Functional Link between the Amygdala and Brown Adipose Tissue Thermogenesis

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ABSTRACT

Introduction:

The amygdala plays a crucial role in emotion. When animals are in emotionally significant situations, their body temperature rises. This increase in body temperature is known to be due to heat production in brown adipose tissue (BAT), which is controlled by the sympathetic nervous system. The amygdala may trigger a sympathetic drive to BAT, contributing to the rise in body temperature. This project aims to identify the role of the amygdala in BAT thermogenesis in rats, specifically whether activation of neurons in the amygdala can cause BAT thermogenesis in anesthetized rats. Known brain circuits controlling BAT thermogenesis include the dorsomedial hypothalamus (DMH) and the medullary raphe. Therefore, this project also investigates whether the medullary raphe mediates amygdala-induced BAT thermogenesis.

Method:

In this project, we measured the electrical signals from the sympathetic nerves controlling the BAT in anesthetized rats (Spargue-Dawley, male). Since BAT is regulated by the sympathetic nervous system, using anesthetized rats allowed us to record these signals. A pharmacological approach was used to control the activity of neurons in the amygdala and the raphe nuclei. Neurons were activated by disinhibition using bicuculline, a GABA receptor antagonist. This activation aimed to determine whether drug-induced BAT thermogenesis could be elicited by stimulating neurons in the amygdala. Subsequently, muscimol, a GABA receptor agonist, was administered to the raphe nuclei to inhibit amygdala-induced BAT thermogenesis. During the experiments, several physiological parameters were recorded, including BAT sympathetic nerve activity, end-tidal CO2, BAT temperature, body temperature, skin temperature, heart rate, and arterial blood pressure. After recording, perfusion and brain extraction were performed for sectioning and immunostaining. Injection sites were observed under a microscope using Horse Radish Peroxidase (HRP) and fluorescent beads to identify the locations of the injections and determine if they corresponded to the observed responses.

Result:

It was found that activating neurons in the amygdala with bicuculline caused an increase in BAT sympathetic nerve activity and BAT temperature. When bicuculline was injected outside the amygdala, the BAT thermogenic response was minimal. Administration of muscimol to the raphe pallidus inhibited amygdala-induced BAT thermogenesis, while drug administration outside this brain region did not produce an inhibitory response.

Conclusion:

These results indicate that the amygdala provides excitatory drives to BAT via the medullary raphe. Future research aims to understand the newly discovered autonomic functions of the amygdala and how its emotional functions trigger BAT thermogenesis in conscious animal experiments. Bicuculline-induced BAT thermogenesis suggests that tonic GABAergic inputs into the amygdala plays an important role in this response. It is also important to elucidate GABAergic system in the amygdala involved in BAT thermogenesis. This project has highlighted new functions of the amygdala and suggested that autonomic functions could serve as valuable biomarkers in amygdala research.

DECLARATION

I certify that this thesis:

1. does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university

2. and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University; and

3. to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Signe: 

Junichi Sakaguchi

Date 31/10/2024

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Lastly, I must also thank the staff of the microscopy laboratory for providing training in various histological techniques.

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INTRODUCTION

Amygdala

Anatomy of amygdala (morphological classification)

The amygdala is a very important part in emotion of the brain, located deep in the left and right cerebrum, inside the temporal lobe, adjacent to the medial side of the hippocampus. Its shape is almond-shaped, and its name is derived from this shape in Greek(Gallagher & Chiba, 1996). Generally, the left and right amygdala are symmetrical, with individual differences in shape and size (Suffren et al., 2022).

The amygdala is composed of multiple nuclei (nuclear clusters), the main nuclei being the central nucleus, basolateral nucleus, and cortical nucleus. The central nucleus is involved in the regulation of emotional responses, while the basolateral and cortical nuclei are involved in the processing and integration of information. The amygdala has close relationships with a wide range of regions in the brain and has been shown to interact with other brain regions such as the basal ganglia, thalamus, hippocampus, and prefrontal cortex, which are important in emotion processing and regulation (Grossman et al., 2022).

Its primary roles include emotion processing, memory formation, and social behaviour and interaction. The amygdala is known to play a particularly important role in relation to emotion. It is involved in the generation and processing of emotions such as fear and stress and is the axis of the mechanism by which such significant events are remembered. It plays a particularly important role in fear conditioning (Watkins et al., 1998).

Although the amygdala is a small structure located deep in the brain, its complex arrangement and interconnections of nuclei play an important role in the regulation of emotion and behaviour. The research and clinical significance of this brain region is that studying the morphological characteristics of the amygdala may lead to a better understanding of aspects such as emotion processing and cognitive function, which may have a positive impact on emotion regulation and social behaviour. In addition, in psychiatric and neuroscience research, abnormalities in amygdala structure and activity may be associated with disorders such as phobias and anxiety disorders, and thus have the potential to find clues to resolving them (Rolls et al., 2023).

Figure 1: This image has been remove due to copyright restrictions

Figure 1*: The amygdala nuclei are categorized into three groups*

: centromedial (orange), deep or basolateral (green), and cortical (brown). The intercalated nuclei (In) are shown in dark gray. The subdivisions are as follows: CEc: Central nucleus, capsular subdivision, CEl: Central nucleus, lateral subdivision, CEm: Central nucleus, medial subdivision, COa: Cortical nucleus, anterior subdivision, Cop: Cortical nucleus, posterior subdivision, BOT: Bed nucleus of the olfactory tract, Bi: Basal nucleus, intermediate subdivision, Bpc: Basal nucleus, parvocellular subdivision, BMmc: Basomedial nucleus, magnocellular subdivision, BMpc: Basomedial nucleus, parvocellular subdivision, Ld: Lateral nucleus, dorsal subdivision, Lvm: Lateral nucleus, ventromedial subdivision, Lvl: Lateral nucleus, ventrolateral subdivision, Md: Medial nucleus, dorsal subdivision, Mv: Medial nucleus, ventral subdivision, In: Intercalated nuclei, Pir: Piriform cortex To simplify the diagram, the magnocellular subdivision of the basal nucleus located more rostrally, the rostral subdivision of the medial nucleus, and the caudally located amygdalohippocampal area are not shown (Knapska et al., 2007).

Function of amygdala (input and output system)

Two main types of input functions exist, divided into sensory input and social information. The amygdala receives sensory information from the thalamus and cortex, including visual, auditory, and olfactory information. The amygdala integrates input information, especially when processing emotionally significant stimuli, such as those that elicit fear or pleasure. It also plays a role in understanding the emotional states of others and regulating social behaviour by processing input to social stimuli and the emotional expressions of others (Dominguez-Borras & Vuilleumier, 2022).

Output functions include the generation and regulation of emotion, memory formation, and behavioural coordination, as described above. The amygdala is deeply involved in the generation and regulation of emotional responses. The amygdala modulates physical responses after receiving emotional stimuli via the autonomic nervous system and endocrine system, to stimuli that elicit emotional responses such as fear, pleasure, and excitement. It plays a particularly important role in fear conditioning, contributing to the formation and storage of fear memories. The amygdala is also involved in the selection and regulation of appropriate behaviour in response to emotional stimuli, for example, it regulates the escape response in the face of danger and the emotional response in social interactions with others (Meyer-Arndt et al., 2022).

The amygdala is part of an intricate neural network that processes and regulates emotions through both incoming and outgoing signals. Its main roles include receiving and integrating sensory information, generating and managing emotions, forming and storing memories, and selecting and regulating appropriate behaviours(Rolls et al., 2023).

Neural pathways involving the amygdala

There are several neural pathways involving the amygdala, each of which plays an important role. The first is the hypothalamus-amygdala pathway. The amygdala is directly linked to the hypothalamus, which regulates autonomic and endocrine responses to emotional stimuli. The hypothalamus is the centre that controls physiological responses, and amygdala activity influences these responses (Kubota et al., 2023). The second is the amygdala-prefrontal pathway, in which the amygdala is also coupled to the prefrontal cortex (ventral thalamus). The prefrontal cortex is involved in decision-making, the regulation of social behaviour and emotions, and coordinates behavioural choices in conjunction with the amygdala's emotional processing (Soto et al., 2019). The hippocampus, the centre of the amygdala-hippocampal pathway, is also involved in memory formation and storage and is important for learning and memory formation, including fear conditioning through pathways with the amygdala. Activity in the amygdala influences hippocampal function and contributes to the storage of memories of emotional events. Furthermore, the amygdala is also closely coupled to the thalamus and plays an important role in the processing and integration of sensory information. Information processed through the amygdala is transmitted via the thalamus to different brain regions to modulate responses to emotional stimuli (Watson et al., 1983).

These neural pathways are crucial for understanding the amygdala’s role in processing emotions, forming memories, and regulating behaviour. The amygdala modulates responses to emotional stimuli through complex neural circuits, aiding in the selection of appropriate behaviours.

Brown Adipose Tissue

Anatomy of Brown adipose tissue (BAT) and its functions

Brown adipocytes are cells specialised in the maintenance of body temperature and energy expenditure, with many endoplasmic reticulum and mitochondria staying inside the cell (Anthony et al., 2019). Mitochondria are rich and internally iron-rich, and the presence of this iron contributes to the formation of brown pigment (Paulo et al., 2021), giving rise to the characteristic brown colouration of the cells. Due to its high vascular density, brown adipose tissue can efficiently supply oxygen and nutrients and maintain high metabolic activity. That supports the energy expenditure required to maintain body temperature. It is also known to be closely related to the autonomic nervous system and is activated by cold stimuli and postprandial nerve stimulation (Nakamura et al., 2022). Brown adipose tissue is generally abundant in neonates and children, and is also found in certain areas in adults, such as the neck, shoulder blades, rib cage and around the kidneys, although the amount of tissue tends to decrease with adulthood. However, they are highly metabolically active and play an important role in the regulation of body temperature(Avram et al., 2005).

Its main function is to generate body heat without shivering in animals and newborns. In contrast to white adipocytes, which contain a single fat droplet, brown adipocytes contain iron, which gives them a brown colour, numerous small droplets and a much larger number of mitochondria. Brown adipose tissue requires more oxygen than most tissues, and brown adipose tissue also has more capillaries clustered together than white adipose tissue. When noradrenaline binds to β3 receptors on brown adipocytes, UCP1 (uncoupling protein 1) is produced and uncouple fatty acid oxidation occurs in mitochondria to produce heat. This is a means of heat production that is not accompanied by exercise (Kataoka et al., 2014).

Thermoregulation

Thermoregulation of the human body is achieved by complex physiological mechanisms. The autonomic nervous system is mainly responsible for this, with the sympathetic nervous system being activated in cold environments, constricting blood vessels and reducing heat release from the skin to maintain body temperature, while in hot environments the parasympathetic nervous system is activated to dilate blood vessels and increase heat release to lower body temperature in human (Nakamura, 2024). In addition, brown adipose tissue is particularly activated by cold stimuli and produces heat through the protein UCP1 in mitochondria, contributing to an increase in body temperature. Temperature sensors are distributed in the skin, body nuclei and peripheral tissues, which detect changes in temperature and send information to the central nervous system to trigger the appropriate response(Nowack et al., 2017). Moreover, the level of metabolic activity also

influences body temperature; after eating or exercising, the metabolism becomes more active, and the body temperature rises. These factors work together to maintain body temperature in response to changes in the external environment and internal metabolic needs(Luo et al., 2011).

Figure 2: This image has been removed due to copyright restrictions.

Figure 2*: A simple diagram illustrates the key steps involved in thermogenesis within the cell and mitochondria.*

Catecholamines are shown in purple, triglycerides in green and yellow, UCP1 is magnified in purple, and the mitochondrial processes are highlighted (Townsend & Tseng, 2012).

Thermogenesis (non-shivering)

Non-shivering heat production involves heat generated by various metabolic processes in the body. Basal metabolism produces constant heat at rest due to cellular activity and enzymatic reactions. Muscle contraction also releases heat during activity, particularly during exercise or in cold environments(Horvath & Wolfrum, 2020; Zhang et al., 2021). Additionally, brown adipose tissue generates heat through a protein called UCP1 during fatty acid metabolism, which is activated by cold or neural stimuli. Diet and metabolic activity further increase heat production during digestion and energy metabolism after meals(Greenhill, 2024; Li et al., 2021). These processes collectively help regulate body temperature through mechanisms like the autonomic nervous system and endocrine system(Coulson et al., 2021; Jastroch, 2023).

Biochemical pathways in BAT

The mechanism of non-shivering heat production in brown adipose tissue (BAT) is primarily driven by the sympathetic nervous system (SNS)(Nisoli et al., 1992). When the SNS is stimulated, it releases norepinephrine, which binds to adrenergic receptors on BAT, mainly β-3 receptors (Thomson et al., 1967). This binding facilitates the production of cyclic AMP (cAMP), which activates protein kinase A (PKA). PKA then phosphorylates various downstream proteins, including hormone-sensitive lipase (HSL) and perilipin A, leading to the breakdown of triglycerides in adipocytes and the release of fatty acids. These fatty acids are metabolized in the mitochondria through β-oxidation, generating energy. Norepinephrine plays a crucial role in regulating energy metabolism and body temperature(Raab & Gigee, 1957). This mechanism differs slightly between humans and rodents, with β-1 receptors being more predominant in humans and β-3 receptors playing a more significant role in rodents (Muzzin et al., 1988).

Neural pathways controlling BAT thermogenesis

As our understanding of the neural pathways of thermoregulation grows, researchers are striving to uncover its complex mechanisms. The neural circuitry of general thermoregulation consists of multiple nuclei and groups of neurons interacting in the brain(Kontos et al., 2013). For example, neurons in the paraventricular nucleus hypothalamus (PVH) are responsible for inhibiting sympathetic outflow of brown adipose tissue (BAT) when body temperature rises(Morrison, 2018). In contrast, glutamate decarboxylase (GAD) immunoreactive neurons project to and around the hypothalamus and are involved in various aspects of thermoregulation(Raam & Hong, 2021). Specifically, sympathetic precursor neurons in the globus pallidus (RP) of the medullary raphe are involved in the inhibitory action of PVH on BAT. In addition, the preoptic area (POA), a key centre in thermoregulation, processes cold and warm sensory information; signals from the lateral parabrachial nucleus (LPB) is sent to the POA, where thermoregulatory responses are coordinated (Yoshida et al., 2003). In addition, inhibitory inputs from the POA are transmitted to sympathetic excitatory neurons in the dorsomedial hypothalamus (DMH) and projected to sympathetic premotor neurons in the globus pallidus (RRP) in the rostral medullary raphe (Blessing, 2005). These neuronal circuits activate sympathetic pre-sympathetic neurons in the thoracic spinal cord, which transmit signals to brown fat cells via sympathetic ganglion cells and post-ganglionic sympathetic fibres. This process allows for the proper regulation of body temperature. Recent research indicates that a comprehensive grasp of these neuronal circuits could pave the way for innovative therapies and interventions to manage heat production and thermal sensation. By activating brown adipose tissue, new strategies for treating metabolic conditions like obesity and diabetes are anticipated(Morrison, 2016).

Figure 3 (a), (b): These images have been removed due to copyright restrictions.

Figure 3: *Pathways for thermoregulation in cold environments.*

(a) Simple pathway loop. (b) Neural pathway during cold environments or fever(Nakamura, 2011).

Autonomic Nervous System

Anatomy of the autonomic nervous system (ANS) and functions

The Autonomic Nervous System (ANS) is the nervous system that regulates autonomously physiological functions in the body. This nervous system is divided into sympathetic and parasympathetic nervous systems, each of which plays a contrasting role(Dieleman et al., 2015; Mertens et al., 2022). The sympathetic nervous system is usually activated during stress and exercise, causing an increase in heart rate, blood pressure and pupil dilation, preparing the body for a state of increased energy expenditure(D & Raju, 2023). The parasympathetic nervous system, on the other hand, is predominant during relaxation and rest, causing a lower heart rate, increased activity in the digestive system and pupil constriction, preparing the body for a state of energy conservation and regeneration. As part of the central nervous system, these systems are regulated by specific area90s of the hypothalamus and the brainstem(Zantvoord et al., 2024). These areas receive sensory input and information about the internal state of the body and send appropriate responses accordingly to the autonomic nervous system. The autonomic nervous system is also activated by emotionally significant conditions, e.g. sudden stress or fear activates the sympathetic nervous system and causes physiological changes in the body(Hirayama et al., 2023). The main neurotransmitters used are acetylcholine and noradrenaline, which bind to specific receptors at nerve endings. Imbalances and dysfunctions of the autonomic nervous system can lead to syndromes such as dysautonomia, chronic stress reactions and autonomic hyperactivity disorder(Teixeira et al., 2015). The autonomic nervous system is essential for the regulation of daily physiological functions and is particularly responsible for the ability to adapt to stress and environmental changes(Togo et al., 2024). It therefore plays an important role in maintaining the body's health and quality of life.

