Central control of thermogenesis in mammals

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Abstract

Thermogenesis, the production of heat energy, is an essential component of the homeostatic repertoire to maintain body temperature in mammals and birds during the challenge of low environmental temperature and plays a key role in elevating body temperature during the febrile response to infection. The primary sources of neurally regulated metabolic heat production are mitochondrial oxidation in brown adipose tissue, increases in heart rate and shivering in skeletal muscle. Thermogenesis is regulated in each of these tissues by parallel networks in the central nervous system, which respond to feedforward afferent signals from cutaneous and core body thermoreceptors and to feedback signals from brain thermosensitive neurons to activate the appropriate sympathetic and somatic efferents. This review summarizes the research leading to a model of the feedforward reflex pathway through which environmental cold stimulates thermogenesis and discusses the influence on this thermoregulatory network of the pyrogenic mediator, prostaglandin E_2, to increase body temperature. The cold thermal afferent circuit from cutaneous thermal receptors ascends via second-order thermosensory neurons in the dorsal horn of the spinal cord to activate neurons in the lateral parabrachial nucleus, which drive GABAergic interneurons in the preoptic area to inhibit warm-sensitive, inhibitory output neurons of the preoptic area. The resulting disinhibition of thermogenesis-promoting neurons in the dorsomedial hypothalamus and possibly of sympathetic and somatic premotor neurons in the rostral ventromedial medulla, including the raphe pallidus, activates excitatory inputs to spinal sympathetic and somatic motor circuits to drive thermogenesis.

Thermogenesis, the production of heat energy, is an essential component of the homeostatic repertoire to maintain body temperature during the challenge of low environmental temperature. In mammals, alert consciousness and normally patterned motor activities only occur when the temperature of the central nervous system is approximately 36-39°C. A fall in cellular temperature reduces enzyme efficiency and diffusion capacity, reducing cellular energy availability and membrane ion fluxes. Below-normal brain temperatures are associated with reduced alertness and with behavioural and physiological disorganization, although without physical damage to the CNS or peripheral tissues. In hibernating mammals, for instance, reduction of body and brain temperature to approximately 5°C is associated with torpor and inactivity, but normal consciousness and behaviour resume with reversal of the hibernation process. Additionally, the heat generated during stimulated thermogenesis contributes to fever, a controlled elevation in body temperature that reduces pathogen viability and stimulates immune cell responses. Since energy consumption during thermogenesis can involve oxidation of lipid fuel molecules, regulation of thermogenesis in response to metabolic signals can also contribute to energy balance and regulation of body adipose stores.

Thermogenesis can occur to a greater or lesser extent in most tissues, since heat generation is a byproduct of the inefficiency of mitochondrial ATP production and of ATP utilization. However, CNS thermoregulatory networks can stimulate thermogenesis in response to a cold

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environment, to a fall in core body temperature or to the presence of pyrogenic cytokines primarily in three tissues: brown adipose tissue (BAT), heart and skeletal muscle. During the rapid, repeated skeletal muscle contractions of shivering and during increases in heart rate, thermogenesis arises primarily from the inefficiency of energy utilization in cross-bridge cycling and calcium ion sequestration and, to a lesser degree, from mitochondrial membrane proton leak in the course of ATP production from fuel substrate oxidation (Rall & Woledge, 1990; Jubrias *et al.* 2008). The muscle contractions of shivering result from rhythmic bursts of activity in the α-motoneurons innervating skeletal muscle fibres. The CNS network generating the cold-evoked bursts of α-motoneuron activity is not well understood, but includes transmission of cutaneous cold afferent signals through the lateral parabrachial nucleus (LPB; Nakamura & Morrison, 2008*a*), integration of thermoregulatory signals in the preoptic area (POA; Zhang *et al.* 1995) and activation of fusimotor neurons that is dependent on neurons in the rostral ventromedial medulla (RVLM; Tanaka *et al.* 2006; Brown *et al.* 2007). The increases in heart rate in response to cold or during fever are sympathetically mediated and involve a neural network that parallels the one controlling the sympathetic activation of BAT thermogenesis.

In contrast to the indirect nature of shivering thermogenesis in skeletal muscles that are normally used to produce movement and posture, non-shivering or adaptive thermogenesis in BAT is the specific metabolic function of this tissue and is accomplished by the heat-generating capacity of a significant facilitated proton leak across the extensive mitochondrial membranes of the brown adipocytes, which occurs because of the high expression of uncoupling protein 1 (UCP1) in BAT mitochondria (Cannon & Nedergaard, 2004). The level of BAT sympathetic nerve activity (SNA) and noradrenaline release and β_3-adrenergic receptor binding to brown adipocytes determine the level of thermogenesis in BAT by regulating both the activity of lipases providing the immediate fuel molecules for BAT mitochondria and the level of expression of BAT mitochondrial UCP1 (Cannon & Nedergaard, 2004).

Brown adipose tissue has developed as an essential thermoregulatory effector in cold defense in rodents and other small mammals (Golozoubova *et al.* 2006), including infant humans, since their large surface area to body mass ratio suggests that basal metabolism alone would yield insufficient heat to maintain body temperature in cold environments. The potential significance of BAT thermogenesis in adult humans and other large mammals has been controversial, at least in part, because evidence for its existence has been lacking. However, several recent observations using positron emission tomographic scanning to assess tissue glucose uptake (reviewed by Nedergaard *et al.* 2007) have demonstrated a remarkable amount of brown adipose tissue in adult humans, and the locations of BAT depots in adult humans bear a striking similarity to those in rodents: a large BAT pad in the vicinity of the scapulae and shoulders and individual pads atop each sympathetic ganglion and surrounding the adrenal glands and kidneys. The curious localization of BAT pads, exemplified by those over each sympathetic ganglion, suggests that, in addition to the defense of core temperature in the cold, BAT may also serve an as yet undescribed function to maintain optimal neuronal and synaptic function in specific locations in situations of reduced core body temperature. Similar to its function in smaller mammals, adult human BAT activity (assessed by glucose uptake) is highly responsive to β-adrenergic agonists (Soderlund *et al.* 2007) and to environmental temperature (Christensen *et al.* 2006). The recent description of adult human BAT is expected to stimulate quantitative assessment of its significance in adult human thermoregulation and energy homeostasis.

This review describes our current understanding of the central thermoregulatory network through which cutaneous cold sensation or reductions in brain and core temperature elicit increases in BAT SNA and thermogenesis. The regulation of BAT thermogenesis in response to environmental temperature constitutes a feedforward reflex (Fig. 1), in that the stimulus of a cold environment in contact with skin thermal receptors is not, itself, affected by the evoked

thermogenic response, but rather, BAT thermogenesis is initiated to counter a 'predicted' fall in body core temperature that would result from the exposure to the cold environment. In contrast, the control of BAT thermogenesis by temperature-sensitive neurons in the brain constitutes a negative feedback reflex in that the neurons activated by the increases in brain temperature resulting from stimulated BAT thermogenesis act, in turn, to inhibit sympathetic outflow to BAT.

Mechanisms for thermoreception

Cutaneous cool thermoreception

Temperature information that is detected by thermoreceptors located in surface and core body parts is transmitted to the POA, which is a thermoregulatory centre located in the rostral pole of the hypothalamus. Environmental temperature has direct and more rapid effects on skin temperature than on the temperatures within the body core. When environmental temperature is reduced, skin temperature rapidly falls, whereas brain and rectal temperatures are not affected or slightly increased in rats (Lomax *et al*. 1964; Bratincsak & Palkovits, 2005), cats (Forster & Ferguson, 1952) and dogs (Hellstrom & Hammel, 1967). Thus, feedforward thermal afferents from the skin provide the POA with signals to rapidly initiate cold-defensive thermogenic responses before environmental thermal challenges affect body core temperature.

The molecular mechanism of cutaneous thermoreception has been extensively investigated. Recent studies suggest that the transient receptor potential (TRP) family of cation channels mediates sensation across a broad physiological range of temperatures. Among the TRP family channels, TRPM8 mediates a dominant part of cold sensation, since TRPM8-deficient nerve fibres show profound loss of cold sensitivity and TRPM8-deficient mice exhibit a reduced ability to avoid innocuous cold temperatures (Fig. 2; Bautista *et al*. 2007;Colburn *et al*. 2007;Dhaka *et al*. 2007). The TRPM8 channel is activated by modest cooling (environmental temperatures <27°C; McKemy *et al*. 2002;Peier *et al*. 2002*a*) and is distributed in the cell bodies, axons and peripheral free nerve endings of a population of primary somatosensory neurons that have small-diameter cell bodies in the dorsal root and trigeminal ganglia (McKemy *et al*. 2002;Peier *et al*. 2002*a*;Bautista *et al*. 2007), consistent with a role for TRPM8 in cold-sensing through C fibres. The TRPM8 channel can also be activated by menthol or icillin (McKemy *et al*. 2002;Peier *et al*. 2002*a*), and application of menthol to the skin evokes warm-seeking behaviour as well as cold-defensive, physiological responses, including BAT and shivering thermogenesis and cutaneous vasoconstriction (Tajino *et al*. 2007). However, the ability of TRPM8-deficient mice to maintain body temperature in cold environments has not yet been tested, although their basal core body temperature in a thermoneutral environment is not different from that of wild-type mice (Bautista *et al*. 2007).

Another potential cold receptor TRP channel is TRPA1, which is activated by colder temperatures (environmental temperatures <17°C) than TRPM8 or by mustard oil and other pungent spice ingredients (Story *et al*. 2003; Bandell *et al*. 2004; Jordt *et al*. 2004). However, contribution of TRPA1 to cold sensation *in vivo* is still controversial. Kwan *et al*. (2006) showed that TRPA1-deficient mice displayed a reduced sensitivity to a cold temperature (0°C), while Bautista *et al*. (2006) found no such deficit in the response to even lower temperatures (environmental temperatures ~-10°C) in TRPA1-deficient mice, and did not observe a delayed onset of shivering when these deficient mice were exposed to cold temperatures. A non-TRP channel-mediated cold-sensing mechanism in which cooling causes depolarization of cold-sensory neurons by closing a background K^+ channel has also been proposed (Viana *et al*. 2002).

Cutaneous warm thermoreception

The TRP family also has warm-sensitive channels, TRPV3 and TRPV4, which are activated by innocuous warm temperatures with a environmental temperature threshold of 33-39 and 25-34°C, respectively (Guler *et al*. 2002; Peier *et al*. 2002*b*; Smith *et al*. 2002; Watanabe *et al*. 2002; Xu *et al*. 2002). The TRPV3 channel is increasingly activated by repeated heating (Peier *et al*. 2002*b*;Xu *et al*. 2002) and, intriguingly, shows different sensitivity to the direction of temperature change, resulting in hysteresis across thermal activation-deactivation cycles (Xu *et al*. 2002), similar to thermosensitive responses exhibited by primary warm afferent fibres (Hensel *et al*. 1960; Iriuchijima & Zotterman, 1960; Hellon *et al*. 1975). The TRPV3 channel is also activated by camphor (Moqrich *et al*. 2005), which sensitizes cutaneous warm sensation, or by flavours derived from oregano, thyme and clove (Xu *et al*. 2006), which elicit a warm sensation when applied to the tongue. Expression of either TRPV3 or TRPV4 is prominent in keratinocytes in skin epidermis, but low in somatosensory ganglia (Guler *et al*. 2002; Peier *et al*. 2002*b*;Xu *et al*. 2006). Although it is still unknown how thermal information detected by these TRP channels in keratinocytes is transmitted to sensory nerves, factors released from stimulated keratinocytes, such as interleukin-1β (Xu *et al*. 2006), might diffuse to adjacent sensory nerves. Mice lacking either TRPV3 or TRPV4 show altered behaviours in discriminating innocuous warm temperatures compared with wild-type mice (Lee *et al*. 2005; Moqrich *et al*. 2005). However, TRPV4-deficient mice exhibit intact circadian changes in body temperature and can defend their core temperature in a cold (4°C) or hot environment (35°C; Liedtke & Friedman, 2003; Lee *et al*. 2005). The effect of TRPV3 deficiency on thermoregulatory responses to environmental thermal challenges remains to be investigated.

