSN neurons. This then would provide insight into the higher-order brain regions involved in regulation of ChBF through the PPG.

Given the apparent role of the PVN and the NTS in mediating vascular responses to fluctuations in systemic BP, if the PVN and the NTS in fact project to the prechoroidal neurons of the SSN, it would suggest the possibility that these inputs regulate ChBF, in part, as a function of systemic BP. Alternatively or in addition, the PVN input to the prechoroidal SSN may be involved in light-mediated control of ChBF. Light-mediated and flicker-mediated ChBF regulation, which may be adaptive responses to the thermal or metabolic demands placed by such stimuli on the retina, 65,74.75 have been demonstrated in pigeons, 76 chickens, 77 monkeys, and humans, 53,78,79 and the suprachiasmatic nucleus (which receives retinal input) is known to project to the PVN. 80 It may be, therefore, that prechoroidal neurons of the SSN receive central inputs by which facial nucleus parasympathetic outflow to the choroid is involved in light-mediated and/or systemic BP-mediated control of ChBF.

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References

24. Suzuki N, Hardebo JE, Kalstrom J, Owmman C. Neuropeptide Y co-exists with vasoactive intestinal polypeptide and acetylcholine in parasympathetic cerebrovascular nerves originating in the sphe-
nopalatine, otic, and internal carotid ganglia of the rat. Neuro-

25. Conterras RJ, Gomez MM, Norgren R. Central origins of cranial
nerve parasympathetic neurons in the rat. J Comp Neurol. 1980;


27. Spencer SE, Sawyer WB, Wada H, Platt KB, Loewy AD. CNS pro-
jections to the pterygopalatine parasympathetic preganglionic
neurons in the rat: a retrograde transneuronal viral cell body

28. Ng YK, Wong WC, Ling EA. A light and electron microscopical
localisation of the superior salivatory nucleus of the rat. J Brains.

29. Tóth IE, Boldogkói Z, Medveczky J, Palkovits M. Lacrimal pregan-
glionic neurons form a subdivision of the superior salivatory nu-
cleus of rat: transneuronal labelling by pseudorabies virus. J Auton
Nerv Syst. 1999;77:45–54.

30. Zhu BS, Gai WP, Yu YH, Gibbins IL, Blessing WW. Preganglionic
parasympathetic sensory neurons in the brainstem contain marks-

31. Zhu BS, Gibbons IL, Blessing WW. Preganglionic parasympathetic
neurons projecting to the sphenopalatine ganglion contain nitric

32. Gai WP, Blessing WW. Human brainstem preganglionic parasympa-
thetic neurons localized by markers for nitric oxide synthesis. Brain.

33. Jansen ASP, Ter Horst GJ, Mettenleiter TC, Loewy AD. CNS cell
projection groups projecting to the submandibular parasympathetic
preganglionic neurons in the rat: a retrograde transneuronal viral cell

34. Ruskell GL. The orbital distribution of the sphenopalatine ganglion
in the rabbit. In: Rothen JW, ed. The Structure of the Eye. Stuttgart,
Germany: Schattauer-Verlag; 1965:335–368.

35. Ruskell GL. The distribution of autonomic post-ganglionic nerve
242.

36. Uddman R, Malm L, Sundler F. The origin of vasoactive intestinal
polypeptide (VIP) nerves in the feline nasal mucosa. Acta Otolary-
gogol. 1980;89:152–156.

37. Ten Tusscher MPM, Klooster J, Baljet B, Van der Werf F, Vrensen
GFJM. Pre- and post-ganglionic nerve fibers of the pterygopalatine
ganglion and their allocation to the eyeball of rats. Brain Res.

38. Nakai M, Tamaki K, Ogata J, Matsui Y, Maeda M. Parasympathetic
cerebrovasodilator center of the facial nerve. Circ Res. 1993;72:
470–475.

39. Van der Werf F, Baljet B, Prins M, Otto JA. Innervation of the
labral gland in the cynomolgus monkey: a retrograde tracing study.

