Presynaptic adrenergic facilitation of parasympathetic neurotransmission in sympathectomized rat smooth muscle

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1. Parasympathetic innervation of rat eyelid tarsal smooth muscle normally inhibits sympathetic neurotransmission prejunctionally without significant direct postjunctional effects. Following surgical sympathectomy, parasympathetic stimulation elicits smooth muscle contraction. This study examined the relative contributions of cholinergic and adrenergic mechanisms mediating these contractions.

2. Electrical stimulation of the superior salivatory nucleus, which activates tarsal muscle parasympathetic nerves, elicited large contractions at 2 days postsympathectomy, which were abolished by atropine and were decreased by 65% by α_1-adrenoceptor blockade or spinal cord transection.

3. Contractions in response to direct cholinergic stimulation by bethanechol at 2 days postsympathectomy were increased following spinal cord transection (C2) and suppressed by the α_1-adrenoceptor agonist phenylephrine, indicating that adrenoceptors on smooth muscle attenuate cholinergic contractions. However, phenylephrine infusion enhanced contractile responses to parasympathetic stimulation.

4. Reverse transcription-polymerase chain reaction revealed α_{1D}-adrenoceptor mRNA within pterygopalatine ganglia.

5. At 5 weeks and 14 months postsympathectomy, adrenergic facilitation was significantly less than at 2 days, whereas prazosin-insensitive muscarinic contraction was increased.

6. We conclude that degeneration of sympathetic innervation is followed rapidly by adrenoceptor-mediated prejunctional enhancement of parasympathetic nerve–smooth muscle neurotransmission, which occurs prior to neuroeffector junction formation as determined previously by electron microscopy. Subsequently, noradrenergic enhancement is diminished as cholinergic neurotransmission becomes established.

Neurons may be strongly influenced by changes in the heterologous neuronal populations with which they interact. Damage and degeneration of one population of nerves may result in an increased terminal arborization or altered neurotransmitter expression (Björklund *et al.* 1985; Terenghi *et al.* 1986; Fan & Smith, 1993). The functional consequences of these changes, however, are not well understood.

The superior tarsal muscle (STM) of the rat provides a useful system for evaluating functional consequences of heterologous denervation (Fig. 1). This eyelid smooth muscle normally receives excitatory noradrenergic innervation from the ipsilateral superior cervical ganglion (Smith *et al.* 1983). The STM is also innervated by parasympathetic neurons of the ipsilateral pterygopalatine ganglion (Simons & Smith, 1994), which receive preganglionic input from the superior salivatory nucleus (SSN) (Spencer *et al.* 1990). Parasympathetic stimulation by itself elicits little change in resting STM tone, whereas stimulation during sympathetic activation reduces contraction. This effect can be blocked by atropine, and because we have been able to demonstrate only excitatory muscarinic receptors on the STM, this attenuation apparently is mediated by inhibitory muscarinic receptors located on the sympathetic nerve varicosities (Beauregard & Smith, 1994). Accordingly, the STM contractile state is normally regulated by noradrenergic sympathetic nerves that elicit smooth muscle contraction, and by parasympathetic nerves that inhibit contractions by suppressing sympathetic neurotransmission.

Following superior cervical ganglion excision in the adult rat, permanent sympathetic denervation of the STM ensues (Smith & Fan, 1996). However, stimulation of the SSN at 5 weeks postsympathectomy elicits robust contractions (Smith & Beauregard, 1993). This is accompanied by sprouting of parasympathetic varicosities, which form close appositions with smooth muscle cells (Smith & Marzban, 1998). These are morphologically similar to presumptive

sites of autonomic neurotransmission identified in other tissues (Luff *et al.* 1991; Hill *et al.* 1993; Cottee *et al.* 1996). Therefore, atypical junctions form between the normally prejunctional inhibitory terminals and muscle cells following sympathetic denervation of the STM.

The objective of the present study was to determine whether parasympathetic sprouting between 2 days and 5 weeks postsympathectomy is accompanied by changes in parasympathetic neuroeffector transmission, and whether transmission is sustained for longer periods. In addition, because parasympathetic neurons show enhanced expression of catecholaminergic traits after sympathectomy (Björklund *et al.* 1985; Terenghi *et al.* 1986; Fan & Smith, 1993), we evaluated the relative adrenergic and cholinergic contributions to parasympathetic neuromuscular transmission. We show here that conversion of the parasympathetic effects from inhibition to excitation occurs rapidly through an initial noradrenergic augmentation of cholinergic neurotransmission. This is due to presynaptic facilitation by circulating catecholamines rather than through release from parasympathetic varicosities. Noradrenergic facilitation becomes less apparent as the density of neuroeffector junctions increases. An abstract of some of these findings has been presented (Krizsan-Agbas *et al.* 1997).

