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Research article

Neurophysiological assessment of sympathetic cardiovascular activity after loss of postganglionic neurons in the anesthetized rat

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ABSTRACT

The goal of this study was to determine the degree of sympathetic postganglionic neuronal loss required to impair cardiovascular-related sympathetic activity. To produce neuronal loss separate groups of rats were treated daily with guanethidine for either 5 days or 11 days, followed by a recovery period. Sympathetic activity was measured by renal sympathetic nerve activity (RSNA). Stereology of thoracic (T13) ganglia was performed to determine neuronal loss. Despite loss of more than two thirds of neurons in T13 ganglia in both treated groups no effect on resting blood pressure (BP) or heart rate (HR) was detected. Basal RSNA in rats treated for 5 days ($0.61 \pm 0.10\ \mu V * s$) and 11 days ($0.37 \pm 0.08\ \mu V * s$) was significantly less than vehicle-treated rats ($0.99 \pm 0.13\ \mu V * s$, $p < 0.05$). Increases in RSNA by baroreceptor unloading were significantly lower in 5-day ($1.09 \pm 0.19\ \mu V * s$) and 11-day treated rats ($0.59 \pm 0.11\ \mu V * s$) compared with vehicle-treated rats ($1.82 \pm 0.19\ \mu V * s$, $p < 0.05$). Increases in RSNA to chemoreceptor stimulation were significantly lower in 5-day treated rats ($1.54 \pm 0.25\ \mu V * s$) compared with vehicle-treated rats ($2.69 \pm 0.23\ \mu V * s$, $p < 0.05$). Increases in RSNA in 11-day treated rats were significantly lower ($0.75 \pm 0.15\ \mu V * s$, $p < 0.05$) compared with both vehicle-treated and 5-day treated rats. A positive correlation of neurons to sympathetic responsiveness but not basal activity was detected. These data suggest that diminished capacity for reflex sympathetic responsiveness rather than basal activity alone must be assessed for complete detection of neurophysiological cardiovascular impairment.

1. Introduction

Adverse effects on the cardiovascular system are one of the leading causes of attrition during drug development (Klein & Redfern, 2015; Laverty et al., 2011; Piccini et al., 2009). Many of these adverse effects are the result of direct effects on the heart or vasculature (Giles et al., 2014; Morton, Houle, & Tomlinson, 2014; Nickel, Sander, & Moon, 2008). For example, direct drug interactions with the K^+ channels in ventricular myocytes can prolong the QT interval and introduce risk of arrhythmia (Piccini, Whellan et al., 2009; Pugsley, Curtis, & Hayes, 2015). However, other adverse effects may reflect drug-induced changes in autonomic control of the cardiovascular system (Becker, 2012; Leung, Barr, Procyshyn, Honer, & Pang, 2012). Because parts of the autonomic nervous system lie outside the blood brain barrier they are susceptible to effects from systemically administered drugs, e.g. adrenergic, cholinergic, as well as some chemotherapeutic and pain medications. This can represent a significant safety concern, especially for neurons that control critical functions like cardiovascular regulation.

The autonomic nervous system is a collection of afferent and efferent neurons that link the central nervous system to peripheral and visceral tissue. The main function of the autonomic nervous system is to maintain homeostasis and to allow mammals to appropriately respond to any perturbations in the environment (Cannon, 1932). The moment-to-moment adjustments of autonomic activity necessary to maintain appropriate cardiovascular activity rely on appropriate levels of tonic sympathetic tone. Sympathetic control of blood pressure (BP) relies on the ability to increase or decrease activity of the sympathetic postganglionic neurons that innervate the vasculature. Although it has been shown that sympathetic neuronal depletion with guanethidine has no appreciable effect on baseline blood pressure (Bennett & Gardiner, 1986; Julien et al., 1990) drugs that diminish the ability of sympathetic postganglionic neurons to respond to acute physiological stimulus may still have serious implications on reflex sympathetic regulation of BP leading to cardiovascular risk (Bhatt, Foote, Smith, Butler, & Steidl-Nichols, 2015; Dampney et al., 2002; Heymans & Neil, 1958; Lowey & Spyer, 1990). Thus it is important to be able to detect adverse effects on the sympathetic nervous system during preclinical testing of new drug candidates.