Thermoregulatory role of peripheral TRPV1

Intriguing effects on body temperature control have been reported for agonists and antagonists of TRPV1, a TRP channel that can be activated by a noxious range of heat (>43°C), by protons (pH ≤ 5.9) or by capsaicin (Caterina *et al*. 1997; Tominaga *et al*. 1998). Peripheral or central administration of capsaicin induces hypothermia (Jancso-Gabor *et al*. 1970; Hori, 1984), an effect beneficial to people living in hot climates, but is ineffective in TRPV1-deficient mice (Caterina *et al*. 2000). Furthermore, administration of potent TRPV1 antagonists induces hyperthermia (Swanson *et al*. 2005; Gavva *et al*. 2007) by both increasing metabolism and reducing heat loss from the body surface, but not by evoking warm-seeking behaviour (Steiner *et al*. 2007), and this hyperthermic effect is probably exerted by antagonizing TRPV1 channels located within abdominal viscera (Steiner *et al*. 2007). Therefore, tonic activation of peripheral TRPV1 channels, effected by non-thermal stimuli at body temperatures below the threshold for TRPV1 activation, could provide afferent signals to lower body temperature; however, TRPV1-deficient mice exhibit no obvious deficit in body temperature control (Szelenyi *et al*. 2004; Iida *et al*. 2005). Further investigations would be required to elucidate the thermoregulatory role of TRPV1.

Thermoreception in the body core

In addition to cutaneous thermoreception, thermoreceptive mechanisms exist in body core structures, including the brain, spinal cord and abdomen. The POA contains abundant neurons whose activity is affected by local brain temperature (Nakayama *et al*. 1961, 1963) and these are described below. Cold and warm receptors are included among the splanchnic and vagus nerve afferent fibres distributed in the abdomen, and their responses to temperature changes are similar to those of cutaneous thermoreceptors (Riedel, 1976; Gupta *et al*. 1979). However, how abdominal thermal information is transmitted to the POA is mostly unknown. Temperature changes in the spinal cord can affect the activity of thermoregulatory neurons in the POA (Guieu & Hardy, 1970). Although this finding implies the existence of thermoreceptive mechanisms inherent to the spinal cord, such as thermosensation by spinal neurons, TRP

channels that are located in the central endings of primary somatosensory fibres in the spinal dorsal horn (Tominaga *et al*. 1998; Bautista *et al*. 2007) are also likely to sense spinal temperature, and the spinal thermal signals could be integrated with cutaneous thermal signals at the spinal cord level. Temperatures in deep body core structures, including the brain, are not as immediately susceptible to changes in environmental temperature as are skin temperatures (Forster & Ferguson, 1952; Lomax *et al*. 1964; Hellstrom & Hammel, 1967; Bratincsak & Palkovits, 2005). Thus, rather than responding directly to changes in environmental temperature, core body thermosensation would be expected to play a role: (a) in setting the basal tone of thermoregulatory effector efferents; (b) in enhancing thermoregulatory responses in situations of extreme thermal environments when the feedforward thermoregulatory responses driven by changes in skin temperature have proven inadequate to prevent changes in brain or body core temperature; and (c) in responding to challenges to thermal homeostasis involving changes in temperature within the body, such as exercise, intake of cold fluids or haemorrhage. Thus, it is important to understand how thermosensory signals from the skin and from the body core are integrated.

Central afferent pathway for thermosensation

Dorsal horn lamina I neurons

Thermal information detected by cutaneous thermoreceptors is transmitted through primary somatosensory fibres to the spinal or trigeminal dorsal horns (Fig. 1), in which lamina I neurons receive most cutaneous thermal signals (Craig, 2002). The best-known thermal somatosensory ascending pathway from lamina I neurons is the spinothalamocortical pathway, in which lamina I neurons directly synapse on neurons in the thalamus that project to the primary somatosensory cortex, leading to perception and discrimination of cutaneous temperature (Craig *et al*. 1994;Craig, 2002). Craig and colleagues have described three main classes of spinothalamic and trigeminothalamic lamina I neurons that were categorized by their responses to cutaneous thermal and mechanical stimuli: nociceptive-specific cells responding to noxious mechanical and heat stimuli; polymodal nociceptive cells responding to noxious mechanical, heat and cold stimuli; and thermoreceptive-specific cells responding linearly to graded, innocuous cooling or warming stimuli and not being activated further in the noxious temperature range (Andrew & Craig, 2001;Craig *et al*. 2001). Considering that cutaneous thermal stimuli that trigger thermoregulatory responses are mostly in the innocuous range, thermoreceptive-specific lamina I neurons probably convey the dominant signals leading to body temperature control. The question of whether the spinothalamocortical pathway contributes to the thermoregulatory responses to defend body temperature during changes in environmental temperature and, if not, what mechanism conveys cutaneous thermal information to the thermoregulatory centre in the POA, has only recently been answered.

Thermoregulatory afferent pathway to the POA

Neuroanatomical and physiological experiments to answer these questions identified a 'missing link' in the central thermal afferent pathway connecting second-order thermal sensory neurons in the spinal dorsal horn to the thermoregulatory integration site in the POA. In functional neuronal tracing experiments, neurons retrogradely labelled following tracer injections into the POA and activated (Fos expression) following cold exposure (4°C) of the animals were found to be densely clustered in the external lateral (LPBel) subnucleus of the mesencephalic LPB with an extension into the central subnucleus (LPBc; Fig. 1, LPB inset; Nakamura & Morrison, 2008*a*). The greatest numbers of double-labelled LPB neurons were found when the tracer injection was centred on the mid-line subregion of the POA, including the median preoptic nucleus (MnPO), suggesting that neurons in the LPBel and LPBc transmit cool thermosensory signals mainly to the MnPO rather than more lateral POA subnuclei, such as the medial (MPO) or lateral POA (LPO; see Fig. 1, POA inset, for anatomical definitions

of the POA subnuclei). The LPB receives numerous projections from the dorsal horn (Cechetto *et al.* 1985;Bernard *et al.* 1995;Feil & Herbert, 1995). Indeed, axonal swellings of dorsal horn neurons were found to be closely associated with postsynaptic structures of POA-projecting LPBel neurons (Nakamura & Morrison, 2008*a*), providing further support for the view that POA-projecting LPBel neurons are activated by direct somatosensory inputs from dorsal horn lamina I neurons.

Physiologically, the LPB-mediated thermosensory pathway to the POA comprises an essential link in the feedforward pathway through which thermal homeostasis is maintained during environmental cold challenges. Either inhibition of local neurons in the LPBel or blockade of their glutamate receptors completely suppresses skin cooling-evoked cold-defense responses, including BAT and shivering thermogenesis and increases in metabolism and heart rate (Fig. 3; Nakamura & Morrison, 2008*a*). Thus, activation of LPBel neurons, probably by glutamatergic inputs from lamina I neurons driven by cutaneous cool signals, is essential for transmission of the cold thermal afferent stimulus to initiate cold defense responses (Fig. 1). Consistent with this notion, rats that have bilateral lesion of the LPB fail to maintain body temperature in a cool environment (17°C; Kobayashi & Osaka, 2003). Glutamatergic stimulation of LPBel neurons with NMDA can evoke increases in BAT thermogenesis, metabolism and heart rate that mimic skin cooling-evoked physiological responses. Such responses triggered by LPBel stimulation are blocked by antagonizing glutamate receptors in the MnPO (Nakamura & Morrison, 2008*a*), suggesting that cutaneous cool signalling to the POA is mediated by glutamatergic inputs from LPBel neurons to the MnPO (Fig. 1). The idea that the MnPO receives glutamatergic inputs for eliciting cold-defensive responses to environmental cooling is also supported by the finding that glutamatergic stimulation of MnPO neurons evokes thermogenic, metabolic and tachycardic responses similar to cold-defensive responses (Nakamura & Morrison, 2008*b*). Whether different thermoregulatory effector tissues are activated by different populations of MnPO neurons or whether a homogeneous population of cool afferent-responsive MnPO neurons influences effector-specific populations of MPO neurons is unknown.

In addition to thermosensory signals coming from the skin through the spinal cord, the LPB receives massive visceral afferent information, via the nucleus of the solitary tract, related to gastric tension, satiety, taste, thirst, blood pressure and temperature (Saper, 2002; Geerling & Loewy, 2008). The LPB subregions that receive these visceral projections from the nucleus of the solitary tract are located primarily caudal to the mesencephalic LPBel that receives thermal somatosensory projections from the dorsal horn (Herbert *et al.* 1990; Nakamura & Morrison, 2008*a*). It is possible, however, that POA-projecting, cool-responsive neurons in the LPBel receive visceral afferent signals through their caudally extending dendritic arbors or through interneurons connecting caudal and rostral LPB subregions. Further studies are needed to determine whether the thermal somatosensory responses of LPBel neurons in the MPO modified by other modalities to provide an integrated signal to the POA, a central site controlling a variety of homeostatic functions.

Recordings from single LPB cells *in vivo* revealed that the firing rate of most neurons in the LPBel that were antidromically identified as projecting to the MnPO increased markedly in response to skin cooling and then returned to the basal level following skin rewarming; responses that paralleled the skin cooling-evoked changes in BAT SNA (Nakamura & Morrison, 2008*a*). The firing rate increase of these cooling-responsive LPBel neurons was linearly related to the skin temperature in the range from 36.6 to 34.4 °C (a range just below the normal body temperature of rats) and was followed by a sustained elevation in firing during further skin cooling. Although skin temperature was still low, LPBel neuronal discharge rapidly returned to basal levels shortly after skin rewarming was initiated (Nakamura & Morrison, 2008*a*). This differential sensitivity to the direction of skin temperature change might be

ascribed to properties of cutaneous thermoreceptors, since a similar response hysteresis to skin cooling and rewarming has been observed in neurons in the thermosensory pathway, including cool-sensory primary fibres (Hensel *et al*. 1960; Iriuchijima & Zotterman, 1960) as well as thermoreceptive-specific lamina I neurons mediating cool signals (Craig *et al*. 2001). Most cool-sensitive LPBel neurons are not activated in response to noxious mechanical stimulus such as tail pinch (Nakamura & Morrison, 2008*a*), providing further support for the idea that LPBel neurons involved in thermoregulatory afferent signalling receive thermal information predominantly from thermoreceptive-specific lamina I neurons rather than other lamina I neurons mediating nociception.

Preliminary data suggest that cutaneous warm signals are also sent to the POA through an LPB-mediated thermal afferent pathway, which involves neurons in a different subnucleus of the LPB. Many POA-projecting neurons that are activated by innocuous heat exposure (environmental temperature of 36°C) are distributed in the dorsal subnucleus of the LPB, which also contains POA-projecting neurons whose firing activity is markedly increased by skin warming (Nakamura & Morrison, 2007*b*). Although the functional contribution of these warm-responsive LPB neurons in thermoregulatory responses is currently unknown, it seems reasonable to assume that cool and warm thermosensory signals, transmitted from the skin through separate LPB pathways, would have opposite effects on POA neurons controlling thermogenesis.