METHODS

Superior cervical ganglionectomy

Female Sprague–Dawley rats (Harlan) at 60–70 days of age (180–220 g) were anaesthetized by inhalation of isoflurane (Abbott Laboratories, Chicago, IL, USA) in a mixture of oxygen and nitrous oxide, or by ketamine hydrochloride (27·5 mg kg^{-1} I.P., Sanofi Winthrop, New York, NY, USA), xylazine hydrochloride

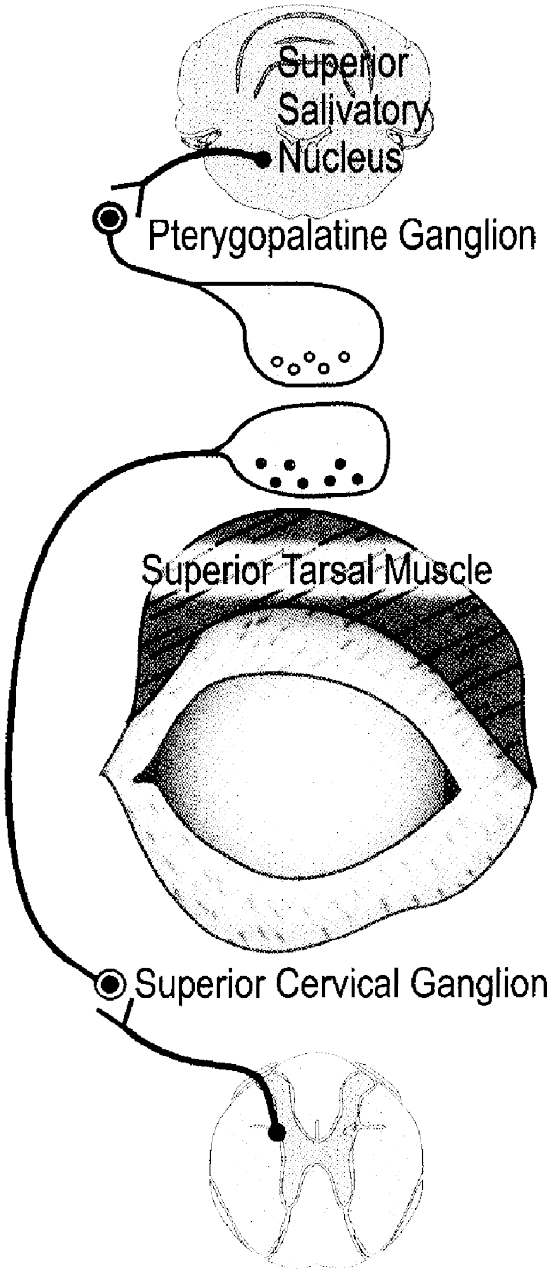


Figure 1. Schematic diagram of the autonomic innervation of the superior tarsal muscle in the rat

Sympathetic noradrenergic excitatory innervation to the superior tarsal muscle derives from postganglionic neurons of the ipsilateral superior cervical ganglion, which receive preganglionic innervation from neurons of the intermediolateral column of the spinal cord. Parasympathetic preganglionic cell bodies are located in the superior salivatory nucleus of the brainstem, and project to postganglionic neurons of the ipsilateral pterygopalatine ganglion. These in turn project to the superior tarsal muscle, where they make neuroeffector junctions with sympathetic nerve varicosities but not smooth muscle cells. If sympathetic innervation degenerates, appositions with smooth muscle cells are formed.

(2·5 mg kg^{-1}, Rompun, Miles, Shawnee Mission, KS, USA) and atropine sulfate (0·24 mg kg^{-1}, Vedco, St Joseph, MO, USA). A ventral mid-line incision was made in the neck and the right superior cervical ganglion removed aseptically (Smith & Beauregard, 1993). This procedure produces complete and permanent loss of STM sympathetic innervation in adult rats (Smith, 1986; Smith & Fan, 1996). All rats recovered without signs of infection or distress. Analysis was conducted in rats with an intact innervation ($n = 45$) and in ganglionectomized animals at 1 day ($n = 4$), 2 days ($n = 55$), 5 weeks ($n = 53$), or 14 months ($n = 3$) following denervation. Surgical procedures and all subsequent experimental manipulations were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center, and conform to local and national statutes.

SSN stimulation

Intact and sympathectomized rats were anaesthetized with urethane (1·25 g kg^{-1} I.P., Sigma) and the right femoral vein and artery were cannulated for drug administration and arterial blood pressure monitoring, respectively. The right facial nerve was cut to eliminate somatic motor innervation to the superior eyelid, and each animal was placed in a stereotaxic frame with body temperature maintained at 37 °C. An incision was made over the sagittal suture and a craniotomy was performed. A semi-micro bipolar concentric electrode (100 μm tip diameter, Rhode Medical Instruments, Woodland Hills, CA, USA) was placed stereotaxically into the SSN at co-ordinates corresponding to 10·3–10·8 mm posterior, 9·5–10·0 mm ventral and 2·4–2·6 mm lateral relative to bregma. Electrode placement in the SSN was verified by porphyrin discharge from the Harderian gland during stimulation. The SSN was stimulated using a constant current of 30 μA, 0·5 ms, 20 Hz for 20–50 s (Grass SD9 stimulator with CCU1A) rather than threshold voltage, as used previously (Smith & Beauregard, 1993; Beauregard & Smith, 1994). Stimulation was maintained until a maximum change in STM tension was observed.

