CNS origins of the sympathetic nervous system outflow to brown adipose tissue

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Bamshad, Maryam, C. Kay Song, and Timothy J. Bartness. CNS origins of the sympathetic nervous system outflow to brown adipose tissue. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R1569–R1578, 1999.—Brown adipose tissue (BAT) plays a critical role in cold- and diet-induced thermogenesis. Although BAT is densely innervated by the sympathetic nervous system (SNS), little is known about the central nervous system (CNS) origins of this innervation. The purpose of the present experiment was to determine the neuroanatomic chain of functionally connected neurons from the CNS to BAT. A transneuronal viral tract tracer, Bartha’s K strain of the pseudorabies virus (PRV), was injected into the interscapular BAT of Siberian hamsters. The animals were killed 4 and 6 days postinjection, and the infected neurons were visualized by immunocytochemistry. PRV-infected neurons were found in the spinal cord, brain stem, midbrain, and forebrain. The intensity of labeled neurons in the forebrain varied from heavy infections in the medial preoptic area and paraventricular hypothalamic nucleus to few infections in the ventromedial hypothalamic nucleus, with moderate infections in the suprachiasmatic and lateral hypothalamic nuclei. These results define the SNS outflow from the brain to BAT for the first time in any species.

Brown adipose tissue (BAT) plays a critical role in the increased heat production occurring with cold exposure (for review see Ref. 19) or overeating (for review see Ref. 20). For example, the ventromedial hypothalamus (VMH) has been implicated in the control of BAT thermogenesis, because electrical or chemical stimulation of the VMH results in morphological and biochemical changes in IBAT as well as changes in the firing rates of the SNS intercostal nerves innervating IBAT (e.g., Ref. 4; 2, 5, 18, 23, 35, 40, 46, 54, 56–58). Complementary lesion studies appear to implicate further the role of VMH in BAT thermogenesis (e.g., Ref. 14; 21, 22, 32, 38, 43, 45, 48, 60, 61). The interpretation of these VMH stimulation or lesion data and of stimulation and lesion data of other brain sites (e.g., Ref. 6; 3, 10, 13, 15, 25, 50) on BAT thermogenesis can be difficult because of the inherent problems of confining the stimulation or lesion to these brain targets of interest. Although several brain regions have been implicated in the regulation of BAT function as a result of these stimulation and lesion studies, no neuroanatomic data exist for the origins of the SNS outflow from brain to BAT.

The present experiment was designed to answer the question: what is the neuroanatomic chain of functionally connected neurons from the CNS to BAT? This was accomplished with the use of a transneuronal viral tract tracer, Bartha’s K strain of the pseudorabies virus (PRV). A similar strategy was successful in defining the CNS origins of the SNS outflow from brain to white adipose tissue (WAT) recently (7).

METHODS

Animals. Adult male Siberian hamsters (Phodopus sungorus sungorus) were obtained from a breeding colony maintained at Georgia State University. The hamsters were initially group-housed (10–12/cage) in polyvinyl cages (48 × 27 × 15 cm) and kept in a light-dark (LD) cycle that simulated long-day, summerlike conditions (16:8 LD with lights on at 0900 and 1100 under a fume hood). The time course for PRV infection was determined by killing the animals at various intervals after PRV injections. Our previous PRV study in WAT of Siberian hamsters (7) showed that infection in brain stem neurons could be detected 96 h after PRV injection into this tissue. The infection reaches the hypothalamus and extends into the forebrain areas by 144 h after PRV infection into this tissue.
injections. Therefore, to induce infections extending into the forebrain areas, hamsters were killed at intervals of 96 h, or 4 days (n = 4 hamsters), and 144 h, or 6 days (n = 4 hamsters). Animals were perfused intracardially with 4% paraformaldehyde in the morning between 0900 and 1100.

