Research Report

Hydralazine administration activates sympathetic preganglionic neurons whose activity mobilizes glucose and increases cardiovascular function

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Abstract

Hypotensive drugs have been used to identify central neurons that mediate compensatory baroreceptor reflex responses. Such drugs also increase blood glucose. Our aim was to identify the neurochemical phenotypes of sympathetic preganglionic neurons (SPN) and adrenal chromaffin cells activated following hydralazine (HDZ; 10 mg/kg) administration in rats, and utilize this and SPN target organ destination to ascribe their function as cardiovascular or glucose regulating. Blood glucose was measured and adrenal chromaffin cell activation was assessed using c-Fos immunoreactivity (ir) and phosphorylation of tyrosine hydroxylase, respectively. The activation and neurochemical phenotype of SPN innervating the adrenal glands and celiac ganglia were determined using the retrograde tracer cholera toxin B subunit, in combination with in situ hybridization and immunohistochemistry. Blood glucose was elevated at multiple time points following HDZ administration but little evidence of chromaffin cell activation was seen suggesting non-adrenal mechanisms contribute to the sustained hyperglycemia. 16 \( \pm \) 0.1\% of T4-T11 SPN contained c-Fos and of these: 24.3 \( \pm \) 1.4\% projected to adrenal glands and 29.7 \( \pm \) 5.5\% projected to celiac ganglia with the rest innervating other targets. 62.8 \( \pm \) 1.4\% of SPN innervating adrenal glands were activated and 29.9 \( \pm \) 3.3\% expressed PPE mRNA whereas 53.2 \( \pm \) 8.6\% of SPN innervating celiac ganglia were activated and 31.2 \( \pm \) 8.8\% expressed PPE mRNA. CART-ir SPN innervating each target were also activated and did not co-express PPE mRNA. Neurochemical coding reveals that HDZ administration activates both PPE\textsuperscript{+}SPN, whose activity increase glucose mobilization causing hyperglycemia, as well as CART\textsuperscript{+}SPN whose activity drive vasomotor responses mediated by baroreceptor unloading to raise vascular tone and heart rate.

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1. Introduction

Immunoreactivity for the protein c-Fos has been used to identify neurons in the central nervous system activated by drugs that cause hypotension (Badoer et al., 1993; Burman et al., 2004; Chan and Sawchenko, 1994; Stornetta et al., 2001) or hypertension (Dampney and Horie, 2003; Li and Dampney, 1994). Activated neurons were found in the nucleus of the solitary tract, the ventrolateral medulla and the lateral horn of the spinal cord, which contains sympathetic preganglionic neurons (SPN), therefore it was postulated that these stimuli identify neurons participating in baroreceptor reflex mediated changes that restore hemodynamic homeostasis (Chan and Sawchenko, 1994; Dampney and Horie, 2003; Minson et al., 1996a, 1996b, 1996c; Minson et al., 1996b, 1997). It should be noted that non-blood pressure related drug effects were never tested in these studies. Nevertheless, in response to hypotensive stimuli c-Fos immunoreactivity (ir) was found in the spinal cord only in SPN, predominantly in T5 to T13 spinal segments (Burman et al., 2001; Fenwick et al., 2006; Minson et al., 1996a, 1996b, 2002). The targets of these activated SPN include the adrenal medulla (Minson et al., 1996a) but must also include the vasculature and heart in order to convey the baroreflex mediated effects. However, hypotensive stimuli also evoke increases in plasma glucose and this seems independent of the hypotensive agent used for example: hydralazine (Sanbar and de Romero, 1969; Satoh et al., 1980), sodium nitroprusside (Boquist, 1989; Staquet et al., 1965), and diazoxide (Altszuler et al., 1977). Increased plasma glucose mediated by sympathetically mediated activation arises predominantly from stimulation of the splanchnic nerve. This nerve directly innervates the adrenal medulla to increase catecholamines and via the celiac ganglia innervates the pancreas, to release glucagon and inhibit insulin secretion, and the liver (Yamaguchi, 1992; Yi et al., 2010). Sympathetic activation of these effectors increases hepatic glycogenolysis and gluconeogenesis (Yamaguchi, 1992; Yi et al., 2010). SPN responsible for both the sympathetically mediated hemodynamic responses and also the hyperglycemia evoked by hydralazine administration have not been neurochemically identified or differentiated.