Analysis of STM responses

Changes in STM tension were recorded (Polyview, Astro-Med Inc., Grass Instruments) by a force displacement transducer (Gould Metrigram, 1 g initial loading tension) attached to the right superior eyelid (Beauregard & Smith, 1994). Rats were killed by an anaesthetic overdose (3·0 g kg^{-1} urethane I.V.) at the completion of the experiment. All values are expressed as means ± S.E.M. Data were analysed by one- or two-way analysis of variance (ANOVA) with *post hoc* analysis by the Student–Newman–Keuls test, or two-way repeated measures ANOVA followed by Student's paired t test with Bonferroni corrections (SPSS or Jandel SigmaStat).

Spinal cord transection

Spinal cord transection was carried out in some urethane-anaesthetized rats to eliminate centrally derived sympatho-adrenal tone. Beforehand, the trachea was intubated, the spinal cord exposed via laminectomy at C1–C2, and the animals ventilated mechanically (Harvard Small Animal Ventilator, 40–50 breaths min^{-1}). Following transection at C2, rats were allowed to recover for approximately 20–30 min or until tarsal muscle tone was stable.

Adrenergic and cholinergic agonists and antagonists

The following drugs were administered as slow bolus injections via femoral vein cannulae: prazosin hydrochloride (1 mg kg^{-1}, Sigma), an α_1-adrenoceptor antagonist; idazoxan hydrochloride (1 mg kg^{-1}, RBI), an α_2-adrenoceptor antagonist; atropine methyl nitrate (0·5 mg kg^{-1}, Sigma) a muscarinic antagonist; and bethanechol chloride (Urecholin; Merck, Sharp & Dohme), a muscarinic agonist. Bethanechol was given as a single standardized dose of 100 μg kg^{-1} to assess effects of co-administered agents or, to define a dose–response relationship, in increasing doses from 30 to 400 μg kg^{-1} until the dose eliciting maximum contraction was exceeded (supramaximum). The α_1-adrenoceptor agonist phenylephrine hydrochloride (5 μg kg^{-1} min^{-1}, American Regent Laboratories, Shirley, NY, USA) was infused intravenously by infusion pump (Razel).

Reverse transcription-polymerase chain reaction (RT-PCR)

Tissue samples (5–10 mg each) from the pterygopalatine ganglion, STM, superior cervical ganglion and heart were obtained from deeply anaesthetized (1·5 g kg^{-1} urethane) rats following vascular perfusion with cold phosphate-buffered saline. Pooled samples from four rats per experiment were homogenized (Omni International) in 1 ml Trizol Reagent (Gibco BRL). Homogenates were mixed with 0·2 ml chloroform, incubated for 5 min at room temperature and centrifuged at 12 000 g for 15 min. RNA was precipitated from the aqueous phase by adding 0·5 ml isopropanol and 10 μg glycogen, chilling at 0 °C for 30 min, and centrifuging at 12 000 g for 15 min. The RNA pellet was washed with 75% ethanol, centrifuged and resuspended in distilled water. The concentration of RNA was measured spectrophotometrically. RNA was treated with DNase (Gibco BRL) to eliminate possible genomic DNA contamination prior to reverse transcription. cDNA was synthesized from 1·5 μg of total RNA, using 0·5 μg oligo(dT)$_{12\text{–}18}$, 200 U SuperScript II reverse transcriptase and 10 U RNA inhibitor (Gibco BRL) as recommended by the manufacturer. The sample was incubated at 42 °C for 50 min and 70 °C for 15 min, followed by addition of 2 U RNase H and incubation at 37 °C for 20 min. A control sample was processed as above except that the reverse transcriptase was omitted to confirm the absence of contaminating genomic DNA.

The cDNAs from the RT reaction were amplified by PCR. A 2 μl sample of the cDNA solution was added to 48 μl of PCR master mixture, consisting of 10 mM Tris-HCl (pH 9·0), 50 mM KCl, 1·5 mM $MgCl_2$, 200 μM dNTP mix, 100 nM each primer and 2·5 U Taq DNA polymerase (Gibco BRL). Amplification was performed in a Twin Block PCR thermal cycler (Ericomp Inc.) The initial denaturation was conducted at 94 °C for 4 min, and was followed by primer annealing for 1 min at 50 °C for α_{1A}-, α_{1B}- and α_{1D}-receptor mRNA and 55 °C for α_2-adrenergic receptor mRNA, primer extension at 72 °C for 1 min and denaturation at 94 °C for 30 s. At the end of forty cycles, the reaction mixture was kept at 72 °C for 10 min and then brought to room temperature. A 20 μl sample of the PCR product was analysed electrophoretically on a 2% agarose gel containing 0·5 μg ml^{-1} ethidium bromide.

Oligonucleotide primers were designed according to cDNA sequences available through GenBank. Primers were eighteen to thirty nucleotides long and had similar A/T and G/C contents so that the annealing temperatures of paired sense and antisense primers were similar. All primer sequences were screened using GenBank to avoid inadvertent homology to other protein sequences.