Sympathetic nerve system (SNS) / parasympathetic nerve system (PNS)

The sympathetic and parasympathetic nervous systems are the two main parts of the autonomic nervous system, which act in concert to regulate functions in the body. The sympathetic nervous system is normally activated during a stress response, causing increased heart rate and blood pressure, vasoconstriction and increased energy metabolism(Seebacher, 2009). In contrast, the parasympathetic nervous system is activated during rest, relaxing the body and aiding recovery through facilitating digestion, slowing the heart rate and dilating blood vessels. The sympathetic nervous system releases noradrenaline via ganglia emanating from the thoracic and lumbar regions of the spinal cord, which act on the heart, blood vessels, respiratory and digestive systems. The parasympathetic nervous system, on the other hand, releases acetylcholine through nerve fibres from the brainstem and lumbar spinal cord and affects the digestive, cardiac, intestinal and urinary

tracts(Togo et al., 2024). These nervous systems often produce opposing responses, but usually work together harmoniously in different parts of the body. When this balance is disturbed, body functions and homeostasis can be disrupted. Prolonged sympathetic hyperactivity can lead to persistent stress reactions and impaired immune function, while parasympathetic hypoactivity can cause problems such as poor digestion and lack of rest.(Togo et al., 2024)

Figure 4: This image has been removed due to copyright restrictions.

Figure 4: Different pre- and postganglionic fibres of the two pathways

: sympathetic in red and parasympathetic in blue. Preganglionic fibres are shown with solid lines, postganglionic fibres with dotted lines, and ganglions are represented as dots (Waxenbaum et al., 2024).

Psychogenic fever (stress-induced hyperthermia)

Psychogenic fever is an increase in body temperature caused by mental factors, also known as stress-induced hyperthermia or emotional hyperthermia, and is excessive heat production above the optimal level for homeostasis(Oka, 2018; Watanabe, 2015). This heat is produced partly by BAT thermogenesis. Specifically, it refers to the phenomenon of a higher-than-normal body temperature due to stress, anxiety, depression or another mental load(Lkhagvasuren & Oka, 2017). Psychogenic fever is often seen in the absence of a clear biological cause, such as a physical illness or infection, and is usually a temporary increase in body temperature under the influence of the sympathetic nervous system as the body responds to stress and the autonomic nervous system is activated(Rey et al., 2017). In cases of prolonged chronic stress or mental strain, body temperature persistently high body temperatures may occur. Specific symptoms include dilated pupils, increased heart rate and increased sweating(Xiong et al., 2016). Such stressful stimuli induce excessive heat production, resulting in hyperthermia; both the hypothalamus and the raphe nuclei are involved in BAT heat production, but how stress signals reach these structures is not yet understood (Grelet et al., 2022).

BAT thermogenesis can be under anaesthetised conditions.

Pharmacological of neurons in the brain can be used to produce excessive heat, acting on skeletal muscle and brown adipose tissue like conditions that are observed in stress-induced hyperthermia. Drug-induced hyperthermia can be elicited in anaesthetised animals with anaesthetic drugs such as chloral hydrate or urethan. Anaesthesia allows the electrical signals of the BAT sympathetic nerves to be observed and recorded, allowing better visualisation of the sympathetic pathways of BAT thermogenesis(Kataoka et al., 2014).

Gamma aminobutyric acid (GABA)

GABAergic receptors and neurotransmitters in amygdala

Neurons that have GABA (γ-aminobutyric acid) or glycine as transmitters and hyperpolarise the cell membrane by increasing chloride ion permeability in the postsynaptic membrane or inhibit membrane potential propagation by a shunt effect are called inhibitory neurons. In the cerebral cortex, approximately 20% of neurons are GABAergic inhibitory neurons(Sharma et al., 2023). They often act in local circuits and have important functions such as regulating output from excitatory neurons, controlling synchrony and preventing overexcitation. They are diverse in terms of morphology, function and expression of marker proteins, and in the cortex, there are morphologically large basket cells, small basket cells, nested basket cells, chandelier cells, spindle cells, double bouquet cells and multi-notch cells(Cao et al., 2010). From the site of synapse formation, there are inhibitory neurons specialising in the dendrites, cell bodies and axonal portions on excitatory neurons, respectively, which are closely associated with their function. During development, GABAergic neurons are produced in the basal ganglia primordium ventral to the telencephalon and migrate to the cortex where they are incorporated into circuits(Jiang et al., 2023).

GABA plays a primarily inhibitory role, maintaining a balance of neural activity by suppressing neuronal excitability GABA is widely distributed throughout the CNS. It is distributed throughout the CNS and plays a particularly important role in the hypothalamus, cerebellum and spinal cord of the brain. In these areas, GABA regulates neural activity, particularly in the control of movement, sleep regulation and emotional processing(Bonanno et al., 2006).

The main mechanism of GABAergic neurotransmission is the binding of GABA-to-GABA receptors located on the membranes of nerve cells; there are several subtypes of GABA receptors, each with different functional properties(Snigirov & Sylantyev, 2021). For example, GABA-A receptors inhibit excitability by bringing chloride ions into the neuron via chloride channels. As drugs, benzodiazepines and barbiturates are used to regulate GABAergic neurotransmission. These drugs act on GABA receptors to enhance the inhibition of neural activity and are known to exhibit anxiolytic and antiepileptic effects(Chen et al., 2014).

Bicuculline

Bicuculline is a compound identified from a neurotoxic alkaloid extract that acts as an antagonist of GABA receptors, with its main action being to increase neuronal excitability by counteracting GABA-A receptors(Zagrodzka et al., 2000).

GABA is the major inhibitory neurotransmitter in the central nervous system and plays an important role in the regulation of neuronal activity; GABA-A receptors inhibit neuronal excitability by bringing chloride ions into the neurons via chloride ion channels(Behrens et al., 2007). Bicuculline, on the other hand, binds to this receptor and inhibits GABA binding, thereby increasing neuronal excitability via disinhibition. This can cause an over-activity of the neuronal circuitry(Ramerstorfer et al., 2015). In Autonomic Neuroscience research, bicuculline is a common tool to activate neurons in the brain to study neuronal circuitry. It is also useful for better understanding of GABAergic neurotransmission. For example, experiments with bicuculline can be used to investigate abnormalities in the GABAergic system of neurons and their effects(Fiske et al., 2006).

Hexamethonium

Hexamethonium is a non-competitive nicotinic acetylcholine receptor antagonist whose main mechanism of action is the blocking of nicotinic acetylcholine receptors in post-sympathetic cells in the autonomic ganglia. This action reduces neuronal excitability, particularly in the sympathetic nervous system. This has the effect of reducing vascular smooth muscle contraction, resulting in a reduction in blood pressure(Tohara et al., 2000).

In medical use, hexamethonium was once widely used to treat hypertension, but due to subsequent pharmacological developments and side effects, it is now mainly used for research purposes(Bernards & Artu, 1991). In neuroscience research, it plays an important role in analysing the function of nicotinic receptors and investigating their effects on the mechanisms of neurotransmission in detail. The main known side effects associated with the use of hexamethonium are decreased gastrointestinal motility, urinary retention, dizziness and fatigue(Ettman et al., 1957). There is also concern about the effects on the autonomic nervous system and circulatory system with long-term use, and safety assessments are underway in this regard.

From a research standpoint, hexamethonium specifically targets nicotinic receptors, offering valuable insights into normal neurotransmission and its dysfunctions. It is regarded as a crucial tool for studying the mechanisms regulating neuronal excitability and for investigating neurodegenerative diseases, especially within neuroscience and neuropharmacology.

Muscimol

Muscimol is a decarboxylated chemical compound of ibotenic acid referred as ibotenate(Cao et al., 2010). It acts as an agonist of GABA, one of the neurotransmitters of the inhibitory system, so that ingestion reduces the frequency of neurotransmitter release, i.e. the brain becomes inactive. Muscimol binds to the same site as GABA on the GABA A receptor complex, in contrast to other GABA agonists such as barbiturates and benzodiazepines, which bind to a different regulatory site, and cerebellum. Muscimol is usually considered a selective GABAA agonist with very high affinity for GABA A-delta receptors, but as it is also a partial agonist of GABA A-rho receptors, its range of action may result from its combined action on multiple GABA A receptor subtypes(Hu et al., 2021).

Interaction of Sympathetic nervous system and GABA

As GABA is a neurotransmitter that inhibits neuronal firing, its function is determined by the neural circuit it inhibits GABA is involved in complex circuits throughout the central nervous system(Kovalev et al., 1982). For one example, GABA is released from striatal neurons in neural pathways that project to the globus pallidus, which in turn extends GABAergic neurons to other brain regions to inhibit unwanted motor signals. GABA signalling in the medulla oblongata is also known to be involved in the maintenance of respiratory rate(Cao et al., 2010), with increased GABA signalling resulting in decreased respiratory rate. In addition, GABA is also found in the spinal cord, where it functions in inhibitory interneurons. These neurons help integrate excitatory proprioceptive signals, allowing the spinal cord to integrate sensory information and produce smooth movements(Cao & Morrison, 2006). This shows that GABA plays a major role in both the brain and spinal cord.

Neurotransmitters are excitatory (glutamate) and inhibitory (GABA) and their balance is essential for correct homeostasis, proper nerve function and cell membrane stability. The neurotransmitter GABA responds to GABAergic receptors within nerve endings where GABAergic receptors bind to GABAergic receptors(Jiang et al., 2023). These sympathetic responses have been shown to be blocked by the NMDA receptor antagonist AP-5, while the GABA antagonist bicuculline causes similar neural activity to glutamate. Bicuculline increases the frequency of glutamate-mediated excitatory responses and increases sympathetic neuronal activity GABA is an inhibitory neurotransmitter and within the CNS GABA influences sympathetic and cardiovascular regulation GABA agonist injections decrease neural activity and antagonists increase neural activity increase neural activity(Kovalev et al., 1982; Morrison et al., 1999). Thus, GABA acts in an inhibitory manner and suppressing GABA causes an excitatory neural response.

Systemic administration of GABA to anaesthetised dogs decreased blood pressure and heart rate, whereas administration of a ganglion blocker (hexamethonium) lifted the GABA-induced inhibition around the heart(Raab & Gigee, 1957). Peripheral GABA receptors are important starters for the responses seen within the vasopressor action of GABA; GABA impairs synaptic transmission (ganglionic transmission), via prevention of bradycardia via preganglionic vagal stimulation; these inhibitory effects of GABA were reversed by bicuculline administration(Tohara et al., 2000). Bicuculline therefore has an inhibitory effect on sympathetic nerves via inhibition of GABA receptors; GABA normally has an inhibitory effect on sympathetic nerves via binding to GABAergic receptors. Bicuculline has an inhibitory effect on excitation(Zagrodzka et al., 2000).

Overview

Hypothesis and Aims

Given that stress increases body temperature and BAT thermogenesis contributes to emotional hyperthermia and that the amygdala participates in emotional processing in the brain, it is hypothesized that the amygdala provides excitatory input to brown adipose tissue, which in turn leads to BAT thermogenesis.

The main objective of my experiment is to determine the role of the amygdala in BAT thermogenesis (non-shivering). To achieve this objective, I will examine the effects of neuronal activation of the amygdala on heat production in BAT.

We also want to determine whether amygdala-induced BAT heat production is mediated via the medullary raphe, a known center of thermoregulatory pathways. To conclude, we will micro-inject bicuculline into the amygdala to determine the activation of neurons in the amygdala on BAT thermogenesis. Muscimol will be micro-injected downstream of the medullary raphe to determine whether the medullary raphe mediates amygdala-induced BAT thermogenesis and map the pathways that follow where the signal goes.

Significance of this research

It is expected that this research will improve and extend our current understanding of the role of amygdala in heat production. While research has been into the emotional and behavioral mechanisms of the amygdala, this study has the potential to reveal new autonomic functions that have yet to be discovered in behavioral experiments. At the end of the study, a map of the amygdala will be produced, showing how autonomic physiological responses are controlled by central amygdala or basolateral amygdala. This may lead to an understanding of the functions controlled by those regions and the discovery of specific targeted therapies.

Also, through this study, if the role of the amygdala in BAT heat production is clear, it may help to find targets for amygdala-related thermotherapy like new approaches to improving body temperature regulation and energy expenditure through stress management and emotional regulation. In addition, additional BAT heat production pathways are expected to be elucidated in the second half of the study, and the various areas are expected to be interconnected. Currently, specific targeted therapies for hyperthermia are limited, but this research may improve our understanding of the amygdala and enable the development of specific therapies targeting these nuclei and their downstream pathways. Furthermore, this research may pave the way for different treatments for patients with stress-induced hyperthermia, which occurs in humans and livestock.

MATERIALS AND METHODS

Animals

SD (Sprague-Dawley, outbred) rats were used. They were supplied by Flinders University Animal Facility. Our experiments and all methods we conduct were approved by AEC (Animal Ethics Committee, approval number 4670.

Preparation surgery

Tracheotomy and the femoral artery and vein Cannulations

In our experiment, all animals we used were under anaesthesia condition. The animals were placed in an anaesthetic induction box. Approximately 3% isoflurane (Veterinary Companies of Australia, Kings Park, Australia) in pure oxygen (Ohemeda Isotec 4, New Jersy USA) was introduced in the box. After confirming that the animal was fully anesthetised, showing loss of withdrawal reflex, the isoflurane vaporiser was reconnected to a special non-invasive mask and the mask was fixed to the animal's nose to keep adequate anaesthesia level. Also, this preparation surgery was conducted on a heat mat (Breville Electrical heat pad, China) to maintain the animal body temperature.

The first step was to shave the hair in the area to be operated on with a hair clipper. Starting from the head, the hair was shaved on the top of the head (from the eyes to the back of the head) (for microinjection into the brain regions), 1-2 cm down from the larynx (tracheotomy location), around the shoulder blades (BAT nerve location), around the coccyx (for fixing animal with a thick string) and the right thigh groin area (Cannulation location). Once the hair was clipped, the rat was placed on its back and its limbs secured with tapes, then the skin was opened with a scalpel at the throat. The fat was cut lengthwise with scissors, and when the muscle layer was visible, it was opened using tweezers or forceps. Open the muscular layer to expose the trachea; prepare two 5-centimetre threads, both of which were to be placed under the trachea. The two threads were placed 1 cm apart at the top and bottom and each was lightly tied. Tie the thread on the oral side tightly, make a cut between the two threads with a scalpel, insert the catheter through the cut into the trachea on the bronchial side, and tie the thread on the pulmonary side tightly to prevent the catheter being removed. The tube of the isoflurane vaporiser was then quickly connected to a tracheal catheter to allow breathing without problems. The threads holding the tracheal catheter was reinforced with a little bit of superglue, and the skin was also closed with superglue.

Next, a catheter was placed into the vein and artery in the right femoral inguinal region. This was done to measure the blood pressure in the artery and the need to administer some medication through the femoral vein. This procedure was almost identical to the procedure for inserting a tracheal catheter, but the catheter was thinner, the thread used was thinner, and the dexterity required was different. It must be done carefully and quickly, because if this procedure was done carelessly, the animal may bleed profusely, and that leads to death in the worst-case scenario.

