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Analysis and Measurement of the Sympathetic and Sensory Innervation of White and Brown Adipose Tissue

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Abstract

Here, we provide a detailed account of how to denervate white and brown adipose tissue (WAT and BAT) and how to measure sympathetic nervous system (SNS) activity to these and other tissues neurochemically. The brain controls many of the functions of WAT and BAT via the SNS innervation of the tissues, especially lipolysis and thermogenesis, respectively. There is no clearly demonstrated parasympathetic innervation of WAT or the major interscapular BAT (IBAT) depot. WAT and BAT communicate with the brain neurally via sensory nerves. We detail the surgical denervation (eliminating both innervations) of several WAT pads and IBAT. We also detail more selective chemical denervation of the SNS innervation via intra-WAT/IBAT 6-hydroxy-dopamine (a catecholaminergic neurotoxin) injections and selective chemical sensory denervation via intra-WAT/IBAT capsaicin (a sensory nerve neurotoxin) injections. Verifications of the denervations are provided (HPLC-EC detection for SNS, ELIA for calcitonin gene-related peptide (proven sensory nerve marker)). Finally, assessment of the SNS drive to WAT/BAT or other tissues is described using the alpha-methyl-*para*-tyrosine method combined with HPLC-EC, a direct neurochemical measure of SNS activity. These methods have proven useful for us and for other investigators interested in innervation of adipose tissues. The chemical denervation approach has been extended to nonadipose tissues as well.

1. INTRODUCTION

Because of the epidemic status of obesity, increased interest in the control of the functions of adipose tissues, both white and brown (WAT and BAT, respectively), has grown considerably in the last 20 years. Although there has long been an appreciation of the control of BAT through the activity of its sympathetic nervous system (SNS) (for reviews, see Bartness, Vaughan, & Song, 2010; Cannon & Nedergaard, 2004; Himms-Hagen, 1991), appreciation of the importance of the SNS innervation of WAT has lagged behind. This is possibly due to early misconceptions of the importance of adrenal medullary epinephrine (EPI) in lipolysis (e.g., Havel & Goldfien, 1959; Wool, Goldstein, Ramey, & Levine, 1954).

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We now know that in the absence of the adrenal medulla, the sole source of circulating EPI, regulated lipolysis occurs in response to food deprivation and cold exposure (factors that increase WAT lipid mobilization, e.g., Paschoalini & Migliorini, 1990; Teixeira, Antunes-Rodrigues, & Migliorini, 1973). We and others have defined the central origins of the SNS outflow from brain to WAT (Adler, Hollis, Clarke, Grattan, & Oldfield, 2012; Bamshad, Aoki, Adkison, Warren, & Bartness, 1998; Shi & Bartness, 2001; Song & Bartness, 2001; Song, Jackson, Harris, Richard, & Bartness, 2005). Destruction of the SNS innervation of WAT blocks lipolysis due to food deprivation, cold, and other stimuli (e.g., Beznak & Hasch, 1937; Cantu & Goodman, 1967; Lazzarini & Wade, 1991; Youngstrom & Bartness, 1998) demonstrating the necessity of the innervation for the control of lipolysis. We and others also have defined the central origins of the SNS outflow from brain to BAT (Bamshad, Song, & Bartness, 1999; Oldfield et al., 2002; Song et al., 2008; Zhang et al., 2011) and destruction of the SNS innervation of BAT blocks or severely diminishes BAT responses to all thermogenic challenges tested to date (for review, see Bartness et al., 2010). Finally, we have demonstrated the sensory inflow to the brain and the central sensory circuits from both WAT (Song, Schwartz, & Bartness, 2009) and BAT (Vaughan & Bartness, 2012) to brain. Our initial studies of the roles of the sensory innervations of WAT and of BAT for physiological responses of these tissues indicate their necessity for normal functioning of the tissues and suggest possible modalities of these neural feedbacks (Foster & Bartness, 2006; Shi & Bartness, 2005; Shi, Song, Giordano, Cinti, & Bartness, 2005; Song & Bartness, 2007; Vaughan & Bartness, 2012). Therefore, testing the necessity of the SNS and sensory innervation of WAT and BAT, as well as other tissues important for energy metabolism (e.g., liver), is apparent.

Because the sympathetic and sensory nerves innervating both WAT and BAT pads travel together in bundles indistinguishable from each other visually by eye, we initially used surgical denervation to test for the contribution of these nerves for normal WAT and BAT physiological responses (Bartness & Wade, 1984; Foster & Bartness, 2006; Rooks et al., 2005; Youngstrom & Bartness, 1998). We recommend surgical denervation as an initial approach because, if done correctly, virtually all innervations are removed.

Previously, chemical denervation of sympathetic (e.g., Johnson & O'Brien, 1976; Levin & Sullivan, 1984; Levin, Triscari, Marquet, & Sullivan, 1984; Mory, Ricquier, Nechad, & Hemon, 1982; Seydoux, Mory, & Girardier, 1981) or sensory (e.g., Cui & Himms-Hagen, 1992a, 1992b; Cui, Zaror-Behrens, & Himms-Hagen, 1990; Kawada, Hagihara, & Iwai, 1986) nerves was performed via systemic injection of sympathetic and sensory neurotoxins resulting in global denervations that are difficult to ascribe specific functional involvement to either WAT or BAT. Therefore, we developed local, selective and effective chemical denervation of the sympathetic and sensory nerve provisions to WAT and BAT pads (Demas & Bartness, 2001a, 2001b; Foster & Bartness, 2006; Rooks et al., 2005; Shi & Bartness, 2005; Shi et al., 2005), the details of which are delineated below.

Thus, the purpose of this chapter is to detail the methods we have used to test the necessity of SNS and sensory innervations of WAT and BAT for their normal functioning. In addition, we describe a direct neurochemical method to assess the sympathetic drive to WAT, BAT or any sympathetically innervated tissue rather than the many inadequate

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surrogates used by researchers to assess such activity such as histological or static measures of neurochemicals found in the sympathetic or sensory nerves. Note that when possible, we give instructions for how to accomplish the methods in laboratory rats and mice, in addition to Siberian hamsters, the latter being our species of choice. These techniques were first applied to Siberians because of their photoperiod induced, naturally occurring obesity and obesity reversal (for review, see Bartness, Demas, & Song, 2002; Bartness & Wade, 1985).