α_1-Receptor subtypes were detected using specific primers for α_{1A} (accession number U13368, 5′-AGAATGTCCTGCGAATCC-3′ (1048–1065) and 5′-TTTCCTGTGACCTTTCCC-3′ (1459–1442), 412 bp product), α_{1B} (accession number M60655, 5′-ATGACA-AAGAATGTGGGG-3′ (586–603) and 5′-GGTAGATGATGATGGG-ATTGAG-3′ (1059–1041), 474 bp product), and α_{1D} (accession number M60654, 5′-CGAAGGTGATGGGTTATGG-3′ (2317–2335) and 5′-TTAGGAGTGTGGGGAAGAGG-3′ (2689–2670), 373 bp product). The presence of α_2-adrenergic receptor mRNA was explored using a primer set designed to recognize identical regions of α_{2A}-, α_{2B}- and α_{2C}-receptors (accession numbers D00818, M32061

and X57659). The sense primer was 5′-TCCATCGTGCACCTG-TGTGCCA-3′ (α_{2A}, 473–494; α_{2B}, 675–696; α_{2C}, 1318–1339), the antisense primer was 5′-TGCAGTAGCCGATCCAGAAG-3′ (α_{2A}, 1334–1315; α_{2B}, 1626–1607; α_{2C}, 2179–2160), and the predicted product size was 861 bp.

In all RT-PCR experiments, glucose 6-phosphate dehydrogenase (G6PD) mRNA was used as a control to monitor extraction and amplification efficiencies. The sense primer was 5′-CAGAACCTC-ATGGTGCTGAGATTT-3′ (666–690) and the antisense primer was 5′-CGCTTGCACTGCTGGTGGAAG-3′ (1201–1181) (Lin *et al.* 1994). The expected product size was 535 bp. Identities of RT-PCR products were confirmed in restriction enzyme cleavage experiments.

Messenger RNA encoding the α_{1D}-receptor was assessed semi-quantitatively in some samples of pterygopalatine ganglia from intact and sympathectomized rats. Samples were processed as described above. However, PCR was carried out for thirty-four cycles, which in preliminary studies was shown to be in the linear region of the product curve. RT-PCR product quantity was estimated in ethidium bromide-stained gels using densitometry and NIH Image software. Product quantity was normalized for differences in extraction and PCR efficiency by correcting for differences in the G6PD signal. Values from right pterygopalatine ganglia, which were sympathectomized, were divided by values for the left, control ganglia, and expressed as a ratio.

RESULTS

Effect of adrenergic antagonists and spinal cord transection on responses to SSN stimulation

Stimulation of the SSN in animals with intact sympathetic innervation elicited either no change or small increases or decreases in STM tension (Figs 2 and 3). Neither the α_1-adrenergic receptor antagonist prazosin, the α_2-receptor antagonist idazoxan (data not shown), nor spinal cord transection affected responses to SSN stimulation (Figs 2 and 3).

Table 1. Times required for parasympathetically stimulated sympathectomized STM to contract and relax

	Maximum contraction (s)	50% relaxation (s)
2 days post-SNX	39 ± 2*	35 ± 4*
5 weeks post-SNX	29 ± 2	15 ± 2
14 months post-SNX	25 ± 8	7 ± 2

Values are times required for sympathectomized STM to attain stable maximum contractile force during SSN stimulation, and to relax to 50% of the maximum following cessation of stimulation. Because of the small and variable responses, it was not possible to obtain measurements from intact preparations. * Times required for maximum contraction and for 50% relaxation were longer than in preparations at 5 weeks or 14 months after sympathectomy (post-SNX) ($P < 0{\cdot}05$).

SSN stimulation in rats 2 days after superior cervical ganglion excision evoked contractions that were significantly greater than those in intact preparations ($P < 0{\cdot}05$, Figs 2 and 3). Contractions reached a maximum at 39 ± 2 s after the onset of stimulation, and relaxed by 50% at 35 ± 4 s after cessation of stimulation (Table 1). Prazosin reduced contractions by 65%, as did spinal cord transection

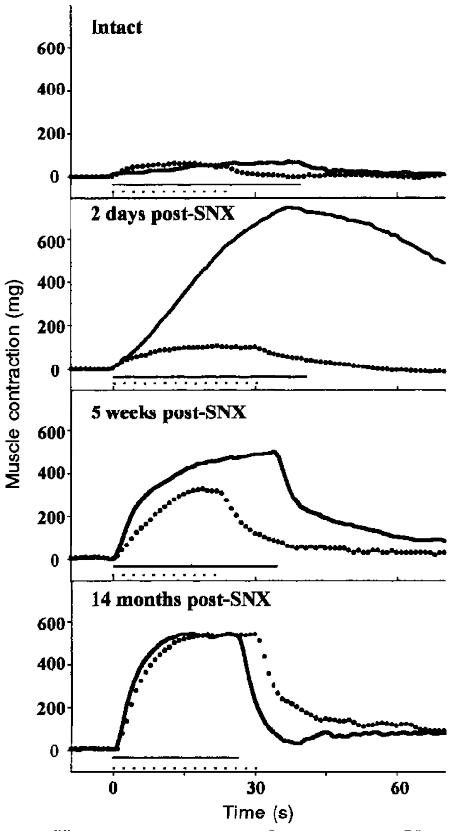


Figure 2. STM contractions elicited by parasympathetic stimulation before and at different times following sympathectomy

Changes in STM tension during SSN parasympathetic stimulation (30 μA, 0·5 ms, 20 Hz) in rats with intact sympathetic innervation (Intact) and at 2 days, 5 weeks and 14 months following ipsilateral superior cervical ganglion excision (post-SNX). Responses from individual animals are shown under control conditions (continuous line) and following attenuation of adrenergic influences (dotted line) by spinal cord transection (Intact, 2 days post-SNX and 5 weeks post-SNX) or by the α_1-adrenergic antagonist prazosin (14 months post-SNX). Bars indicate the duration of electrical stimulation.