The rat was placed ventral side up, tail and hindlimb were toward me, and tape the hindlimb as wide as possible. A large artery and vein were in the inguinal region of the right hindlimb, and an incision was made in the skin at the location where the strongest pulse can be felt by pressing with a finger. Connective tissue, fat, and muscle layers were carefully opened using scissors and forceps. Once the vein and artery were visible, the surgical site was opened with a hook to allow sufficient space. Using thin tweezers, diverge the vein from the artery to secure a place to place the thread. The first step was to start with the vein: two 10-15 cm sutures were threaded under the vein as in the trachea and temporarily fastened with 1-2 cm. The threads at the distal end were tied tightly to prevent unravelling and to stop the blood flow. The central thread was tied loosely, and a scissor clamp was used to pull the knot toward the centre of the vein to constrict the blood flow in the vein and prevent bleeding. The catheter was cut at a 45° angle with a scalpel to allow smooth insertion of the catheter into the vein and to prevent the sharp tip from piercing the vessel wall. A small incision was made in the vein with micro scissors to secure the catheter insertion opening. The catheter filled with Ringer's solution was inserted slowly into the vein, twisting it slightly from the distal end toward the central end. The catheter was inserted up to the base of the thin tube, making sure that Ringer solution can be injected smoothly into vein without any leaks. The loose knot was tightened on the central side and tie it tightly, then a thread was wrapped around the catheter junction and tie a double knot. The procedure for arterial cannulation was the same as for venous cannulation, except that a three-way stopcock was inserted between the catheter and syringe. The syringe leading to the artery was also filled with Ringer's solution containing heparin to prevent coagulation of the blood. After both cannulations, the skin was closed over the wound with a superglue.

Anaesthetic infusion

The concentration of 500 mg/mL urethane and 40 mg/mL alpha-chloralose was determined using previous experiments (Ootsuka et al., 2009). The volume of the solution mix was calculated based on the rat's body weight (urethane 750mg/kg and alpha-chloralose 60mg/kg). This mix was administered intravenously using a rate selector (Sage Instruments, Australia). After the infusion, the intravenous line was flushed with Ringer's solution to ensure all remaining anaesthetics was administered, and isoflurane inhalation was stopped post-infusion.

Burr holes on skull

Animal was placed on a stereotaxic frame (Kopf, David Kopf Instruments, Tujunga, USA) to secure the head with both ears on ear bars (Kopf, David Kopf Instruments, Tujunga, USA) and front teeth on incisor bars. A scalpel incision was made in the parietal skin and the membranes and blood on the skull were removed using a cotton swab or thick tweezers. Once the scalp was exposed and sufficient exposed area was secured, a dot was placed with a marker at the bregma, where the frontal bone meets the parietal bone, to serve as a base point for determining the injection point stereotaxically. To microinject drugs into the amygdala and the medullary raphe nucleus in our experiments, the three points to be drilled (left and right Amygdala and medullary raphe) were determined based on the brain atlas, previous research and the body weight of the animal we used. The exact drill position

varies from one experimental individual to another, but approximately 2.4 mm dorsally and 5 mm laterally right and left from the bregma was the drilling point for microinjections in the amygdala and 10 mm straight dorsally from the bregma was the point for the raphe nucleus. Burr sites calculated from rat atlas book (Paxinos and Watson, 2006).

Drilling was done carefully with a small electric drill. Pressure applied to the skull with the drill bit was carefully controlled to avoid penetrating the dura mater under the skull and damaging the brain. When drilling, saline solution was applied at the points of the hole to release frictional heat. This prevents damage to the brain and tissue. Once the burr holes were made, wet cotton balls were placed on top to keep the brain from drying out until the microinjection was performed.

Exposure sympathetic nerves connecting BAT

The scapula was touched with the fingers to identify the incision site, and a longitudinal incision was made between the left and right scapulae from rostral to caudal with a scalpel. Using forceps and scissors, the fat layer was cut centrally from caudal to rostral. Once all fat layers were cut, the Sulzer vein can be seen branching to the left and right brown adipose tissue sides. The Sulzer vein on the right side was cut with scissors, and the skin on the right side was lifted and secured to separate the fat and muscle into two layers, further exposing the BAT on the right side. The thoracic trapezius muscle was cauterized using an electrical scalpel (Symmetry Surgical, Canada) to prevent bleeding during muscle separation. Once a certain extent of BAT was exposed, the skin on the right side of the back was pulled to create a pool. Ringer solution was injected from behind the BAT to locate and separate the BAT nerve. Once the nerve was separated, a 2 cm piece of threaded fibre was threaded under the nerve, tied so that it does not come undone, and the end side was cut off with micro scissors. The separated nerve was kept moist with a cotton ball soaked in Ringer's solution to prevent deactivation of the nerve due to dryness. The left side of the shoulder blade incision was used for BAT temperature measurement. Also, left side of the BAT was confirmed, and then the BAT temperature probe was inserted. The exposed nerve was immersed in paraffin oil (Faulding remedies, Australia), and was placed on the silver-chloride electrode for recording. A grounding electrode was placed between the skull and scalp to reduce noise during recording.

Paralysis of the animal

After all preparatory operations were done, the rats were paralysed with a paralysing agent to prevent possible movement induced by stimulating neurons in the brain. The paralysing agent (+)-Tubocurarine chloride pentahydrate (d-tubo) (0.3mg in 1mL ringer) (Sigma, product number 6989-98-6) was administered 0.15 mL intravenously through the catheter. This was administered every 60 minutes for the duration of the experiment. Between dosing, an adequate anaesthesia level was

assessed, and additional anaesthetic was given if necessary. The animals were artificially ventilated with a ventilator (Rodent ventilator model 683, Harvard Apparatus, USA) and breathed at 60 cycles/min, 10 ml/kg.

Water jacket and cooling system

During the experiment, the animals' body trunk was wrapped in a water jacket (Biomedical Engineering, Flinders University) to maintain a constant body temperature by perfusing warm water (around 40°C) from water bath (Haake D1, Sigma-Aldrich, Germany) into the jacket using a pump (Masterflex, Radnor, PA, USA).

The water was changed to ice-cold water to decrease skin temperature, to confirm that BAT SNA was increased by cold exposure. Once the activation response was confirmed, we proceeded to the stage of drug administration to the amygdala.

Drug administration

Bicuculline microinjection

To activate neurons in the amygdala micro-injected 1(*S*),9(*R*)-(-)-Bicuculline methiodide (bicuculline) (sigma, product number 40709-69-1), an antagonist of GABA receptorsinto the Amygdala. We determined whether this activation increased BATSNA and BAT temperature. A solution of 2 µl of bicuculline-methiodide was mixed with 1 µl of Read beads (Fluospheres carboxylate modified microspheres, red) and an appropriate amount of Horseradish Peroxide (HRP) (ThermoFisher Scientific, Australia). This solution was withdrawn into a glass pipette for microinjection. The injection pipette was attached a stereotaxic manipulator prepared on a stereotaxic frame. Approximate injection locations were anterior-posterior (AP): -2mm from Bregma, medial-lateral (ML): ±5mm from the midline, dorsal-ventral (DV): -7.2mm from the cortex surface.

This coordinate values were determined with the rat atlas by Paxinos and Watson (2006) and reviewed beforehand adjusted by body weight described in "The Rat Brain in Stereotaxic Coordinates" by Yang et al. (2018).

Muscimol microinjection

Muscimol was injected into medullary raphe to determine if this brain area was involved amygdala-induced BAT thermogenesis. The stereotaxic coordinate to the medullary raphe was approximately AP: -12mm from Bregma, ML: 0mm from the midline, DV: -9.6mm from the dorsal surface of the cortex. The solution for microinjection of Muscimol was made by mixing 10ul of pre-aliquoted muscimol with 1-2ul of READ Beads and an appropriate amount of HRP. The concentration of

muscimol was 1nmol in 100nl of Ringer solution. The injection procedure of muscimol was same as Bicuculline injection, using a glass pipette. Muscimol injection was performed in the second part of the study.

Measuring BAT nerve activities

The electrical discharges from the nerves were amplified (x10,000, AC Preamplifire, Digitimer, UK) and filtered (bandpass filter, 1Hz-1kHz, Digitimer, UK). In addition, the electrical signals of neural activity were assessed as sound through speakers so that they could be recognized by the auditory sense.

During the experiment, BAT sympathetic nerve activity, end tidal CO2 (EtCO2), BAT temperature, body temperature, skin temperature, and arterial blood pressure were recorded with 'Labchart 8'.

Hexamethonium intravenous injection

At the end of experiments, hexamethonium was injected through the femoral vein catheter (12mg in 1ml per animal) to confirm that the nerves used in the experiment were sympathetic nerve. Hexamethonium binds competitively with acetylcholine in autonomic ganglia and blocks acetylcholine's nicotinic effects. The loss of neural activity was used as confirmation that it was sympathetic.

Perfusion

This procedure was carried out on a perfusion tray within a fume hood. With the animal positioned ventral side up, a No. 20 scalpel was used to make a 4-6 cm incision along the midline of the chest from the rostral to the caudal end. The xiphoid process was exposed and lifted with clamp scissors, and the heart was revealed by cutting the ribs with surgical scissors to avoid damaging the large blood vessels around it. The clamp scissors holding the xiphoid process were pulled towards the head, and a small pair of surgical scissors was used to make an incision into the left ventricle of the heart. A catheter was inserted through this incision, passing from the left ventricle to the right atrium and then to the aorta. The entire heart was clamped with the clamp scissors to prevent the catheter from being pulled out. The right atrium was cut with scissors to relieve blood flow and pressure. washing solution (1M phosphate-buffered saline and 10% NaNO2) was performed through the catheter and kept flowing until the blood draining from the right ventricle was colourless. Formaldehyde solution (1M phosphate-buffered saline and 4% formaldehyde) was then perfused through the catheter and allowed to flow until the entire body became rigid. After the perfusion, the animal's cervical spine was cut with surgical scissors, and the scalp was cut by inserting scissors through the skin of the occipital region. Once the skull was exposed, the skull bone was peeled off with a bone cutter, the brain was

separated from the skull, placed in formaldehyde solution, and stored in a 4°C refrigerator. After overnight storage, the brain was transferred to a 30% sucrose-PBS solution for preservation.

Brain sectioning and mounting

The excess rostral and caudal edges of the brain were removed with a razor blade, except for the brain region where the intracerebral microinjection was made and the surrounding area. The brain was placed caudally into a silicone mould and immersed in an optimal cutting temperature (OCT) compound (Sakura, tissue-tek OCT compound, Queensland, Australia); the silicone mould containing the brain was placed in a 100 ml beaker and 35 ml of isopentane (TCI, Tokyo) was poured between the silicone side and the beaker. Immerse it in liquid nitrogen and wait until the OCT compound hardens, maintaining a temperature of about -30°C. The frozen brain was sliced to a thickness of 50um with a cryostat (Cryocut 19900, Leica Microsystem Pty, Ltf, North Rude, NSW, Australia) in the CMPH microscopy room. When it was sectioned, brain slices were placed in 48 wells plates. The brain sections were divided to 2 groups.

After sectioning, one of the groups was mounted on slide glasses, which were written the animal No., experimental area, sample No., date and name of experimenter. Before mounting, the samples were washed with 0.1M PBS for 10 mins 3 times on a shaker to remove OCT compound. A petri dish and a small brush was used to mount brain slices onto the slide glasses. A surfactant, Triton solution, was filled to about half the depth of petri dish and slide glasses were dipped into it to wet the surface. Then six brain sections were placed on slide glasses, starting from the front of the brain, one at a time. When finished mounting, dry them and put cover glasses onto them with a few drops of 100% buffer glycerol. Finally, the slide glasses were covered by cover glasses using transparent nail polish, and they were stored till microscope observation.

Visualising injection sites

Injections sites was visualising with 3,3'-diaminobenzidine (DAB, Sigma, product number 202-110-6)) reaction with HRP contained in injectate. Due to carcinogenic of DAB, it was handled with care. DAB (25g) was dissolved in 1ooml of PBS with a sonication machine. Once the solution was fully dissolved, it was added to the plate containing the brain slices, which were then immersed in the solution for a period of 10 minutes. Then, 7.5 µl of 30% hydrogen peroxide was added to the plate containing the DAB solution using a pipette and shaken until the sample shows a discoloration reaction. Once the reaction was visible, the DAB solution was replaced with PBS. The brain sections were washed with PBS (10min, three times). All DAB waste liquid from this process was collected in a 500 ml beaker, and the DAB was later deactivated with bleach. After that, the sections were

mounted in the same way as the other groups, dried overnight without a cover glass, and then stained with neutral red (see below).

The sample slides, which had brain sections mounted, were placed in metal slide holders, immersed in distilled water for 30 seconds, and then in neutral red solution (2.5 g neutral red powder, 1.53 g sodium acetate, 0.6 mL acetic acid) (Sigma product number 209-035-8) for 10 minutes. Then, the slides were transferred to distilled water to remove excess neutral red solution, and then immersed in 70% ethanol solution, 95% ethanol solution, and 100% ethanol solution for 5 to 10 minutes each. Next, they were washed with a fresh 100% ethanol solution and immersed in a 100% xylene solution for 10 minutes. They were then transferred to another fresh 100% xylene solution. Once the cover glass was ready, the sample slide was removed from the xylene solution, a few drops of mounting medium (Dibutylphthalate Polystyrene Xylene (DPX) (Trajan, Victoria, Australia)) were placed on the sample slide, and the cover glass was placed on top and allowed to dry naturally. Once the slides were dry, write the animal number, site of observation, and sample information such as date and investigator's name on the slides, and store them in the slide case until observation.

The samples were then observed using a fluorescence microscope and bright-field microscope, mainly used Slide Scanner VS200 (Olympus LS, Tokyo Japan) to identify the injection sites.

Data Analysis

All data obtained during the experiment were converted from LabChart 8 (AD Instruments, Castle Hill, NSW, Australia) to Igor files, and figures were created using Igor Pro (version 9.0.5).

BATSNA was Fourier-transformed to determine the magnitude of nerve activity (the log base 10 of Power of BATSNA) (Ootsuka et al., 2009).

The average values of all parameters before cold exposure or drug administration (with the time of cold stimulation or drug administration set as 0 minutes) were calculated using the mean values from -6 minutes to -1 minute. For the parameters after cold stimulation or drug administration, the maximum values (changes) were determined by averaging the values over 30 seconds before and after the peak. Additionally, the change in all parameters was calculated as [((mean value after cold exposure and drug administration - mean value before cold exposure and drug administration) / mean value before cold exposure and drug administration) \times 100]. All these calculations were performed using IgorPro, and the numerical data were recorded in Excel.

Finally, these quantified data were entered into Prism 9 to generate a bar chart, and a paired t-test was performed to analyse the statistical significance of the results ($p<0.05$, mean ±SD).