2. SURGICAL DENERVATION OF WAT AND BAT NERVES

Sympathectomy has been used widely as a means to eliminate postganglionic input to the tissue of choice. We typically have waited ~12 weeks to ensure that consequences of the denervation (axonal degeneration and cell death a.k.a. Wallerian degeneration) are complete (Bartness et al., 1986; Bowers et al., 2004; Foster & Bartness, 2006; Hamilton, Bartness, & Wade, 1989; Shi & Bartness, 2005; Shi et al., 2005; Youngstrom & Bartness, 1998). However, we have observed significant cell denervation-induced increases in white adipocyte proliferation in inguinal WAT (IWAT) as early as 10 days post denervation (Bartness et al., 1986; Bowers et al., 2004; Foster & Bartness, 2006; Hamilton et al., 1989; Shi & Bartness, 2005; Shi et al., 2005; Youngstrom & Bartness, 1998). Therefore, one should conduct time course studies to document the destruction of the sympathetic/sensory innervations (see Sections 5 and 6 for verification methods) for each tissue tested to determine the optimal time to conduct any post-axotomy measures.

Surgical denervation removes both sympathetic output and sensory input from adipose tissues; therefore, to verify successful surgical denervation it is necessary to measure norepinephrine (NE) content (typically by HPLC-EC as current enzyme-linked immunosorbent assay (ELIA) methods, even the currently available ultra-sensitive ELIAs, are not sensitive enough), the principal neurochemical released by sympathetic nerves (described in Section 5), or by ELIA/Western blot for tyrosine hydroxylase (TH) or immunohistochemical assessment of TH-immunoreactivity (Foster & Bartness, 2006). It needs to be emphasized that none of those measures are an assessment of sympathetic activity. Sympathetic activity can only be assessed directly either electrophysiologically, or by norepinephrine turnover (NETO), the latter described in Section 5. As for verification of the extent and specificity of the sensory innervation of WAT and BAT, a marker of sensory nerves such as calcitonin gene-related peptide (CGRP) can be measured via ELIA (described in Section 6), or immunohistochemically. To our knowledge, the only direct method to measure the activity of sensory nerves innervating adipose tissues is electrophysiologically, which we have done for WAT in response to glucoprivation (Song et al., 2009) and we (Murphy et al., 2013) and others (Niijima, 1998, 1999; Shi et al., 2012; Xiong et al., 2012) have done for direct intra-WAT leptin injection.

2.1. Identification of nerves innervating WAT and BAT for surgical denervation

We have performed unilateral and bilateral nerve cuts of inguinal IWAT, epididymal WAT (EWAT), retroperitoneal WAT (RWAT), and inter-scapular BAT (IBAT) to test for depot-specific effects on anatomy and physiology (Bartness et al., 1986; Bowers et al., 2004; Foster & Bartness, 2006; Hamilton et al., 1989; Shi & Bartness, 2005; Shi et al., 2005; Youngstrom & Bartness, 1998).

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The nerves innervating BAT are more easily discernible than those innervating WAT. For IBAT, there are five intercostal nerves that unilaterally innervate each IBAT lobe in laboratory rats (Foster, Depocas, & Zaror-Behrens, 1982; Foster, Depocas, & Zuker, 1982), laboratory mice (Sidman & Fawcett, 1954), and Siberian hamsters (Klingenspor, Meywirth, Stohr, & Heldmaier, 1994). These can be seen and severed without the aid of a dissecting microscope, although visualization and axotomy is improved with magnification.

Because of the difficulty in visualizing the nerves innervating WAT, we frequently use one to two drops of 1% toluidine blue directly onto tissue (Foster & Bartness, 2006; Shi & Bartness, 2005; Shi et al., 2005) and sever any nerves that appear to enter the fat pad. Use of a dissecting microscope is mandatory for visualization and axotomy of WAT innervation.

EWAT nerves are encased in a neurovascular pedicle and pass through the inguinal canal (Correll, 1963). Nerves innervating EWAT are best visualized after testis and associated EWAT are pulled intact from the abdominal cavity through a ventral incision (described in Shi et al., 2005; Song et al., 2009). Any inguinal nerves that innervate IWAT in addition to accessory nerves present nearby that appear to be connected to skin can be visualized after a ventral to dorsal semicircular incision in the skin near the flank.

2.2. Required materials for all denervation protocols

- 95% ethanol
- Sterile gauze
- Povidone-iodine (topical antiseptic, e.g., Betadine)
- At least one of the following to handle skin and/or fat. All available from Roboz Surgical Instrument Co., Gaithersburg, MD
  - Tissue forceps (Graefe: jar width 4.5 mm, length 41/4″; Cat #: RS-8248)
  - Curved serrated forceps (full curve; Cat #: RS-5137)
- Sharp surgical scissors (e.g., Cat #: RS-6702; Roboz Surgical Instrument Co., Gaithersburg, MD)
- Nitrofurozone (antibacterial powder to minimize wound infection, e.g., nfz Puffer; Hess & Clark, Lexington, KY)
- 9 mm wound clips (Stoelting, Wood Dale, IL)
- Wound clip applicator to close skin (EZ Clip ™ Applier; Stoelting, Wood Dale, IL)
- Wound clip remover (Cat #: RS-9263; Roboz Surgical Instrument Co., Gaithersburg, MD)
- Vicryl suture to close musculature (Ethicon, Somerville, NJ)
- Razor or depilatory cream (e.g., Nair®) to clear hair from target area
- Analgesic and/or anti-inflammatory agent (e.g., ketoprofen, buprenorphine)

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2.3. Surgical denervation protocol

2.3.1 Required materials

- Materials listed in Section 2.2
- Dissecting microscope
- At least one of the following microdissecting forceps. All available from Roboz Surgical Instrument Co., Gaithersburg, MD
  - Microdissecting angled fine sharp points forceps, length 4″ (Cat #: RS-5095)
  - McPherson: straight smooth tying platform forceps (Cat #: RS-5068)
  - Graefe: serrated, straight forceps (Cat #: RS-5110)
- At least one of the following microdissecting scissors. All available from Roboz Surgical Instrument Co., Gaithersburg, MD
  - McPherson-Vannas: microdissecting straight, sharp spring scissors (Cat #: RS-5600)
  - Vannas: scissors angled on edge (Cat #: RS-5618)
- *Note*: For help with identifying adipose depots, see Cinti (1999)

1. Anesthetize animal and remove fur from target area. Wipe area with 95% ethanol-soaked sterile gauze followed by additional application of Betadine to the area.
2. *For all fat pads*, use the following descriptions to locate nerves and if difficult use dye to visualize and then sever the nerves.

   For IBAT, make a midline incision in the skin along the upper dorsal surface to expose both IBAT pads (Fig. 11.1A). Gently expose the medial, ventral surface of both pads to visualize nerves beneath pad (Fig. 11.1B). Nerves may appear in bundles of two to three. If seen under the microscope, the bundles can be gently dissociated to view individual nerves (Fig. 11.1C; 5 nerves/BAT pad). Care should be taken not to cut the large Sulzer's vein that is located medially to both pads (Fig. 11.1D).