Immunocytochemical procedures. The brains were post-fixed overnight in 4% paraformaldehyde followed by overnight incubation in 25% sucrose. Brains were cut coronally into 50-µm-thick sections on a freezing microtome and stored in five vials of 0.1 M sodium phosphate buffer with 0.1% sodium azide. One of every five sets of stored sections was incubated in a pig polyclonal antibody to PRV (donated by Dr. Kenneth Platt, Iowa State Univ.) overnight at room temperature. The PRV antibody was diluted 1:30,000 in a buffer containing 2% normal rabbit serum and 0.3% Triton X-100. The sections were then incubated in biotinylated rabbit anti-pig secondary antibody (Sigma) at a 1:100 dilution for 2 h at room temperature. The sections were then incubated in avidin-biotin horseradish peroxidase complex (Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA) at a 1:200 dilution for 1 h at room temperature. PRV-infected neurons were visualized with 3,3'-diaminobenzidine. The sections were cleared with xylene and placed under coverslips with DPX. The specificity of the immunocytochemical staining for the pig anti-PRV antibody used in this study has been validated previously (51).

The animals that had PRV infections in the brain stem and forebrain neurons were checked for signs of lysis of the infected neurons in the spinal cord. The spinal cords were removed from the infected animals killed on day 6, the longest postinjection interval. The cords were post-fixed overnight in 4% paraformaldehyde followed by overnight incubation in 25% sucrose. The entire length of the spinal cord, including all sections of the cervical, thoracic, and lumbar regions, was cut coronally into 50-µm-thick sections on a freezing microtome. The sections were stored in 0.1 M sodium phosphate buffer with 0.1% sodium azide. They were processed immunocytochemically following the same procedure as described above for brain sections.

Histological quantification. The PRV-labeled neurons in brain sections were localized and quantified using Image Tracer software (Translational Technology) and a stage-mounted position transducer system (MD3 Microscope Digitizer, Minnesota Datametrics). Camera lucida pictures of each brain section, stained with cresyl violet, were drawn and scanned into the computer. A digital image of each drawing was projected onto the computer screen with the Image Tracer program. The image of each brain section was registered with the same section on the microscope stage with the use of the stage transducers. For all animals, the position of PRV-labeled neurons on each brain section was visualized with the microscope and marked in the exact position on the computerized image of that section. The number of marked PRV-labeled neurons was counted in each nucleus for each animal. The absolute number of infected neurons was combined for ipsilateral and contralateral sides of the injection site for each brain region and analyzed statistically using a two-way ANOVA (brain site × time postinjection). Mean percentages of total infected cells for the brain stem, midbrain, and forebrain areas were calculated. Post hoc comparisons were done using Duncan’s new multiple range tests (29).

RESULTS

Of eight hamsters injected with PRV, five became infected. One of the animals killed on day 4 and two of the animals killed on day 6 had no infections. There was no sign of illness in the infected animals with exception of slight weight loss in day 6 animals.

Spinal cord. Quantification of virus distribution within the spinal cord was not conducted; however, the entire length of the spinal cords of day 6 animals, those with the longest postinjection survival period, were inspected microscopically to screen for lysis of the infected cells. In addition, the presence of infected cells in the ventral horns also was determined, because such infections would indicate spread of the virus from the IBAT pad to underlying musculature. The spinal cords were sectioned coronally, and the cervical, thoracic, and lumbar regions were not marked before cord removal; thus it was not possible to determine the exact level of the cord at which neurons were infected. Lysis did not occur in any of the infected neurons, nor were there any cases of infected cells in the ventral horns. Infected neurons were seen only in a well-defined cluster located in the intermediolateral cell group and the central autonomic nucleus of the spinal cord ipsilateral to the injection site (i.e., the SNS preganglionics; Fig. 1).

Data presentation. The distribution of infected neurons is presented schematically in Fig. 2. These seven levels of the neuroaxis were chosen because the densest infections were seen at these levels. Figure 3 is composed of representative microphotographs from infected animals at low and high magnifications. The letter-number designations correspond as closely as possible to the letter designations for each level of the neuroaxis in Fig. 2.