RESULTS

Exposure to cold temperature

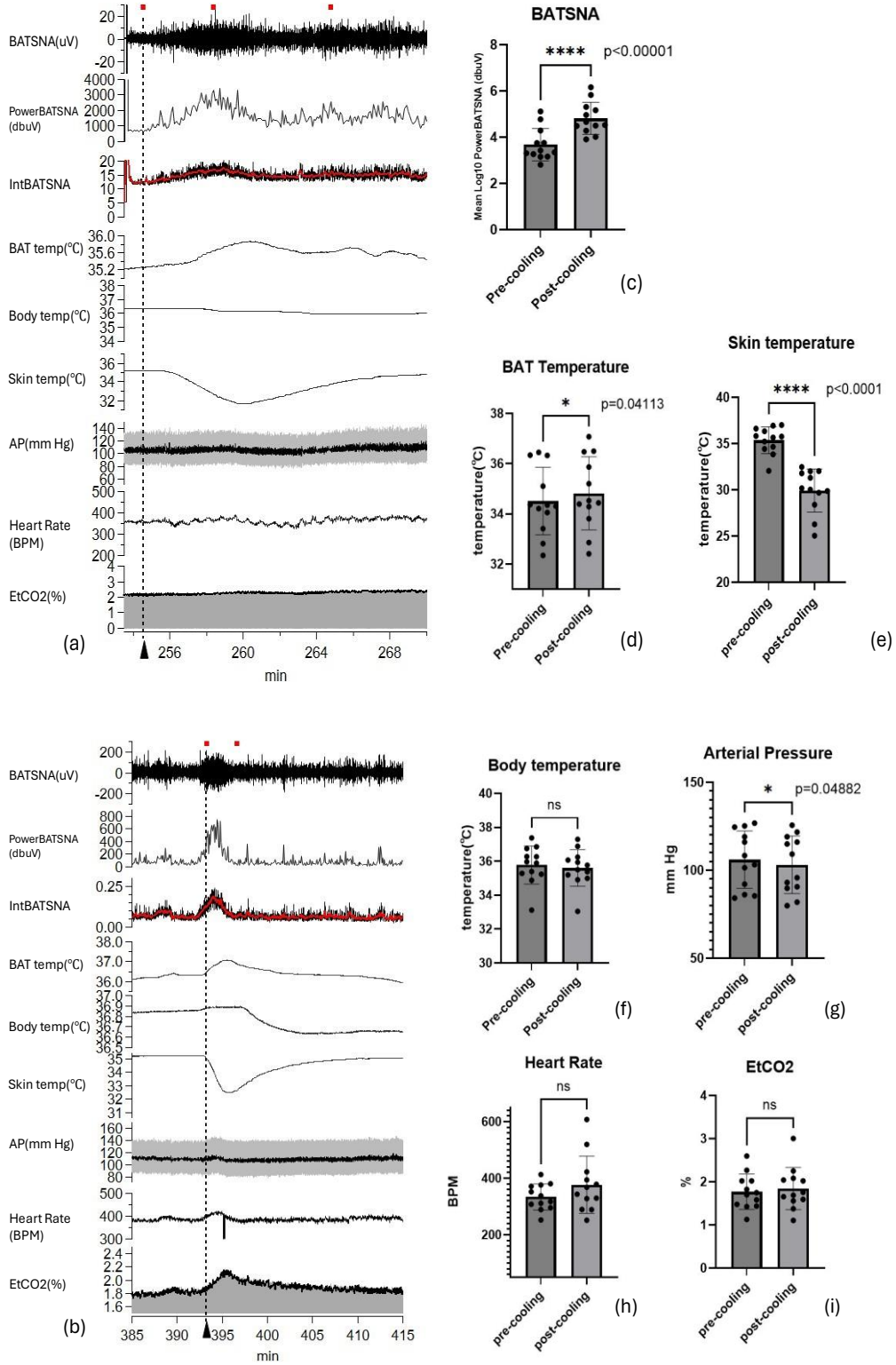


Figure 5*: cold exposure stimulating*

(a), (b) all parameters of cooling responses (2 examples); Changes in mean values, pre-cooling against post-cooling for all parameters. (c)Log10PowerBATSNA, (d)BAT Temperature, (e)Skin Temperature, (f)Body Temperature, (g)Arterial Pressure, (h)Heart Rate, (i)End tidal CO2

Cold exposure was performed to confirm that the nerves exposed from the BAT were sympathetic. Cold water was run through a water jacket to reduce skin temperature by 5.4 ± 1.8°C (n=12). This cooling caused an increase in neural activity in all experiments, confirming that the exposed nerves were sympathetic (Figure 5 (a) (b)). The mean increase in neural activity was also highly significant. The peak value of mean BAT SNA increased by 1.1±0.3 dbuV ($p<0.0001$, paired t-test) (Figure 5 (c)). Also, the increase in mean BAT temperature and decrease in mean arterial pressure were also shown to be significant. The mean BAT temperature increased by 0.3±0.3 ℃ ($p<0.05$, paired t-test) (Figure 5 (d)). The mean arterial pressure decreased by 3±3 mmHg ($p<0.05$, paired t-test) (Figure 5 (g)).

Skin temperature was intentionally reduced, which of course created a significant difference, confirming that the cold exposure was functioning correctly. The mean skin temperature decreased by -5.4±1.8 ℃ ($p<0.0001$, paired t-test) (Figure 5 (e)).

During the cold exposure, there were no significant differences in the animals' body temperature, heart rate or expiratory terminal carbon dioxide. Body temperatures changed by -0.2±0.3° C ($p=0.1607$, paired t-test) (Figure 5(f)). Heart rate changed by 43±58 BPM ($p=0.1304$, paired t-test) (Figure 5 (h)). EtCO2 changed by 0.1±0.1 % ($p=0.1398$, paired t-test) (Figure 5 (i)).

Microinjection of Bicuculline into the Amygdala

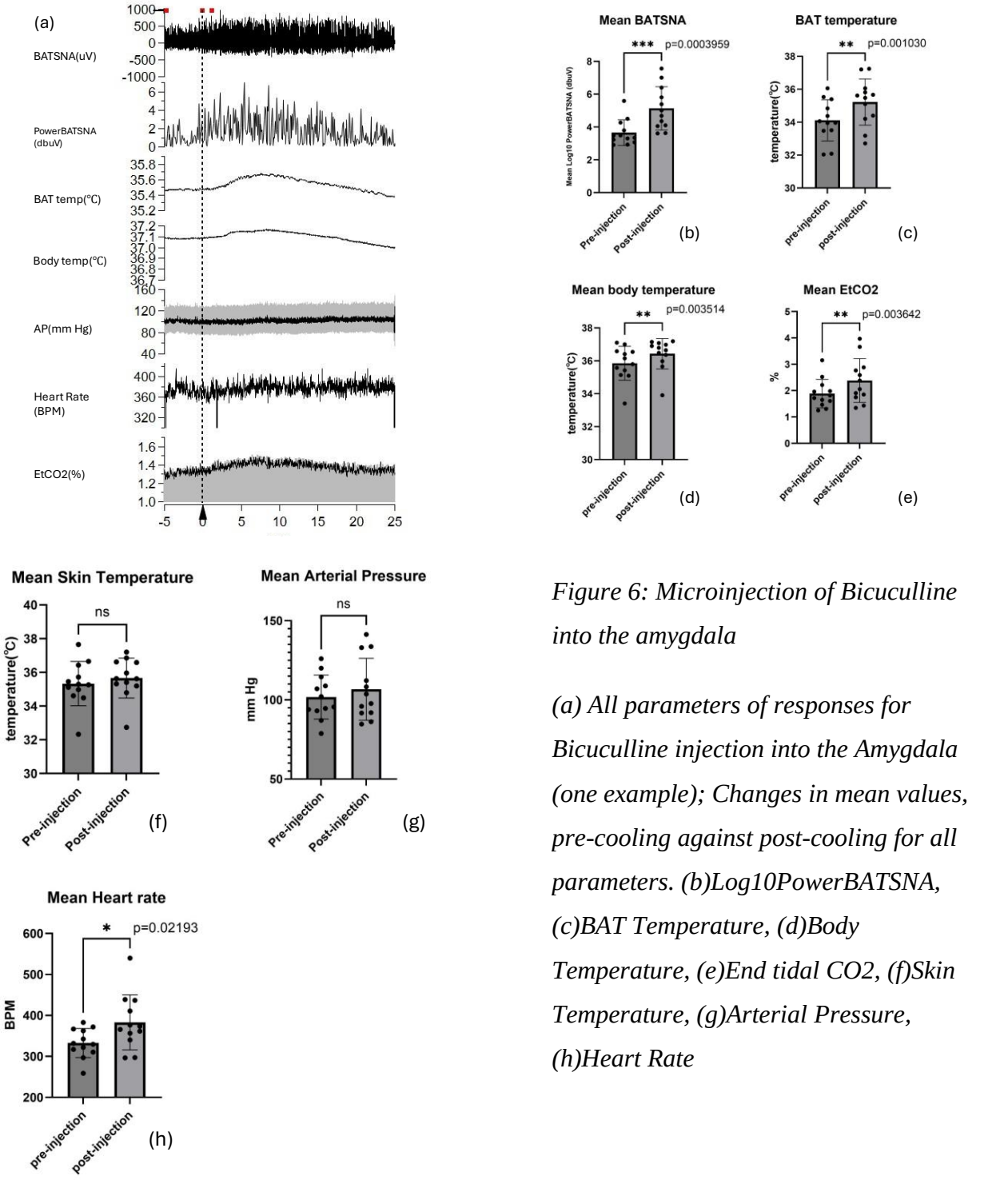


Figure 6: Microinjection of Bicuculline into the amygdala

(a) All parameters of responses for Bicuculline injection into the Amygdala (one example); Changes in mean values, pre-cooling against post-cooling for all parameters. (b)Log10PowerBATSNA, (c)BAT Temperature, (d)Body Temperature, (e)End tidal CO2, (f)Skin Temperature, (g)Arterial Pressure, (h)Heart Rate

In this experiment, bicuculline was injected into the left and right amygdala, and the second injection is shown as 0 minutes in the above figure. The injection of bicuculline into the Amygdala induced BATSNA thermogenesis (Figure 6 (a)).

Significant differences were found in the three key thermogenesis indices in BAT: BATSNA, BAT temperature and the increase in end-tidal CO2, with the increase in mean BATSNA being statistically significant with a mean of 1.5±0.6 dBμV ($p<0.001$, paired t-test) (Figure 6 (b)). Mean BAT temperature increased by an average of 2.7±0.9 °C ($p=0.0010$, paired t-test), indicating a statistically significant difference (Figure6 (c)). The increase in mean end-tidal CO2 was statistically significant with an average of 0.5±0.3 % ($p=0.0036$, paired t-test) (Figure 6 (e)). The average reaction duration for the increase in BATSNA caused by microinjection of bicuculline was 48.2 ± 9.7minutes (n=11). The average onset latency from the time of microinjection to the onset of brown adipose tissue reaction was 1.9 ± 0.3 minutes (n=11).

Other statistically significant changes in mean body temperature and mean heart rate readings were also recorded before and after injection: mean body temperature increased by 0.6 ± 03°C ($p=0.0035$, paired t-test) (Figure 6 (d)); mean heart rate increased by 50 ± 41 BPM ($p=0.0219$, paired t-test) (Figure 6 (h)). Mean skin temperature and mean arterial pressure value both increased, but not statistical significance. After the microinjection, skin temperature increased by a mean of 0.3 ± 0.4 °C ($p=0.1109$, paired t-test) (Figure 6 (g)), and mean arterial pressure increased by 5 ± 6 mmHg ($p=0.0825$, paired t-test) (Figure 6 (h)).

Figure 7*: Brain injection mapping with Rat Brain Atlas corresponding BATSNA waves*

Figure removed due to copyright restriction

Paxinos, G. and Watson, C., 2014. The rat brain in stereotaxic coordinates.

Amsterdam: Elsevier

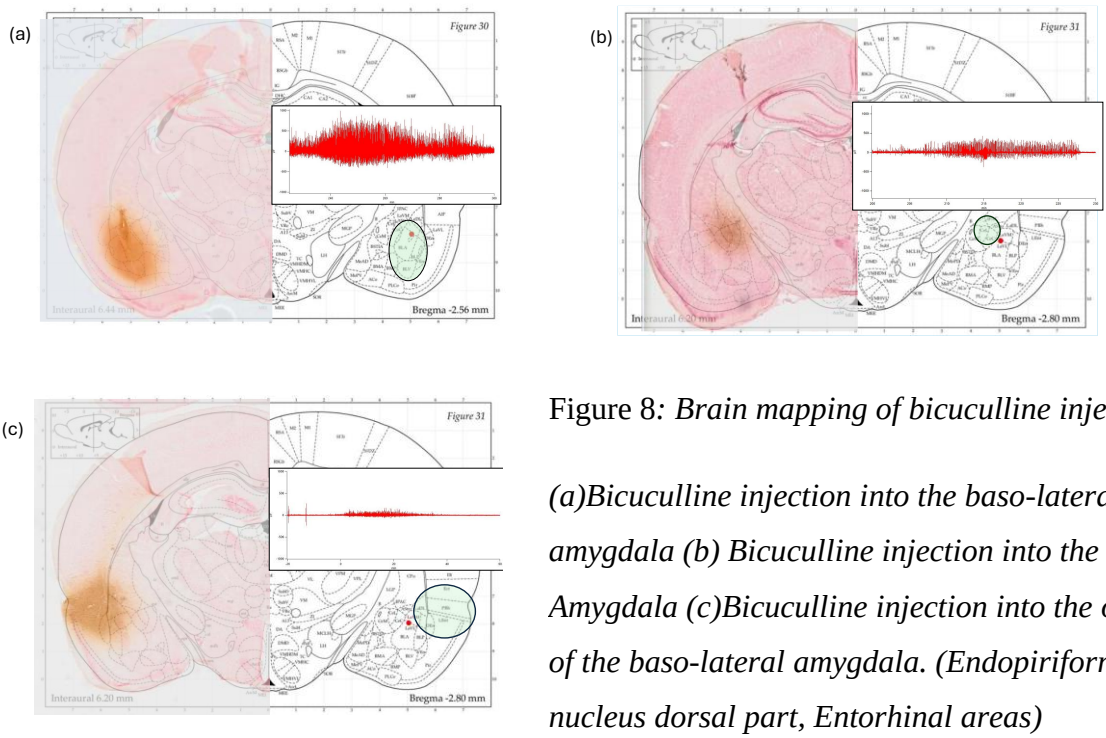


Figure 8*: Brain mapping of bicuculline injection*

(a)Bicuculline injection into the baso-lateral the amygdala (b) Bicuculline injection into the central Amygdala (c)Bicuculline injection into the outside of the baso-lateral amygdala. (Endopiriform nucleus dorsal part, Entorhinal areas)

Bicuculline injections were made at approximately AP-2.1 mm from Bregma, ML±5 mm from midline and DV-7.8 mm from the surface of cortex, but some variation was expressed between experimental individuals (Figure 7). The site of administration was examined by causing a DAB brown colour reaction with HRP added to the bicuculline solution (Figure 8). BATSNA response was greatest when bicuculline was administered around the basolateral amygdala (Figure 8 (a)). There was also a clear BATSNA response when bicuculline was administered near the central amygdala (Figure 8 (b)). No significant sympathetic activity was observed regarding animal individuals in which bicuculline was not administered to the amygdala (Figure 8 (c)).

The brain regions where BATSNA responses were observed following bicuculline administration included the basolateral amygdala group (anterior, posterior, ventral, amygdaloid intramedullary gray) and the central amygdala group (lateral, medial division). Conversely, no responses were observed in the cerebral cortex, caudate putamen, and globus pallidus, which is consistent with the results obtained from DAB staining (Figure 7).

Microinjection of Muscimol into Medullary Raphe

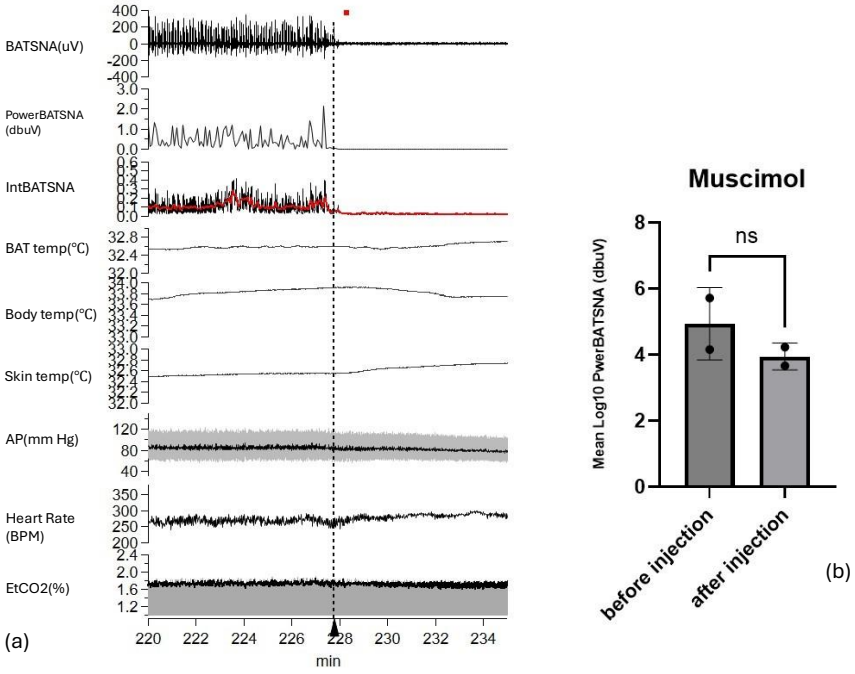


Figure 9*: Microinjection of muscimol into medullary raphe*

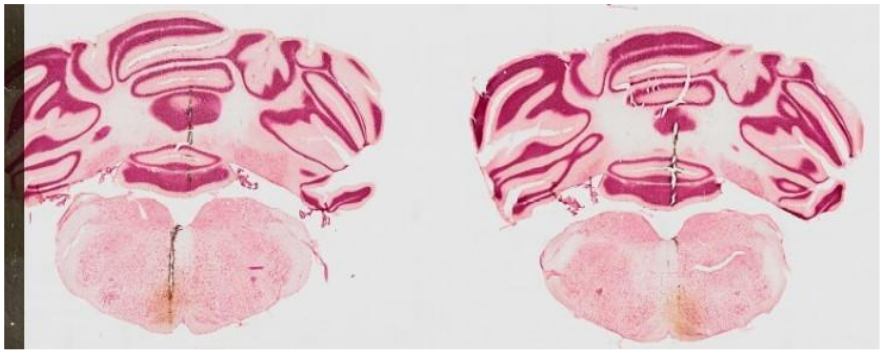
(a) Muscimol injection into the medullary raphe after injection of Bicuculline into the Amygdala. (b) Mean log10 PowerBATSNA pre-injection vs post-injection of Muscimol

Muscimol administration into the medullary raphe counteracted the BATSNA response induced by bicuculline injections into the amygdala in this individual animal (Figure 9 (a)). Mean BATSNA decreased by 0.1±6.3 dB μ V from post-bicuculline values (p=0.2940, paired t-test) (Figure 9 (b)). Also, an inhibitory response in the activated BAT sympathetic nerves was observed (Figure 9(a), Figure10 (a) (b)). Muscimol was administered near the medullary raphe, as can be seen by fluorescence microscopy (Figure 10 (c)).