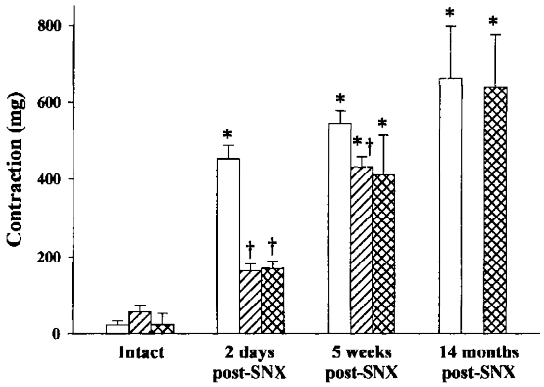


Figure 3. Effect of adrenergic blockade and spinal cord transection on responses to SSN stimulation

STM responses to electrical stimulation of the SSN in rats with intact innervation (Intact) and 2 days, 5 weeks and 14 months after excision of the ipsilateral superior cervical ganglion (post-SNX). Responses were obtained from untreated control preparations (□) and after spinal cord transection at C1–C2 in one subset of animals (▨), or prazosin (▩) in another group. Two-way ANOVA reveals variation among experimental groups ($P < 0{\cdot}001$), treatments ($P < 0{\cdot}001$), and a group–treatment interaction ($P < 0{\cdot}001$). * $P < 0{\cdot}05$ *vs.* intact preparations under comparable conditions by Student–Newman–Keuls test. † $P < 0{\cdot}05$ *vs.* control responses.

($P < 0{\cdot}05$, Figs 2 and 3), while idazoxan caused no further reduction (not shown). Responses after prazosin or spinal cord transection did not differ significantly from those of intact rats under the same conditions. Atropine abolished contractions when administered alone or following prazosin.

At 5 weeks after sympathectomy, SSN stimulation elicited contractions with similar magnitudes to those at 2 days postsympathectomy (Figs 2 and 3), but which contracted and relaxed more rapidly ($P < 0{\cdot}05$, Table 1). Contractile responses after prazosin (Fig. 3) or idazoxan were not significantly different from control responses. Contractions after spinal cord transection (Figs 2 and 3) were comparable to those after prazosin, but were lower than control responses ($P < 0{\cdot}05$). Responses after prazosin and spinal cord transection were greater than those of intact rats and rats 2 days after superior cervical ganglion excision ($P < 0{\cdot}05$). Atropine abolished contractions remaining after prazosin, and prevented contractile responses when administered alone, as reported previously (Smith & Beauregard, 1993).

In rats 14 months after sympathectomy, SSN stimulation elicited contractions that were comparable to those at 2 days and 5 weeks after sympathectomy, and the time courses for contraction and relaxation were comparable to those at 5 weeks (Table 1). Prazosin had no detectable effect on STM contractions at this time (Figs 2 and 3).

Effects of cholinergic muscarinic receptor activation on STM tone

A standardized bethanechol dose of 100 μg kg^{-1} yielded modest contractions in intact rats (Fig. 4), whereas the same dose at 2 days postsympathectomy evoked larger contractions ($P < 0{\cdot}05$, Fig. 4). This heightened response was not accompanied by an increase in the contraction elicited by a supramaximum dose of bethanechol, but was associated with increased sensitivity to this agent as reflected in decreased bethanechol ED_{50} values (Table 2). At 5 weeks after superior cervical ganglion excision, contractions elicited by 100 μg kg^{-1} bethanechol remained elevated ($P < 0{\cdot}05$, Fig. 4) and ED_{50} reduced ($P < 0{\cdot}05$, Table 2). Tension generated by a supramaximum dose of bethanechol was now significantly lower than that at 2 days after superior cervical ganglion excision ($P < 0{\cdot}05$).

Effects of spinal cord transection on cholinergic contractions

Spinal cord transection increased the magnitude of the response to 100 μg kg^{-1} bethanechol in preparations with intact sympathetic innervation ($P < 0{\cdot}05$, Fig. 4) and at 2 days post-superior cervical ganglion excision, although the bethanechol response at that time was greater than in the intact muscle ($P < 0{\cdot}05$, Fig. 4). However, at 5 weeks after

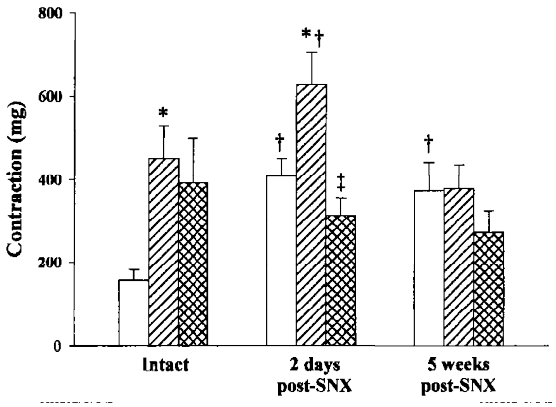


Figure 4. Effects of bethanechol alone and following spinal cord transection, without and with phenylephrine, on STM tension

STM responses to bethanechol (100 μg kg^{-1} I.V.) in rats with intact innervation (Intact) and 2 days or 5 weeks after excision of the ipsilateral superior cervical ganglion (post-SNX). Bethanechol was administered alone (□), following spinal cord transection (▨), and in spinal cord-transected rats during phenylephrine application (5 μg kg^{-1} min^{-1} I.V.; ▩). Responses varied as a function of treatment (bethanechol, spinal cord transection and phenylephrine, $P = 0{\cdot}005$), with an interaction between groups and treatments ($P = 0{\cdot}027$). * $P < 0{\cdot}05$ *vs.* bethanechol alone. † $P < 0{\cdot}05$ *vs.* the intact preparation under the same conditions. ‡ $P < 0{\cdot}05$ *vs.* bethanechol plus spinal cord transection.