Brain stem. Figures 2, A–C, and 3, A–C, show the distribution of and examples of, respectively, some of the infected neurons in brain stem at day 6 after injection of the virus into IBAT. At this level of the neural axis, the infection was bilateral and uniform.

In day 4 animals, the virus invaded most of the ventrally located nuclei in the brain stem. Labeling was sparse among dorsally located nuclei. For example, among dorsally located nuclei, only a few infected neurons were found throughout the rostrocaudal extent of the dorsal aspects of the nucleus of the solitary

Fig. 1. Photomicrograph illustrating distribution of pseudorabies virus (PRV)-labeled cells in spinal cord of day 6 hamster (longest postinjection period). Bar = 200 µm. CC, central canal; IML, intermediolateral nucleus.
tract (Sol) and the medial aspects of the medial vestibular nucleus (MVe). At the most caudal levels of the brain stem (bregma = −14.30 to −13.30 mm), infection was found in low levels in the neurons of the lateral reticular nucleus and medullary reticular nuclei, dorsal and ventral aspects. At this level, a few infected neurons were found in the raphe pallidus nucleus (Rpa). At the level of the C1 epinephrine cells (C1) and rostroventrolateral reticular nucleus (RVL) regions (bregma = −13.30 to −12.72 mm), intense labeling was seen in the intermediate reticular nucleus (IRt), with moderate labeling of the C1/RVL neurons. Some of the neurons of the parvocellular reticular nucleus also were labeled. More rostrally, the infection in the IRt and C1/RVL regions had intensified. At the level of the MVe, neurons were heavily infected in the C1/RVL region, the caudal raphe, including the Rpa and raphe obscurus nucleus (ROb), and all aspects of the gigantocellular reticular nucleus (Gi). At the level of the facial nucleus, PRV infected the noradrenergic cells (A5) region. The caudal raphe nucleus, the Gi, the lateral paragigantocellular nucleus (LPGi), and the raphe magnus remained heavily infected at this level.

In day 6 animals, the pattern of distribution of infected neurons was quite similar to that of day 4 animals; the infection was contained within the same nuclei. In the majority of nuclei examined, however, intensity of infection was much more severe in day 6 vs. day 4 animals. For example, the virus that had infected the dorsal aspects of the Sol in day 4 animals was transmitted to the lateral and ventral portions of the Sol by day 6. In some regions, such as the caudal raphe (ROb/Rpa), the virus infected neurons in the entire rostrocaudal extent of the nuclei.

Table 1, which shows the mean ± SE of infected neurons in each brain region across days, has been provided to illustrate the difference in degree of infection between day 4 and day 6 animals. The average number of infected neurons combined for all brain stem regions was significantly higher in day 6 than in day 4 animals (F = 30.10, P < 0.001). The difference in average number of infected neurons among brain regions across the two postinjection intervals approached significance (P = 0.07). The mean percentage of total infected cells across all brain stem regions in day 6 hamsters showed that 20.9% of total infected cells were found in the IRt. The A5 had 15.2%, the Rpa/ROb had 12.2%, the LPGi had 11.0%, and the C1 had 10.3% of the total number of infected cells. The lowest mean percentage of total infected cells was in the Gi at 4.3%.