(a)

Figure removed due to copyright restriction

(b)



(c)

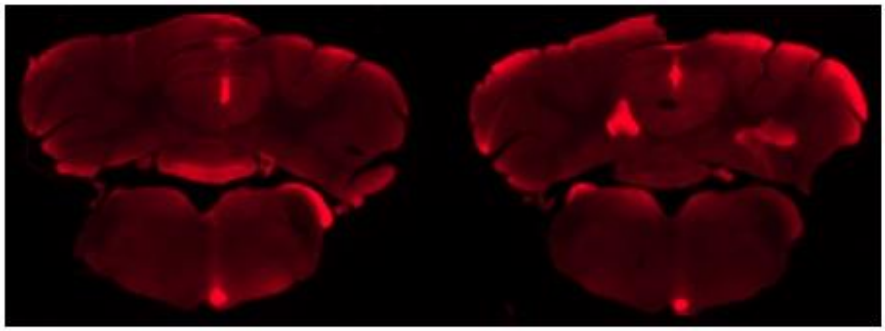


Figure 10*: Brain sections of medullary raphe*

(a)Paxinos, G. and Watson, C., 2014. The rat brain in stereotaxic coordinates. Amsterdam: Elsevier. (b)Muscimol injected; HRP/Neutral Red staining (c)Fluorescent image/ BEADS

(b)

(a)

Figure removed due to copyright restriction

(c)

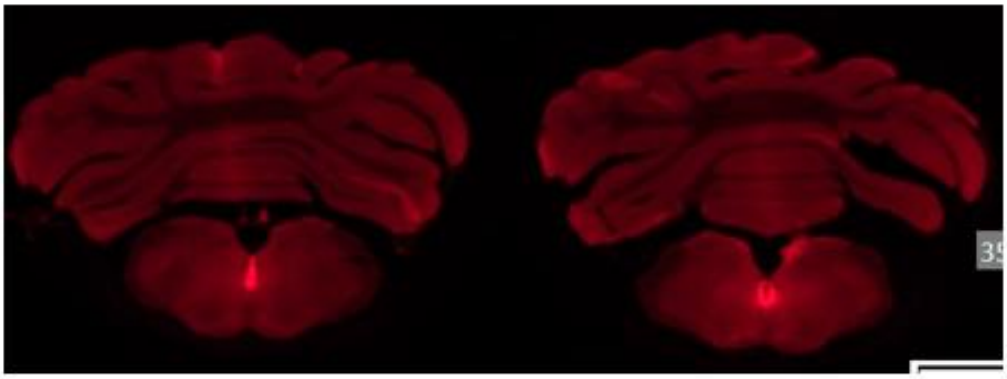


Figure 11*:Brain sections of medullary raphe*

(a) Muscimol injected; HRP/ Neutral Red staining (b) Clarified injection site. Paxinos, G. and Watson, C., 2014. The rat brain in stereotaxic coordinates. Amsterdam: Elsevier. (c) Fluorescent image/ BEADS

In this experimental subject, the injection site of muscimol was shifted dorsally (Figure 11 (a) (b)). As shown in the fluorescent images, it was administered to the caudal interstitial nuclei (Figure 11 (c)). Muscimol did not penetrate to the raphe nuclei. During this time, the BAT sympathetic nerve activity (BATSNA) was not inhibited, and active responses were still observed (Figure 12).

Figure removed due to copyright restriction

Figure 12*: Visualization of Muscimol Penetration Area and BAT Nerve Activity*

Mapping results of muscimol injections into the raphe nuclei. The region where muscimol was administered did not caused inhibition of the amygdala-elicited BATSNA; raphe magnus nuclei, raphe obscurus nuclei, gigantocellular reticular nuclei, dorsal paragigantocellular nuclei, predorsal bundle, prepositus hypoglossal nuclei, medial longitudinal fasciculus, caudal interstitial nuclei. The region where muscimol was administered did cause inhibition of the amygdala-elicited BATSNA; the raphe pallidus, inferior olive medial nuclei, inferior olive dorsal nuclei, and medial lemniscus (Figure 12).

DISCUSSION

Main Observations

When bicuculline was injected into the amygdala, an increase in neural activity in brown adipose tissue was recorded. Bicuculline injection into the amygdala resulted in significant changes in BATSNA, BAT temperature, end-expiratory CO2, body temperature and heart rate. In contrast, no significant changes were found in skin temperature and mean arterial pressure. Furthermore, administration of bicuculline to sites outside the amygdala did not cause a significant response in the sympathetic nerves of brown adipose tissue, suggesting that bicuculline acts on the amygdala and not on other sites. The main injections where the increased neural activity response was greater were in or near the basolateral part of the amygdala.

Brown adipose tissue activation induced by bicuculline injections into the amygdala was reversed by muscimol injection into the rostral medullary raphe nucleus(Morrison et al., 1999). Inhibition of neurons in this nucleus suppressed the BATSNA activated after bicuculline administration and no longer responded to cold stimuli. This indicates that the medullary raphe nucleus is part of the neural pathway that mediates the amygdala-induced BAT thermogenesis (Nakamura, 2015; Nason & Mason, 2006).

Cooling Cycle

The main purpose of the cold exposure was to determine whether the nerves-isolated in the BAT area were sympathetic nerves (Ootsuka et al., 2009). As previous studies have shown that BAT heat production and an increased BATSNA occurs in cold environments, the cold exposure was used to determine whether the animals under test were capable of BAT heat production and whether the parameters measured changed as reported previously (Ootsuka et al., 2007). In every experiment, what was observed in this process was a decrease in skin temperature first, followed by the activation of the BAT heat production pathway.

For BAT to trigger heat production, skin temperature receptors need to send a cold signal to the BAT's thermogenic neural pathway (Nowack et al., 2017). The time lag between BAT body temperature and end-expiratory CO2 was also noted, and BATSNA was activated before BAT body temperature and end-tidal CO2 increased. This indicates that BAT neural activity responds earlier than the activation of other BAT-related indicators. This was also observed in previous studies, indicating that BAT thermogenesis caused by acute cold exposure is mainly mediated the sympathetic nervous system, with little involvement of hormonal system(Nowack et al., 2017). The

increase in end-tidal CO2 is proportional to the increase in metabolic rate (Page, 2011). This is the response seen when metabolism is accelerated by the heat-producing response of BAT.

BAT temperature and body temperature, these two measurements, are similarly elevated. This suggests that the increase in BAT temperature corresponds to an increase in body temperature(Brizuela et al., 2019). This increase in body temperature was observed after a decrease in skin temperature, indicating that this phenomenon occurs because skin temperature receptors sense the cold stimulus and resist the stimulus. This is supported by changes in skin temperature during the heating phase (Chung et al., 2024). As skin temperature increased, BATSNA, end tidal CO2, BAT temperature and body temperature began to decrease. These decreases continued until they returned to the readings taken before the cold stimulus was applied.

The role of the amygdala in BAT thermogenesis

Bicuculline injections into the amygdala showed a similar trend to that seen during the cold cycle. Significant differences in BATSNA, BAT temperature, and end tidal CO2 were observed before and after the injection, with the major difference being no significant change in blood pressure. This indicates that there is functional excitatory input from the amygdala to the BAT (Mesquita et al., 2016; Nakamura, 2015).

Among the brain regions where bicuculline was administered, the areas with the highest BATSNA were the basolateral amygdala group (anterior, posterior, ventral, amygdaloid intramedullary gray) (Grossman et al., 2022; Knapska et al., 2007). Although the central amygdala group also showed a clear increase in neural activity when bicuculline was administered, the response was not as pronounced as in the basolateral amygdala (Kubota et al., 2023). Conversely, the region around the amygdala that can be definitively excluded from contributing to BAT thermogenesis is the cerebral cortex. The cerebral cortex in rats is also responsible for higher cognitive functions. In particular, the prefrontal cortex is known to be involved in decision-making, planning, and regulating social behaviour. These regions interact with the amygdala, but bicuculline, when administered to the cerebral cortex, does not affect the sympathetic nervous system (Mares et al., 2018).

Bicuculline was used to activate neurons in the amygdala. The main excitatory neurotransmitter in the brain is glutamate. In research exploring the brain circuits involved in autonomic nervous system function, bicuculline is often used because it can induce a response even when glutamate does not(Carrive & Kuwaki, 2017), and so it was also used in this study. It would be useful to investigate whether the same type of thermogenesis occurs in brown adipose tissue when glutamate agonists are used (Cao & Morrison, 2006). If glutamate is shown to be involved, this could lead to

research into the role of glutamate in the amygdala in the mental thermogenesis that occurs in awake animals.

Bicuculline is an antagonist of the GABA-A receptor. The fact that the disinhibition of neurons in the amygdala with bicuculline caused BAT thermogenesis indicates that there is a spontaneous and continuous GABAergic input to the amygdala at least under anaesthetic conditions. This GABAergic input may play an important role in psychogenic BAT thermogenesis. As mentioned earlier, the results of this study suggest that the basolateral amygdala is the main site of action of bicuculline within the amygdala complex. Neurons in the central nucleus of the amygdala are primarily GABAergic (Janak & Tye, 2015). There are GABAergic neurons within the basolateral amygdala (Izadi & Radahmadi, 2022). It is a future task to explore the GABAergic neuronal pathway to the basolateral amygdala that is involved in thermogenesis in brown adipose tissue.

The brain regions where muscimol was injected and a sympathetic inhibitory response was observed included not only the raphe pallidus but also the inferior olive nuclei. This raises uncertainty about whether the raphe pallidus is the sole brain region affected by muscimol that releases inhibitory signals for BAT neural activity. Since GABAergic receptors are also present in the inferior olive nuclei, it is possible that the effects of muscimol were influenced by this region (Paul & Das, 2024). To determine whether muscimol acts exclusively on the raphe pallidus nuclei, further experiments are necessary. These should aim to achieve precise injection sites and administer low concentrations of muscimol to ensure it does not spread to brain regions other than the raphe pallidus.

This research has revealed that the amygdala is indeed involved in BAT thermogenesis, and that the medullary raphe, which is the hub of the main thermoregulatory neural circuit in the lower brainstem, mediates this response. Previous studies have shown that the main brain regions that form the central nervous circuit involved in BAT thermogenesis are the preoptic area, the dorsomedial hypothalamus, and the rostral medullary raphe nucleus (pallidus)(Morrison & Nakamura, 2019; Nakamura & Morrison, 2022) In addition, it has also been shown that the lateral habenula (Ootsuka et al., 2017), as well as the pathway from the dorsal peduncular cortex and dorsal tenia tecta to the DMH (Kataoka et al., 2020), are important in emotional hyperthermia. In addition to the increase in brown adipose tissue thermogenesis, the suppression of heat dissipation by vasoconstriction of thermoregulatory skin vessels contributes to emotional hyperthermia(Mohammed et al., 2014). Inhibiting neurons in the amygdala suppresses the vasoconstriction response of the skin caused by emotional stress This study shows that the amygdala may be included in the central circuit of emotional hyperthermia.

Limitations

In this study, both bicuculline and muscimol were used at a concentration of 1 nmol in 100 nl, with this concentration consistently applied and an injection volume of 100 nl used as a standard. This concentration is relatively high compared to other studies (Mesquita et al., 2016). Using a high concentration increases the likelihood that the administered drug will spread to non-target areas, potentially activating or inhibiting regions of no interest or causing contradictory results. However, it was evident that bicuculline contributed to the activation of BAT sympathetic nerve activity in the amygdala, as animals that received bicuculline injections outside the amygdala and did not penetrate the amygdala showed minimal BAT neural activity (Mesquita et al., 2016). Additionally, high concentrations can affect response time, typically increasing it. While a longer response time is desirable, it might have been better to use a lower concentration and focus on more precise targeting of brain regions.

This project faced several constraints, one of which was time related. Due to the complexity of the surgical procedures involved, which required 2-3 months to master, we were unable to secure enough samples to obtain reliable data. The limited timeframe available to complete the project impacted the sample size. The data obtained from the experiments conducted within this period were used for analysis and served as the basis for the project's conclusions. Although the sample size met the minimum requirements for statistical analysis, additional experiments are necessary to enhance statistical significance.

The insufficient experimental period also contributed to the lack of gender variation in the subjects analyzed in this project. The experiments were conducted using only male rats, and the results were analyzed accordingly. It remains unclear whether there are differences in BAT thermogenesis between males and females, necessitating further research to determine if such differences exist.

The variety of rat strains used was also limited by the time constraints, resulting in the exclusive use of Sprague-Dawley rats. Although plausible results were obtained with this strain, other strains might exhibit different responses under this research design. The project did not allow for experiments to verify significant differences between rat strains.

The precise location of the brain region for injection also varies depending on the body weight of the animals used in the experiments. The accuracy of these calculations may have been a limiting factor. In some experimental subjects, the amygdala was correctly identified, and bicuculline was administered to the amygdala. However, in some subjects, there was evidence of administration

outside the amygdala. These results allowed for the measurement of latency and the visualization of the relationship between injection site and BAT neural activity.

The same approach was applied for the administration of muscimol to the raphe nuclei, where the injection site was calculated based on the weight of the experimental animals. However, inaccuracies in these calculations may have led to deviations in the injection site.

The most significant constraint during this project was the relocation of the laboratory. This move had a substantial impact on the progress of the experiments, requiring considerable time for the reinstallation of equipment and environmental readjustments. Additionally, the animal facility responsible for providing experimental animals also relocated, resulting in a delay of approximately three months to secure the animals again. Consequently, these factors led to delays in the experimental schedule and a shortened data collection period.

Future Directions

New findings from the present study can be utilized to further understand BAT thermogenesis in the context of emotion regulation mediated by the amygdala. There is no consensus that the amygdala is directly connected to the medullary raphe nucleus, therefore it is highly likely that BAT thermogenic signals from the amygdala to the medullary raphe is mediated by other relay nuclei. There have been reports suggesting that the dorsomedial hypothalamus, which is the pivotal hub of the thermoregulatory pathway, receives neuronal projections from the amygdala (Nikolenko et al., 2020). The next step is to investigate the involvement of the DMG in the amygdala-induced BAT thermogenesis. It is also important to investigate whether the amygdala is involved stress-induced BAT thermogenesis.

Researching the relationship between stress-induced BAT thermogenesis and the amygdala could lead to the development of new treatments to mitigate stress-related damage in livestock and aid patients with stress disorders. This study indicates that the amygdala can be a target for pharmacological treatment. Therefore, the connection between the amygdala and stress-induced BAT thermogenesis may expand treatment options for stress-related damage in livestock and patients with stress disorders.

Furthermore, this project has revealed a link between the amygdala and autonomic functions. While the amygdala has been extensively studied for its role in emotional and behavioural regulation, its role in the autonomic nervous system remains underexplored. Future research focusing on the autonomic functions of the amygdala may uncover new functions and roles.

Conclusion

This project aimed to determine whether the amygdala is involved in brown adipose tissue (BAT) thermogenesis. Additionally, it examined whether the amygdala could elicit a drug-induced BAT thermogenesis response. This objective was tested by administering bicuculline into the amygdala to see if drug treatment could induce BAT thermogenesis. Bicuculline is an antagonist of GABA receptors, and disinhibition could potentially trigger a BAT thermogenesis response. The results indicated that bicuculline injections into the amygdala produced a BAT thermogenesis response comparable to or greater than that induced by cold exposure. This response included significant increases in PowerBATSNA, BAT temperature, and end-tidal CO2 measurements, all key indicators of BAT thermogenesis. In contrast, injections outside the amygdala did not elicit a significant sympathetic nerve activity response in BAT.