A range of neurochemicals have been described in SPN, particularly in those projecting to the adrenal gland and celiac ganglia, including pituitary adenylate cyclase activating polypeptide (PACAP) mRNA, pre-proenkephalin (PPE) mRNA, nitric oxide synthase (NOS), cocaine and amphetamine-regulated transcript (CART) and calretinin (Edwards et al., 1996; Fenwick et al., 2006; Hinrichs and Llewellyn-Smith, 2009; Kumar et al., 2010; Parker et al., 2013). We have shown previously that glucoprivation, induced by 2-deoxy-D-glucose, which does not change blood pressure, activates all SPN projecting to the adrenal glands and celiac ganglia that express PPE mRNA and only activates adrenergic chromaffin cells (Parker et al., 2013) suggesting PPE mRNA codes SPN regulating glycemic but not cardiovascular responses. This is in keeping with the findings that enkephalergic terminals preferentially target adrenergic chromaffin cells (Holbert et al., 1995, 1996, 1998; Peltot-Huikko et al., 1987) and that adrenal SPN responsive to glucopenia were not responsive to baroreflex reflex activation (Cao and Morrison, 2000). Conversely, PPCART mRNA was not found in any adrenal or celiac ganglia projecting SPN activated following glucoprivation (Parker et al., 2013) suggesting these SPN are not involved in regulating glycemia. This was not surprising as it has been suggested that CART-ir SPN predominantly have cardiovascular targets (Gonzalez et al., 2010) including noradrenergic chromaffin cells and post ganglionic neurons innervating the vasculature. It is perhaps surprising that adrenal chromaffin cells activated in response to hypotensive stimuli have not been identified particularly considering hypotensive stimuli have been correlated with increases in both plasma catecholamines, at least at early time points (Altszuler et al., 1977; Madden et al., 2006; Staquet et al., 1965; Vollmer et al., 2000).

The objective of this study was therefore to determine the effects of hydralazine on the sympathoadrenal and sympathoceliac pathways. The major aim was to describe the neurochemical phenotype of SPN that project to the adrenal medulla and celiac ganglia activated following hydralazine administration. We hypothesized that SPN projecting to the adrenal gland activated following hydralazine would include those which express PPE mRNA and those which express CART-ir in order to regulate the release of adrenaline and noradrenaline, respectively. Similar phenotypes would also distinguish SPN that project to the celiac ganglia to influence glucose mobilization or hemodynamic homeostasis. Multi-label immunohistochemistry, in combination with in situ hybridization, was used to investigate for the first time the expression of neurochemicals in activated SPN with known projection targets following hydralazine administration. We also assessed the activation of adrenal chromaffin cells using c-Fos-ir and sensitive measures of catecholamine synthesis, i.e. phosphorylation of tyrosine hydroxylase which indicate increased capacity for catecholamine release (Damanhuri et al., 2012) across multiple time points.

2. Results

2.1. HDZ administration increased blood glucose over time

In order to confirm that blood glucose levels increased following hydralazine (HDZ) administration as described previously (Sanbar and de Romero, 1969; Satoh et al., 1980), measurements were made following injection at: 20 min (HDZ, 13.1 ± 1.4 mmol/l; n = 5; vs control 10.2 ± 0.6 mmol/l; n = 5, p < 0.08), 60 min (HDZ 16.3 ± 1.1 mmol/l; n = 6; vs control 9.8 ± 0.6 mmol/l; n = 6, p < 0.001) and 120 min (23.8 ± 2.8 mmol/l; n = 7; vs 10.6 ± 0.8 mmol/l; n = 7 p < 0.001).