Midbrain. Figures 2D and 3D show the distribution of and examples of, respectively, some of the infected neurons in brain stem at day 6 after injection of the virus into IBAT. The infection also was bilateral and uniform at this level of the nervous axis. The most heavily infected area in the midbrain was the central gray (CG). Infections throughout the rostrocaudal extent of the CG were found in hamsters killed 4 days after PRV injections. The most intense labeling was found in the caudal regions, at the level of the pyramidal tract (Py) in the ventral portions of the CG. More rostrally, at the level of pontine nuclei (Pn; bregma = −8.00 to −7.64 mm), PRV-infected neurons were seen in lateral and ventral portions of the central gray (CGLV) and surrounding areas and posterior to the dorsal raphe nucleus (DR). A few scattered PRV neurons were found in the lateral dorsal central gray (CGLD), but none were found in the dorsal central gray (CGD) or medial central gray.
forebrain regions ($F = 4.37, P < 0.05$), with significantly larger numbers of infected neurons in magnocellular and parvocellular portions of the PVN (combined) and in the MPA ($P < 0.05$). Specifically, 6 days after PRV injections, 38.7% and 32.3% of the total number of infected neurons were found in the PVN and the MPA, respectively. In contrast, only 1.6% of the total number of infected neurons was found in the VMH. Moderate PRV infection was seen in the LH (9.5%) and in the SCN (8.8%). The areas with the lowest percentage of total infected cells were the BNST (4.2%) and the lateral septum (LS; 2.2%).
DISCUSSION

These results provide the first neuroanatomic description of the SNS outflow from the brain to BAT in any species. The pattern of labeling observed in the present study after PRV was injected into IBAT resembled the pattern of labeling seen after PRV injections into WAT of Siberian hamsters and laboratory rats (7) and of labeling seen after PRV injections into the adrenal medulla of laboratory rats (53) or Siberian hamsters (unpublished observations). Collectively, these suggest that some of the labeling seen after injections of the virus into IBAT labeled part of the general SNS outflow from the brain to the periphery (52). We found, however, that although it was similar to the labeling after PRV injections into WAT or the adrenal medulla, there were some differences in the degree of labeling of certain brain structures. Specifically, higher percentages of cells were infected in the A5 and caudal raphe (Rob and Rpa collectively, Table 1) regions of the brain stem after BAT (15.2% and 12.2%, respectively) vs. WAT (4% and 5%, respectively) (7) injections of the virus. There also were more infected neurons in the LH and the BNST in animals injected with PRV into IBAT than there were in animals injected with the virus into WAT (7).

Previous attempts to identify the CNS origins of the SNS innervation of IBAT used nonneuroanatomic techniques (i.e., stimulation or lesions) and measured changes in IBAT morphology, biochemistry, or neurophysiology. The VMH has been implicated in most of these studies (for review see Ref. 20), yet we found little or no neural connections between the VMH and IBAT using this transneuronal viral tract tracer (see Table 1 and Fig. 1G). One possible reason for the discrepancy between the results of the nonneuroanatomic studies and those of the present neuroanatomic study is that the targeted stimulation or destruction of the VMH secondarily affected the caudally projecting neurons of the hypothalamic PVN that course near and around the VMH (24, 28, 33). When these PVN neurons are stimulated or destroyed more directly, BAT physiology. The VMH has been implicated in most of the targeted stimulation or destruction of the VMH secondarily affected the caudally projecting neurons of the hypothalamic PVN that course near and around the VMH (24, 28, 33). When these PVN neurons are stimulated or destroyed more directly, BAT physiological, biochemical, and/or morphological thermal responses are affected (e.g., Ref. 9; 3, 10, 13). Therefore, previous attempts to affect BAT thermogenesis through manipulations of the VMH may have altered these caudally projecting PVN neurons known to make direct connections with the spinal preganglionics (for review see Ref. 31). In this manner, BAT thermogenesis may ultimately have been affected.

Concerning the infections seen in the magnocellular region of the PVN, it should be noted that we believe this labeling was not a result of vascular transport of PRV to, for example, the posterior pituitary. This interpretation is based on the inability of PRV injected directly into peripheral venous or arterial blood to induce CNS infections, even at long postinjection survival times (30). Rather, it seems more likely that the scattered spinally projecting parvicellular neurons located within the magnocellular area (36) as well as the merging of the dorsal and ventral parvicellular spinally projecting areas more caudally within the hamster PVN (36) are the origins of the infections seen in the magnocellular PVN in the present study. The magnocellular neuronal infections seen in the present study and the lesser infections seen in other studies wherein PRV was injected into peripheral tissues (52, 53), including our own study after injections of virus into WAT (7), could be explained by transsynaptic spread of PRV within and between the nucleus subdivisions.