Next, the study tested whether the BAT thermogenesis response from the amygdala was linked to the known BAT thermogenesis pathway by administering muscimol into the raphe nuclei. Muscimol, a GABA receptor agonist, was expected to bind to GABA receptors in the raphe nuclei and inhibit the BAT thermogenesis response. If muscimol could inhibit the amygdala-induced BAT thermogenesis response, it would suggest that the two brain regions are part of the same BAT thermogenesis pathway. The results showed no significant differences in the data due to the small sample size. However, in subjects where the drug was administered near the raphe nuclei, an inhibitory response in BAT sympathetic nerve activity was observed. Muscimol injections outside the raphe nuclei did not show significant inhibition of the increased BAT sympathetic nerve activity. This suggests that the bicuculline-induced BAT thermogenesis response was inhibited by muscimol injections into the raphe nuclei, supporting the involvement of the amygdala in BAT thermogenesis. The amygdala is upstream in the pathway, while the raphe nuclei are downstream. Therefore, it can be concluded that the amygdala is involved in the BAT thermogenesis pathway.

The next step is to understand whether the amygdala has emotional input into the BAT thermogenesis pathway. Additionally, further research is needed to explore other autonomic functions of the amygdala that have yet to be discovered.

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APPENDICES

Q&A from Viva

Q. What is the (definition of) Hyperthermia? Is it different from a fever?

Hyperthermia and fever are both conditions characterized by elevated body temperature, but they differ significantly in their causes and mechanisms("Fever / Hyperthermia," 1994).

Fever is a regulated response to infection or illness, typically induced by the immune system. When the body detects pathogens, it releases substances called pyrogens, which signal the hypothalamus to raise the body's temperature set point. This increase helps the body fight off infections by enhancing immune function.

Hyperthermia, on the other hand, is an uncontrolled rise in body temperature that occurs when the body cannot dissipate heat effectively. This can happen due to environmental factors (like extreme heat), vigorous exercise, or certain medical conditions. Unlike fever, hyperthermia is not a regulated response and can lead to serious health risks, such as heat exhaustion or heat stroke.

Also, in humans, hyperthermia is characterized by a body temperature exceeding 37.5–38.3 °C (99.5–100.9 °F), depending on the source, and occurs without any alteration in the body's temperature set point. Typically, normal body temperature can reach up to 37.7 °C (99.9 °F) in the late afternoon. Hyperthermia signifies an increase above the expected temperature range, which can vary from mild to severe; temperatures exceeding 40 °C (104 °F) can be life-threatening(Edwards, 2006).

In summary, while fever is a protective mechanism against infection, hyperthermia is a potentially dangerous condition resulting from the body's inability to cool itself(Singh & Hasday, 2013).

Q. Why did we measure the end tidal CO2?

End-tidal carbon dioxide ($EtCO_2$) is an indicator of the concentration of carbon dioxide at the end of exhalation. It is primarily used to assess the efficiency of respiration and the state of ventilation, playing a crucial role in the management of individuals using mechanical ventilation. Normal

$EtCO_2$ values reflect the body's carbon dioxide elimination status, providing useful information for understanding an animal's respiratory condition(Mur et al., 2024).

The reason for measuring end-tidal carbon dioxide is to obtain an accurate measurement of carbon dioxide concentration that represents in PCO2 in pulmonary vein. ~~Since the beginning and middle of exhalation contain carbon dioxide that has entered the trachea, measuring the end-tidal exhalation allows for the measurement of carbon dioxide concentration expelled from the base of the lungs~~ At the beginning and middle of exhalation, the breath contains air that has not been involved in gas exchange and does not reflect the carbon dioxide concentration in the body. By measuring the end of exhalation after that gas has been expelled, it is possible to measure the actual carbon dioxide concentration expelled into the lungs through gas exchange~~.~~

(Page, 2011).

Q. How about giving heat stimulation instead of cold exposing in this research?

The purpose of the cold exposure was to identify the sympathetic nerves that control brown adipose tissue, not to examine the role of the amygdala in brown adipose tissue thermogenesis caused by cold exposure. Therefore, while the proposal is interesting, we believe it is not the issue to be examined in this study.

Reports from previous studies indicate that the amygdala is activated by heat stimuli(Becerra et al., 1999). Additionally, since an increase in temperature has been associated with higher levels of anxiety disorders(Nakagawa et al., 2020; Soares, 2011), it can be said that heat stimuli may cause psychological stress. Therefore, it is worthwhile to ~~investigate the~~investigate the role of the amygdala in the stress response caused by heat exposure in future studies~~..~~

~~In my current experiment, I conducted cold exposure to activate brown adipose tissue and confirmed sympathetic nervous activity. However, since the thermogenic response of brown adipose tissue is activated when the body requires excess heat~~(Cannon & Nedergaard, 2004)~~., it seems unlikely that brown adipose tissue would be activated by heat stimuli. Therefore, while heat~~

~~stimuli may be meaningful for confirming amygdala activity, they may not be appropriate for assessing the activity of brown adipose tissue.~~

Q. What is the perspective of injecting muscimol into the amygdala instead of the medullary raphe?

Previous research has shown that inhibiting neuronal activity in the amygdala suppresses the vasoconstriction of skin blood vessels that occurs in response to stress. Therefore, it is expected that if musimol is administered to the amygdala of an awake animal, it will suppress the rise in body temperature caused by psychological stress. In anesthetized animals, psychological stress stimuli would be meaningless because the animal is unconscious. Therefore, it is thought that administering musimol to the amygdala of anesthetized animals would have no effect.

In my experiment, I injected bicuculline into the amygdala to stimulate the sympathetic nervous system and activate brown adipose tissue. This induced thermogenesis in the brown adipose tissue, allowing me to observe amygdala-induced increases in BAT temperature(Mesquita et al., 2016). Additionally, to confirm whether the medullary raphe mediates this thermogenic neural pathway, I injected muscimol into the medullary raphe to suppress sympathetic nervous activity(Cao et al., 2010). The principle by which this drug suppresses sympathetic activity is that muscimol is like the inhibitory neurotransmitter GABA, which binds to GABA receptors and selectively allows Cl^- to pass through ion channels(Morrison et al., 1999). This results in hyperpolarization of the neurons, making it more difficult to generate action potentials and leading to inhibitory effects on neurotransmission(Hu et al., 2021).

Several prior experiments have reported the administration of this drug to the amygdala, but most of them are behavioural studies(Coleman-Mesches & McGaugh, 1995; Helmstetter & Bellgowan, 1994; Holahan & White, 2004; Salome et al., 2007), and I could not find any instances of muscimol being administered to the amygdala in experiments related to thermoregulation. Since GABA receptors are present in the amygdala, I believe that the thermogenic response of brown adipose tissue induced by bicuculline administration to the amygdala should also be suppressed by microinjections of muscimol into the amygdala(Stefanits et al., 2018). However, one of the objectives of my current experiment was to confirm whether the medullary raphe mediates the

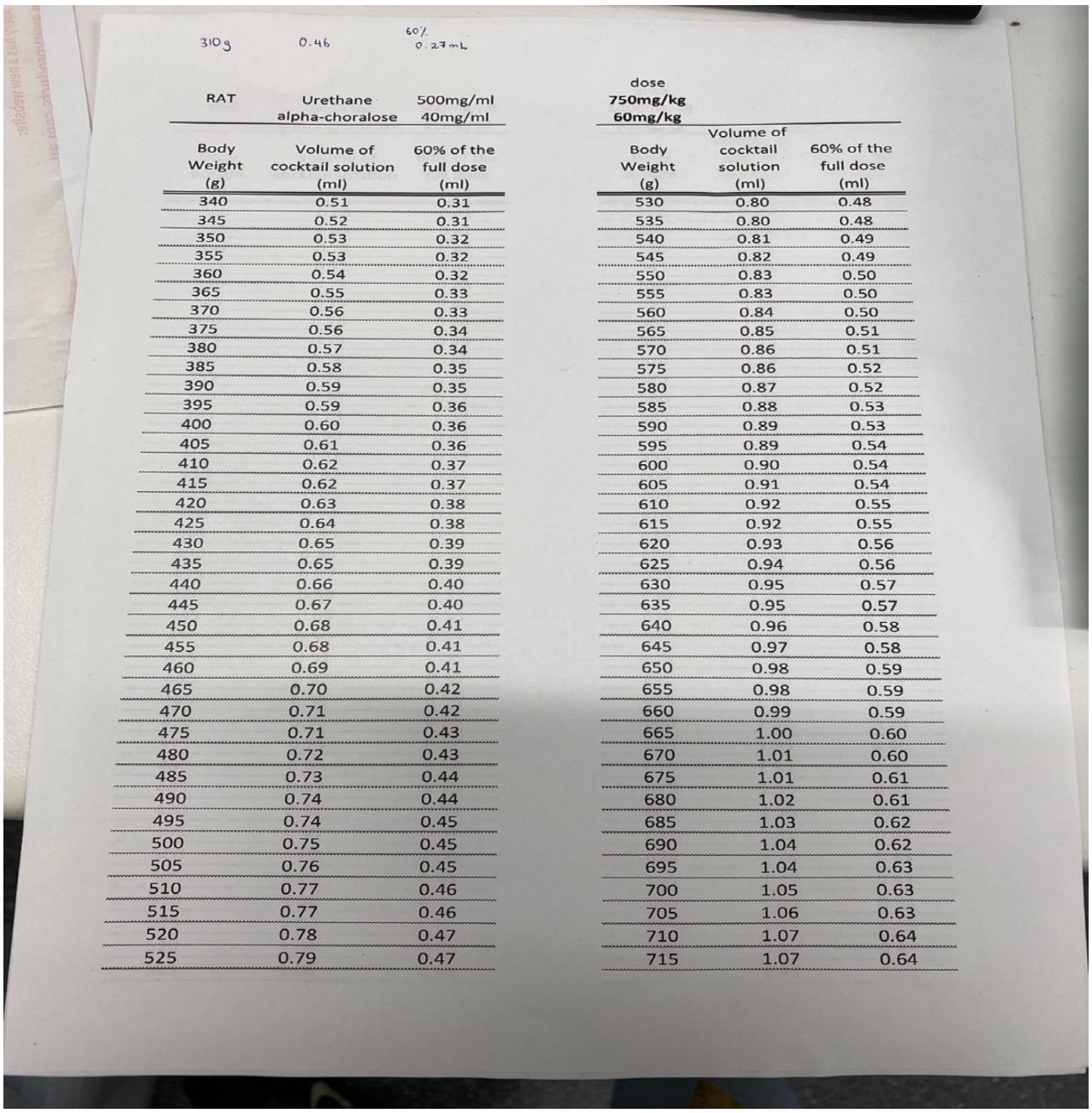
thermogenic pathway of brown adipose tissue originating from the amygdala, so I injected the inhibitory muscimol into the medullary raphe.

Appendices Figures

Appendices Figure 1: Experimental detail sheet

Animal No.	Species	Sex	Date of	Body Weight(g)	Experiment detail
L092	SD rat	Male	2/11/2023	330	Bicuculline into the Amygdala, Hexamethonium i.v.
L098	SD rat	Male	23/11/2023	340	Bicuculline into the Amygdala, Hexamethonium i.v.
L099	SD rat	Male	24/11/2023	341	Bicuculline into the Amygdala, Hexamethonium i.v.
L135	SD rat	Male	22/03/2024	222	Bicuculline into the Amygdala, Hexamethonium i.v.
L136	SD rat	Male	29/03/2024	272	Bicuculline into the Amygdala, Hexamethonium i.v.
L137	SD rat	Male	4/4/2024	303	Bicuculline into the Amygdala, Hexamethonium i.v.
L146	SD rat	Male	10/4/2024	306	Bicuculline into the Amygdala, Hexamethonium i.v.
L147	SD rat	Male	23/04/2024	331	Bicuculline into the Amygdala, Hexamethonium i.v.
L148	SD rat	Male	6/5/2024	303	Bicuculline into the Amygdala, Hexamethonium i.v.
L149	SD rat	Male	16/05/2024	327	Bicuculline into the Amygdala, Hexamethonium i.v.
L151	SD rat	Male	24/05/2024	296	Bicuculline into the Amygdala, Muscimol into medullary Raphe
L152	SD rat	Male	29/05/2024	321	Bicuculline into the Amygdala, Muscimol into Medullary Raphe

Appendices Figure 2: Anaesthetic dosage sheet



310g 0.46 60% 0.27mL

RAT	Urethane alpha-choralose	500mg/ml 40mg/ml
Body Weight (g)	Volume of cocktail solution (ml)	60% of the full dose (ml)
340	0.51	0.31
345	0.52	0.31
350	0.53	0.32
355	0.53	0.32
360	0.54	0.32
365	0.55	0.33
370	0.56	0.33
375	0.56	0.34
380	0.57	0.34
385	0.58	0.35
390	0.59	0.35
395	0.59	0.36
400	0.60	0.36
405	0.61	0.36
410	0.62	0.37
415	0.62	0.37
420	0.63	0.38
425	0.64	0.38
430	0.65	0.39
435	0.65	0.39
440	0.66	0.40
445	0.67	0.40
450	0.68	0.41
455	0.68	0.41
460	0.69	0.41
465	0.70	0.42
470	0.71	0.42
475	0.71	0.43
480	0.72	0.43
485	0.73	0.44
490	0.74	0.44
495	0.74	0.45
500	0.75	0.45
505	0.76	0.45
510	0.77	0.46
515	0.77	0.46
520	0.78	0.47
525	0.79	0.47

dose 750mg/kg 60mg/kg		
Body Weight (g)	Volume of cocktail solution (ml)	60% of the full dose (ml)
530	0.80	0.48
535	0.80	0.48
540	0.81	0.49
545	0.82	0.49
550	0.83	0.50
555	0.83	0.50
560	0.84	0.50
565	0.85	0.51
570	0.86	0.51
575	0.86	0.52
580	0.87	0.52
585	0.88	0.53
590	0.89	0.53
595	0.89	0.54
600	0.90	0.54
605	0.91	0.54
610	0.92	0.55
615	0.92	0.55
620	0.93	0.56
625	0.94	0.56
630	0.95	0.57
635	0.95	0.57
640	0.96	0.58
645	0.97	0.58
650	0.98	0.59
655	0.98	0.59
660	0.99	0.59
665	1.00	0.60
670	1.01	0.60
675	1.01	0.61
680	1.02	0.61
685	1.03	0.62
690	1.04	0.62
695	1.04	0.63
700	1.05	0.63
705	1.06	0.63
710	1.07	0.64
715	1.07	0.64

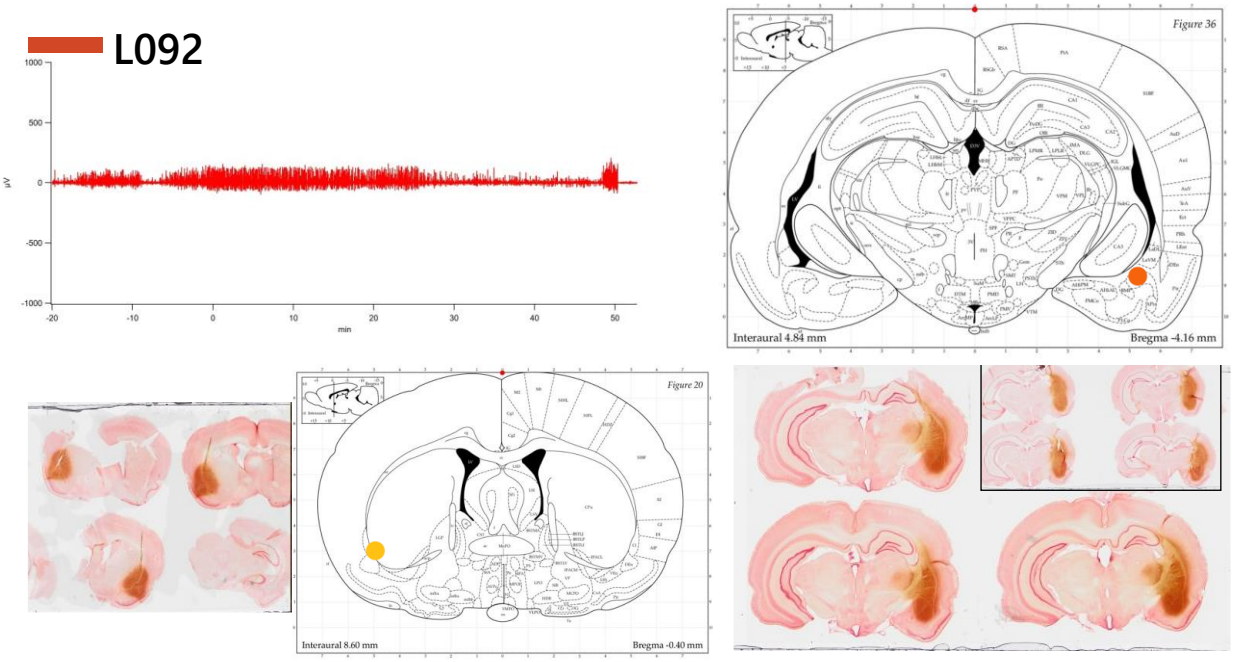
Appendices Figure 3: Neural electrical signals