2.2. Plasma catecholamines were not elevated 120 min following HDZ administration

Several previous studies have demonstrated that plasma catecholamines are increased early following HDZ administration (Madden et al., 2006; Vollmer et al., 2000) so in the current study plasma catecholamines were determined 120 min after injection (adrenaline HDZ 0.4 ± 0.2 ng/ml; n = 7 vs saline 0.5 ± 0.1 ng/ml; n = 7; noradrenaline HDZ 0.4 ± 0.1 ng/ml; n = 7 vs saline 0.6 ± 0.1 ng/ml; n = 7).
2.3. HDZ administration did not evoke c-Fos expression in adrenal medulla chromaffin cells

Activation of adrenal chromaffin cells was determined by c-Fos-ir. Chromaffin cells did not express c-Fos-ir 120 min following saline injection (Fig. 1a–d, n=7). Surprisingly, c-Fos-ir was not present in any chromaffin cells 120 min following HDZ injection (n=7), except in one animal where a few faint c-Fos-ir cells were seen located in CART-ir but not PNMT-ir chromaffin cells (Fig. 1e–h). As we have previously reported following 2DG (Parker et al., 2013), c-Fos-ir was expressed in almost all PNMT-ir but not CART-ir chromaffin cells (n=4) and illustrate this for comparison (Fig. 1i–l).

2.4. HDZ administration increased Ser31TH phosphorylation but did not change the phosphorylation of Ser19TH, Ser40TH, MAPK or total TH protein in the adrenal medulla

Released adrenal catecholamines are initially replenished by increasing the activity of the enzyme tyrosine hydroxylase, induced by its phosphorylation (Dunkley et al., 2004). In keeping with minimal c-Fos expression of the adrenal medulla, no changes in the phosphorylation of Ser19TH or Ser40TH were observed at any time point (5 min; n=4) (20 min; n=6) (60 min; n=6) (120 min; n=3) compared to control (5 min; n=4) (20 min; n=5) (60 min; n=5) (120 min; n=3) (Fig. 2a and c). However, phosphorylation of Ser31 was increased at 60 min (4.75 fold; n=6, p<0.0001) and returned to baseline by 120 min with no change at 5 (n=4) and 20 min (n=6) following HDZ injection (Fig. 2b).

As mitogen-activated protein kinase (MAPK) activity is strongly linked to Ser31 phosphorylation (Dunkley et al., 2004), pMAPK relative to total MAPK protein levels were quantified. No significant changes were seen at any time point when HDZ was compared to saline (Fig. 2e). Not surprisingly, total TH protein did not change relative to β-actin protein (20 min; n=4) (60 min; n=6) (120 min; n=6) following HDZ injection compared to saline controls (20 min; n=4) (60 min; n=6) (120 min; n=5) (Fig. 2d) indicating that TH protein levels were unchanged.

2.5. SPN were activated following HDZ treatment

In order to determine whether or not HDZ administration activated SPN, the number of ChAT-ir SPN that expressed c-Fos-ir was determined in the T4-T11 segments of the spinal cord. Following HDZ treatment 16±0.1% (460/2977 total neurons;
n=3) SPN contained both markers. No c-Fos-ir, ChAT-ir cells were seen in control animals (0/1708 total neurons; \( n=3; p<0.0001 \)).

2.6. SPN that project to the adrenal gland and celiac ganglion were activated following HDZ administration

In order to identify the targets of SPN activated following HDZ treatment we determined whether or not c-Fos-ir SPN projected to the adrenal gland or celiac ganglia (identified by the presence of the retrograde tracer, cholera toxin subunit B; CTB). Fig. 3a–f shows a representative example in which CTB labeled SPN, projecting to the adrenal gland and/or celiac ganglia, were seen surrounded by CART-ir fibers. Many contained c-Fos-ir and a proportion of these also expressed PPE mRNA (see Section 2.7).

Of all c-Fos-ir neurons identified, 24.3\( \pm \)1.4% projected to the adrenal gland, 29\( \pm \)5.5% projected to the celiac ganglia and the rest (53.9\( \pm \)2.1% of 1144 c-Fos-ir neurons; \( n=3 \)) projected elsewhere or were interneurons. Of the adrenally projecting SPN population, 62.8\( \pm \)1.4% (277/441 total neurons; \( n=3 \)) contained c-Fos-ir following HDZ injection. Of SPN projecting only to the celiac ganglia, 53.2\( \pm \)8.6% (332/712 total neurons; \( n=3 \)) contained c-Fos-ir following HDZ injection. In contrast following saline injection, very few SPN projecting to the adrenal gland (0.05\( \pm \)0.03%, 4/815 total neurons; \( n=4 \)) or celiac ganglia (0.02\( \pm \)0.02%, 6/1082 total neurons; \( n=4 \)) expressed c-Fos-ir (\( p<0.0001 \)).