Spinothalamocortical pathway

The spinothalamo-cortical pathway for thermal perception and localization does not contribute to the feedforward mechanism for triggering involuntary thermoregulatory responses to environmental cold challenges. Rats with lesions of the thalamic regions that receive thermal somatosensory signals from the spinal cord exhibit no deficits in sympathetic thermogenic responses to skin cooling, but lack the skin cooling-evoked changes in primary somatosensory cortical EEG activity that are observed in intact animals, indicating that autonomic cold-defensive responses do not require a thalamic relay (Nakamura & Morrison, 2008*a*). Consistently, even after removal of the neocortex, dorsal hippocampus and most of the striatum, animals still maintain the ability to initiate a metabolic increase in response to skin cooling (Osaka, 2004*b*). Therefore, there are at least two thermosensory pathways ascending from the dorsal horn: the spinothalamocortical pathway, leading to perception and discrimination of cutaneous thermal sensation; and the spinoparabrachiopreoptic pathway, transmitting feedforward information on cutaneous temperature to the POA that is required for defending body temperature against changes in environmental temperature (Fig. 1). Anatomical studies suggest that dorsal horn lamina I neurons can provide cutaneous thermal signals to both the thalamus and LPB through their axon collaterals (Hylden *et al*. 1989; Li *et al*. 2006). Separate sensory afferent pathways for thermal perception and for thermoregulation may allow thermosensory signals going through the thalamus to the neocortex to be modulated by sensory signals of a different modality, such as vision or audition (Driver & Noesselt, 2008), while those to the POA via the LPB could be integrated with information on the status of other homeostatic variables, such as body fluid osmolarity and sodium and water balance (De Castro e Silva *et al*. 2006; Geerling & Loewy, 2006; De Luca *et al*. 2007) or food intake and fuel availability (Wang *et al*. 1999; Yamamoto & Sawa, 2000; Wilson *et al*. 2003). Additionally, the spinothalamocortical pathway may play a role in the appropriate induction of behavioural thermoregulatory responses, such as seeking a warm or cold environment.

Preoptic area mechanisms in thermoregulation

Response to thermal afferents from the skin

Based on the aforementioned findings, the POA receives feedforward cool signals from the skin that are probably mediated by glutamatergic inputs from cool-responsive LPBel neurons. The fact that the POA subregion receiving thermosensory cold signals is confined to the MnPO is suggested by the findings that the projections from LPBel neurons activated by skin cooling terminate mainly in a median part of the POA (Nakamura & Morrison, 2008*a*) and that glutamatergic stimulation or disinhibition of the MnPO with nanoinjections of NMDA or bicuculline, respectively, evokes physiological responses mimicking cold-defensive responses, while the same stimulation of the MPO or LPO does not (Nakamura & Morrison, 2008*b*). Similarly, disinhibition of neurons in the MnPO elicits shivering (K. Nakamura & S. F. Morrison, unpublished observations). Furthermore, inhibition of MnPO neurons completely blocks the activation of BAT thermogenesis and increases in metabolism and heart rate that are all evoked by skin cooling (Nakamura & Morrison, 2008*b*), indicating that activation of MnPO neurons is an essential process in the central mechanism for eliciting cold-defensive responses to environmental cold challenges (Fig. 1).

In a thermoneutral environment, when cold-defensive responses are not needed, inhibitory projection neurons in the MPO are postulated to tonically inhibit neurons in caudal brain regions, such as the dorsomedial hypothalamus (DMH) and rostral ventromedial medulla, including the rostral raphe pallidus nucleus (rRPa), whose activation leads to stimulation of cold-defense responses (Fig. 1). That such a disinhibitory mechanism is a fundamental aspect of the control of thermoregulatory effectors for cold defense is based on the findings that: (a) a coronal transection just caudal to the POA evokes BAT thermogenesis (Fig. 4*B*;Chen *et al.* 1998); (b) lesion of the MPO, but not of the ventral LPO, evokes hyperthermia by increasing metabolism and by stimulating shivering thermogenesis and heat conservation through cutaneous vasoconstriction (Szymusiak & Satinoff, 1982); and (c) inhibition of neurons in the MPO, but not those in the MnPO or LPO, increases body core temperature, EMG activity (shivering), metabolism and heart rate (Osaka, 2004*a*;Zaretsky *et al.* 2006). Furthermore, BAT and shivering thermogenesis as well as increases in metabolism and heart rate that are evoked by skin cooling are blocked by antagonizing GABA$_A$ receptors in the MPO (Osaka, 2004*a*;Nakamura & Morrison, 2007*a*). Thus, skin cooling-evoked responses are postulated to require a local circuit in the POA in which cutaneous cool signals that are received by MnPO neurons provide a GABA input to the inhibitory projection neurons in the MPO to reduce their tonic activity, resulting in disinhibition of neurons in caudal brain regions whose excitation stimulates thermoregulatory effectors for cold defense (Fig. 1). Consistent with this hypothesis, increases in BAT thermogenesis, metabolism and heart rate that are evoked by stimulation of MnPO neurons are all reversed completely by antagonizing GABA$_A$ receptors in the MPO (Nakamura & Morrison, 2008*b*). The existence of GABAergic interneurons in the MnPO that innervate the MPO projection neurons, although not yet directly demonstrated, is supported by the anatomical observations that: (a) some MnPO neurons innervate the MPO (Uschakov *et al.* 2007); (b) the MnPO contains many GABAergic neurons (Nakamura *et al.* 2002;Gong *et al.* 2004); and (c) many neurons in the MnPO, rather than the MPO or LPO, are activated (express Fos protein) in response to reduced environmental temperature (Bratincsak & Palkovits, 2004). Furthermore, microdialysis determinations of extracellular GABA in the POA of freely moving rats were elevated during cold exposure and reduced during heat exposure (Ishiwata *et al.* 2005).

Response to local brain temperature

Not only skin cooling but also cooling the local environment of POA neurons evokes sympathetic thermogenesis in BAT as well as shivering thermogenesis (Hammel *et al.* 1960;

Imai-Matsumura *et al*. 1984). Nakayama and colleagues made important progress by obtaining the first single-cell recording from thermosensitive neurons that are abundantly distributed in the POA (Nakayama *et al*. 1961, 1963), most of which are activated by warm temperatures (warm-sensitive neurons; Boulant & Dean, 1986; Fig. 4*A*). Warm-sensitive POA neurons are tonically active at thermoneutral temperatures (Nakayama *et al*. 1961, 1963), and the POA contains warm-sensitive neurons whose tonic discharge is reduced by skin cooling and also whose thermosensitivity to preoptic temperature is increased when the skin is cooled (Boulant & Hardy, 1974). We therefore propose (Fig. 1) that tonically active, warm-sensitive neurons located in the MPO are inhibited by reductions in local temperature and by inputs from cool-sensitive cutaneous thermoreceptors relayed via GABAergic MnPO neurons and that they function as MPO projection neurons to inhibit thermogenic neurons in the DMH (Nakamura *et al*. 2005) and in rRPa (Nakamura *et al*. 2002). However, whether warm-sensitive neurons project axons outside of the POA remains unknown.

The neurophysiological mechanism underlying the thermosensitivity of warm-sensitive neurons in the POA continues to be investigated. The suggestion that a heat-induced membrane depolarization allows warm-sensitive neurons to reach their discharge threshold potential and then determines their discharge frequency (Kobayashi *et al*. 2006) contrasts with the concept that a temperature-dependent (i.e. warming-dependent) facilitation of the rate of rise of a depolarizing (pacemaker) prepotential in warm-sensitive neurons shortens the intervals between action potentials and thereby increases the firing rate (Boulant, 2006). In the latter case, a transient, outward hyperpolarizing K^+ current (A-type potassium current) helps maintain a hyperpolarized membrane for a brief time after an action potential, and the heat-induced increase in the inactivation rate of the A-type potassium current allows the prepotential to depolarize at a faster rate (Boulant, 2006). Ion channels that could contribute to the thermosensitivity of warm-sensitive neurons, such as hyperpolarization-activated cyclic nucleotide-gated channels, background potassium leak channels and TRPV4, are distributed in the POA and these, except for TRPV4, are localized in the cell bodies of many POA neurons, but are also distributed ubiquitously in the brain (Guler *et al*. 2002; Wechselberger *et al*. 2006). Identification of the molecule(s) responsible for the thermosensitivity of POA neurons awaits further investigation, and the identification of specific anatomical markers for thermosensitive neurons would be a major discovery in this field.

Prostaglandin EP3 receptor-containing neurons in the POA in fever

Neurons in the POA are critically involved in the elaboration of fever as well as in thermoregulatory responses. Fever is a controlled elevation in body temperature that plays a significant role in the acute phase reaction stimulated by endogenous pyrogens released during infection, providing an optimal hyperthermic environment for mounting host defenses against invading bacteria and viruses while reducing pathogen viability. Prostaglanin E_2 (PGE_2), which is synthesized in the brain vasculature in response to immune signals (Elmquist *et al*. 1997; Matsumura *et al*. 1998; Yamagata *et al*. 2001), acts as a powerful endogenous pyrogenic mediator in the POA. An earlier study in which PGE_1 was injected into various sites throughout subcortical brain regions indicated that the POA is the sole region that can sense the E-series of prostaglandins to produce fever (Stitt, 1973; Williams *et al*. 1977). In a later study, detailed prostaglandin-sensitive sites within the POA were located by nanoinjecting a small dose of PGE_2 (1 ng in 10 nl vehicle) and showed that such sites are in the MPO and MnPO (Scammell *et al*. 1996). In these POA subregions, the EP3 subtype of PGE receptor is localized on many neuronal somata and dendrites (Fig. 1, POA diagram; Nakamura *et al*. 1999, 2000). Although mRNA expression for the EP1 and EP4 subtypes is also detected in the POA (Oka *et al*. 2000), analyses of mice lacking each of the known PGE receptor subtypes showed that only EP3 receptor-deficient mice completely failed to show a febrile response to PGE_2, interleukin-1β or endotoxin (Ushikubi *et al*. 1998), and EP1 receptor-deficient mice showed a

partial attenuation of endotoxin-induced fever (Oka *et al.* 2003). Furthermore, genetic deletion of the EP3 receptor specifically in neurons distributed in the MnPO and MPO suppressed most of the febrile response to PGE_2 or endotoxin (Lazarus *et al.* 2007). These lines of evidence indicate that the EP3 receptors in somatodendritic portions of POA neurons are the principal target site of PGE_2 for its pyrogenic action and that activation of these receptors by PGE_2 triggers the neuronal processes for fever induction.

The neuronal population expressing EP3 receptors in the POA provides direct projections to both the DMH and rRPa (Nakamura *et al.* 2002, 2005) and, as evidenced by pseudorabies viral tracing, EP3 receptor-expressing POA neurons multisynaptically innervate BAT (Yoshida *et al.* 2003). Since the majority of EP3-expressing POA neurons are GABAergic (Nakamura *et al.* 2002), the finding that antagonizing $GABA_A$ receptorsinthe DMH or rRPa evokes fever-like responses, including BAT thermogenesis and tachycardia (Morrison, 1999; Morrison *et al.* 1999; Zaretskaia *et al.* 2002; Cao *et al.* 2004), suggests that disinhibition of BAT and cardiac sympathoexcitatory neurons in these sites could contribute to febrile thermogenesis. Coronal transection just caudal to the POA evokes BAT thermogenesis (Fig. 4*B*; Chen *et al.* 1998), and inhibition of POA neurons with a muscimol nanoinjection elicits hyperthermic, cardiovascular and neuroendocrine responses similar to those evoked by a PGE_2 nanoinjection into the same site (Zaretsky *et al.* 2006). Binding of PGE_2 to EP3 receptors can inhibit neuronal activity by coupling to inhibitory GTP-binding proteins (Narumiya *et al.* 1999), and the tonic activity of most warm-sensitive neurons in the POA is inhibited by the E-series of prostaglandins (Schoener & Wang, 1976; Ranels & Griffin, 2003). Nonetheless, some spliced variants of EP3 receptors can also couple partially to stimulatory GTP-binding proteins (Negishi *et al.* 1995) and some warm-sensitive neurons in the POA are activated by PGE_2 (Matsuda *et al.* 1992). Together, these data suggest a model (Fig. 1) in which EP3-expressing POA neurons, potentially the population of warm-sensitive POA neurons described above, normally maintain a tonic GABAergic inhibition of thermogenic neurons in the DMH and/or the rRPa and, during infection, local or systemic PGE_2 binds to their EP3 receptors and reduces their tonic firing which, in turn, leads to disinhibition of thermogenic neurons in caudal brain regions and activation of thermoregulatory effectors to increase heat production and reduce heat loss (Nakamura, 2004; Nakamura *et al.* 2005).