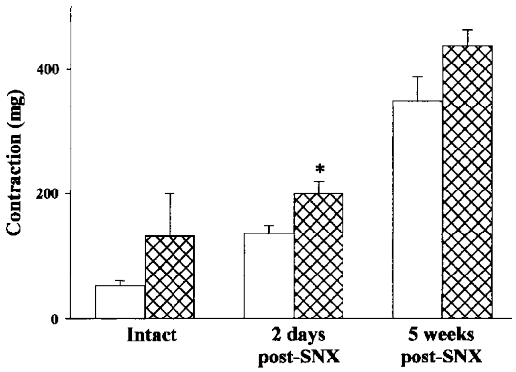


Figure 5. Effects of an α-adrenergic agonist on STM contractions elicited by parasympathetic stimulation
STM contractile responses to electrical stimulation of the SSN in spinal cord-transected rats before (□) and during intravenous infusion of phenylephrine (▨). Measurements were made from intact rats and 2 days or 5 weeks following sympathectomy (post-SNX). Responses during phenylephrine infusion were increased overall relative to control responses ($P = 0{\cdot}001$). * $P < 0{\cdot}05$ *vs.* spinal cord-transected controls.

ganglionectomy, spinal cord transection did not affect the response to bethanechol, and this was now lower than at 2 days after superior cervical ganglion excision ($P < 0{\cdot}05$) and comparable to the response in the intact rat (Fig. 4).

Effects of α_1-adrenergic agonist on cholinergic contractions

Phenylephrine was infused intravenously at a rate that increased tarsal muscle tone to approximately its pre-spinal cord transection level (an increase in tension of approximately 200 mg; see Methods). Phenylephrine decreased the response to bethanechol ($P < 0{\cdot}05$, Fig. 4). The reduction was greatest at 2 days postsympathectomy, where cholinergic contractions were reduced by 50 % ($P < 0{\cdot}05$).

Effects of α_1-adrenergic agonist on STM contractions elicited by SSN stimulation

The effects of phenylephrine infusion on parasympathetically mediated STM contractions were evaluated in spinally transected rats (Fig. 5). Overall, phenylephrine increased contractions ($P = 0{\cdot}001$, main treatment effect), with an increase of 46 % at 2 days postsympathectomy achieving significance by *post hoc* analysis ($P < 0{\cdot}05$).

Expression of α-adrenoceptor mRNA in parasympathetic neurons

mRNA extracted from heart, which expresses α_{1A}-, α_{1B}- and α_{1D}-receptor subtypes (Rokosh *et al.* 1994), confirmed the sensitivity of the RT-PCR analysis (Fig. 6). The STM, which is known to possess excitatory α_1-receptors (Smith *et al.* 1983) showed strong expression of α_{1D}-receptor mRNA, very weak α_{1A}-receptor mRNA expression, and no α_{1B}-receptor mRNA (Fig. 6). Analysis of the SCG confirmed the presence of α_1-receptor mRNA, although expression in these Sprague–Dawley rats was confined to the α_{1D}-receptor, suggesting strain differences from adult Wistar–Kyoto rats, which are reported to express α_{1A}-adrenoceptor mRNA (Vidovic & Hill, 1997). Pterygopalatine ganglia expressed mRNA sequences for only the α_{1D}-receptor.

RT-PCR analysis of α_2-receptor mRNA showed strong expression in the STM and superior cervical ganglion, but no detectable signal in the pterygopalatine ganglion (Fig. 7).

Semi-quantitative evaluation of the relative amounts of RT-PCR product corresponding to the α_{1D}-receptor mRNA in pterygopalatine ganglia showed no obvious changes in

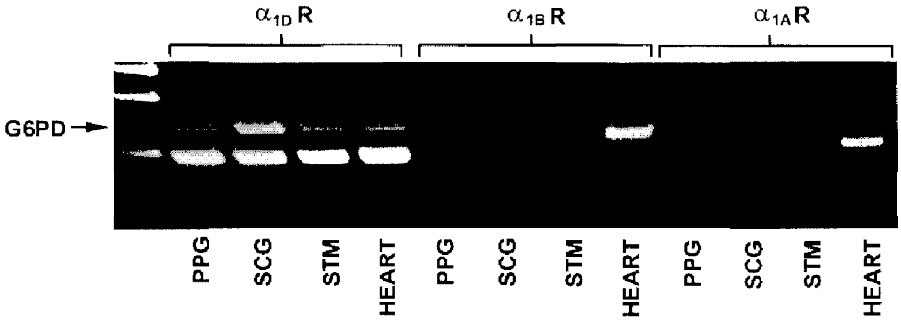


Figure 6. α_1-Adrenergic receptor mRNA in parasympathetic ganglia and other tissues
RT-PCR products corresponding to mRNA encoding the α_{1D}-, α_{1B}-, and α_{1A}-adrenergic receptors in pterygopalatine ganglion (PPG), superior cervical ganglion (SCG), superior tarsal muscle (STM) and heart. The α_{1D}-receptor reaction mixture included primers for the housekeeping gene glucose 6-phosphate dehydrogenase (G6PD). A molecular size ladder is included in the far left-hand lane, with bands corresponding to 300 (bottom), 400, 600 and 800 bp (top).