2.7. SPN projecting to the adrenal gland or celiac ganglion activated following HDZ administration contain PPE mRNA or CART-ir

In order to identify the neurochemical phenotype of neurons activated following HDZ administration and their targets we determined whether c-Fos-ir neurons projecting to the adrenal gland or celiac ganglia expressed PPE mRNA and/or CART-ir. The density of terminal labeling surrounding SPN prevented the accurate counting of all CART-ir neurons therefore, where clear CART-ir neurons could be distinguished, these were qualitatively assessed with respect to activation and target.

It was evident that many SPN expressing c-Fos-ir contained CART-ir. In addition, 24.1\( \pm \)5.2% (275/1144 c-Fos-ir neurons; \( n=3 \)) of c-Fos-ir neurons in the spinal cord expressed PPE mRNA. However, where clear somatic CART-ir was present no evidence of PPE mRNA was ever seen suggesting they are not co-localized in SPN.

Many c-Fos-ir SPN that projected to the celiac ganglia contained CART-ir (Fig. 3d) with fewer projecting to the adrenal gland (data not shown). SPN activated following HDZ treatment that projected to either the adrenal gland or the celiac ganglia expressed PPE mRNA: 29.9\( \pm \)3.3% (85/277 c-Fos-ir neurons; \( n=3 \)) of c-Fos-ir neurons projecting to the adrenal gland contained PPE mRNA and 31.2\( \pm \)8.8% (114/332 c-Fos-ir neurons; \( n=3 \)) of c-Fos-ir neurons projecting to the celiac ganglia expressed PPE mRNA.

3. Discussion

This study demonstrates that SPN whose activity increase either glucose mobilization or vascular tone and heart rate are both stimulated following administration of HDZ, a blood pressure lowering agent. The major finding is the identification of the neurochemical phenotypes of these two functionally distinct populations of SPN, which project to the adrenal medulla and/or celiac ganglia. In addition, we have shown that HDZ administration does not evoke c-Fos-ir or strongly phosphorylate TH in adrenal chromaffin cells, suggesting that the...
adrenal medulla is not strongly stimulated or this stimulus is attenuated early and indicates that the sustained hyperglycemia measured is likely mediated by other mechanisms. Data presented here suggest that sympathetic activation of the liver and/or pancreas via the celiac ganglia may be responsible.

Our study found that approximately 16% of T4-T11 SPN were activated following HDZ administration which was similar to the 17% of SPN calculated from the data reported by Minson et al. (2002) when sodium nitroprusside (SNP) was used. Other studies have also described c-Fos-ir SPN following hypotensive stimuli (Burman et al., 2001; Fenwick et al., 2006; Minson et al., 1997) but did not identify the targets of these neurons. Of the activated SPN in the present study, 25% projected to the adrenal glands, 30% projected to the celiac ganglia and the remainder (45%) projected to other targets or were interneurons.

Approximately 63% of SPN that projected to the adrenal gland were activated following HDZ treatment which was similar to the 60% of the SPN emerged in the data reported by Minson et al. (2002) when SNP was used. Other studies have also observed c-Fos-ir SPN following hypotensive stimuli (Burman et al., 2001; Fenwick et al., 2006; Minson et al., 1997) but did not identify the targets of these neurons. Of the activated SPN in the present study, 25% projected to the adrenal glands, 30% projected to the celiac ganglia and the remainder (45%) projected to other targets or were interneurons.