Innervation of BAT by the SCN has been suggested by an increase in IBAT thermogenesis after electrical stimulation of the retinohypothalamic tract that innervates the SCN (1) or by glutamate injections directly into the SCN (6). In the present study, a substantial number of infected neurons were visualized in the SCN after injection of PRV into IBAT. It is tempting to speculate that the SCN-BAT connection may be involved in the circadian timing of torpor bouts in Siberian hamsters, because SCN lesions block the expression of the torpor-associated rhythmic daily decreases in body temperature in this species (42).

We also found extensive labeling of PRV-infected neurons in the MPA. These data may help explain why electrical (59) or chemical (11) stimulation of the MPA increases IBAT thermogenesis and the firing rate of the sympathetic nerves that innervate IBAT (11, 59), respectively. The roles of the MPA and the SCN in BAT thermogenesis, however, are not well understood.
Although the pattern of infection in the present study after PRV injections into IBAT was more similar than different compared with the pattern after virus injections into the adrenal medulla of laboratory rats (52, 53), there were some notable differences, especially in the forebrain. These sites included the SCN and MPA as well as the LS and the BNST. These areas also were infected after PRV injections into WAT pads in Siberian hamsters and laboratory rats (7). One possible explanation for these differences may be that we used a longer...
In conclusion, the results of the present study suggest that the general SNS outflow to the periphery (52) also is involved in the SNS innervation of BAT (specifically, IBAT). In addition, other CNS sites identified as being connected neuroanatomically to BAT, such as the BNST, SCN, MPA, and LS, also are parts of the SNS innervation of BAT (7).

Perspectives

Our understanding of the central control of peripheral metabolic functions is hampered by the inability to trace the chain of neurons originating in the brain and terminating in peripheral glands and organs. The use of transneuronal viral tract tracers, such as the PRV, permits the definition of neural circuits, such as those involved in the central control of peripheral metabolism, within the same animal. The work to date delineating the SNS innervation of a variety of peripheral tissues, including BAT (in the present study), WAT (7), the adrenal medulla (26, 52, 53), the kidney (47), and other peripheral tissues, such as the heart (26, 27, 55), suggests a general SNS outflow from the CNS to the periphery (52). A critical question is raised because of these similarities: how is this general SNS outflow from the brain to the periphery regulated under conditions where there are differential SNS drives on peripheral tissues? Moreover, given the present findings for BAT and our previous findings for WAT (7), a more specific question can be posed: how can there be both separate and simultaneous SNS drives to BAT and WAT? An example of the separate SNS control of these adipose tissues is starvation or severe food restriction. In these conditions of reduced caloric intake, the SNS drive to BAT, and consequently BAT thermogenesis, is decreased (44), and the SNS drive to WAT, and consequently WAT lipolytic activity, is increased (34). An example of the simultaneous SNS control of these adipose tissues is cold exposure. In this condition, the SNS drive to BAT, and consequently BAT thermogenesis, is increased (37, 62); the SNS drive to WAT, and consequently WAT lipolytic activity, is also increased (16, 17). Because of the relatively separate postganglionic SNS innervation of the epididymal and inguinal WAT pads, there may be an analogous separate postganglionic SNS innervation of WAT and BAT. Alternatively, the differences that are seen in the degree of innervation of WAT and BAT by the SNS in some CNS structures should not be overlooked as a means by which this differential control of the SNS drive on adipose tissues or, for that matter, other tissues and organs innervated by the SNS may be controlled.