A role for DMH neurons in driving thermogenesis

The observation that transection of the neuraxis immediately caudal to the POA (approximately 1.3-1.8 mm caudal to bregma) increases BAT SNA and BAT thermogenesis (Chen *et al.* 1998) suggests that the efferent output of the POA is inhibitory to thermogenesis. In contrast, transections made in the midbrain, just caudal to the hypothalamus (approximately 4.0-4.5 mm caudal to bregma), do not increase basal levels of BAT thermogenesis in normothermic animals (Rothwell *et al.* 1983) and, in fact, reverse PGE_2-evoked increases in BAT SNA and thermogenesis (Morrison *et al.* 2004; Rathner & Morrison, 2006). Therefore, a simple model consisting of only a long inhibitory pathway from the POA neurons to medullary sympathetic premotor neurons does not provide an adequate explanation of the pathways necessary for mediating BAT thermogenesis in response to removal of the efferent output of the POA or to PGE_2 within the POA, though such a pathway may contribute to BAT thermogenesis. Instead, data from experiments involving transection of the neuraxis suggest that a hypothalamic area is necessary for BAT thermogenesis in response to PGE_2 acting within the POA.

Several areas of the hypothalamus, including the paraventricular hypothalamus (PVH), the ventromedial hypothalamus (VMH), the posterior hypothalamus (PH) and the dorsomedial hypothalamus/dorsal area of the hypothalamus (DMH/DA), have been implicated in thermogenesis; however, in studies involving the PVH, VMH and PH, the anatomical specificity of the observations has been called into question (Dimicco & Zaretsky, 2007) owing

to the large injection volumes used, as well as the lack of appropriate anatomical control injections, or the use of electrical stimulation, electrolytic lesion or other methodological approaches that affect not only cell bodies but also fibres of passage. In contrast, several lines of evidence support a role of neurons in the DMH/DA in thermogenesis. Administration of endotoxin or cold exposure increases the expression of Fos in neurons of the DMH/DA (Elmquist *et al.* 1996; Cano *et al.* 2003; Sarkar *et al.* 2007). Furthermore, disinhibition of neurons within the DMH by blockade of GABA$_A$ receptors within the DMH increases BAT SNA (Cao *et al.* 2004) and thermogenesis (Fig. 5*A*; Zaretskaia *et al.* 2002), suggesting a tonic GABAergic inhibitory input to thermogenic neurons within the DMH (Fig. 1). This tonic GABAergic input to neurons within the DMH may originate in the POA, as evidenced by the observation that POA-derived GABAergic axon swellings make close appositions with DMH neurons, including those that project to the rRPa (Fig. 1, DMH inset; Nakamura *et al.* 2005). In addition, inhibition of neurons in the DMH/DA blocks febrile (Fig. 5*B*; Zaretskaia *et al.* 2003; Madden & Morrison, 2004; Morrison *et al.* 2004; Nakamura *et al.* 2005) and cold-evoked excitation of BAT SNA and thermogenesis (Nakamura & Morrison, 2007*a*), as well as shivering thermogenesis (Tanaka *et al.* 2001). Blockade of ionotropic glutamate receptors within the DMH also blocks the increase in BAT SNA and thermogenesis evoked by PGE$_2$ within the POA (Madden & Morrison, 2004), suggesting that a glutamatergic input to neurons within the DMH is essential for these febrile responses, though the source of this input has yet to be determined. Taken together, these data suggest a model in which GABAergic inhibitory inputs from POA to sympathoexcitatory neurons in DMH are reduced by skin or core cooling or by PGE$_2$ acting on EP3 receptors within the POA.

Since neurons in the DMH do not project directly to sympathetic preganglionic neurons, these neurons probably contribute to thermogenic sympathetic outflow by influencing the activity of the sympathetic premotor neurons responsible for thermogenesis. Neurons in the rostral ventromedial medulla, including the rRPa are the putative sympathetic premotor neurons for BAT thermogenesis (see discussion below) and, indeed, glutamate receptor activation within the rRPa is necessary for the increase in BAT SNA and thermogenesis evoked by disinhibition of neurons within the DMH (Cao & Morrison, 2006). However, the pathway(s) responsible for transmitting the thermogenic drive from the DMH to the rRPa is unclear. A direct monosynaptic pathway from neurons in the DMH to the rRPa has been implicated in transmitting thermogenic drive (Fig. 1), as suggested by the finding that some DMH/DA neurons that are labelled by microinjection of a retrograde tracer into the rRPa express Fos in response to thermogenic stimuli, such as endotoxin administration or stress (Sarkar *et al.* 2007). Further indirect evidence for the involvement of a monosynaptic pathway from neurons in the DMH to the rRPa in thermogenesis is the observation that some DMH neurons that are labelled by microinjection of a retrograde tracer into the rRPa receive close GABAergic appositions (putative synapses) from neurons in the POA (Fig. 1, DMH inset; Nakamura *et al.* 2005).

In addition to the evidence for a direct monosynaptic pathway from the DMH/DA to the rRPa, both anatomical and physiological lines of evidence suggest that the caudal periaqueductal grey (cPAG; approximately 8.0-8.7 mm caudal to bregma) may play a role in transmitting a thermogenic signal from the DMH to the rRPa. For example, some of the DMH/DA neurons that are labelled by microinjection of a retrograde tracer into the cPAG express Fos in response to cold exposure (Yoshida *et al.* 2005). Furthermore, some neurons in the cPAG are labelled by microinjection of pseudorabies virus into BAT (Cano *et al.* 2003). Many cPAG neurons project directly to the medullary raphe (Hermann *et al.* 1997), and neurons within the cPAG express Fos in response to cold (Cano *et al.* 2003); however, a conclusive demonstration of cold-evoked Fos expression in the neurons within cPAG that project to the raphe is lacking. Physiological evidence suggesting a role of the cPAG in non-shivering thermogenesis includes the observation that microinjection of the excitatory amino acid receptor agonist, D,L-

homocysteic acid, into the cPAG increases BAT temperature, without a concomitant increase in core temperature (Chen *et al.* 2002); however, in this study the animals were not paralysed, so there remains the possibility that the thermogenesis was due to shivering in the underlying muscle. In addition, in awake rats the increase in body temperature evoked by microinjection of bicuculline into the DMH is attenuated by ~50% by prior microinjection of muscimol into the cPAG (de Menezes *et al.* 2006). In contrast, when BAT SNA and thermogenesis were directly measured in anaesthetized and paralysed rats, during skin cooling the microinjection of muscimol into the cPAG did not reverse the skin cooling-evoked increase in BAT SNA and thermogenesis (Nakamura & Morrison, 2007*a*). There are many potential explanations for the apparent controversy concerning the role of neurons within the cPAG in thermogenesis, indicating the need for further investigation of the pathways transmitting the thermogenic drive from the hypothalamus to premotor neurons.

The rostral ventromedial medulla contains BAT, cardiac and somatic premotor neurons controlling thermogenesis

Within the hierarchical organization of the central network controlling thermogenic thermoregulatory effectors, medullary neurons play key roles as premotor neurons, providing excitatory input to spinal motor neurons, for the circuits regulating sympathetic BAT thermogenesis, heart rate and somatic shivering thermogenesis.

The locations of premotor neurons in sympathetic thermogenic pathways and characterization of their transmitter phenotypes have been determined anatomically through viral retrograde transynaptic transport studies involving tracer injections into the interscapular BAT pad. Neurons in the rostral ventromedial medulla, centred in the rRPa and ventral raphe magnus and extending over the pyramids to the parapyramidal (PPy) area, were consistently infected, including neurons in those studies with short post-inoculation times that are consistent with their having become infected by virtue of their synaptic contact with BAT sympathetic preganglionic neurons (Bamshad *et al.* 1999; Cano *et al.* 2003; Yoshida *et al.* 2003). Other brainstem regions with spinally projecting neurons also became infected following virus injections into BAT and these, as well as the rRPa area, overlapped regions previously shown with conventional retrograde tracing to provide inputs to the intermediolateral cell column (IML) containing sympathetic preganglionic neurons (SPNs). Although the existence of a variety of innervated cell types in all tissues (brown adipocytes and vascular smooth muscle in BAT, for instance) may account for a portion of this anatomical dispersion of potential premotor neurons, there may also be varying degrees of influence from several premotor populations onto the SPNs controlling specific effector cell types. A comparison of the localization of Fos induced by cold exposure, which activates BAT thermogenesis, with the locations of virally-labelled neurons following inoculations of BAT provided function-based evidence that the rRPa and the ventromedial parvicellular subdivision of the paraventricular hypothalamic nucleus were the two potential premotor populations having a principal role in mediating the descending regulation of the spinal sympathetic circuit controlling BAT thermogenesis (Cano *et al.* 2003). Viral retrograde tracing to identify central sympathetic pathways controlling the heart (Ter Horst *et al.* 1996) found the heaviest labelling in the rostral ventromedial medulla. Additionally, a potential role for neurons in the rostral ventromedial medulla, including the rRPa, in shivering thermogenesis is suggested by the intense viral retrograde labelling in this region following inoculation into skeletal muscle (Kerman *et al.* 2003).

Anatomical studies have indicated that rostral ventromedial medullary neurons, including those in rRPa and surrounding the pyramids, that project to the IML to influence the discharge of BAT SPNs contain one or more of the following markers: (a) the vesicular glutamate transporter 3 (VGLUT3), potentially indicative of glutamatergic neurons (Nakamura *et al.*

2004*a*; Stornetta *et al*. 2005); (b) serotonin (5-HT) or tryptophan hydroxylase, a synthetic enzyme for 5-HT (Cano *et al*. 2003; Nakamura *et al*. 2004*a*; Stornetta *et al*. 2005); and (c) glutamic acid decarboxylase-67 (GAD-67), a marker for GABAergic neurons (Stornetta *et al*. 2005; Fig. 1, rRPa inset). In addition to serotonin, IML-projecting neurons located in the rRPa and the PPy can contain thyrotrophin-releasing hormone and substance P. The fact that VGLUT3-expressing and serotonin-containing neurons in the rostral ventromedial medulla are functionally related to the control of cold-defense effectors, such as BAT, cardiac or shivering thermogenesis, is suggested by the findings that a significant percentage of VGLUT3-containing neurons in the rRPa express Fos in response to cold exposure or intracerebroventricular PGE_2 (Nakamura *et al*. 2004*a*) and that physiologically identified, putative serotonergic neurons in the rRPa increase their firing rate in response to PGE_2 administration or cold exposure (Martin-Cora *et al*. 2000).

Physiologically, activation of neurons in the restricted region of the rostral ventromedial medulla containing the rRPa, the PPy and ventral portions of raphe magnus increases BAT SNA, BAT thermogenesis and heart rate. Disinhibition of rRPa neurons following blockade of local $GABA_A$ receptors with nanoinjections of bicuculline into rRPa elicits a large and sustained increase in BAT SNA and BAT temperature and a sympathetically mediated tachycardia in anaesthetized rats with external body temperature support (Fig. 6*A*; Morrison *et al*. 1999;Cao & Morrison, 2003) and a marked increase in heart rate with no change in body temperature in awake rats (Zaretsky *et al*. 2003). Increases in muscle EMG activity and shivering are also elicited by disinhibition of rostral ventromedial medullary neurons (Nason & Mason, 2004). These data indicate that when a normothermic body temperature is maintained with an external heat source, the rRPa neurons controlling BAT SNA, the sympathetic outflow to the cardiac sino-atrial node and the discharge of motoneurons for skeletal muscle receive a tonic, GABAergic inhibition and that relief of this inhibition allows a potent increase in rRPa neuronal discharge. The finding that disinhibition of rRPa neurons increases their activity suggests the existence of on-going or bicuculline-activated excitatory inputs to these rRPa neurons or of a complement of membrane ion channels supporting intrinsic activity in these rRPa neurons. The physiological relief of a tonically active inhibitory input to BAT sympathetic premotor neurons in rRPa, potentially from warm-sensitive POA projection neurons (Fig. 1), is postulated to contribute to the stimulation of BAT thermogenesis in cold defense and in fever. The absence of an increase in body temperature following bicuculline administration into rRPa in awake animals in a room temperature environment is consistent with an on-going level of activity in rRPa neurons to drive BAT thermogenesis needed to maintain body temperature. Nanoinjections into rRPa of agonists for either NMDA or non-NMDA glutamate receptors evoke brief, but intense activation of BATSNA and increases in heart rate (Madden & Morrison, 2003). Thus, rRPa neurons capable of increasing the sympathetic drives to BAT and to the heart express NMDA and non-NMDA subtypes of glutamate receptors.