In light of these findings, noradrenergic and adrenergic chromaffin cells were expected to be activated following HDZ administration following HDZ treatment with about a third expressing prepro-enkephalin (PPE) mRNA. Glucoprivation, which results in glucose mobilization, activated all PPE mRNA expressing SPN innervating the adrenal glands and only adrenergic chromaffin cells (Parker et al., 2013). As HDZ administration also activated PPE mRNA expressing SPN innervating the adrenal gland these were likely candidates to evoke the hyperglycemia. This is further supported as adrenally projecting SPN activated by 2-deoxy-D-glucose (2DG) are insensitive to baroreceptor stimulation (Morrison and Cao, 2000) suggesting that mechanisms unrelated to baroreflex unloading activated the PPE expressing SPN. On the other hand CART-ir SPN that projected to the adrenal gland were also activated by HDZ treatment in the present study, in keeping with previous reports (Fenwick et al., 2006), although no target was defined in the previous study. CART-ir terminals innervate only noradrenergic chromaffin cells (Gonsalvez et al., 2010) suggesting these SPN regulate adrenal noradrenaline release which might contribute to compensatory vascular and cardiac effects caused by HDZ induced hypotension (Pelayo et al., 2002). Together these data support the idea that functionally defined subgroups of SPN controlling adrenaline or noradrenaline release from the adrenal medulla can be defined by their neurochemistry. Therefore, activation of SPN innervating the adrenal gland following HDZ administration likely regulates both cardiovascular targets and glucose mobilization.

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...tissue perfusion pressures. ...stimulus for glucose mobilization following HDZ administration. Further- innervation of the adrenal medulla and the liver were explained by multiple direct actions that HDZ has on chromaf ...

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...blunting of the adrenal medullary response and particularly which contrasts with the robust activation seen following 2DG administration (Parker et al., 2013; Ritter et al., 1998), may be explained by multiple direct actions that HDZ has on chromaffin cells. HDZ reduces exocytosis (Machado et al., 2002) reducing catecholamine secretion by blocking calcium influx to the cells (Nakanishi et al., 1986) possibly via an IP3 dependent mechanism (Yang et al., 2005). It appears that this may overcome the effects of sympathetically-released acetylcholine thus preventing the sustained activation required for c-Fos production. HDZ also reduces the activity of TH and dopamine beta hydroxylase: by its metal chelating activity and as a result of allosteric actions (Houchi et al., 1986; Morita et al., 1986; Songkittiguna et al., 1980).

Together these data suggest that some catecholamine is released from the adrenal medulla early following HDZ administration mediated by activation of SPN identified above. The catechola-

...response is abrogated due to direct actions of the drug on the chromaffin cells. The present study demonstrated that plasma glucose increased for at least 2 h following HDZ treatment raising the likelihood that other glucose mobilizing pathways were activated. In keeping with this 53% of SPN projecting to the celiac ganglia, of which 30% expressed PPE mRNA, were activated by HDZ administration. As glucoprivation activated all PPE mRNA expressing SPN that supply the celiac ganglia (Parker et al., 2013), PPE mRNA containing SPN activated in the present study could regulate hepatic glycogenolysis and gluconeogenesis. These anatomical data are supported by functional data which show that removal of the adrenal glands and hepatic denervation together abolished hyperglycemia in response to hemorrhagic hypotension in dog (Briand et al., 1989a, 1989b) suggesting that both the sympathetic innervation of the adrenal medulla and the liver were activated by the hypotension (Briand et al., 1990). Furthermore ganglionic blockade prevented hyperglycemia evoked by HDZ administration (Sunaga and Oghara, 1990). The stimulus for glucose mobilization following HDZ administra-

...are in keeping with the idea that neurochemically distinct subgroups of SPN innervating the celiac ganglia influence functionally specific outputs.

...is important to note however that about half of all SPN activated following HDZ administration projected to targets other than the adrenal gland or celiac ganglia. It is likely that many of these also express CART in keeping with the need to increase vascular tone (Gonsalvez et al., 2010) in order to compensate for the blood pressure lowering effects of the drug.

3.1. Conclusions

HDZ administration activates 16% of SPN to mediate both the well described baroreceptor reflex mediated changes that restore hemodynamic homeostasis as well as to induce hyperglycemia. The early increase in glucose is likely influenced by PPE expressing SPN innervating adrenergic chromaffin cells with later increases likely mediated by PPE expressing SPN innervating the celiac ganglia. Increases in vascular tone and heart rate could be mediated by CART containing SPN that innervate the adrenal glands, celiac ganglia and other targets. The neurochemical phenotypes and ultimate targets of other SPN activated by hypotension require further investigation. Interestingly, HDZ is an anti-hypertensive medication used in select populations of patients and our acute data showing the activation of sympathetic pathways which mobilize glucose suggests that with repeated administration HDZ could potentially contribute to insulin resistance. In this light it is curious that the incidence of new onset diabetes is found to increase in long term studies of patients prescribed different anti-hypertensive drugs (Mancia et al., 2006; Taylor et al., 2006), including hydralazine (Bhalla, 2008), although there is some controversy (Jong et al., 2009). Knowledge of the phenotypes of SPN that control specific functions is essential to understanding sympathetic nervous system functions.