Histopathology is not typically employed to identify changes in postganglionic neurons during drug development due to the small size of the tissues involved, leaving in-vivo functional assessments as the best means of detection. However, the sensitivity of preclinical models of

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cardiovascular function to changes in the activity of postganglionic sympathetic neurons is not known. Therefore, the purpose of this study was to compare basal and reflex cardiovascular-related sympathetic activity after sympathetic postganglionic neuronal loss. To do this we treated rats with high doses of guanethidine, a peripherally restricted adrenergic neuron blocking agent, to create selective impairment of sympathetic postganglionic neurons (Burnstock, Evans, Gannon, Heath, & James, 1971; Heath & Burnstock, 1977; Johnson, Cantor, & Douglas, 1975; Johnson, O'Brien, & Werbitt, 1976; Juul & Sand, 1973; Manning, Powers, Schmidt, & Johnson, 1983; Schmidt et al., 1988). Following a 20–28 day recovery period, to eliminate pharmacological and acute toxicological effects of guanethidine, we measured cardiovascular-related sympathetic activity and reflex responsiveness of left renal sympathetic nerve activity (RSNA). We then performed unbiased stereological assessment of neuron number and neuron size in the left T13 sympathetic ganglia.

2. Methods

All procedures were approved by the Pfizer Committee on Animal Care and Use and were consistent with federal law and NIH regulations. Male Sprague–Dawley rats (Charles River, Raleigh, NC) weighing between 400 and 500 g were randomly assigned to 3 groups. To produce variable degrees of neuronal loss 2 separate groups were treated with guanethidine (100 mg/kg/day, i.p.); one for 5 days (n = 12) and another for 11 days (n = 10). The 3rd group of rats was treated with vehicle (saline 1 ml/kg/day, n = 10) for 11 days. A dose of 100 mg/kg/day was chosen for the current study in order to develop a model in which neuronal loss could be induced in a relatively short period of time (1–2 weeks). Preliminary studies, not described here, revealed that 100 mg/kg/day produced clear evidence of neuronal degeneration after as few as 5 daily doses, while lower doses did not; consistent with previous reports (Heath & Evans et al., 1972) that a dose of 100 mg/kg/day causes neuronal depletion within these shorter time periods.

Because 100 mg/kg/day guanethidine treatments decreased food and water intake rats were weighed daily and monitored for signs of dehydration, malaise, and general discomfort. After the final guanethidine treatment rats were given a 20–28 day recovery period to allow the acute pharmacological effects of guanethidine to dissipate. In addition to recovery from the acute pharmacological sympathetic blockade, this recovery period also eliminated the acute inflammatory response that occurs with high dose guanethidine during treatment and which may be involved in the mechanism causing neuronal injury and death (Manning et al., 1983).

2.1. Tissue processing, histology, stereology, and electron microscopy

At the conclusion of the recordings rats were perfused transcardially with 400 ml of physiological saline, followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The left T13 sympathetic ganglia, which contain the cell bodies of the renal sympathetic nerves and correspond to the RSNA, were collected. The formaldehyde-fixed T13 sympathetic ganglia were manually dehydrated and then individually embedded in paraffin.