Table 2. Maximum force and receptor sensitivity to muscarinic stimulation of intact and sympathectomized STM

	Maximum contraction (mg)	ED_{50} ($\mu g\ kg^{-1}$)
Intact	894 ± 62	212 ± 21
2 days post-SNX	1005 ± 79	108 ± 112 [b]
5 weeks post-SNX	673 ± 46 [a]	66 ± 7 [b]

STM maximum tension generated by a supramaximal dose of bethanechol, and bethanechol dosage yielding a 50% maximum contraction (ED_{50}) are shown. [a] Maximum contraction at 5 weeks after sympathectomy (post-SNX) was less than that of intact preparations and at 2 days following sympathectomy ($P < 0{\cdot}05$). [b] Bethanechol dosage producing 50% maximum contraction was reduced at 2 days and 5 weeks postsympathectomy relative to intact animals ($P < 0{\cdot}05$).

Table 3. Semi-quantitative analysis of relative amounts of α_{1D}-adrenergic receptor RT-PCR product in pterygopalatine parasympathetic ganglia from intact and sympathectomized rats

Preparation	Product ratio
Intact control	0·99
1 day post-SNX	0·86
2 days post-SNX	1·05
5 weeks post-SNX	1·03

Results are expressed as the ratio of PCR product in the right (experimental) pterygopalatine ganglion to that in the left (intact). Determinations were made on pooled homogenates from 4 pairs of ganglia in each group. PCR was conducted for 34 cycles, which preliminary studies showed to be in the linear range of the product curve. Relative product amounts were measured densitometrically, and values were normalized for differences in extraction/reaction efficiency as determined using primers for the housekeeping gene G6PD.

expression among unoperated rats and those sympathectomized for 1 day, 2 days or 5 weeks (Table 3).

DISCUSSION

Rapid facilitation of parasympathetic neurotransmission after sympathectomy

The rapidity with which parasympathetic neurotransmission is established after sympathectomy could not be predicted from ultrastructural findings. At 2 days after surgical sympathectomy, numbers of nerve varicosities in the tarsal muscle are reduced by 97% and those remaining are relatively distant (> 400 nm) from smooth muscle cells (Smith & Marzban, 1998). These structural features seem incompatible with the idea that the excitation obtained at 2 days is primarily due to transmission via close neuromuscular junctions, and at least two other mechanisms may participate.

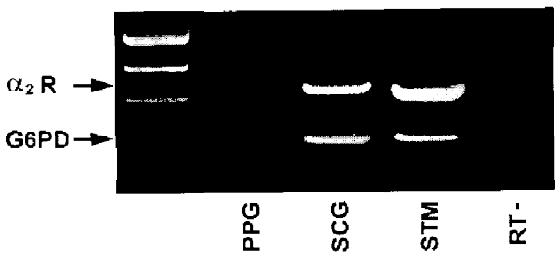
First, the bethanechol ED_{50} decreased by half within 2 days, suggesting that postdenervation increases in cholinergic receptor sensitivity and density, which occur by 1 week postsympathectomy in rat parotid gland (Asking & Ekstrom, 1979; Pimoule *et al.* 1980), are established quickly in the STM. Second, α_1-adrenoceptor blockade reduced contractions dramatically at 2 days after sympathectomy, implying that catecholamines are involved in the parasympathetic nerve–smooth muscle neuroeffector transmission. These could derive either from parasympathetic varicosities or from other sources, and could facilitate neurotransmission by interacting with postjunctional or prejunctional receptors.

Because parasympathetic neurons express enzymes that synthesize noradrenaline (Björklund *et al.* 1985; Fan & Smith, 1993), catecholamine release cannot be ruled out. However, previous studies have failed to demonstrate stored catecholamines (Landis *et al.* 1987) or mRNA encoding the noradrenaline transporter or vesicular monoamine transporter proteins (R. Zhang & P. G. Smith, unpublished observations) within parasympathetic neurons. Because atropine completely blocked contractions in the present study, adrenergic facilitation cannot be due to parasympathetic release of catecholamines, and augmentation of acetylcholine release is a more plausible explanation.