4. Experimental procedure

4.1. Animals

All experimental procedures were approved by the Macquarie University Animal Ethics Committee and carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Experiments were performed on adult male Sprague-Dawley rats (n=51; 300-500 g, 10–20 weeks old), obtained from the Animal Resource Centre, Perth, WA, Australia and housed at the Central Animal House Facility, Macquarie University. One day prior to experimentation rats were housed singly in a temperature controlled room (21 ± 1 °C) with ad libitum access to food and water for a minimum of 12 h in order to minimize the stress of isolation.
4.2 Injection of retrograde tracers into the adrenal gland and celiac ganglion

A subset of animals (n=7) were anesthetized with a mixture of the sedative medetomidine (0.75 mg/kg; ip) and the anesthetic ketamine (75 mg/kg; ip) and then administered the non-steroidal anti-inflammatory Carprofen (2.5 mg/kg sc.) and the antibiotic Cephazolin (0.55 g/kg im.). The right adrenal gland and celiac ganglion were exposed using a retroperitoneal approach and injected with 1–2 μl of fluorescently conjugated cholera toxin subunit B (CTB) either Alexa Fluor (R) 488 nm (C-22841; 1% in saline; Invitrogen Molecular Probes, Australia) for adrenal glands or Alexa Fluor (R) 555 nm (C-22843; 1% in saline; Invitrogen Molecular Probes, Australia) conjugate for celiac ganglia as described previously (Parker et al., 2013). The animals were left to recover for 7 days.

4.3 Hypotensive or vehicle challenge

Animals received an intraperitoneal injection of vehicle (0.4 ml; 0.9% NaCl; n=23), 10 mg/kg hydralazine (HDZ; n=24; Sigma Chemical Co. St Louis, MO, USA) or 2-deoxy-d-glucose (2DG; n=4; 400 mg/kg; Sigma Chemical Co. St Louis, MO, USA) and food and water were withheld until perfusion or decapitation. In conscious or lightly anesthetized (50 mg/kg sodium pentobarbital; intravenous) adult male Sprague-Dawley rats it has been well established that HDZ (10 mg/kg) reduces mean arterial pressure (MAP) to 50–70% (~50–70 mmHg) of resting level MAP (~90–125 mmHg) within 5–10 min and is sustained at 2 h (Fenwick et al., 2006; Springell et al., 2005; Stornetta et al., 2001; Vollmer et al., 2000). 2DG was used to compare high levels of selective adrenergic chromaffin cell activation as we have shown previously (Parker et al., 2013).

4.4 Perfusion and tissue collection

A subset of animals were sacrificed by decapitation following anesthesia (sodium pentobarbital; 100 mg/kg ip.) at four time points: 5 min (n=5 HDZ; n=5 saline), 20 min (n=6 HDZ; n=6 saline), 60 min (n=6 HDZ; n=5 saline) and 120 min (n=7 HDZ; n=7 saline) following HDZ or saline injection as described previously (Bobrovskaya et al., 2010). Adrenal glands were collected and snap frozen for Western blot analysis.

Animals which had received spinal cord injections of tracer were randomly selected to receive saline (n=4) or HDZ (n=3) and at 120 min following injection were anesthetized with sodium pentobarbital (100 mg/kg ip.) and perfused transcardially using 300 ml of ice-cold Dulbecco’s Modified Eagle Medium (Sigma-Aldrich, Australia) followed by 300 ml of ice cold fixative (4% paraformaldehyde/0.1 M phosphate buffer; pH 7.4; Sigma-Aldrich, Australia). Untreated animals were randomly selected to receive saline (n=3), HDZ (n=3) or 2DG (n=4) and were perfused 120 min following treatment as described above. Spinal cords from T4-T11 and non-injected adrenal glands were removed for immunohistochemistry (IHC) and placed overnight in the perfusion fixative at 4°C prior to sectioning. Spinal cord segments were divided parasagittally and all tissues were sectioned (40 μm in thickness) on a vibrating microtome (VT1200S; Leica Microsystems, North Ryde, NSW, Australia) into pots containing phosphate buffered saline with 0.1% Tween-20 (Sigma-Aldrich, Australia).