Conversely, inhibition of the activity in neurons in the rRPa reverses the increases in BAT SNA, BAT heat production and heart rate elicited by every thermogenic stimulus that has been tested. For example, the elementary cold defense stimulus of skin cooling elicits responses including increases in BAT SNA, BAT temperature (a measure of thermogenesis), expired CO_2 (reflecting a stimulation of oxidative metabolism such as in BAT and the heart) and heart rate (Fig. 6*B*, top panel). Nanoinjection of glycine into the rRPa (Fig. 6*B*, bottom panel) to inhibit local neurons produces a rapid and complete reversal of the skin cooling-evoked increase in BAT SNA (Fig. 6*B*, middle panel) and an immediate waning of the accompanying metabolic and cardiac responses, despite the sustained reduction in skin temperature (Nakamura & Morrison, 2007*a*). Inhibition of rostral ventromedial medullary neurons produces dramatic falls in body temperature in the awake rat (Fig. 6*C*;Zaretsky *et al*. 2003), consistent with the possibility that BAT sympathetic premotor neurons in the rRPa and BAT thermogenesis are actively contributing to the maintenance of core temperature in a room

temperature environment. Inhibition of rostral ventromedial medullary neurons also attenuates cold-evoked shivering responses (Brown *et al.* 2007) and eliminates the activation of fusimotor neurons produced by skin cooling (Tanaka *et al.* 2006). The effects of inhibiting neurons in rRPa have led to the conclusion that the activity of sympathetic premotor neurons in rRPa for BAT and for the heart provides the supraspinal excitatory drive to spinal sympathetic circuits that is both necessary and sufficient for the thermogenic and tachycardic responses to thermoregulatory, febrile and a variety of neurochemical mediators that increase body temperature. Although more detailed investigations are needed, the rostral ventromedial medulla also appears to contain bulbospinal neurons whose activity is required for cold-evoked shivering thermogenesis. In addition to cold defense, other thermogenic stimuli whose stimulated BAT thermogenesis and tachycardia are reversed or prevented by inhibition of neural activity in the rRPa region of the rostral ventromedial medulla include the pyrogenic mediator, PGE_2 (Nakamura *et al.* 2002;Madden & Morrison, 2003;Morrison, 2003;Ootsuka *et al.* 2008); disinhibition of neurons in the DMH (Cao *et al.* 2004) or in the lateral hypothalamus (Cerri & Morrison, 2005); activation of central μ-opioid receptors (Cao & Morrison, 2005), central melanocortin receptors (Fan *et al.* 2007) or preoptic corticotrophin-releasing factor receptors (Cerri & Morrison, 2006); and systemic administration of the adipose tissue hormone, leptin (Morrison, 2004).

Blockade of glutamate receptors in the rRPa is also effective in reversing cold-evoked and pyrogen-mediated stimulations of BAT thermogenesis and of heart rate (Madden & Morrison, 2003; Nakamura & Morrison, 2007*a*), as well as those elicited by disinhibition of neurons in the DMH (Cao & Morrison, 2006). These findings implicate glutamate receptor activation in the tonic excitation of BAT and cardiac sympathetic premotor neurons that may be revealed by relief of their tonic inhibitory inputs (Fig. 1) or in their excitation from rostral inputs, such as neurons in the DMH (Fig. 1). Together with the anatomical evidence for direct projections from the rRPa region to IML neurons, including the SPNs, that control BAT thermogenesis, these functional data indicate that the rRPa region contains the principal population of BAT sympathetic premotor neurons providing the final common bulbospinal pathway for the sympathoexcitatory drive to the spinal network controlling BAT SNA (Fig. 1).

In addition to BAT sympathetic premotor neurons in the medulla and neurons in the cPAG that may contribute to their excitation, the brainstem also contains sources of inhibition of BAT thermogenesis. The existence of a tonically active inhibition of BAT thermogenesis from neurons in the region of the pontine retrorubral field (Fig. 1) was indicated by the large increases in BAT temperature that followed transections of the neuraxis in the vicinity of the pontomedullary junction (Rothwell *et al.* 1983), but which were absent if the transection was made rostral to the pons, but caudal to the DMH (Rothwell *et al.* 1983). Neither the exact location of the neurons mediating this inhibition nor the physiological basis for its control has been determined. The rostral ventromedial PAG in the midbrain contains a population of neurons whose disinhibition can reverse the BAT stimulation in response to PGE_2 in the POA and that following disinhibition of neurons in the DMH (Rathner & Morrison, 2006). The BAT sympathoinhibitory effects of these neurons appear to be mediated through a reduced excitability of neurons in rRPa; however, the pathways controlling this inhibition are unknown.

The brainstem also contains the pathways mediating the inhibition of BAT thermogenesis in response to arterial hypoxia, a reflex to restrict oxygen consumption in the face of reduced oxygen availability or compromised oxygen diffusion and transport in the blood. Systemic hypoxia or bolus systemic injections of sodium cyanide produce a prompt and complete reversal of the BAT SNA activations evoked by hypothermia and by PGE_2 in the POA, and this response to hypoxia is eliminated by section of the carotid sinus nerves or by inhibition of second-order arterial chemoreceptor sensory neurons in the commissural region of the nucleus of the solitary tract (Madden & Morrison, 2005). Interestingly, hypoxia also eliminates the

BAT SNA activation resulting from bicuculline nanoinjection into the rRPa, suggesting that activation of a GABAergic input to BAT sympathetic premotor neurons in rRPa is unlikely to mediate the hypoxic inhibition of BAT thermogenesis. Indirect evidence points to a possible role for a spinal inhibitory mechanism. Similar to arterial hypoxia, disinhibition of neurons in the rostral ventrolateral medulla (RVLM) reduces the BAT SNA activation following bicuculline into the rRPa (Morrison, 1999), and both anatomical (Stornetta *et al*. 2004) and electrophysiological studies (Deuchars *et al*. 1997) support the existence of a bulbospinal inhibitory pathway to SPNs from the RVLM. The pathway for the hypoxic inhibition of BAT metabolism between the nucleus of the solitary tract and the BAT SPNs remains to be investigated.

Spinal sympathetic and somatomotor mechanisms in thermogenesis

The discharge of BAT SPNs that determines the level of BAT SNA and BAT thermogenesis, as well as the rhythmic bursting characteristic of BAT SNA, is governed by their supraspinal and segmental inputs as well as those to the network of spinal interneurons that influence BAT SPN excitability. A significant fraction of the BAT sympathetic premotor neurons in rRPa are glutamatergic and/or serotonergic neurons (Fig. 1, rRPa inset). Consistent with these findings from viral retrograde tracing studies, 5-HT-containing (Bacon & Smith, 1988;Vera *et al*. 1990) and VGLUT3-containing terminals synapse on SPNs (Stornetta *et al*. 2005) or make close appositions with SPN dendrites (Fig. 1, IML inset; Nakamura *et al*. 2004*a*,b). Sympathetic preganglionic neurons contain ionotropic glutamate receptors (Aicher *et al*. 2000), and several subtypes of 5-HT receptors are located within the IML (Marlier *et al*. 1991;Thor *et al*. 1993;Maeshima *et al*. 1998;Doly *et al*. 2004), although the specific neuronal subtypes expressing these receptors have not been determined. Viral inoculations of sympathetically innervated tissues (Brooke *et al*. 2002), including interscapular BAT (Cano *et al*. 2003), consistently label a population of spinal interneurons in the vicinity of the IML. Spinal GABAergic interneurons would appear to be among this population, since they influence the discharge of SPNs (Deuchars *et al*. 2005). That such interneurons could receive inputs from the BAT premotor area in the rostral ventromedial medulla is suggested by the demonstration that VGLUT3- and GAD-67-containing terminals synapse on GABAergic neurons in the IML (Stornetta *et al*. 2005).

In general, SPNs are activated by glutamate receptor agonists (Coote *et al*. 1981) and by 5-HT (Lewis & Coote, 1990; Pickering *et al*. 1994). Nanoinjection of glutamate or NMDA into the upper thoracic IML activates BAT SNA and BAT thermogenesis (Nakamura *et al*. 2004*a*; Madden & Morrison, 2006). Figure 7*A* illustrates the brief, but marked increases in BAT SNA consistently evoked by nanoinjections of NMDA into the IML in the fourth thoracic segment, which contains a high concentration of SPNs for interscapular BAT (Cano *et al*. 2003). Furthermore, blockade of glutamate receptors in the upper thoracic IML suppresses the increase in BAT thermogenesis evoked by disinhibition of rRPa neurons (Fig. 7*C*; Nakamura *et al*. 2004*a*). These findings support the view that the glutamatergic premotor pathway from the rRPa to the IML is an essential mechanism in the control of BAT thermogenesis.

Nanoinjection of serotonin into the fourth thoracic IML can also activate BAT SNA and BAT thermogenesis (Madden & Morrison, 2006). Additionally, when nanoinjection of NMDA into the IML is preceded by local injection of a dose of 5-HT that does not activate BAT SNA by itself, the BAT SNA response to subsequent glutamate receptor activations is markedly enhanced (Fig. 7*A*; Madden & Morrison, 2006). Furthermore, such potentiation by serotonin allows a subthreshold dose of NMDA into the IML to evoke BAT SNA (Madden & Morrison, 2006). This serotonergic potentiation of glutamate receptor-mediated increases in BAT SNA is blocked by antagonists of 5-HT$_{1A}$/5-HT$_7$ receptors (Fig. 7*B*; Madden & Morrison, 2008). A functional role for spinal serotonergic neurotransmission in supporting the elaboration of

cold-defense responses is suggested by the increase in spinal 5-HT turnover (an index of 5-HT release) during cold exposure (Passerin *et al*. 1999) and by the effectiveness of 5-HT receptor antagonism in the T3-T6 IML in preventing the cooling-evoked stimulation of interscapular BAT SNA (Madden & Morrison, 2007). These data demonstrate that the serotonergic innervation provided by raphe neurons to IML neurons, including SPNs, plays a critical role in eliciting physiologically relevant levels BAT thermogenesis in cold defense. The significant role of serotonin-containing neurons in normal cold-defense responses is also supported by the recent finding that mice that lack almost all central serotonergic neurons show blunted BAT thermogenesis during cold exposure (Hodges *et al*. 2008). The mechanisms of the interaction between glutamatergic and serotonergic neurotransmission in the IML remain to be elucidated.

While the parsimony of a model consisting only of supraspinal glutamatergic and serotonergic excitatory inputs to SPNs is attractive, other data suggest that the neurochemical phenotypes of the VGLUT3-containing and tryptophan hydroxylase-containing neurons might not be so straightforward and that more complex spinal circuitry may also influence BAT SNA and thermogenesis. Although exclusively asymmetric (putatively excitatory) synapses for VGLUT3-containing terminals have been described within the IML (Nakamura *et al*. 2004*b*), another study indicates that while the majority (~60%) of the VGLUT3-containing terminals in the IML synapse on SPNs, they form approximately equal numbers of asymmetric and symmetric synapses, suggesting the possibility that these inputs may be either excitatory or inhibitory (Stornetta *et al*. 2005). Supporting the suggestion that some of the VGLUT3-containing inputs from the ventral medullary raphe to the IML are inhibitory, this study found that the majority of the spinally projecting VGLUT3-containing neurons of the ventral medullary raphe contain GAD-67 and some of the VGLUT3-containing terminals within the IML also contain GAD-67 and make symmetric synapses (Stornetta *et al*. 2005).