   For IWAT, make an incision in skin dorsally on the flank from a point near the tail and lateral to the spinal column. Continue the incision rostrally along the dorsum adjacent to the spinal column to a point just rostral to the hind limb, then laterally and ventrally to a point ~2 cm from the ventral midline (Fig. 11.2A and B). Separate the IWAT pad from the abdominal wall and overlying skin by blunt dissection, keeping intact the major blood vessels leading into or through the pad (Fig. 11.2C–F).

   For EWAT, make a single abdominal midline incision in the skin. Through this incision, EWAT pads can be accessed and gently lifted or pushed to visualize nerves (Fig. 11.3A and B). For better visualization, the testis and associated EWAT can be pulled from the peritoneal cavity and laid on 0.9% NaCl-soaked sterile

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gauze placed on the animal's ventrum for better visualization. Some of the nerves innervating EWAT appear to run along the main artery providing the testis with blood. Do not damage either of these arteries because both are integral to normal testicular function (Fig. 11.3C). After surgery, close the peritoneal cavity with absorbable sutures (Vicryl).

For RWAT, make an incision in the skin on the ventral surface close to the midline then make an incision into the peritoneal cavity to expose the area medial to the kidneys. RWAT extends longitudinally down the spine and between vertebrae. It is separated from the perirenal depot by a peritoneal fold (Frontini & Cinti, 2010). Gently lift the kidney and cut the three nerve bundles that innervate RWAT (Bowers et al., 2004). After surgery, close the peritoneal cavity with absorbable sutures (Vicryl).

3. For all initial incisions to access the fat pads, care should be taken with the depth of the incision to avoid damaging the underlying blood vessels and musculature. Nerves identified at ×4 magnification that terminate in the pad should be cut in two or more locations and if possible, remove the nerve sections between the cuts to assure denervation and the unlikely possibility of nerve regrowth.
4. Throughout the surgery, keep the target fat pad moist with 0.9% NaCl-soaked sterile gauze.
5. If using a contralateral pad for a within-animal control (unilateral denervation model; Bartness & Bamshad, 1998; Bartness & Song, 2007), the sham procedure should include fat pad manipulation and nerve identification with the exception that the nerves are left intact. Because of laterality of the innervations, alternate the side for denervation or sham surgery (or chemical denervation, see Sections 3 and 4).
6. After surgery the sham or denervated pad is replaced (if pulled from the peritoneal cavity or moved to the side) and rinsed with sterile 0.9% NaCl.
7. The incision in the skin is closed with sterile wound clips, and nitrofurozone powder is applied to the wound surface to minimize the risk of bacterial infection.

3. CHEMICAL DENERVATION OF ADIPOSE TISSUE USING 6-HYDROXY-DOPAMINE

We previously used intra-fat injections of guanethidine, which is toxic to sympathetic nerves at high concentrations (Burnstock, Evans, Gannon, Heath, & James, 1971). Although we had excellent, nearly axotomy-like complete SNS denervations, as evidenced by nearly or nondetectable NE content (Demas & Bartness, 2001a, 2001b), newer preparations are less reliable; therefore, we now use intra-fat injection of the catecholaminergic neurotoxin, 6-hydroxy-dopamine (6-OHDA; Knyhar, Ristovsky, Kalman, & Csillik, 1969; Ungerstedt, 1968). 6-OHDA has been used by others for chemical sympathectomy in a variety of preparations (Benarroch, Schmelzer, Ward, Nelson, & Low, 1990; Depocas, Foster, Zaror-Behrens, Lacelle, & Nadeau, 1984; Dobbins, Szczepaniak, Zhang, & McGarry, 2003; Joost & Quentin, 1984). Using 6-OHDA for local SNS denervation was inspired by the sympathetic denervation of the testes via direct injection of the toxin into the gonads

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(Mayerhofer, Amador, Steger, & Bartke, 1990). Therefore, we tried and have successfully sympathetically denervated WAT with direct injections of 6-OHDA (Foster & Bartness, 2006; Giordano, Morroni, Santone, Marchesi, & Cintiet, 2006; Rooks et al., 2005). Previously, 6-OHDA has been injected subcutaneously resulting in a reduction in BAT NE content (denervation) (Depocas et al., 1984; Thureson-Klein, Lagercrantz, & Barnard, 1976), but BAT denervation in light of the global sympathetic denervation that occurs by this type of systemic approach makes interpretation of results exceedingly difficult. Thus, direct intra-WAT or BAT injections of 6-OHDA are both restrictive and selective.

3.1. Chemical denervation protocol

3.1.1 Required materials

- Materials listed in Section 2.2
- 6-OHDA (6-hydroxy-dopamine hydrochloride; Cat #: H4381; Sigma-Aldrich, St. Louis, MO)
- L-Ascorbic acid (Cat #: A5960; Sigma-Aldrich, St. Louis, MO)
- Sodium chloride
- N_2 gas tank
- Hamilton syringe, 10 μl model (Hamilton Company, Reno, NV)

1. To prepare 6-OHDA, first make a solution of 0.15 *M* NaCl containing 1% ascorbic acid (AA). This AA/saline solution should be gassed in a light-tight container with N_2 for 10 min before adding 6-OHDA. Based on previous work (Foster & Bartness, 2006; Giordano et al., 2006; Rooks et al., 2005), we have found that different fat pads and other organs require slightly different concentrations of 6-OHDA (see directly below).
    a. 6-OHDA is light- and temperature-sensitive so keep solution on ice and covered during surgeries and replace every 2–3 h
2. Anesthetize animal and remove fur from target area. Wipe area with 95% ethanol-soaked gauze followed by application of Betadine.
3. *For all fat pads*, make incisions using descriptions in Section 2.3. With all incisions, care should be taken with the depth of the incision so as not to damage the underlying fat pad and vasculature. Once pad is gently separated from skin, use a 10 μl microsyringe to inject the following across the respective pads:
    - *EWAT: Siberian hamsters*: 20 loci with 2 μl injections of 9 mg/ml 6-OHDA solution or vehicle. *Mice*: 10 loci across the pad with 2 μl injections of 8 mg/ml 6-OHDA in 0.01 *M* PBS containing 1% AA.
    - *IWAT: Siberian hamsters*: 40 loci with 2 μl injections of 4 mg 6-OHDA in 100 μl vehicle or vehicle. *Mice*: 12 loci with 2 μl injections of 9 mg/ml 6-OHDA in PBS containing 1% AA (Harris, 2012; Rooks et al., 2005).