4.5 Site-specific TH phosphorylation and TH protein in whole adrenal gland

Each adrenal gland was weighed and homogenized in 40 volumes of homogenization buffer (2% SDS, 2 mM EDTA, 50 mM Tris (pH 6.8)), sonicated on ice (3 times x 30s at 10,000 A), then boiled for 5 min and centrifuged for 20 min. Supernatants were carefully removed and protein concentration was determined by BCA assay (Thermo Fisher Scientific, IL, USA). 100 μl of supernatant was mixed with 5 μl of 10% dithiothreitol (DTT) and 35 μl of sample buffer (40% glycerol, 50 mM Tris, minimal bromophenol blue, pH 6.8) to make up a final protein concentration of 2 μg/μl in each sample. 30 μg from each sample was run on 10% Criterion Tris–HCl precast gels and then transferred onto nitrocellulose membrane as previously described (Bobrovskaya et al., 2010). Membranes were immunoblotted with total or phospho-specific TH antibodies overnight at 4°C. The levels of pSer19TH, pSer31TH, pSer40TH, total TH (tTH) protein and β-actin protein were determined using specific antibodies which have been characterized previously (Bobrovskaya et al., 2010; Gordon et al., 2009). Secondary antibodies (goat anti-rabbit, goat antimouse or rabbit anti-sheep immunoglobulin) were applied for 1 h at room temperature. The immunoblots were visualized and quantified on an LAS4000 imaging system (GE Health Care, Little Chalfont, UK) using the ECL-advanced detection reagent (GE Health Care, Little Chalfont, UK). Densitometry was used to measure the protein band. All analysis was done using Fujifilm Multigauge v3.0 software (Tokyo, Japan) and expressed as a fold increase relative to basal. Loading controls were performed by analyzing the amount of total TH protein and β-actin protein in each sample. Site-specific TH phosphorylation was expressed as the ratio of TH phosphorylation at Ser19, Ser31 or Ser40 to total TH protein, to account for variability in total TH protein between samples. Total TH protein levels were expressed as the ratio of TH protein to β-actin as β-actin levels are largely invariable and commonly used as a housekeeping protein.

4.6 Measurement of blood glucose and plasma catecholamines

Blood was collected from all animals <5 min following anesthesia. Blood glucose levels were determined at 20, 60 and 120 min post HDZ using a glucometer (Roche AccuCheck performa glucometer; Mannheim, Germany). Blood samples collected 120 min post HDZ were centrifuged for 10 min at 1800 rpm (4°C) and then 10 min at 2700 rpm (4°C) to separate plasma from red blood cells, leukocytes, and platelets and stored at −80°C. Plasma catecholamines were extracted and measured on a HTEC-500 Complete Stand-Alone HPLC-ECD system (Eicom Corporation, Kyoto Japan) as described previously (Bobrovskaya et al., 2010; Damanhuri et al., 2012; Parker et al., 2013) with slight modifications on the method of Anton and Sayre (1962). The specific retention times were determined using adrenaline and noradrenaline standards. The amount of catecholamine from each sample was
4.7. Combined in situ hybridization (ISH) and immunohistochemistry (IHC)