40. LeDoux MS, Zhou Q, Murphy RB, Greene ML, Ryan P. Parasympa-
thetic innervation of the meibomian glands in rats. Invest Ophthal-


42. Rotto-Perclay DM, Wheeler JG, Osorio FA, Platt KB, Loewy AD.
Transneuronal labeling of spinal interneurons and sympathetic
preganglionic neurons after pseudorabies virus injections in the rat

HJ. Neurotransmitter organization of the nucleus of Edinger-West-
phal and its projection to the avian ciliary ganglion. Vis Neurosci.

44. Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates.

45. LeDoux MS, Ryan P. Motor and trigeminal blink circuitry in the rat

46. Mayer B, Mathias J, Bohme E. Purification of Ca 2+ /calmodulin
dependent nitric oxide synthase from porcine cerebellum. Fed Eur

47. Johnson CD, Epstein ML. Monoclonal antibodies and polyvalent
antiserum to chicken choline acetyltransferase. J Neurochem.

48. Reiner A. Catecholaminergic innervation of the basa galla in
mammals. In: Smets WJAJ, Reiner A, eds. Phylogeny and Devel-
opment of Catecholamine Systems in the CNS of Vertebrates.

49. Anderson KD, Reiner A. The extensive co-occurrence of substance
P and dynorphin in striatal projection neurons: an evolutionarily
conserved feature of basal ganglia organization. J Comp.

50. Medina L, Veeman CL, Reiner A. Evidence for a possible avian
dorsal thalamic region comparable to the mammalian ventral an-
terior, ventral lateral, and oral ventroposterolateral nuclei. J Comp.

51. Zin-Ka-Jeu S, Roger M, Arnault P. Direct contacts between fibers
from the ventrolateral thalamic nucleus and frontal cortical neu-
rons projecting to the striatum: a light-microscopy study in the rat.

52. Reiner A, Veeman CL, Medina L, Jiao Y, Del Mar N, Honig MG.
Pathway tracing using biotinylated dextran amines. J Neurosci

of superoxide dismutase in interneurons versus projection neu-
rons in patch versus matrix neurons in monkey striatum. Brain.
Res. 1996;708:59–70.

huntingtin in striatal and cortical neurons in rats: lack of correla-
tion with neuronal vulnerability in Huntington’s disease. J Neuro-

selective histochemical marker for striatal neurons containing both
somatostatin- and avian pancreatic polypeptide (APP)-like

G. Differential distribution of NADPH-diaphorase and neutral
nitric oxide synthase in the rat choroid plexus: a histochemical and

and choroidal blood vessels by the pterygopalatine ganglion in

endings form nucleus of Edinger-Westphal in pigeon ciliary gan-
854.

59. Smets WJAJ, Reiner A. Phylogeny and Development of Catechol-
amine Systems in the CNS of Vertebrates. New York: Cambridge
University Press; 1994.

60. Nemoto T, Konno A, Chiba T. Synaptic contact of neuropeptide

61. Pickard GE, Smeraski CA, Tomlinson CC, et al. Intravital injec-
tion of the attenuated pseudorabies virus PRV Btha results in
infection of the hamster suprachiasmatic nucleus only by retro-

62. Suzuki N, Hardebo JE, Owman C. Origins and pathways of cere-
brovascular vasoactive intestinal polypeptide-positive nerves in the
rat: a retrograde transneuronal viral cell body labeling study.

63. Bill A, Sperber GO. Control of retinal and choroidal blood flow.

64. Steinle JJ, Krizsan-Agbas D, Smith PG. Regional regulation of cho-
roidal blood flow by autonomic innervation in the rat. Am J

65. Agassandian K, Fazan VPS, Cassell LH, Lin LH, Talman WT.
Anterograde and retrograde tracers define projections between nucleus
tractus solitarii and superior salivatory nucleus in rats. Soc

66. Hosoya Y, Matsushita M, Sugira Y. Hypothalamic descending affer-
ents to cells of origin of the greater petrosal nerve in the rat, as