For stereology serial sections (20 μm) were cut longitudinally through the entire T13 sympathetic ganglion. Every 1st section of each series of 3 sections (interval: 60 μm) was mounted separately on microscope slides (3 sections per slide, 8–12 sections per ganglion). Sections were stained with hematoxylin and eosin (H&E) for neuronal identification. One set of 8–12 sections was sampled in a systematic-random manner from the total number of sections through each rat T13 ganglia. Stereology data was collected with assistance from a commercially available computerized stereology system (Courchesne et al., 2011; Harry et al., 2014; Mouton et al., 2012; Stoner et al., 2014). Total numbers and mean neuron volumes (MNV) of H&E neurons in each ganglion were quantified at high magnification [100 × oil (n.a. 1.4 objective)] using the optical fractionator (Gundersen, 1986; West, Slomianka, & Gundersen, 1991) and rotator (Tandrup, Gundersen, & Jensen, 1997) techniques. Unbiased counting rules were followed for inclusion/exclusion planes and guard volumes for neuron counts. Inclusion criteria for counting H&E neurons included the presence of cell bodies with a clear nucleolus within a nuclear membrane and high cytoplasm:nucleus (C:N) ratio. Special care was taken to exclude non-neuronal cells as previously described (Gundersen, 1986; West et al., 1991). One T13 ganglia from the vehicle group was excluded as statistical outlier due to an atypically low number of neurons (583 neurons vs. mean 11,682 neurons for vehicle group), most likely from incomplete ganglia collection at tissue harvesting.

A T13 ganglion from a vehicle-treated and an 11 day guanethidine-treated rat were selected for further histological and ultrastructural evaluation. Tissues were fixed in Karnovsky's fixative (0.1 M phosphate buffered 2% formaldehyde + 2.5% glutaraldehyde), post-fixed in 0.1 M phosphate-buffered 1% OsO_4, dehydrated through graded ethanols and embedded in Spurr's epoxy resin. Thick sections (0.6 μm) were prepared for light microscopic examination and stained with Toluidine Blue (1% aqueous) or triple-stained as follows: 1% p-phenylenediamine (methanolic), eosin Y (5%, ethanolic), and 1% Toluidine Blue O (aqueous). For electron microscopy, thin sections (~90 nm) were prepared for examination and stained with uranyl acetate (10% aqueous) and Reynold's Lead Citrate. The thin sections were examined on a Hitachi H-7100 transmission electron microscope and representative neurons were digitally recorded (Advanced Microscopy Techniques, Woburn, MA).

2.2. Acute surgical preparation

Rats were initially anesthetized with isoflurane in O_2. An adequate depth of anesthesia was confirmed by the absence of a withdrawal response to a noxious stimulus (tail pinch). The trachea was cannulated and rats were mechanically ventilated using a rodent ventilator (CWE, Ardmore, PA). Expired O_2 and CO_2 concentration were monitored with a gas analyzer (CWE, Ardmore, PA) and CO_2 was maintained at 5–7% by adjusting the respiratory rate. The left carotid artery was cannulated and the BP was measured directly with a pressure transducer. The left jugular vein was cannulated for intravenous injection of drugs. Isoflurane exposure was discontinued after urethane (1.25 g/kg, i.v., Sigma) was administered. This dose of urethane has proven to produce a level of deep surgical anesthesia that persists for several hours with minimal effects on sympathetic nervous system activity (Field, White, & Lang, 1993; Silva & Schreihofer, 2011; Xing & Pilowsky, 2010). During renal nerve recordings (see below) rats were treated with neuromuscular blocking agent gallamine triethiodide (40 mg/kg i.v.). During neuromuscular blockade the depth of anesthesia was determined by either the corneal reflex before and during the recovery from paralysis or by the variability of RSNA and BP when rats were paralyzed. Body temperature was maintained at 37–38 °C with a heating lamp. RSNA, BP, and heart rate (HR) were simultaneously recorded with Cambridge Electronic Design Micro1401 hardware and Spike 2® software (Cambridge, UK.).

2.3. Renal sympathetic nerve recording

Preparation for RSNA recording has been described elsewhere in detail (Chau, Kim, & Schramm, 1997). Briefly, the left kidney was exposed through a left flank incision via a retroperitoneal approach. The renal nerve was carefully dissected from the renal vasculature and surrounding tissue with the aid of an operating microscope. The peritoneum was immersed in warm mineral oil and the renal sympathetic nerve mounted on a stainless steel bipolar hook electrode connected to a differential amplifier with a bandpass of 300–3000 Hz. The sympathetic activity was further processed by rectification and lowpass filtering at