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- *RWAT: Rats*: 10 loci with 2 μl injections of either 9 mg/ml 6-OHDA in 0.01 *M* PBS containing 1% AA or vehicle.

4. Once the needle is inserted into the adipose tissue, fluid should be expelled slowly over a ~30 s period for each injection. The tip of the needle should be held in place for ~30–60 s to avoid reflux.
5. The incision is closed with sterile wound clips and nitrofurozone powder is applied to minimize sepsis.
6. Administer an analgesic and monitor animal appropriately.
7. Determine the postsurgical time in pilot studies for significant reductions in sympathetic and sensory nerve markers. Generally, allow anywhere from 10 days to 4 weeks post injection for testing the denervations (Foster & Bartness, 2006; Giordano et al., 2006; Harris, 2012; Rooks et al., 2005).

4. LOCAL SENSORY DENERVATION OF ADIPOSE TISSUE USING CAPSAICIN

Capsaicin, the pungent part of red chili peppers, selectively destroys small, unmyelinated mostly C-fiber sensory nerves (Jansco, Kiraly, & Jansco-Gabor, 1980; Jansco, Kiraly, Joo, Such, & Nagy, 1985). We have shown that injections of capsaicin into WAT selectively destroys sensory nerves, as documented immunohistochemically by significant decreases in, CGRP, but not TH (Foster & Bartness, 2006), the rate-limiting enzyme in NE synthesis and a proven sympathetic nerve marker.

Other investigators have tested the effects of IBAT sensory denervation via repeated, large doses of systemic capsaicin in neonatal or adult rats resulting in denervation of all peripheral tissues (Cui & Himms-Hagen, 1992a, 1992b; Cui et al., 1990; Himms-Hagen, Cui, & Sigurdson, 1990; Melnyk & Himms-Hagen, 1994). This approach results in capsaicin-desensitization, as the animals no longer respond to the hypothermic effects of an acute capsaicin injection (e.g., Jancso-Gabor, Szolcsanyi, & Jancso, 1970). Acute intraperitoneal (ip) injections of capsaicin also create lesions in some central structures that have a weak blood–brain barrier, such as the nucleus of the solitary tract (Castonguay & Bellinger, 1987). Due to these unclear interpretations regarding whole animal capsaicin-desensitization, we choose to use local injections into fat as above for SNS denervations using 6-OHDA. This approach was inspired by the direct application of capsaicin to the vagus nerve to promote vagal afferent denervation (Raybould, Holzer, Reddy, Yang, & Tache, 1990; Yoneda & Raybould, 1990).

4.1. Sensory denervation protocol

4.1.1 Required materials

- Materials listed in Section 2.2
- Hamilton syringe, 10 μl model (Hamilton Company, Reno, NV)
- Hot block (Isotemp Heat block, Fisher Scientific, Pittsburgh, PA)

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- Heating pad and pump (Gaymar T Pump & Multi-T pad, Braintree Scientific, Inc., Braintree, MA)
  - *Note*: surgery takes about ≥1 h
- Olive oil (Cat #: O1514; Sigma-Aldrich, St. Louis, MO)
- Capsaicin (Cat #: M2028; Sigma-Aldrich, St. Louis, MO). *Note*: All of the capsaicin is used to make the stock solution and is not measured out on a balance due to risk of skin, eye, and/or respiratory irritation
- Capsaicin stock solution: Add 1.25 ml of 100% ethanol to 250 mg bottle of capsaicin to make the final concentration of 200 μg/μl and keep in dark at 4 °C
- Capsaicin working solution: Dilute stock solution in olive oil at 1:10 (stock:olive oil) to yield a final concentration of 20 μg/μl

1. Make capsaicin stock solution.
2. Before surgery, make fresh working solution under a fume hood. Warm solution to 37 °C using hot block or water bath; make sure solution is covered during warming. Change out for fresh solution if crystals begin to form in container with working solution. Anesthetize and prepare animal by removing hair in target area. Wipe area with 95% ethanol-soaked sterile gauze followed by application of Betadine.
3. Expose fat pads and keep them moist. Be careful when moistening tissue after injecting capsaicin as it can spread to unwanted areas.
4. Change gloves between handling capsaicin and handling the next animal.
   a. If at any point, there is a possibility there may be capsaicin solution on gloves, change gloves.
   b. Wear a mask at all times when handling capsaicin.
5. *For all adipose tissues*, make incisions using descriptions in Section 2.3. For all fat pads, inject across 20–30 s and wait for ~30 s with tip of needle in the adipose tissue and then move to the next injection site in that pad. *Test that fluid is being expelled often*. Occasionally, olive oil solution will clog the needle; therefore, use 100% ethanol to flush needle and syringe if necessary between injections. Once pads are gently separated from skin, inject the following volumes across the respective pads:
   - *IBAT: Siberian hamsters*: inject with 30 microinjections (1 μl/injection) of 20 μg/μl of capsaicin or vehicle (1:10, ethanol: olive oil).
   - *IWAT: Siberian hamsters and mice*: inject with 40 microinjections (1 μl per injection) of 20 μg/μl of capsaicin or vehicle (1:10, ethanol: olive oil).
   - *EWAT: Siberian hamsters*: inject with 40 microinjections (1 μl per injection) of 20 μg/μl of capsaicin or vehicle (1:10, ethanol:olive oil).
6. During the injections minimize the handling of the adipose tissue.

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7. Determine the postsurgical time in pilot studies for significant reductions in sympathetic and sensory nerve markers. We typically allow 21 days before conducting tests in Siberian hamsters.

5. ASSESSMENT OF SYMPATHETIC DENERVATION USING NETO OR CONTENT AS MEASURED BY HPLC-EC

NE content is measured to verify the effectiveness of the surgical or chemical sympathetic denervations. We use reverse-phase HPLC-EC following our modifications (Bowers et al., 2004) to the method of Mefford (1981). NETO is used as a direct neurochemical measure of sympathetic drive; as noted above, there is no surrogate for this method of assessment except for direct measures of sympathetic nerve activity electrophysiologically. The benefit of NETO, however, is that there is no restriction on the number of tissues where NETO can be measured from the same animal (e.g., various WAT pads, IBAT, muscle, liver, etc.).