It is also noteworthy that some of the putatively inhibitory inputs within the IML synapse on GABAergic dendrites (Stornetta *et al*. 2005), providing a potential anatomical substrate for a pathway that increases the activity of SPNs through disinhibition. Similarly, 5-HT terminals form close appositions with GABAergic interneurons in the central autonomic area (Conte *et al*. 2007). Physiologically, 5-HT in the spinal cord potentiates sympathetic outflow to BAT at least in part via 5-HT$_{1A}$ receptors and, based on the known inhibitory signal transduction systems for 5-HT$_{1A}$ receptors, this effect could be mediated via inhibition of GABA inputs to BAT SPNs (Madden & Morrison, 2008).

Further complexity in the spinal neurocircuitry regulating BAT SNA and thermogenesis may also be introduced by other neurochemical inputs. For example, catecholamines could be postulated to modulate the activity of SPNs regulating BAT, especially considering the excitatory and inhibitory effects of catecholamines on SPNs (Coote *et al*. 1981; Miyazaki *et al*. 1989) and the observation of a dense dopamine *β*-hydroxylase innervation of SPNs that are labelled by pseudorabies virus injection into BAT (Cano *et al*. 2003). In addition, substance P terminals innervate SPNs (Vera *et al*. 1990), substance P directly excites the majority of SPNs (Cammack & Logan, 1996) and intrathecally administered substance P affects thermoregulation (Dib, 1987), suggesting a potential role for substance P in modulating sympathetic outflow to BAT. While the present data clearly demonstrate that the spinal cord is much more than a simple relay circuit driving BAT SNA and thermogenesis, the precise details of the spinal neurocircuitry involved in the regulation of thermogenic effector systems await future studies.

The ventral horn of the spinal grey matter contains both *α*-motoneurons and fusimotor (*γ*)-neurons projecting to skeletal muscle fibres and to muscle spindles, respectively. During shivering, two patterns of EMG activity occur to varying degrees: (a) a continuous, low-amplitude activity representing an increase in tone in type I muscle fibres specialized for lipid

fuel consumption; and (b) bursts of large-amplitude activity, indicating synchronous activation of type II muscle fibres preferentially consuming carbohydrate fuel (Haman, 2006). These two patterns of EMG activity presumably are the result of similar patterns of activation of the respective α-motoneurons innervating these muscle fibre types. During shivering, the dynamic fusimotor system activates muscle spindles (Schafer & Schafer, 1973*a*), and this cooling-evoked activation of fusimotor neurons is significantly dependent on the activity of neurons in the rostral medullary raphe (Sato *et al.* 1990; Tanaka *et al.* 2006). The increase in Ia afferent activity arising from muscle spindle activation could elicit an increase in α-motoneuron discharge and the muscle tone corresponding to the first pattern of EMG activity in shivering. The mechanisms underlying synchronous discharge of the populations of α-motoneurons producing the large-amplitude bursts in EMG activity are unknown, although an instability in the stretch reflex loop and a 'pacemaker'-like function of spinal motor control circuits have been proposed (Schafer & Schafer, 1973*b*).

Summary and future directions

Thermogenesis is regulated in BAT, the heart and skeletal muscle by what appear to be parallel networks in the central nervous system which respond to the feedforward afferent signals from cutaneous and core body thermoreceptors and to feedback signals from brain thermosensitive neurons to activate specific populations of sympathetic and somatic efferents. We have summarized the research leading to a model (Fig. 1) of the feedforward reflex pathway through which environmental cold stimulates thermogenesis, including the influence on this thermoregulatory network of the pyrogenic mediator, PGE_2, to increase body temperature. The cold thermal afferent circuit from cutaneous thermal receptors ascends via second-order thermosensory neurons in the medullary and spinal dorsal horn to activate neurons in the LPBel which drive GABAergic interneurons in the MnPO to inhibit warm-sensitive, inhibitory output neurons of the MPO. The resulting disinhibition of thermogenesis-promoting neurons in the DMH and possibly of sympathetic and somatic premotor neurons in the rostral ventromedial medulla, including the rRPa, activates excitatory inputs to spinal sympathetic and somatic motor circuits to drive thermogenesis.

Although significant recent research progress has enhanced our understanding of the central control of thermogenesis and body temperature, several areas remain poorly understood. As with the circuits controlling other autonomic functions, the sources and mechanisms responsible for the tonic discharge and the synchronous bursting that characterizes the sympathetic outflow to thermogenic effectors remain to be delineated. The neural basis for the tremor-like discharge in somatic motor neurons during shivering is not known. The sites and mechanisms underlying the critical integration of thermogenesis with other homeostatic systems regulating oxygen and fuel substrate availability and energy balance remain to be investigated. The projection neurons connecting important regions in the central thermogenic pathways remain to be characterized, including the inhibitory output neurons of the POA, the excitatory output neurons in the DMH and the sympathetic premotor neurons in the rRPa and PPy regions of the medulla. The marked differences in temperature response thresholds among thermoregulatory effectors suggests that significant differences should be expected among the populations of warm-sensitive POA projection neurons that control these effectors. The microcircuitry of the POA and the role and regulation of the pontine inhibitory area for thermogenesis also represent significant lacunae in our understanding of the functional organization of the model circuit for central control of thermogenesis that we have proposed. We hope that this review will inspire research into some of these questions.

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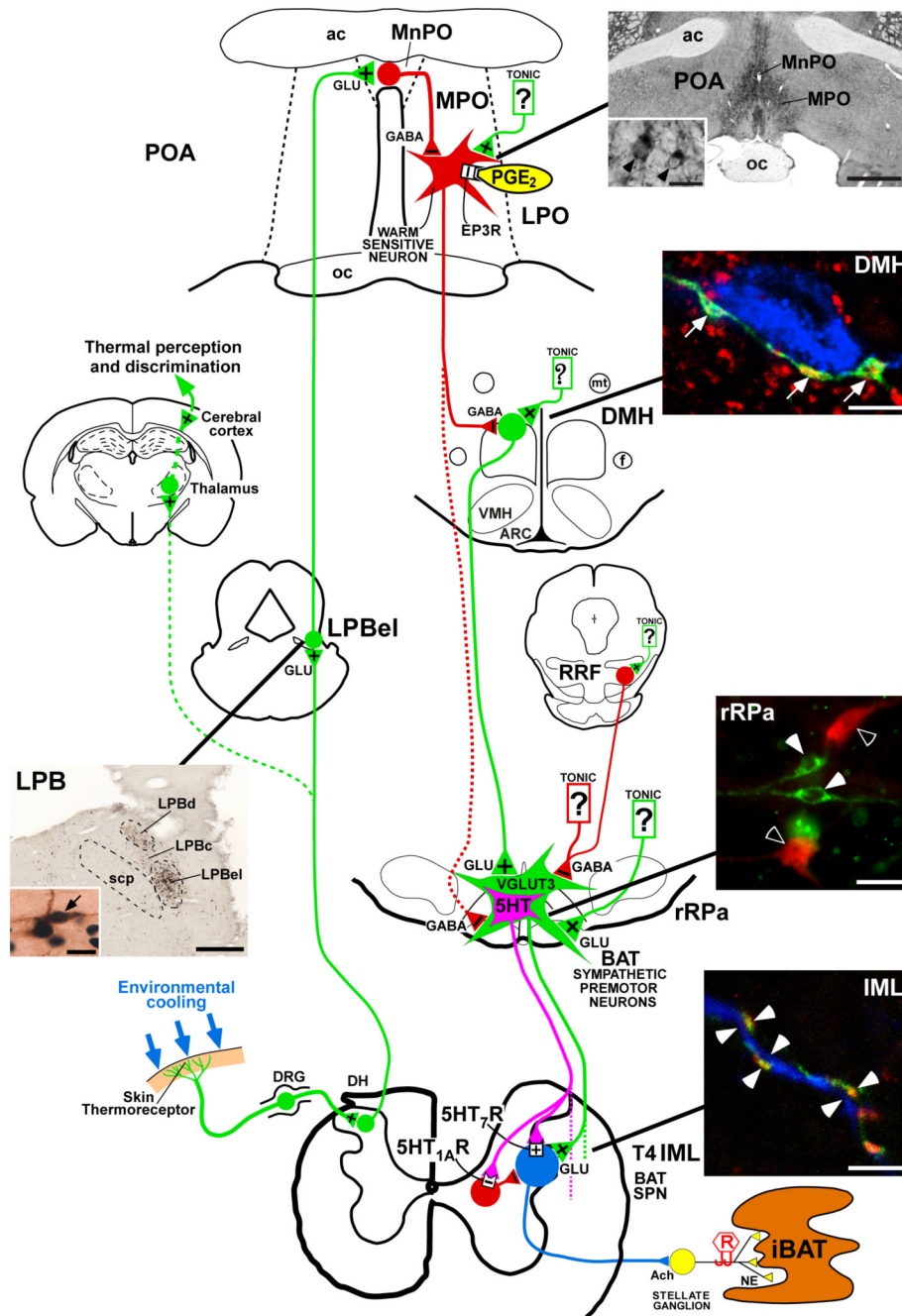
Figure 1. Schematic diagram summarizing the proposed model for the feedforward reflex circuit for cold-evoked brown adipose tissue thermogenesis

Photomicrographs of the lateral parabrachial nucleus (LPB), the preoptic area (POA), the dorsomedial hypothalamus (DMH), the rostral raphe pallidus (rRPa) and spinal intermediolateral nucleus (IML) illustrate the anatomical substrates for key neurochemical and synaptic aspects of the proposed thermoregulatory circuit. In the LPB panel, (LPBel), central (LPBc) and dorsal (LPBd) subnuclei of the LPB contain neurons that are retrogradely labelled with a tracer (brown) from the median preoptic (MnPO) subregion of the POA. Retrogradely labelled neurons in the LPBel and LPBc, but not those in the LPBd, also express Fos (blue-black nuclei) following cold exposure of the animals (arrow, inset); scp, superior cerebellar