The authors thank Drs. Arthur Loewy, Michal Stöck, Patrick Card, and Frank Gordon for helpful discussions of these data. We also thank Dr. Kenneth Platt for providing the PRV antibody, Dr. Arthur Loewy for providing the initial supply of the virus, and Dr. Teryl Frey for providing the current supply of PRV. Finally, we thank Drs.

Table 1. Average number of transneuronally labeled neurons in brain of Siberian hamsters after injection of PRV into IBAT

<table>
<thead>
<tr>
<th>Brain stem</th>
<th>Postinjection Intervals</th>
<th>4 days ((n = 3))</th>
<th>6 days ((n = 2))</th>
</tr>
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<tbody>
<tr>
<td>Sol</td>
<td>2.7 ± 2.7</td>
<td>107.0 ± 37.0</td>
<td></td>
</tr>
<tr>
<td>ROb/Rpa</td>
<td>79.0 ± 27.9</td>
<td>130.0 ± 11.0</td>
<td></td>
</tr>
<tr>
<td>Llrt</td>
<td>2.0 ± 2.0</td>
<td>89.0 ± 17.0</td>
<td></td>
</tr>
<tr>
<td>Cj/RVL</td>
<td>85.7 ± 36.2</td>
<td>109.5 ± 4.5</td>
<td></td>
</tr>
<tr>
<td>Lrt</td>
<td>23.3 ± 24.1</td>
<td>222.5 ± 103.5</td>
<td></td>
</tr>
<tr>
<td>LPGi</td>
<td>55.7 ± 30.0</td>
<td>117.5 ± 63.5</td>
<td></td>
</tr>
<tr>
<td>GiA</td>
<td>6.0 ± 3.5</td>
<td>45.5 ± 14.5</td>
<td></td>
</tr>
<tr>
<td>A5</td>
<td>32.3 ± 14.2</td>
<td>162.5 ± 8.5</td>
<td></td>
</tr>
<tr>
<td>Midbrain</td>
<td>5.7 ± 3.2</td>
<td>43.5 ± 32.5</td>
<td></td>
</tr>
<tr>
<td>CG</td>
<td>0.7 ± 0.7</td>
<td>13.5 ± 10.5</td>
<td></td>
</tr>
<tr>
<td>VMH</td>
<td>1.3 ± 0.7</td>
<td>22.5 ± 10.5</td>
<td></td>
</tr>
<tr>
<td>Arc</td>
<td>71.7 ± 47.5</td>
<td>322.5 ± 143.5</td>
<td></td>
</tr>
<tr>
<td>SCN</td>
<td>0.7 ± 0.7</td>
<td>73.5 ± 57.5</td>
<td></td>
</tr>
<tr>
<td>LH</td>
<td>6.0 ± 2.3</td>
<td>79.0 ± 56.0</td>
<td></td>
</tr>
<tr>
<td>MPA</td>
<td>1.3 ± 0.9</td>
<td>269.5 ± 175.5</td>
<td></td>
</tr>
<tr>
<td>BNST</td>
<td>35.5 ± 23.5</td>
<td>19.5 ± 15.5</td>
<td></td>
</tr>
<tr>
<td>LS</td>
<td>18.0 ± 7.0</td>
<td></td>
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</table>

All values are means ± SE; \(n = \) no. of rats. PRV, pseudorabies virus; IBAT, interscapular brown adipose tissue; Sol, nucleus of the solitary tract; ROb/Rpa, raphe obscurus nucleus/raphe pallidus nucleus; Llrt, lateral reticular nucleus; Cj/RVL, C1 epinephrine cells/rostroventrolateral reticular nucleus; LPGi, lateral paragigantocellular nucleus; GiA, gigantocellular reticular nucleus, alpha part; A5, norepinephrine cells; CG, central gray; VMH, ventromedial hypothalamic nucleus; Arc, arcuate; SCN, suprachiasmatic nucleus; LH, lateral hypothalamic area; MPA, medial preoptic area; BNST, bed nucleus of the stria terminalis; LS, lateral septum.
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