5.1. Alpha-methyl-*para*-tyrosine method to measure sympathetic drive (NETO)

NETO is measured using the alpha methyl-*para*-tyrosine (AMPT) method (Cooper, Bloom, & Roth, 1982). AMPT is a competitive inhibitor of TH. Without available TH and thus no new NE, the endogenous tissue levels of NE decline at a rate proportional to initial NE concentrations (Cooper et al., 1982). NETO is measured over the last 2–4 h of a test, regardless of the stimulus applied (e.g., central or peripheral drugs/hormones/ neurochemicals, cold exposure, food deprivation, etc.). For WAT or BAT, which are less heavily sympathetically innervated than some tissues (e.g., heart), the 4 h time period seems best. Because AMPT is a severe behavioral depressant that is expected to markedly affect many physiological/biochemical responses, only NETO should be measured in these animals, with a parallel set of animals used for non-NETO measures.

It is important to emphasize that handling and other manipulations (previous surgery, intraventicular injection, peripheral injection) can increase sympathetic drive to WAT or BAT. Therefore, based on our experience, animals should be handled daily for ~1–3 weeks for 5 min to adapt them to the handling associated with the AMPT injection procedure and thereby minimize stress-induced increases in sympathetic drive (Brito, Brito, Baro, Song & Bartness, 2007; Brito, Brito, & Bartness, 2008).

The assumption for NETO is that the decline across the NETO test time is linear; therefore, this should be demonstrated first by using three time points (e.g., 0, 2, and 4 h; see Fig. 11.4). Once established in the laboratory for tissues of interest in pilot experiments, we have found that the 0 and the longest time point (typically 4 h) are sufficient. Half the animals are killed by decapitation without anesthesia. This is necessary because anesthesia can cause NE release from sympathetic terminals in the tissue, but rapid decapitation without anesthesia does not (Depocas & Behrens, 1977; Popper, Chiueh, & Kopin, 1977). Animals should be divided into two time point groups on measures relevant to the study. One group (time 0 h) will be killed with no injection of AMPT, whereas the second group will be killed later (typically time 4 h) after initial AMPT injection.

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5.1.1 Required materials

- Syringes and needles for AMPT injections (appropriate needles for ip injections and syringes for calculated volume)
- Glacial acetic acid (Fisher Scientific, Pittsburgh, PA)
- AMPT methyl ester hydrochloride (Cat #: M3281-1G; Sigma-Aldrich, St. Louis, MO)
  - Solution: Add 1 μl of glacial acetic acid per 1 mg of AMPT to make the initial AMPT dose. Then, for the subsequent doses dilute the initial dose with 0.15 *M* NaCl.
- Balance to weigh animals day before test to calculate volume for AMPT injections.
- Microbalance for weighing tissue on test day.
- Dissection tools
  - For example, tissue forceps (Graefe: jar width 4.5 mm, length 4¼″; Cat #: RS-8248), curved serrated forceps (full curve; Cat #: RS-5137) or sharp surgical scissors (e.g., Cat #: RS-6702). All available from Roboz Surgical Instrument Co., Gaithersburg, MD.
- Weighing papers (Cat #: 09-898-12A; Fisher Scientific, Pittsburgh, PA) or aluminum foil for weighing tissue
  - Use adipose tissue weight to calculate mg tissue/ml NETO (see below for comment).
- Sharp scissors for rapid decapitation (Fiskars scissors, small animals) or guillotine (rats).

1. One group will be a 0 h time point group that receives no AMPT ip and are immediately decapitated for tissue harvest (see Fig. 11.4). The second set of animals will receive AMPT (hamsters: 250 mg AMPT/kg; 25 mg/ml; mice: 300 mg AMPT/kg; rats 250 mg AMPT/kg; Levin, 1995) injected ip.
2. Two hours later (or the halfway time point), the animals will be injected with a supplemental dose of AMPT (hamsters: 125 mg AMPT/kg; 25 mg/ml; mice: 150 mg AMPT/kg; rats: 125 mg AMPT/kg; Levin, 1995) to assure the maintenance of TH inhibition.
3. Two hours later (or at the last time point, e.g., 6 h later for brain, given the short half-life of NE in that tissue; Levin, 1995), the animals are decapitated and tissues are harvested.
4. During harvest, it is critical to do this rapidly and to keep them cold. Therefore, the headless carcass should be packed in crushed ice and the tissues of interest should be bathed frequently with ice cold 0.15 *M* NaCl during dissection. Tissues are weighed, placed in a suitable container (e.g., aluminum foil) labeled with animal number and tissue, and snap frozen in liquid nitrogen for subsequent storage at −80 °C until assayed for NE content to determine NETO.

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5.2. Preparation of samples for HPLC-EC (NETO and NE content)

CATs are thermal and light labial and will therefore degrade easily. Handling them on ice and shielding from light throughout the preparation of samples ensures longevity of CATs. After extraction (see below), the liquid samples should be stored at −80 °C in acidic conditions (perchloric acid and ascorbic acid (PCA/AA); recipe below). While processing the samples, especially during bullet blending (see below), make certain that the sample is kept cold. To reduce electrochemical "noise" during subsequent HPLC analysis, HPLC-grade water should be used for all solutions.

5.2.1 Required materials

- Microbalance for weighing tissue
- Dissection and mincing tools (see Section 5.1)
- 70% PCA (Cat #: AC42403; Fisher Scientific, Pittsburgh, PA)
- L-Ascorbic acid (Cat #: A5960; Sigma-Aldrich, St. Louis, MO)
- Dihydroxybenzylamine (DHBA) (Sigma-Aldrich, St. Louis, MO)
- Pipets and appropriate tips
- Tris (Trizma base; Cat #:T1503-1 kg; Sigma-Aldrich, St. Louis, MO)
- Aluminum oxide (Al_2O_3; Sigma-Aldrich, St. Louis, MO)
- Multiflex round tips (1–200 μl; Sorenson, Salt Lake City, UT)
- Eppendorf® Safe-Lock tubes (Fisher Scientific, Pittsburgh, PA).
- 2 ml conical screw cap tubes (Fisher Scientific, Pittsburgh, PA)
- Bullet Blender (Next Advance, Averill Park, NY) and accessories below:
  - 50 μl scoop for Bullet Blender beads (Next Advance, Averill Park, NY)
  - Zirconium oxide beads (0.5 mm) for Bullet Blender (Next Advance, Averill Park, NY)
- Parafilm (Fisher Scientific, Pittsburgh, PA)
- Degree Vertical Multi-Function Rotator (Grant-Bio, Grants Pass, OR)

1. Make PCA/AA. Add 0.3 mg of L-ascorbic acid (AA) for every milliliter of 0.2 *M* PCA. Solution is light-sensitive and can be kept at room temperature.
2. Weigh out 0.015–0.250 g of frozen tissue and add to new 2 ml Eppendorf® Safe-Lock tubes containing 790 μl of PCA/AA and one scoop (~0.15 g) of Bullet Blender beads.
3. Make stock solution of DHBA (1,000,000 ng/ml), aliquot and store at −80 °C.
4. Add 10 μl of the internal standard, DHBA in the appropriate concentration to each sample as followed (values based on hamster/mice tissues, but should be empirically determined for the animals and tissues used):