peduncle; scale bars represent 0.5 mm and 15 μm (inset). Reproduced with permission from Nakamura & Morrison, 2008*b*). In the POA panel, immunohistochemistry for EP3 receptors (EP3R) shows the localization of these receptors in cell bodies (inset, arrowheads) and dendritic fibres of neurons that are distributed in the MnPO and medial preoptic (MPO) subregions of the POA; ac, anterior commissure; oc, optic chiasm; LPO, lateral preoptic area; PGE2, prostaglandin E2; GLU, glutamate; scale bars represent 1 mm and 20 μm (inset). Reproduced with permission from Nakamura *et al*. (1999). In the DMH panel, axon swellings of POA neurons (green) that are positive for a marker of GABAergic terminals (red) are closely apposed (arrows) to neurons that are retrogradely labelled with a tracer (blue) from the rRPa; ARC, arcuate nucleus; f, fornix; mt, mammillothalamic tract; VMH, ventromedial hypothalamic nucleus; RRF, retrorubral field; scale bar represents 5 μm. Reproduced with permission from Nakamura *et al*. (2005). The rRPa panel shows double immunofluorescence labelling for vesicular glutamate transporter 3 (VGLUT3)-positive (green, white arrowheads) and serotonin (5-HT)-positive neurons (red, open arrowheads); BAT, brown adipose tissue; scale bar represents 30 μm. The IML panel shows that axon swellings of rRPa neurons (green) that are positive for VGLUT3 (red) are closely associated (white arrowheads) with dendritic fibres positive (blue) for a marker of sympathetic preganglionic neurons (SPNs); Ach, acetylcholine; DH, dorsal horn; DRG, dorsal root ganglia; iBAT, interscapular BAT; NA, noradrenaline; R, recording electrode; scale bar represents 5 μm. Reproduced with permission from Nakamura *et al*. (2004*a*).
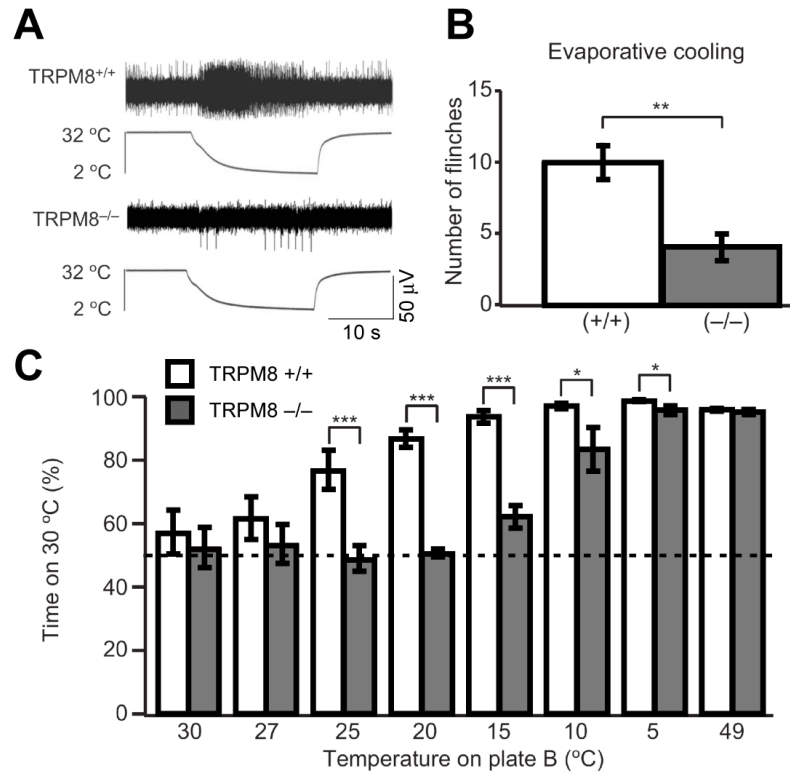
Figure 2. Mice deficient in TRPM8 exhibit profoundly diminished responses to cold
A, the discharge response of cutaneous C fibres to a cold ramp that is seen in wild-type mice (top) mostly disappears in TRPM8-deficient mice (bottom). *B*, licking and flinching in response to evaporative cooling was measured for 1 min following application of acetone to the hindpaw. The TRPM8-deficient mice displayed significantly decreased behaviour compared with wild-type littermates. *C*, wild-type and TRPM8-deficient littermates were allowed to choose between adjacent surfaces adjusted to 30°C *versus* a range of temperatures, as shown. The percentage of time spent at 30°C over a 5 min period is shown. The TRPM8-deficient mice show a clear, but not complete, deficit in their ability to discriminate between cold and warm surfaces. Graphs display means ± s.e.m.; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. Modified with permission from Bautista *et al.* (2007)
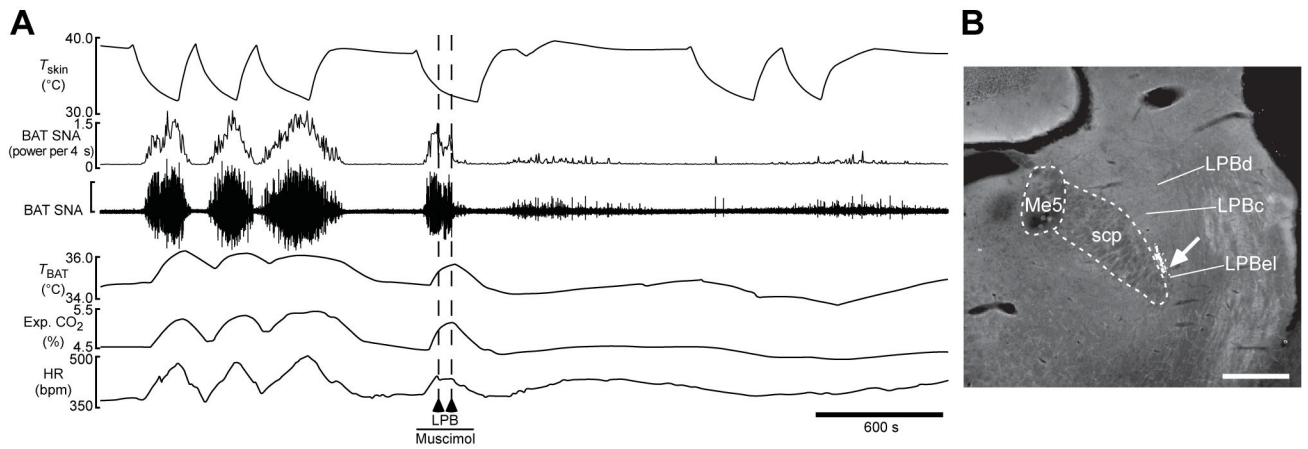
Figure 3. Inhibition of neuronal activation in the lateral parabrachial nucleus (LPB) blocks skin cooling-evoked thermogenic, metabolic and cardiac responses
A, increases in brown adipose tissue sympathetic nerve activity (BAT SNA), BAT temperature (T_{BAT}), expired (Exp.) CO_2 and heart rate (HR) that were evoked by reducing temperature of rat trunk skin (T_{skin}) were no longer observed after bilateral nanoinjections (dashed lines) of muscimol into the external lateral part of the LPB (LPBel); vertical scale bar represents 100 μV for BAT SNA. *B*, representative view of a nanoinjection site in the LPBel as identified with fluorescent beads (arrow). Abbreviations: LPBc, central part of the LPB; LPBd, dorsal part of the LPB; and Me5, mesencephalic trigeminal nucleus; scale bar represents 0.5 mm. Modified with permission from Nakamura & Morrison (2008*a*).
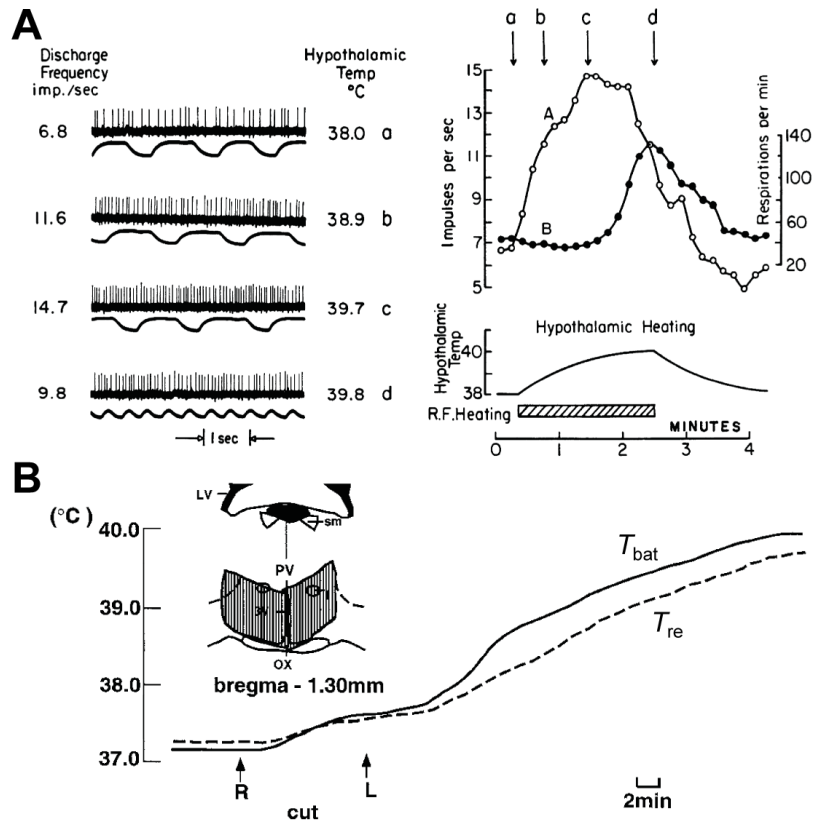
Figure 4. The preoptic area (POA) contains warm-sensitive neurons and maintains a tonic inhibition of neurons in caudal thermogenic brain areas

A, Nakayama and colleagues reported the first unit recording from warm-sensitive neurons in the POA and anterior hypothalamus of anaesthetized cats. Discharge frequency of this POA neuron and respiration rate increased in response to local heating by radio frequency current. Reproduced from the classic paper of Nakayama *et al.* (1963) with permission. *B*, Effects of knife cuts at the level immediately caudal to the POA on the interscapular BAT temperature (T_{bat}) and rectal temperature (T_{re}, core temperature). Knife cuts applied on the right (R) and left (L) sides increased BAT thermogenesis. Inset shows the extent of the knife cuts (hatched area). Abbreviations: 3V, third ventricle; LV, lateral ventricle; OX, optic chiasm; PV, paraventricular thalamic nuclei; and sm, stria medullaris thalami. Modified with permission from Chen *et al.* (1998).
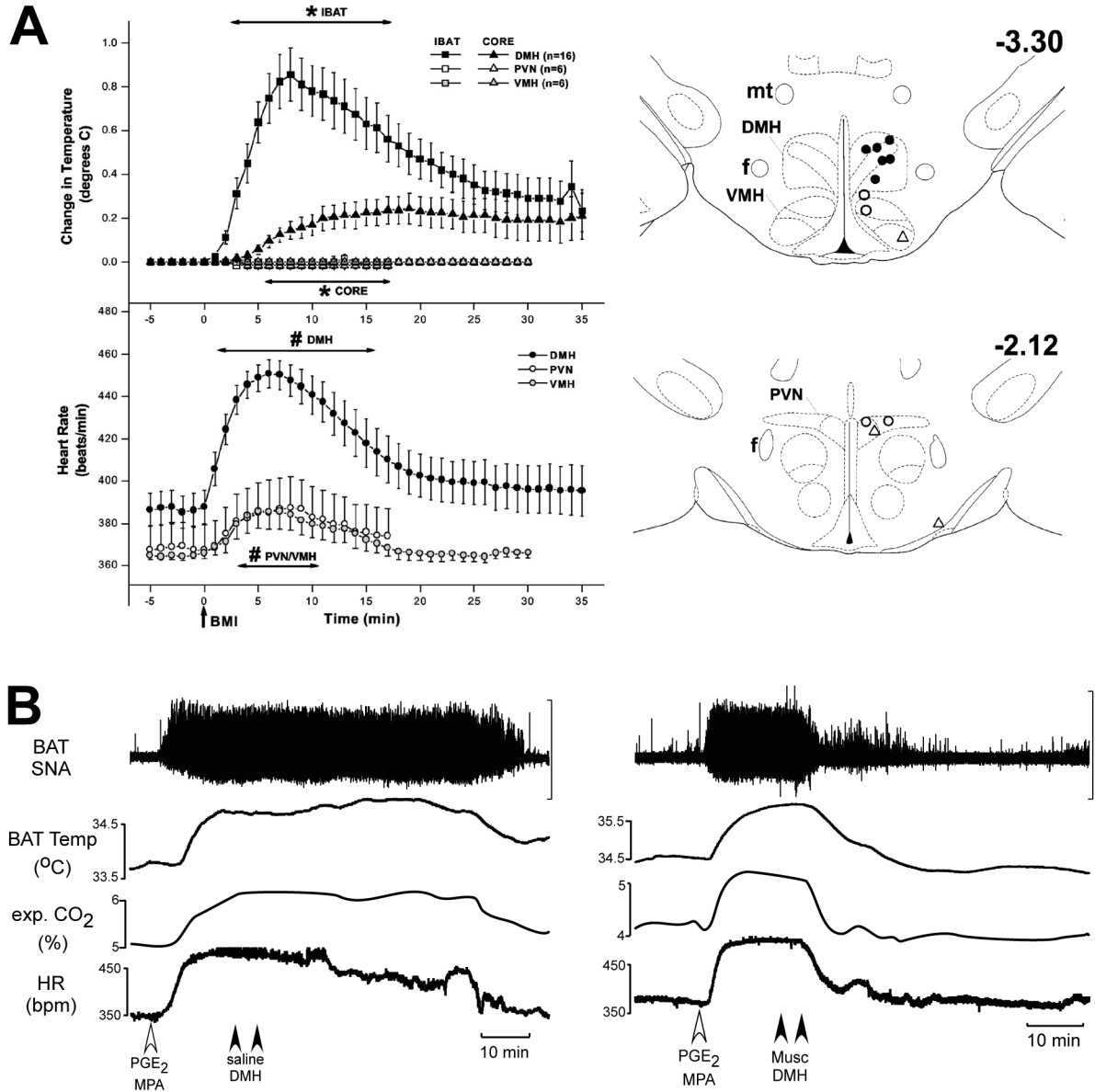
Figure 5. Disinhibition of neurons within the dorsomedial hypothalamus (DMH) increases thermogenesis in brown adipose tissue (BAT), and inhibition of neurons within the DMH reverses febrile-evoked BAT sympathetic nerve activity (SNA) and thermogenesis

