Hypothalamic and zona incerta neurons in sheep, initially only responding to the sight of food, also respond to the sight of water after intracerebroventricular injection of hypertonic saline or angiotensin II

Suriyaphun S. Mungarndee*, Basil A. Baldwin, Chittin Chindadoungratana, Naiphinich Kotchabhakdi

Neuro- Behavioural Biology Center, Institute of Science and Technology for Research and Development, Mahidol University at Salaya, Nakorn Pathom 73170, Thailand

Accepted 5 October 2001

Abstract

Extracellular single-unit recordings were made from neurons in the lateral hypothalamus (LH) or zona incerta (ZI) of conscious sheep. A small population of neurons (12/83) were found which responded with increased firing rate when the animal looked at food but did not respond when the sheep looked at water. The effects of rapidly inducing intense thirst by the intracerebroventricular (i.c.v.) injection of hypertonic (0.85 M) saline or 200 ng of angiotensin II, or a mixture of the two dipsogenic stimuli, on the response of neurons initially responding only to the sight of food were investigated. Following i.c.v. injection of the dipsogenic stimuli the neurons began to respond strongly to the sight of water. The results demonstrated that changing the animal’s motivational state alters the response of some neurons in the LH and ZI and suggests that the neuronal response is influenced by the animal’s dominant need at the time of testing. © 2002 Elsevier Science B.V. All rights reserved.

Theme: Neural bases of behaviors

Topic: Ingestive behaviors

Keywords: Lateral hypothalamus; Zona incerta; Extracellular recording; Hypertonic saline; Angiotensin II; Intracerebroventricular; Sheep

1. Introduction

The hypothalamic region is known to play a vital role in the regulation of ingestive behavior [9,19] and neurons have been found in the lateral hypothalamus (LH) and substantia innominata (SI) of monkeys which change their

Abbreviations: AD, anterior dorsal nucleus; AHD, dorsal hypothalamus; AHL, lateral hypothalamus; AM, anterior medial nucleus; AMB, basal amygdala; AmC, central amygdala; AmCo, cortical amygdala; AmL, lateral amygdala; AmM, medial amygdala; AV, anterior ventral nucleus; CC, corpus callosum; Cd, caudate nucleus; CF, field of forel; C. Int, internal capsule; Cl, claustrum; C Mm, medial mammillary body; DFS, forel of supramammillary decussation; FMT, mamillothalamic tract; fsc, sub-callosal fasciculus; Fx, fornix; GC, substantia grisea centralis; GL, lateral geniculate nucleus; GLv, lateroventral geniculate nucleus; HbL, lateral habenula nucleus; Hbm, medial habenula nucleus; Hip v, hippocampus; Imd, intermediodorsal nucleus; IV, interventral nucleus; LD, lateral dorsal nucleus; LME, external lamina medullaris; LP, lateral posterior nucleus; MD, medial nucleus (pars dorsalis); MV, medial nucleus (pars ventralis); NCL, central lateral nucleus; NCMD, central medial nucleus; NHDM, dorsomedial hypothalamus; NHVM, ventromedial hypothalamus; NI, interstitial nucleus; NIAM, interanteromedial nucleus; NPC, paracentral nucleus; NPI, parataenialis nucleus; Ped. Cer., cerebral peduncle; Pf, parafascicularis nucleus; PVT, paraventricular nucleus thalamus; Re, reuniens nucleus; Ret, reticularis nucleus; Rh, rhomboideus nucleus; Sn, substantia nigra; SLM, stria medullaris thalamus; ST, stria terminalis; TO, optic tract; VA, anteroventral thalamus; VL, ventral lateral thalamus; VPM, medial posteriorventral thalamus; VPL, lateral posteriorventral thalamus; ZI, zona incerta

*Corresponding author. Tel.: +66-2-441-9321; fax: +66-2-441-9743.
E-mail addresses: stssc@mahidol.ac.th or yru2sam@hotmail.com (S.S. Mungarndee).