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- WAT, liver, skeletal muscle—10 ng of DHBA/10 μl (1 μg/ml)
- BAT, pancreas—50 ng of DHBA/10 μl (5 μg/ml)
- Heart—100 ng of DHBA/10 μl (10 μg/ml)
- Adrenal—2.0 μg of DHBA/10 μl (200 μg/ml)
- Plasma—2 ng of DHBA/10 μl (200 ng/ml)

5. Thoroughly mince with surgical scissors if tissue is >0.03 g.
6. Tightly wrap the cap of the tubes with a small piece of parafilm. Only Eppendorf Safe-Lock tubes should be used with the Bullet Blender. Do not wrap the entire tube. Place tubes in Bullet Blender. It is unnecessary to balance tube placement.
7. Using a Bullet Blender at speed setting #8, disrupt tissue at 1 min intervals, letting samples sit on ice for 2–3 min in between each run. To ensure the tubes are held tightly into place, lay a few napkins on top of the samples to seal. This should be repeated until tissue is fully homogenized, seemingly opaque with no visible large pieces remaining. Sonication is not a desirable method for homogenization of these tissues due to the thermolability of catecholamines by sonication-induced heat generation. For samples not minced in step 4, bullet blend 1–2 min longer. Maximum total bullet blending time is usually 5–6 min and a minimum of 3 min. For samples that are not completely homogenized at the end of 5–6 min, place back in Bullet Blender and bullet blend separately for 30 s, holding tube into place with hands. BAT will be more difficult to homogenize and will require more blending time.
8. Remove parafilm and centrifuge at 8000×*g* for 20 min at 4 °C. For BAT, centrifuge at 10,000×*g* for 20 min at 4 °C.
9. Carefully remove the homogenate under the fat layer and add to sterile 2 ml conical screw cap microcentrifuge tubes containing ~150 g of *ACTIVATED* alumina (Al_2O_3). Use a 1000 μl pipette and appropriate tip to remove ~750 μl, then a 200 μl pipette with a gel loading tip (multiflex round tips 1–200 μl) to aspirate out the remaining fluid, allowing the Bullet Blender beads to act as a filter.

   Note: Alumina can be purchased already activated from Sigma-Aldrich (Cat #: 199974). Alumina can be activated for catecholamine extraction using the following steps:

   - Add 100 g of AL_2O_3 to a 1 l beaker. Add 500 ml of 2 N HCl. Heat continuously and shake for 45 min at 90–100 °C.
   - Decant HCl to remove supernatant and small (thin) particles (after ±5 min off heat).
   - Wash the precipitate (the remaining Al_2O_3) with 250 ml of 2 N HCl for 10 min at 70 °C. Decant HCl to remove supernatant and small (thin) particles (after ±5 min off heat).
   - Wash with 500 ml 2 N HCl for 10 min at 50 °C. Decant HCl.

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- Wash ±10 times with pure, Milli-Q water using ~200 ml each time until pH 3–4. Decant water.
- Heat at 120 °C for 1 h.
- Heat at 200 °C for 2 h.
- To cool, put on vacuum desiccator at 37 °C.

10. Add 1 ml of 0.5 *M* Tris. pH to 8.6. Adjust with 70% PCA.
11. Mix samples well using Degree Vertical Multi-Function Rotator for 20 min at 4 °C on speed 7, then vortex for 20 s to ensure the binding of CATs to the alumina.
12. Centrifuge at 8000×*g* for 2 min at 4 °C. For BAT centrifuge at 10,000×*g* for 2 min at 4 °C.
13. Remove and discard the supernatant. To avoid alumina aspiration, use a 1000 μl pipette to remove and discard ~850 μl of supernatant followed by using a 200 μl pipette using redi-tip 200 μl pipette tip for the remaining 150 μl.
14. Add 1 ml of HPLC-grade water to each tube to wash the alumina. Invert 10 times by hand then vortex for 5 s.
15. Centrifuge at 8000×*g* for 1 min at 4 °C. For BAT, centrifuge at 10,000×*g* for 1 min at 4 °C.
16. Repeat steps 12–15.
17. Add 200 μl of PCA/AA to elute the CATs. Allow samples to spin on Degree Vertical Multi-Function Rotator for 10 min in 4 °C fridge then vortex for 20 s to ensure desorption.
18. Centrifuge at 8000×*g* for 1 min at 4 °C.
19. Remove 180 μl of supernatant and store in ×80 °C until run on the HPLC-EC. Be careful not to transfer alumina with the supernatant in order to prevent clogging of HPLC column during HPLC analysis.

5.3. HPLC-EC protocol (and NE content)

Settings and suggestions will vary with the HPLC-EC system used. The following is for the Coulochem II (ESA/DIONEX) system. Separation is performed by injecting 50 μl of sample onto a C-18 reverse-phase column (model HR-80; DIONEX/Thermo Fisher Scientific, Rockford, IL). Mobile phase is Cat-A-Phase II (Product: 45-0216; DIONEX/Thermo Fisher Scientific, Rockford, IL) at a flow rate of 1 ml/min. In-line filters are recommended to prevent accumulation of alumina in the analytical column. Coulometric electrochemical detection (Coulochem II; DIONEX/Thermo Fisher Scientific, Rockford, IL) settings are as follows: *guard cell* (DIONEX model 5021; Thermo Fisher Scientific, Rockford, IL) +350 mV, *analytical cell* (DIONEX model 5011; Thermo Fisher Scientific, Rockford, IL) *cell 1* +10 mV, and *cell 2*–300 mV. A three-point external standard curve is used for quantitation. Standards containing known concentrations of NE, E, and DA are injected every 10 unknowns. A single-point internally standardized method using dihydroxybenzylamine

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(DHBA; added during sample preparation) is used to control for extraction efficiency. Typical retention times for NE, E, DHBA, and DA on this system with settings/processing as above are 4, 4.5, 7, and 9.5 min, respectively.

NE concentrations are determined for the pre- and post-AMPT treatment groups. The total tissue weight of the adipose depot, the amount of adipose tissue used for catecholamine extraction, and the NE content obtained from HPLC-EC quantitation are used to calculate the concentration of NE per gram of adipose for both baseline and AMPT-injected animals (e.g., time=4 h; Brodie, Costa, Dlabar, Neff, & Smooker, 1966). Then, the NE content of the total adipose tissue (calculating NETO at fat pad level) is calculated by multiplying the adipose tissue mass (at time of harvest) and the concentration of NE per gram of adipose tissue for both baseline and AMPT-injected animals (e.g., time=4 h).