Cholinergic transmission may be augmented through either pre- or postjunctional actions. Because STM expresses α-receptor mRNA and responds to α-receptor agonists by contracting (Smith *et al.* 1983), adrenergic facilitation could occur through direct effects on smooth muscle. If so, contractions induced by direct cholinergic stimulation

Figure 7. Absence of α_2-adrenergic receptor mRNA in pterygopalatine ganglion

RT-PCR for mRNA encoding the α_2-adrenergic receptors in homogenates from pterygopalatine ganglion (PPG), superior cervical ganglion (SCG) and superior tarsal muscle (STM). A control experiment in which the reverse transcription step was omitted (RT−) is also presented. Primers for G6PD were included in reaction mixtures. The far left-hand lane contains molecular size standards corresponding to 400 (bottom), 800, 1200 and 2000 bp (top).



should be augmented by an α-receptor agonist. Because phenylephrine depressed rather than enhanced the response to bethanechol at 2 days postsympathectomy, a post-junctional site of action is unlikely.

Despite the depressive effect on the smooth muscle cholinergic response, phenylephrine significantly enhanced the contractions in response to parasympathetic nerve stimulation, with the greatest effect at 2 days after sympathectomy. This indicates a prejunctional site of action, and our RT-PCR findings of α_{1D}-receptor mRNA within pterygopalatine ganglia is consistent with such a mechanism. Presynaptic α_1-adrenergic facilitation of cholinergic transmitter release has been reported for other systems, including somatic motor nerves (Snider & Gerald, 1982) and parasympathetic nerves innervating the bladder (Somogyi *et al.* 1995). Therefore, prejunctional α_1-receptor-mediated facilitation of acetylcholine release represents a credible explanation for the prazosin-sensitive enhancement of parasympathetic neurotransmission after short-term sympathectomy.

The enhancement of parasympathetic responses by α_1-adrenoceptor agonism implies that the prejunctional receptors are activated by an endogenous ligand that either is released during parasympathetic stimulation or is present constitutively. As sympathetic nerve degeneration is complete by 2 days after ganglionectomy (Morgan & Hansen, 1978), modulation by sympathetic nerves is unlikely. Evidence that circulating plasma catecholamines are responsible comes from acute spinal cord transection experiments. Plasma catecholamine levels are normally maintained by tonic discharge of medullary neurons that project to preganglionic spinal cord neurons, which in turn innervate sympathetic postganglionic neurons and adreno-medullary chromaffin cells. By interrupting activity in this pathway, acute spinal cord transection reduces plasma catecholamines (Tibbs *et al.* 1979). Because spinal cord transection at 2 days post-sympathectomy reduced contractions to an extent comparable to complete α-adrenergic blockade, plasma catecholamines represent the most likely endogenous ligands activating prejunctional α-receptors. Why plasma catecholamines are able to activate prejunctional α_1-receptors rapidly and strongly after sympathectomy is unclear. However, our finding that α_{1D}-receptor mRNA is not increased after sympathectomy suggests that it is due to factors other than increased receptor synthesis.

In conjunction with ultrastructural studies, which failed to find closely apposed varicosities at 2 days after sympathectomy (Smith & Marzban, 1998), these observations are consistent with the idea that neurotransmission occurs over relatively long distances in the acutely sympathectomized STM. The slower time course with which contractions achieve their maxima would seem to be consistent with additional diffusion time required to attain sufficient neurotransmitter concentrations to activate receptors on distant smooth muscle cells. However, this mode of transmission appears to be highly reliant upon augmented acetylcholine release, as it is strongly depressed by α_1-adrenoceptor blockade. It would appear, therefore, that relatively large distances between parasympathetic nerves and smooth muscle normally serve to limit autonomic neurotransmission, but this can be overcome when acetylcholine release is increased through prejunctional receptor modulation.

The role of prejunctional α_1-receptor facilitation of parasympathetic neurotransmission is diminished after long-term sympathectomy

While contractions in response to parasympathetic stimulation are of comparable magnitude from 2 days to 14 months postsympathectomy, the mechanisms mediating these contractions vary. At 5 weeks, the contractile response was attenuated less by either spinal cord transection or prazosin than at 2 days postsympathectomy. This could be due either to greater α-adrenoceptor-mediated depression of cholinergic contraction at the level of the muscle, thereby offsetting the prejunctional enhancement, or to an abatement of the prejunctional facilitation. Because phenylephrine depressed the bethanechol response to a greater degree at 2 days than at 5 weeks, greater attenuation of muscarinic muscle contraction cannot explain this change. Therefore, α_1-receptor prejunctional facilitation appears to play a diminishing role with increased time after sympathectomy.

In conjunction with this decrease, the prazosin-independent component of parasympathetic neurotransmission increased, and this was associated with more rapid muscle contractions. Ultrastructural analyses show that between 2 days and 6 weeks postsympathectomy the number of parasympathetic varicosities within the STM increases nearly 5-fold, with approximately 5% of the varicosities forming close appositions (< 100 nm) with smooth muscle cells (Smith & Marzban, 1998). Therefore, the increase in the prazosin-independent component of neurotransmission probably reflects increased total numbers of varicosities and closer appositions.

While the increased cholinergic component can be explained by parasympathetic sprouting, it is less clear why the adrenergic modulation abates with time after sympathectomy. One possibility is that prejunctional α_1-receptor activity is not maintained following sympathectomy. Another is that facilitation is maintained, but this becomes less important as increased numbers of varicosities in close apposition lead to more efficient neurotransmission. Adrenergic enhancement of acetylcholine release therefore may be of particular importance in situations where innervation is marginal, as is the case at 2 days post-sympathectomy when nerve density is low and junctional distance is large.

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