In-situ hybridization for custom synthesized and validated prepro-encephalin (PPE) riboprobe incorporated with digoxigenin (DIG)-11-UTP (Roche Applied Science, Mannheim, Germany) was performed (final concentration 1000 ng/ml) in conjunction with fluorescent immunohistochemical (IHC) detection as described previously (Bowman et al., 2013; Kumar et al., 2010; Parker et al., 2013). No alkaline phosphatase reaction product is seen after hybridization with our PPE mRNA sense probe, or in the absence of antisense DIG-riboprobes in rat spinal cord tissue (Kumar et al., 2010; Parker et al., 2013). Table 1 details the antibodies used. Polyclonal sheep anti-digoxigenin (DIG) antibody recognizes the DIG-11-UTP incorporated into the riboprobe during in vitro transcription. Polyclonal anti-choline acetyltransferase (ChAT) antibody was used to identify SPN, which were determined by size and location in nests within the intermediolateral cell column (ML) of the spinal cord (Fenwick et al., 2006; Hinrichs and Llewellyn-Smith, 2009; Parker et al., 2013). ChAT is also found in ventral horn motoneurons which were not analyzed. Polyclonal rabbit anti-c-Fos antibody was used to identify activated neurons. c-Fos immunoreactivity was rarely seen in tissue from saline injected animals. Polyclonal sheep anti-phenylethanolamine N-methyltransferase (PNMT) was used to mark adrenergic adrenal chromafin cells (Parker et al., 2013; Phillips et al., 2001). Monoclonal mouse anti-cocaine-and amphetamine-regulated transcript (CART) was used to mark subpopulations of SPN as well as adrenal chromafin cells which lack PNMT-ir (Couceyro et al., 1997; Dun et al., 2006; Gonsalvez et al., 2010; Koylu et al., 1997).

4.8. Data analysis

In animals that did not receive tracer injections but were perfused, SPN were identified by ChAT immunoreactivity (-ir) and then quantitatively analyzed for the presence or absence of c-Fos-ir. In the animals which received tracer injections, each CTB labeled neuron was counted and reported separately by the sympathetic outflow which they innervated. SPN were then analyzed for the presence or absence of c-Fos-ir, CART-ir and PPE mRNA. The distribution of CART-ir was used to help determine the location of SPN as it is strongly expressed in the intermediolateral cell column (IML) and is also found in the central autonomic area (CAA), and dorsolateral funiculus (DLF) (Dun et al., 2000; Fenwick et al., 2006; Gonsalvez et al., 2010). Qualitative analysis of PNMT, CART and c-Fos-ir was also determined in adrenal glands by examining their distribution.

T4 marks the rostral extent of the adrenal and celiac projecting SPN (Strack et al., 1988). Since different fluorophores labeled the CTB tracers, SPN containing either tracer was easily detected in the same section simultaneously. All CTB labelled SPN projecting to the adrenal glands were counted. A population of SPN contained both CTB fluorophores, most likely arising from the fact that adrenal nerves passing through the celiac ganglia plexus took up CTB. All neurons double labelled

<table>
<thead>
<tr>
<th>Table 1 – Primary and secondary antisera.</th>
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<tr>
<td><strong>Primary antisera</strong></td>
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<tr>
<td>DIG AP conjugate</td>
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<tr>
<td>ChAT</td>
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<tr>
<td>c-Fos</td>
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<tr>
<td>CART</td>
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<td>PNMT</td>
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<tr>
<td><strong>Secondary antisera</strong></td>
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<tr>
<td>Rabbit IgG Cy3</td>
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<tr>
<td>Rabbit IgG Dylight 649</td>
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<td>Mouse IgG Alexa Fluor® 350</td>
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<tr>
<td>Mouse IgG Alexa Fluor®488</td>
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<tr>
<td>Goat IgG Alexa Fluor®488</td>
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<tr>
<td>Sheep IgG Alexa Fluor® 647</td>
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Processed sections were wet mounted on glass slides in TBS buffer and coverslipped for viewing using an AxioImager Z2 microscope under epifluorescence and brightfield illumination (Zeiss, Gottingen, Germany). Mosaic images of each parasagittal spinal cord section or adrenal medulla cross section were captured at 10 × magnification using AxioVision software (Version 4.8). Each tile in the mosaic was imaged separately but sequentially. Color channels were overlaid in the resultant image file for analysis. Images were acquired and adjusted for brightness and contrast using Axiovision software (Version 4.8, Carl Zeiss, Germany).

calculated by the peak height/area ratio relative to DHBA using PowerChrom v2.6.3 software (eDAQ Pty Ltd, NSW Australia).
with Alexafluor-488 and Alexafluor-555 were quantified and presumed to be adrenal projecting only and were therefore excluded from celiac ganglia quantification. Co-expression with peptide mRNA was calculated as a percentage of CTB positive and/or c-Fos-ir SPN. All data is presented as mean±SEM. Data was compiled using Graphpad Prism software (Version 5.04) and figures were produced using Corel-Draw software (Version 14). Student’s t-test was used for statistical analysis where p<0.05 was considered significant.

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