We use the following equation based on the method of Brodie et al. (1966): $k = (\log[NE]_0 - \log[NE]_4)/(0.434 * 4)$ and $K = k*NE_0$. To obtain the rate of NE efflux (k) that is used to obtain NETO, take the log NE content in the total fat tissue mass (log[NE]) and subtract the log of NE content of the total tissue mass at baseline and at “time=4 h” animals ($\log[NE]_0 - \log[NE]_4/(0.434 * 4)$. The NETO ($K$) is obtained by multiplying the rate of NE efflux by the initial NE content of baseline animals (Brito et al., 2007, 2008; Shi, Bowers, & Bartness, 2004; Youngstrom & Bartness, 1995).

6. ASSESSMENT OF SENSORY DENERVATION USING CGRP ELIA

Direct injection of capsaicin into WAT and BAT selectively and effectively destroys small unmyelinated C-fiber sensory nerves. This can be tested by assessing levels of the sensory nerve-associated peptides CGRP and/or substance P for effectiveness of the denervation and TH (ELIA) or NE (HPLC-EC) for selectivity. In our experience (unpublished observations), we find equivalent values for the percent depletions of SP and CGRP following intra-WAT or intra-BAT capsaicin injections. Therefore, we usually assess only CGRP. The presence of these sensory peptides has been identified immunohistochemically at the level of both WAT and BAT depots (Foster & Bartness, 2006; Giordano et al., 1998; Giordano, Morroni, Santone, Marchesi, & Cinti, 1996; Norman, Mukherjee, Symons, Jung, & Lever, 1988; Shi et al., 2005).

6.1. Required materials

- Microbalance for weighing tissue
- Dissection tools (see Section 5.1)
- Weighing papers (Fisher Scientific, Pittsburgh, PA; Cat #: 09-898-12A) or aluminum foil for weighing tissue
- Sharp scissors for rapid decapitation (Fiskars scissors, small animals) or guillotine (rats)
- Bullet Blender (Next Advance, Averill Park, NY) and accessories (see Section 5.2)
- 2 *M* acetic acid

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- 2 ml conical screw cap tubes (Fisher Scientific, Pittsburgh, PA)
- Eppendorf® Safe-Lock tubes (Fisher Scientific, Pittsburgh, PA)
- Lyophilizer (We use the FreeZone 6 l Shell Freezer model from Labconco, Kansas City, CO)
  - Accessories we use for FreeZone lyophilizer: Fast-freeze flask, Fast-freeze flask top and Lyph-lock flask adapter tube (Labconco, Kansas City, CO). Assemble these items together and use to hold samples for attachment to lyophilizer
- CGRP ELIA kit (Cat # 589101; Cayman Chemical, Ann Arbor, MI)
- Spectrophotometer

6.2. Preparation of samples

We typically use fresh adipose tissue for homogenization before the CGRP ELIA and it is unnecessary to fix tissue. If tissue will not be processed the same day as sacrifice, then weigh tissue, place in aluminum foil, snap freeze, and store at −80 °C. When using tissue that has been frozen, place frozen tissue in Safe-Lock tube with 2 *M* acetic acid and one scoop of zirconium beads and follow the homogenization steps (see below).

6.2.1 Protocol for homogenizing adipose tissue with Bullet Blender

1. Use 150–300 mg of tissue for analysis and place into a Safe-Lock microcentrifuge tube.
2. Add 750 µl 2 *M* acetic acid into each tube; for tissue <0.40 g, 400 µl is enough.
3. Thoroughly mince with surgical scissors if tissue is >0.03 g.
4. Add one scoop of zirconium oxide beads (0.5 mm) to each tube.
5. Close the microcentrifuge tubes and place tubes into the Bullet Blender.
6. Using the Bullet Blender at speed setting # 6, disrupt tissue at 1 min intervals. This should be repeated until tissue is fully homogenized, relatively opaque and containing no visible large pieces.
7. Heat at 90 ºC for 10 min in a hot block.
8. Centrifuge samples at 4 °C at 13,000 × g for 15 min.
9. Aspirate about 725 µl of the infranatant under the fat cake and add to sterile 2 ml conical screw cap microcentrifuge tube. Use a 200 µl pipette with a gel loading tip (multiflex round tips 1–200 µl) to aspirate out the remainder allowing the Bullet Blender beads to act as a filter. Place conical screw cap tube immediately on dry ice to freeze and store at −80 °C until ready to lyophilize.

6.2.2 Preparation of samples for ELIA using lyophilizer

1. Remove samples from −80 °C.

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2. Lyophilize samples according to manufacturer's directions for operation.
3. If using a FastFreeze flask, unscrew caps about halfway to allow vacuum suction access to the samples. Do not place the flask in freezer (−20 to −80 °C) or dry ice while placing samples in flask (use box grid). Use wet ice while placing samples into flask or refrigerate (4 °C) if loading more than one flask as the flasks will crack if placed in freezer.
4. When samples are all lyophilized, screw on caps tightly and place in −80 °C until assaying CGRP by ELIA.
5. Follow directions in CGRP ELIA kit to test for CGRP content.

7. EXPRESSION OF DATA

There are numerous arguments for how any or all of the above data should be expressed. For verification of sympathetic (NE content) or sensory (CGRP or SP content), the data can be expressed as the substance amount/mg tissue or substance amount/mg protein. As long as one is making comparisons between similar tissues (IBAT controls and IBAT denervated) with relatively the same amount of tissue, the total amount of the substance/pad seems more appropriate because physiology does not work on a relative basis. For example, a small change in a substance found in a large tissue (e.g., a liver enzyme) might be statistically nonsignificant when expressed per mg protein or mg tissue, but because of the size of the tissue (e.g., liver or muscle), physiologically this may be highly significant. Alternatively, the data can be expressed as a % of the control tissue (i.e., % depletion) based on total or relative values. When analyzing NETO, we also take the view that physiology does not work in a relative manner and we therefore express the data per adipose depot (e.g., Brito et al., 2007, 2008).

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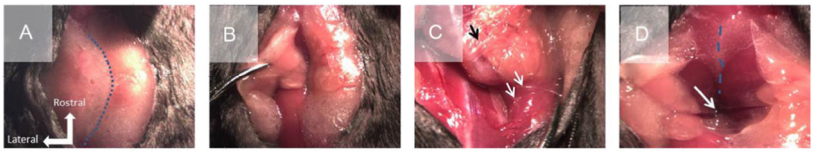

Figure 11.1.