1Co-corresponding author.
E-mail address: scnk@mahidol.ac.th (N. Kotchabhakdi).
firing rate when a hungry animal looks at food. These neurons have been extensively investigated by Rolls and co-workers [7,28–30,32,33]. Similarly, a small population of neurons in the LH and zona incerta (ZI) of sheep have been found to respond when a hungry animal looks at food [16,17,20] or a sodium depleted sheep looks at salt [16]. In both sheep and monkeys it has been shown that these neurons cease to respond when the animal is satiated and will extinguish their responses when the sight of food is not paired with ingestion [7,17,24,28,29]. In sheep, the magnitude of the neuronal response and number of trials to extinction are related to the animal’s preference for the food it is shown [17]. If the sheep or monkey is satiated on a particular food, the neurons will cease to respond to the sight of that food but will immediately respond to the sight of a novel food [17,29]. In both monkeys and sheep the neurons can be induced to respond differentially to non-food objects depending on whether or not they have been paired with food reward in a classical conditioning procedure [17,24].

There is evidence mentioned by Rolls [28,30] that when monkeys are both hungry and thirsty some hypothalamic neurons will respond to the sight of both food and water. In sheep, depleted of sodium by loss of parotid saliva, neurons have been located in the ZI that respond to the sight of both food and salt solutions when the animals were hungry and depleted of sodium [16]. Neurons responsive to the sight of water were also found in this study [16]. It was possible to reduce the response to salt solution but leave intact the response to food by allowing the animals to drink salt solution. These experiments suggested that, for some neurons in the ZI, changing the sheep’s motivational state produced changes in the responses of these neurons. The ZI is known from lesion studies and unit recording to be involved in food and fluid intake [8,10,12,14,38].

In the present experiments a novel approach has been used to study the effects of rapid changes in the animal’s motivational state on the response of neurons in the LH or ZI to the sight of food or water. The question considered is whether a neuron that responds to the sight of food but not of water would respond to the sight of water if the sheep was suddenly made thirsty? This would indicate whether these neurons can rapidly change their response and whether they are specific for food or react according to the animal’s needs. Intense thirst can be rapidly and reliably induced in animals by intracerebroventricular (i.c.v.) administration of hypertonic saline or the dipsogenic neuropeptide angiotensin II (ANG II) [5,6,11,12,26,40]. Hypertonic saline stimulates osmoreceptors while ANG II acts upon specific receptors [11,26,40]. In some experiments either ANG II or hypertonic saline was given i.c.v. and the effects of showing the sheep water, on the responses of neurons which initially only responded to the sight of food, were recorded. In other experiments a combined stimulus of 200 ng of ANG II dissolved in 0.85 M hypertonic saline was given i.c.v. and the effect on neuronal responses to the sight of water was observed.

2. Materials and methods

2.1. Experimental animals

Three adult female sheep (Ovis aries) were used. They were all of mixed native breed, hornless, aged 1–3 years and weighing 35–40 kg. They were housed in individual cages in an animal house with visual, auditory and olfactory contact with other sheep. They were fed on chopped hay plus 0.5 kg concentrates of pelleted sheep food. Water was available ad libitum and each animal had access to a mineral lick. All the sheep were tame and used to humans. On days when they were being tested the sheep were not fed pellets before the recording but water was available ad libitum. All experiments began between 0930 h and 1600 h. Animal care and surgical or experimental procedures were carried out following the guidelines of the National Research Council Guide for Care and Use of Laboratory Animals and under veterinary supervision.

2.2. Surgical procedures

After an adaptation period of 15 days the sheep were anesthetized and operated on using sterile precautions. Anesthesia was induced by intravenous injection of Thiopentone, a short-acting barbiturate, at a dose of 15–20 mg/kg body wt. of a 2.5% solution. The sheep was then intubated with a cuffed endotracheal tube and anesthesia maintained using halothane (1.5–2.0% in oxygen) via a closed circuit apparatus with absorption of carbon dioxide. Using lateral X-rays for guidance a hole 2 cm in diameter was trephined to be above the hypothalamic region. The dura was not punctured. A hole about 2 mm diameter was also drilled in the skull at a site 15 mm posterior to bregma and 10 mm lateral to the midline. This was used for insertion of a guide-tube cannula directed towards the lateral ventricle.