Normal electrical signals of nerves

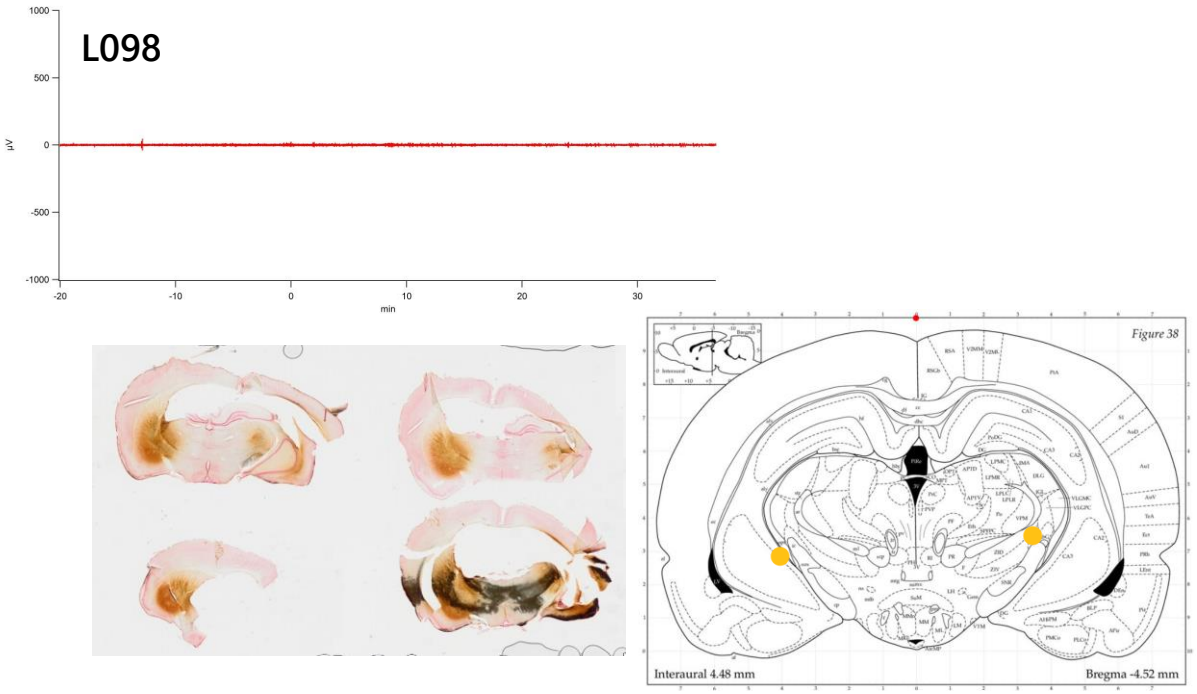
Electrical signals of nerves with noises

Appendices Figure 4: (a)-(k) BATSNA and Visualized injection sites

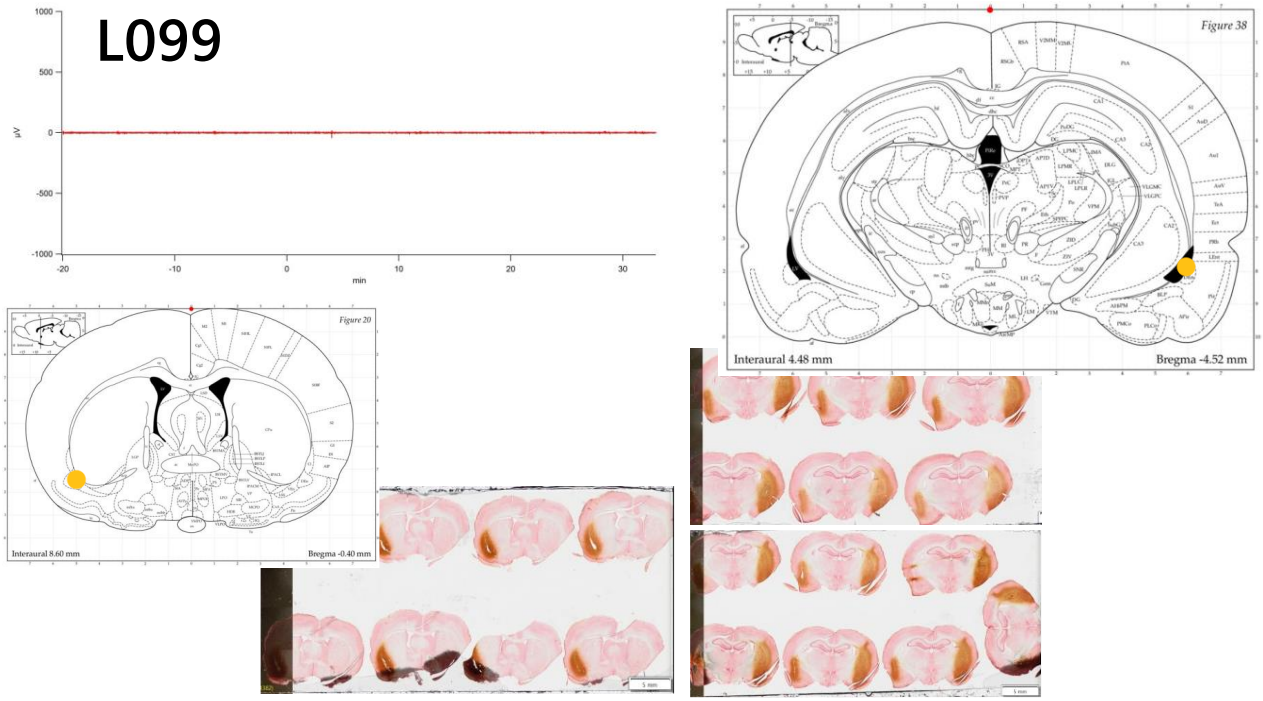
(a)


L092
1000
500
0
-500
-1000
μV
-20
-10
0
10
20
30
40
50
min
Figure 36
Interaural 4.84 mm
Bregma -4.16 mm
Figure 20
Interaural 8.60 mm
Bregma -0.40 mm

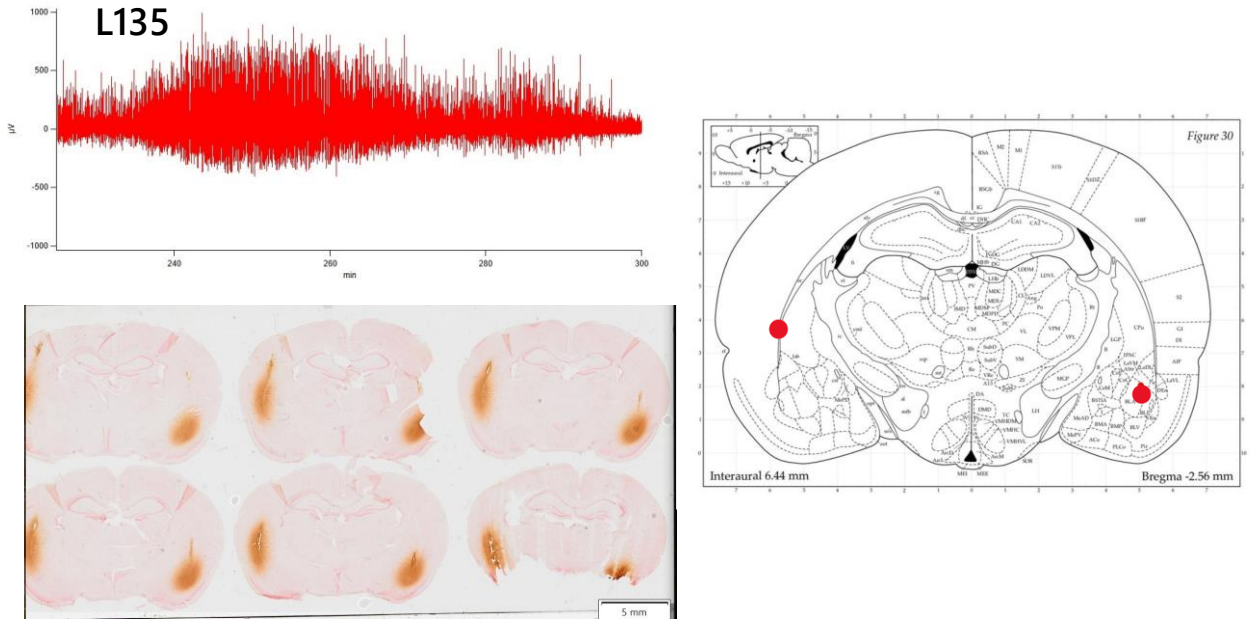
(b)


L098
1000
500
0
-500
-1000
μV
-20
-10
0
10
20
30
min
Figure 38
Interaural 4.48 mm
Bregma -4.52 mm

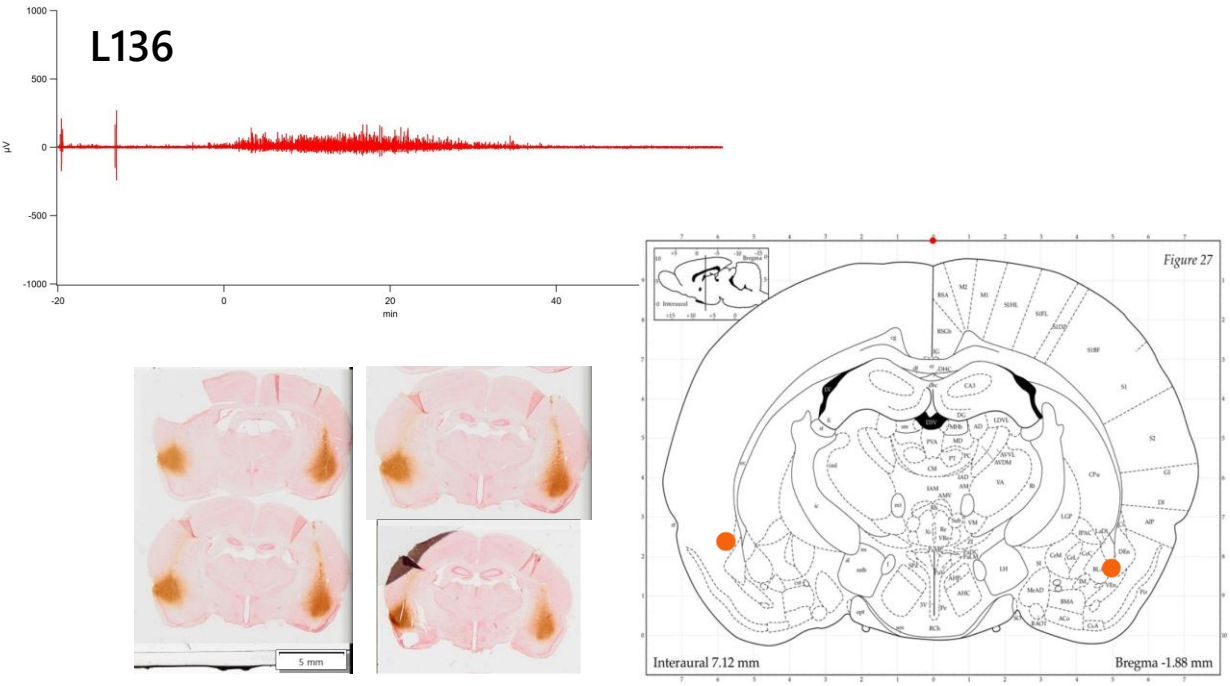
(c)


L099
Figure 20
Interaural 8.60 mm
Bregma -0.40 mm
Figure 38
Interaural 4.48 mm
Bregma -4.52 mm

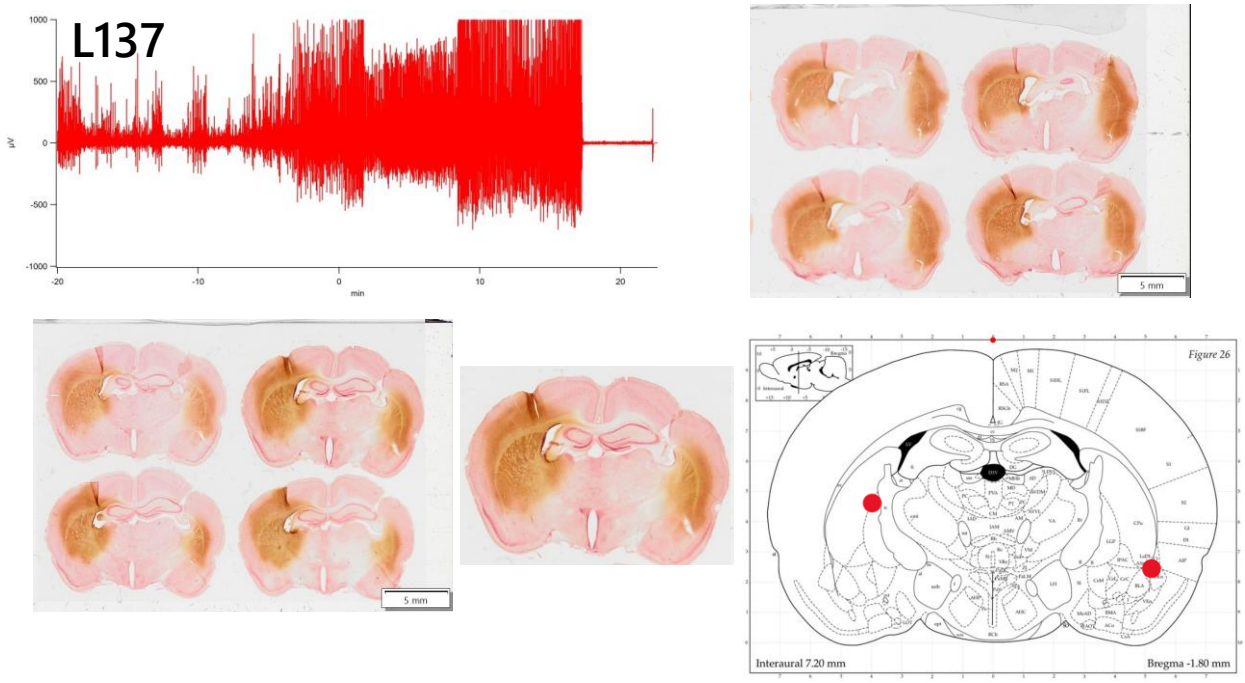
(d)