A, left panel, increases in interscapular BAT (IBAT; squares) and core (triangles) temperatures (top panel) and heart rate (HR, bottom panel) following unilateral microinjection of bicuculline (BMI) into the DMH (filled symbols), the paraventricular hypothalamus (PVN; open symbols) or the ventromedial hypothalamus (VMH; shaded symbols). *A*, right panel, schematic coronal sections at two levels of the hypothalamus depicting representative injection sites in the DMH, the VMH and the PVN that were effective (filled symbols) or ineffective (open symbols) for increasing both IBAT temperature and heart rate. Numbers indicate distance from bregma in millimetres. f, fornix; mt, mammillothalamic tract. Reproduced with permission from Zaretskaia *et al.* (2002). *B*, left panel, microinjection of PGE_2 into the medial preoptic area (MPA) (open arrowhead) increased BAT SNA, BAT temperature, expired CO_2, and HR (bpm, beats min^{-1}). Bilateral nanoinjections of saline vehicle into the DMH (filled arrowheads) had

no effect on any of the measured variables. *B*, right panel, in a different rat, bilateral nanoinjections of muscimol into the DMH (filled arrowheads) completely reversed the PGE_2-evoked responses. Vertical scale bars represent 80 μV for BAT SNA. Reproduced with permission from Madden & Morrison (2004).
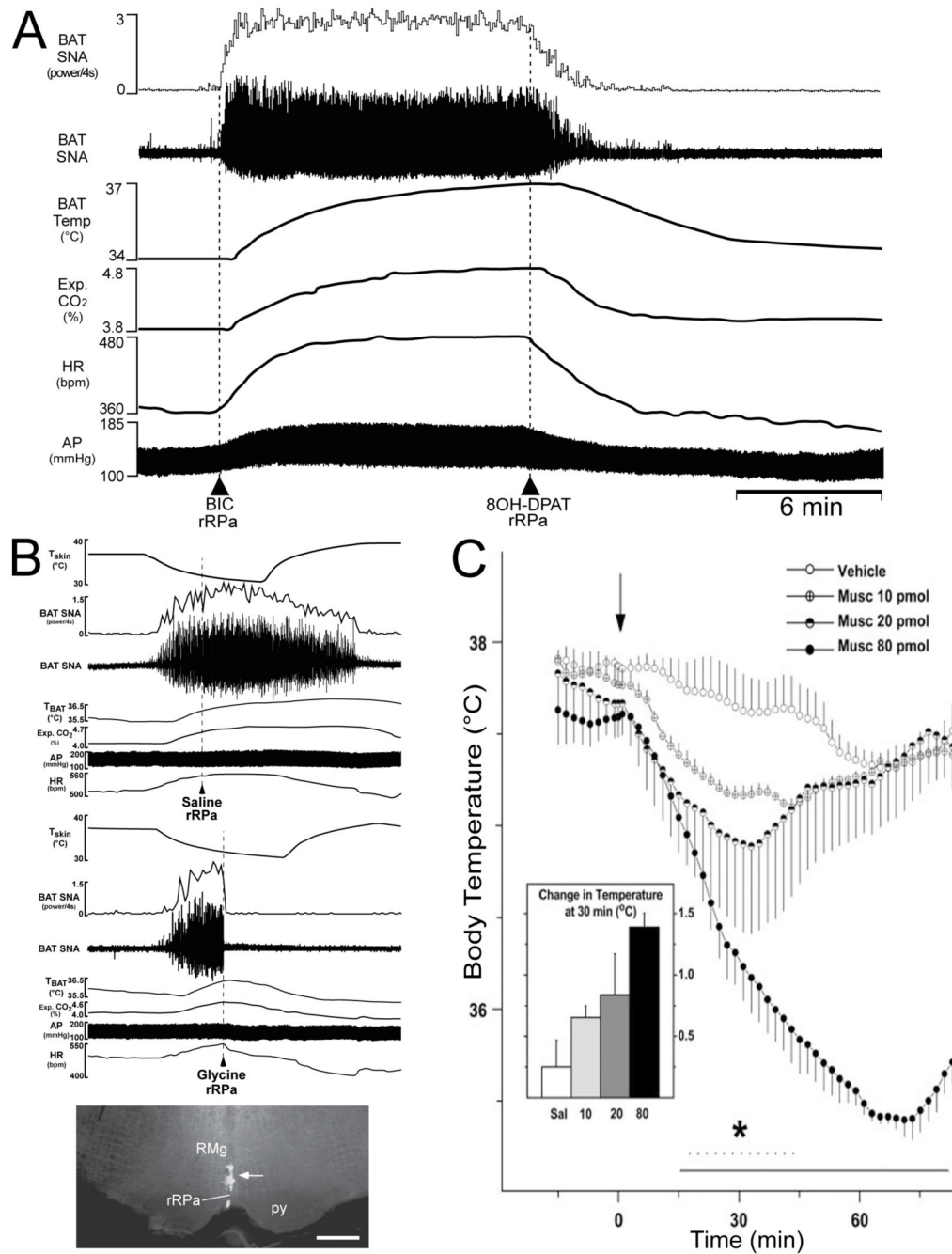
Figure 6. Effects of disinhibition and inhibition of rostral raphe pallidus (rRPa) neurons on BAT thermogenesis and body temperature

A, blockade of GABA$_A$ receptors with bicuculline (BIC) in the rRPa dramatically activates BAT sympathetic nerve activity (SNA) and increases BAT temperature, expired CO_2, heart rate (HR) and arterial pressure (AP). All of these responses are reversed by nanoinjection of the 5-HT$_{1A}$ agonist, 8-hydroxy-2-(di-N-propylamino)tetralin hydrobromide (8-OH-DPAT), into rRPa. *B*, the increases in BAT SNA, BAT temperature, expired CO_2 and HR produced by a reduction in skin temperature (T$_{skin}$) are blocked by inhibition of neural activity in the rRPa with nanoinjection of glycine. *B*, bottom panel, deposit of fluorescent beads (arrow) indicates the glycine injection site in rRPa. Abbreviations: RMg, raphe magnus; and py, pyramid.

Reproduced with permission from Nakamura & Morrison (2007*a*)). *C*, falls in body temperature following nanoinjection of muscimol (Musc, arrow) into the rostral ventromedial medulla to inhibit local neurons in awake rats. Asterisk indicates period of significant falls in core temperature. Reproduced with permission from Zaretsky *et al*. (2003).
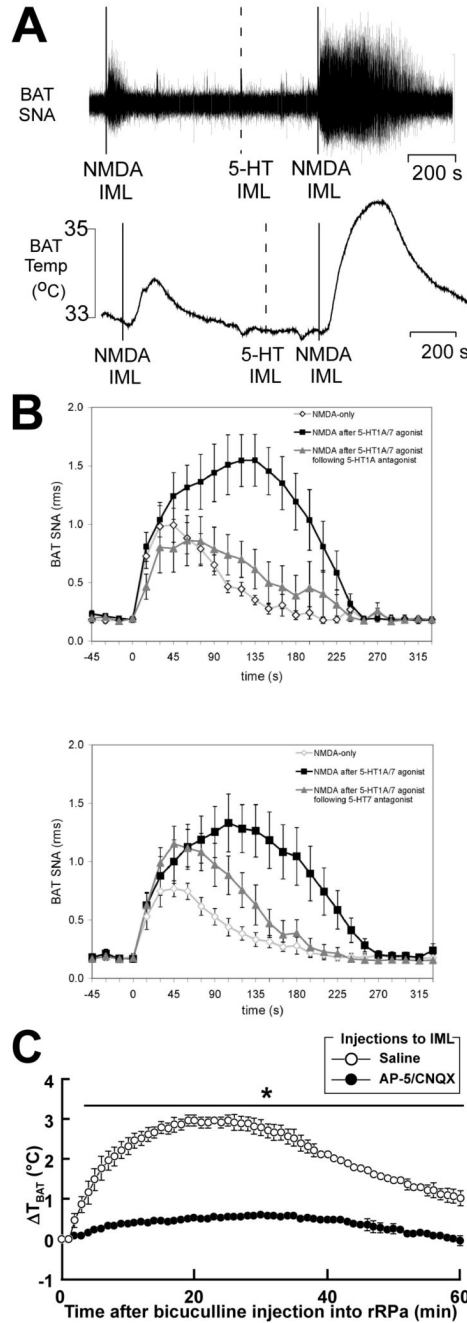
Figure 7. Effects of activation and blockade of spinal glutamate and serotonin receptors on sympathetic activation of BAT and BAT thermogenesis
A, microinjection of 5-HT into the T4 intermediolateral nucleus (IML) potentiated (+285%) the NMDA-evoked increase in BAT sympathetic nerve activity (SNA) and in BAT temperature. Vertical scale bar represents 20 μV for BAT SNA. Reproduced with permission from Madden & Morrison (2006). *B*, the time courses of the inhibition by selective 5-HT receptor antagonists of the 5-HT$_{1A/7}$ receptor agonist 8-OH-DPAT-mediated potentiation of the increase in BAT SNA evoked by microinjection of NMDA into the T4 IML. The 5-HT$_{1A}$ receptor antagonist, WAY-100635, attenuated both the amplitude and the duration of the 8-OH-DPAT potentiation. The 5-HT$_7$ receptor antagonist, SB269970, attenuated the

duration but not the amplitude of the 8-OH-DPAT potentiation. Nanoinjection of NMDA only into the T4 IML ($\diamondsuit$), NMDA into the T4 IML after 8-OH-DPAT into the T4 IML ($\blacksquare$) and NMDA into the T4 IML following 8-OH-DPAT into the T4 IML after WAY-100635 or SB269970 into the T4 IML ($\blacktriangle$). Reproduced with permission from Madden & Morrison (2008). *C*, nanoinjections of glutamate receptor antagonists into the IML blocked BAT thermogenesis triggered by bicuculline-induced disinhibition of rRPa neurons. Changes in BAT temperature (ΔT_{BAT}) are shown after bicuculline injection into the rRPa in rats microinjected with a mixture of 2-amino-5-phosphonovaleric acid (AP-5, 5 mM) and 6-cyano-7-nitroquinozline-2,3-dione (CNQX, 5mM) or saline into the bilateral T2-T6 IML, using 200 nl/site, every 0.8 - 1.0 mm. The changes in BAT temperature were significantly different between the AP-5/CNQX- and saline-pretreated groups during the time period denoted by a horizontal bar with an asterisk ($P < 0.05$). Reproduced with permission from Nakamura *et al*. (2004*a*).