IBAT nerve identification. The orientation of all pictures is the dorsal, inter-scapular surface. (A) Picture of both IBAT lobes. Dotted line delineates left from right IBAT. (B) Forceps revealing the ventral surface of the left IBAT lobe. (C) Picture of right IBAT and associated intercostal nerves supplying sympathetic and sensory innervation (black arrow, 3 nerves; white arrow, 1 nerve each). D. Sulzer's vein draining both BAT lobes (white arrow). Dotted line depicts midline and medial borders of both BAT lobes.

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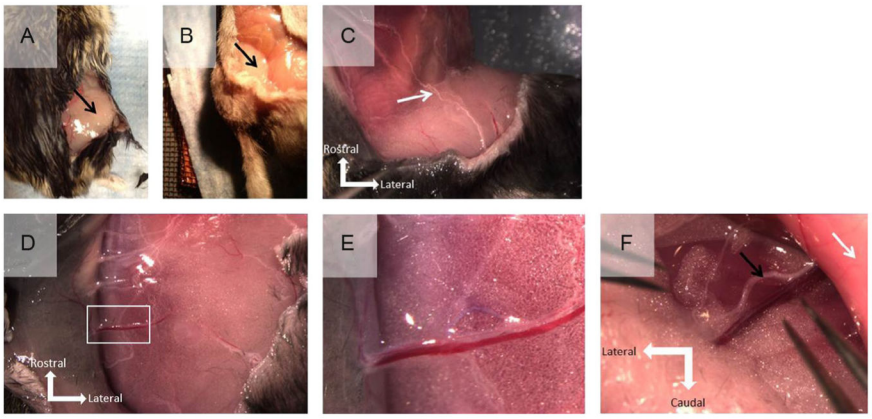

Figure 11.2.

IWAT nerve identification. Dorsal (A) and ventral (B) views of animal. Black arrow denotes IWAT pad location. (C) Dorsolateral view of nerves innervating (white arrow) fat and skin. (D) Medial border of the right IWAT (midline to the left of picture). (E) Magnification of white box in D depicting nerves on either side of blood vessel. (F) Ventral surface showing nerve that bifurcates sending one branch to leg and one to IWAT. Do not cut this nerve (black arrow). Peritoneal cavity to the right of this picture (white arrow).

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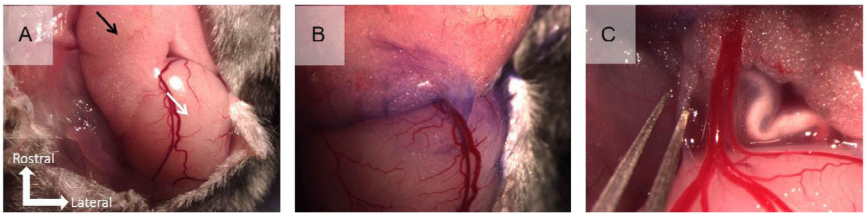

Figure 11.3.

EWAT nerve identification. (A) The right testis (white arrow) and right EWAT (black arrow). (B) Toluidine blue dye highlighting nerve innervating EWAT. (C) Forceps holding nerve to EWAT that should not be cut. If cut, loss of testis function occurs.

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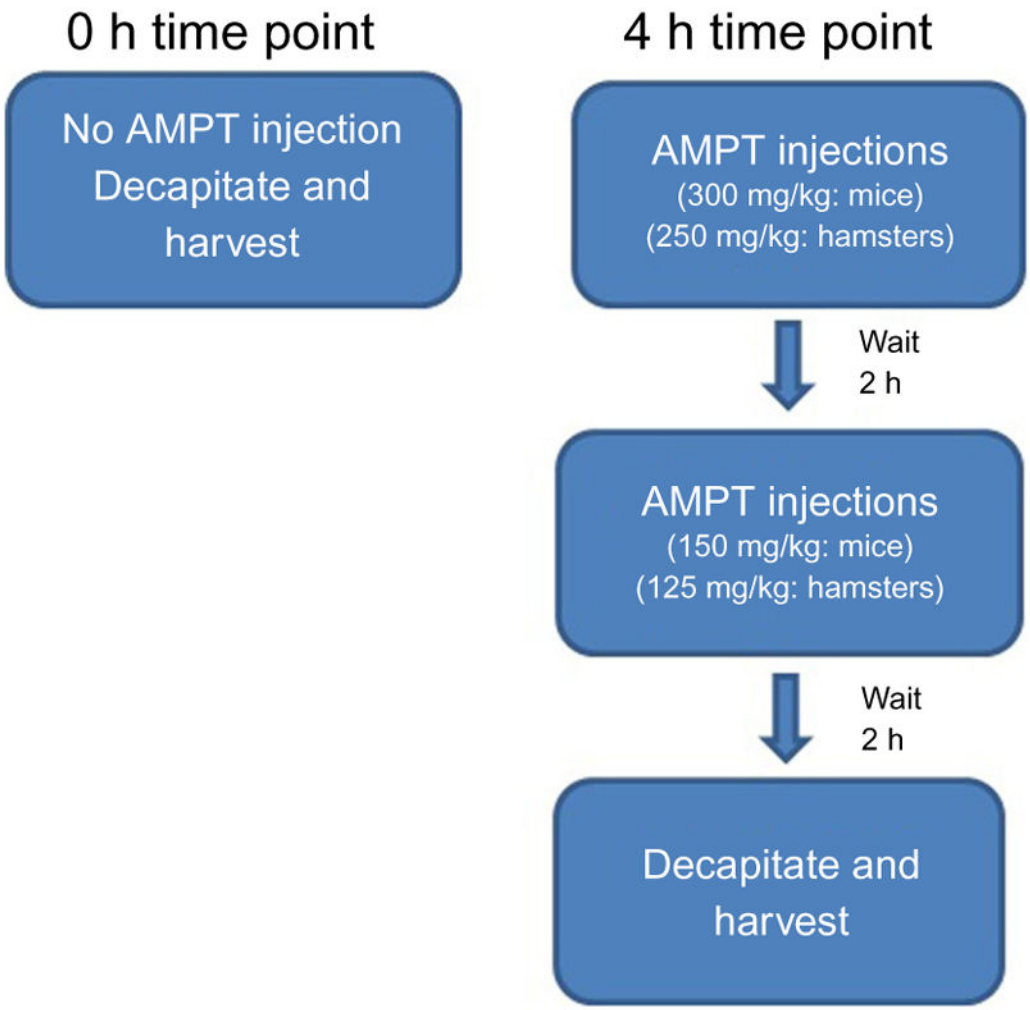

Figure 11.4.
Timeline of AMPT injections for NETO measurement.