L135
Figure 30
Interaural 6.44 mm
Bregma -2.56 mm
5 mm

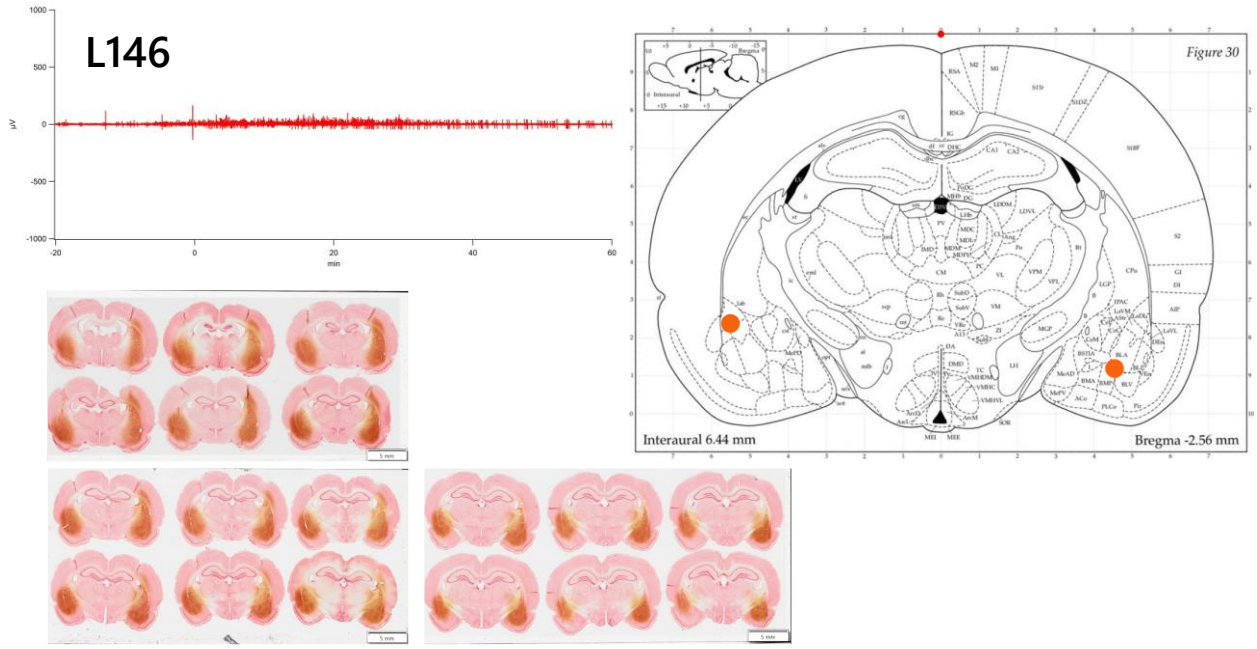
(e)


L136
1000
500
0
-500
-1000
μV
-20
0
20
40
min
5 mm
Figure 27
Interaural 7.12 mm
Bregma -1.88 mm

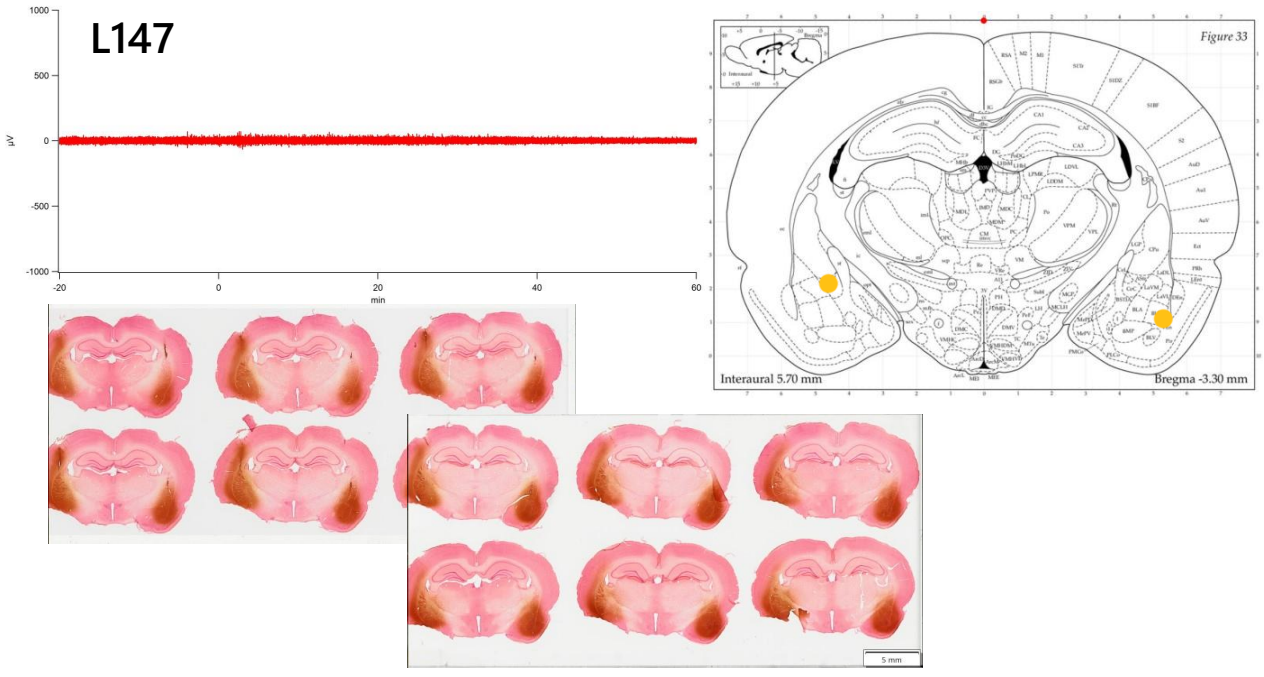
(f)


L137
1000
500
0
-500
-1000
μV
-20
-10
0
10
20
min
5 mm
5 mm
Figure 26
Interaural 7.20 mm
Bregma -1.80 mm

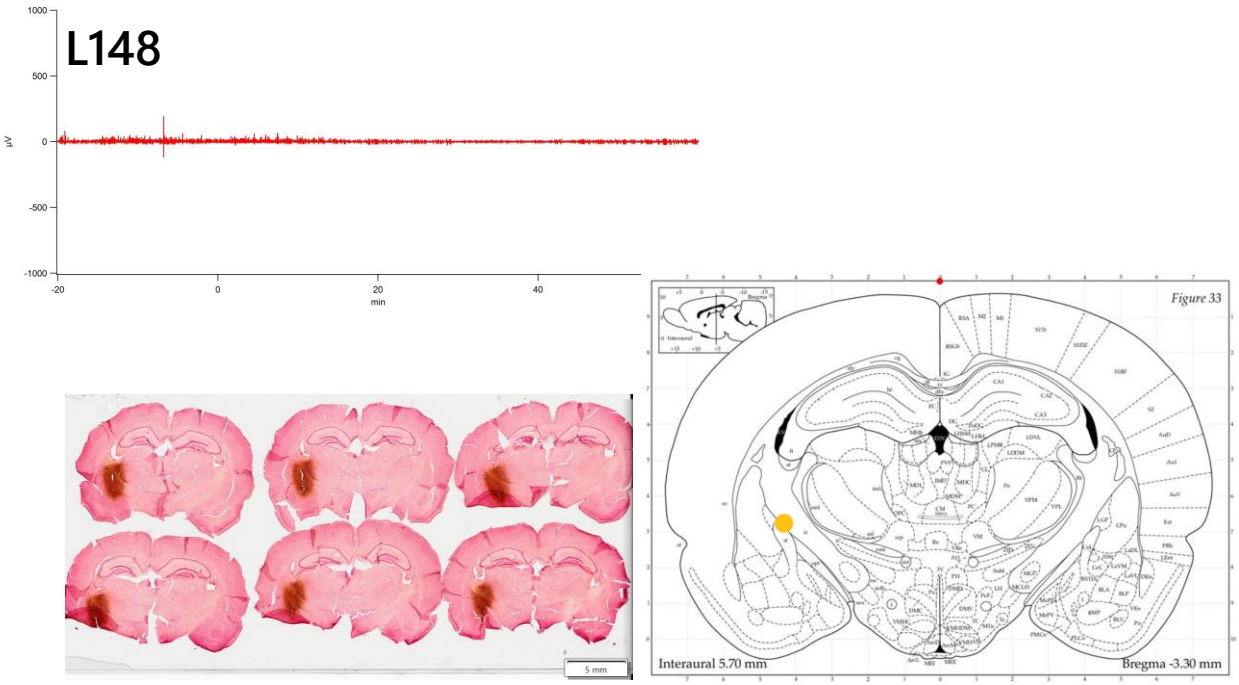
(g)


L146
Figure 30
Interaural 6.44 mm
Bregma -2.56 mm

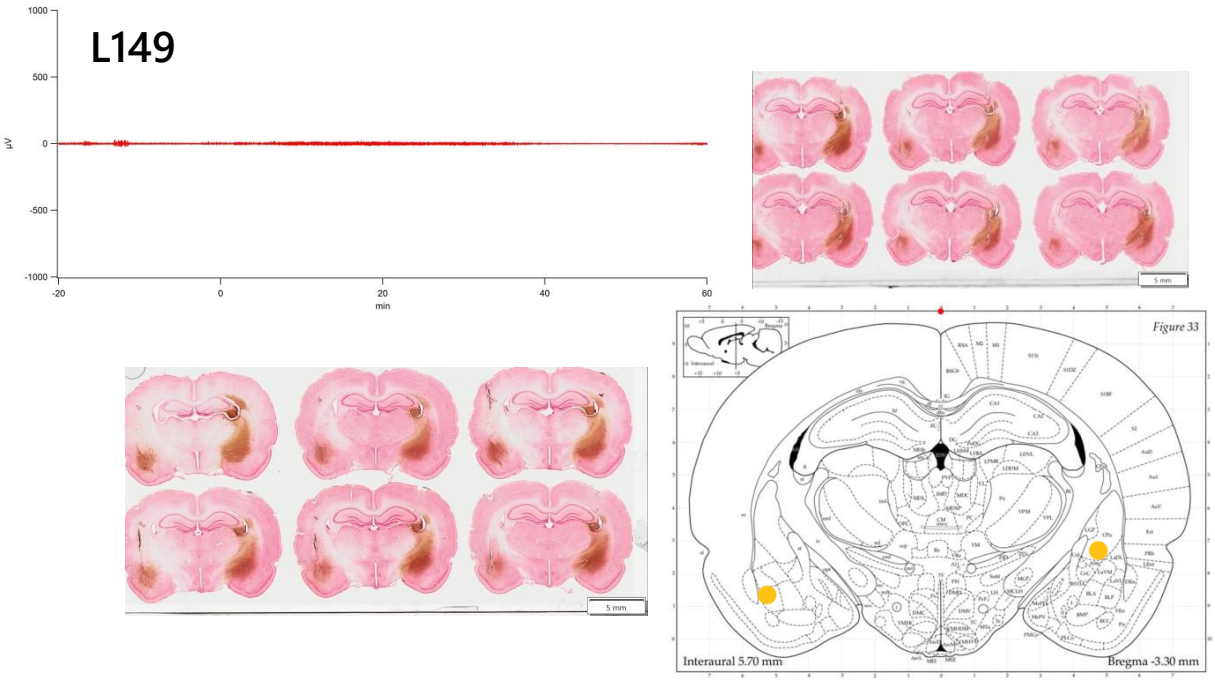
(h)


L147
Figure 33
Interaural 5.70 mm
Bregma -3.30 mm

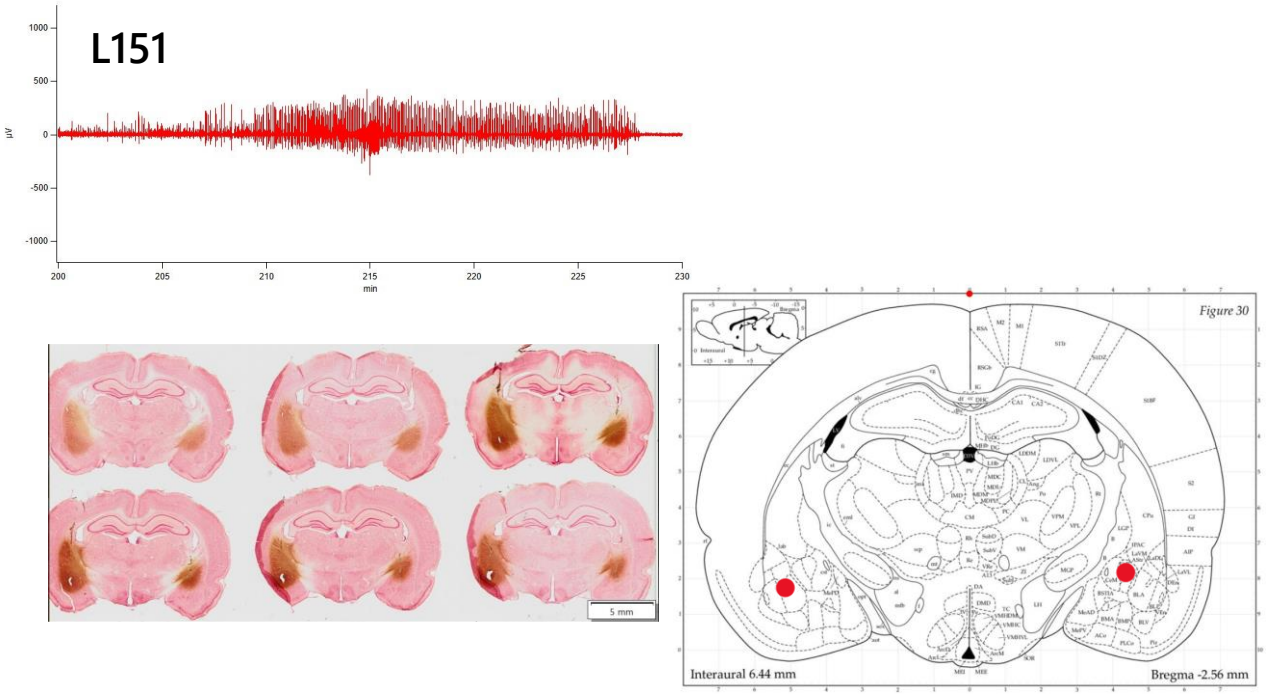
(i)


L148
1000
500
0
-500
-1000
µV
-20
0
20
40
min
5 mm
Figure 33
Interaural 5.70 mm
Bregma -3.30 mm

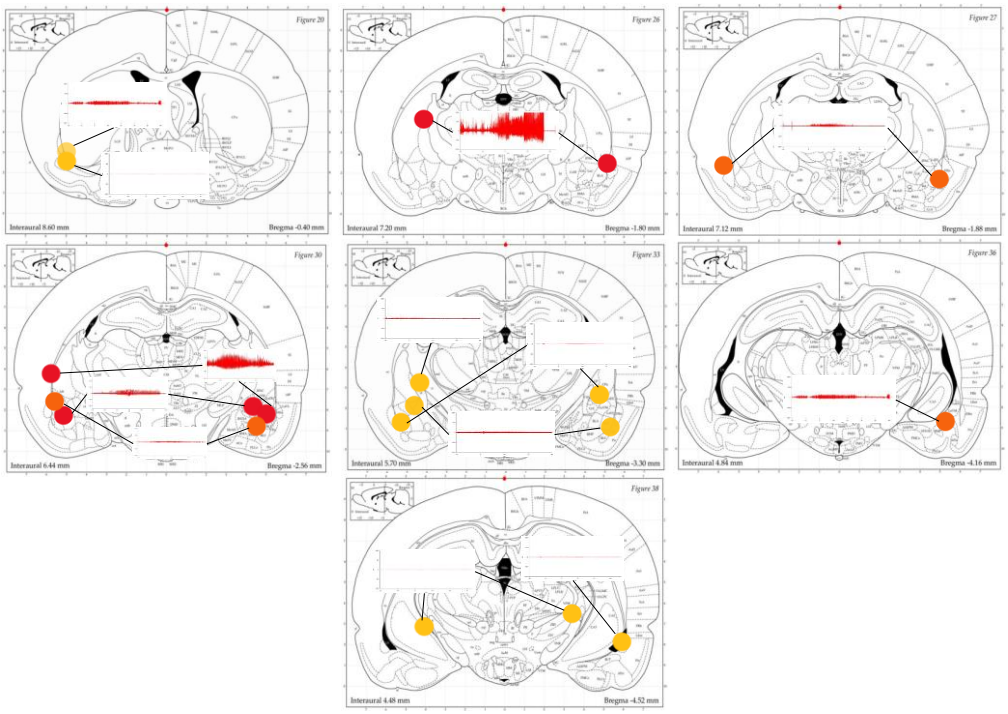
(j)


L149
1000
500
0
-500
-1000
µV
-20
0
20
40
60
min
5 mm
5 mm
Figure 33
Interaural 5.70 mm
Bregma -3.30 mm

(k)



Appendices Figure 5: Summary of injection sites and BATSNA



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