A stainless steel cylinder 2 cm diameter × 3 cm deep was attached to the skull with dental cement anchored by stainless steel screws. A 1.0-cm long guide-tube cannula made from an 18G thin wall Becton Dickinson procedure needle was inserted through the previously drilled hole and anchored in place with acrylic cement. The hub of the needle was plugged with a stainless steel obturator. The stainless steel cylinder and the ventricular cannula were protected by a stainless steel cap attached to the skull with dental acrylic. This cap had attachments on it by which the sheep’s head could be held rigid during recording sessions by inserting metal rods into through the attachments and holding them in clamps. A prophylactic dose of penicillin G (100 000 I.U.) were given i.m. after surgery and topical
antibiotic was sprayed around the implant. The cylinder was filled with aureomycin mastitis suspension to prevent infection and containing steroids to inhibit granulation of the dura. Two weeks were allowed for recovery.

2.3. Single unit recordings

Single unit recordings were made from the sheep while they were comfortably restrained in a canvas hammock. The animals’ heads were held rigidly by pressing metal rods through the protective steel cap and attaching the rods to a metal frame. This method of restraint differs from those previously used for horned sheep [2,15]. The nylon cap was removed from the stainless steel well and the aureomycin removed with sterile swabs. The exposed dura was then anesthetized by topical application of a 5% (w/v) solution of tetracaine hydrochloride and a Trent–Wells xy table and hydraulic microdrive were attached to the top of the well. Extracellular recordings of single unit activity were made using glass-coated tungsten microelectrodes (impedance 1–5 MΩ). The electrodes were introduced into the brain via a stainless steel guide tube (19G) which just penetrated the dura. The sheep showed no signs of pain or discomfort when the anesthetized dura was penetrated by the guide tube or when the electrode entered the brain. During recordings the animals would eat and drink normally.

Unit activity was amplified and filtered (500–5 kHz band pass) using Neurolog Digitimer modules and displayed on an oscilloscope and the activity was also heard on an audio monitor. Unit neuronal activity was displayed on-line by a MacLab™ computer recording system which also stored the data for subsequent detailed off-line analysis. The software (spike histogram extension, AD Instruments, Australia) was used to analyze data off-line and provided a dual height and width window discriminator which enabled single units to be selected from multiple unit recordings and assured that the same unit was being analyzed. Rate meter records could also be obtained and linked to stimulus events.

2.4. Experimental procedures

Three sheep were used in these experiments and each animal was recorded from on at least five occasions, but never more than twice in the same week. Recording sessions lasted up to 6 h. Only neurons which responded to the sight of food and not to water and were stable enough for prolonged recording were selected for further detailed study. The location of neurons successfully used for detailed study was noted by reading the xy co-ordinates in the Trent–Wells micropositioner table and the depth determined by direct measurement of how far the upper end of the electrode had descended and also by noting the readings on the hydraulic microdrive. As described in Section 2.6, this method enabled marker lesions to be made at these sites at the end of the series of experiments when the sheep was sacrificed. A detailed description of the apparatus and methods used for unit recording in conscious sheep has been published [15].

Food and water were each placed in separate colored plastic bowls fitted with long handles. The bowls were different colors and food or water was always placed in the same bowl so that the sheep could easily learn to distinguish the contents at a distance. The sheep had not been deprived of chopped hay but the sheep nuts placed in the bowl were a highly preferred food which the animal would usually eat if offered.

When a stable unit was found its response to various stimuli was investigated. The stimuli consisting of food, water or a nonsense object (e.g. a videocassette) was shown to the sheep. The objective was to find neurons which responded to the sight or visual approach of food but did not respond to water. The procedure for stimulus presentation is outlined below using food as an example. When it was established that the unit was stable and not responding to general arousing stimuli, a `clinical test' was begun with a 5-s period of baseline control being recorded. A green colored plastic bowl containing some sheep nuts was shown statically to the sheep at a distance of about 1 m for 5 s. This procedure established whether the neuron being recorded from responded to the static visual presentation of food. A response could readily be detected from the activity on the screen of the oscilloscope on the MacLab computer and was particularly prominent on the audio monitor. After 5 s of static presentation the green bowl containing sheep nuts was slowly moved towards the sheep’s mouth over a 5-s period and when the bowl reached the animal’s mouth it was allowed to eat if it wished to do so. Eating intensity was scored 0 to 4 (grade 4 means avidly eating while 0 means ignoring the food) and feeding was allowed for 10 s. During this period the neuronal activity was recorded. A record of the xy coordinate and depth of each neuron was made. When a neuron was found which responded to the sight or visual approach of food but not to water, it was a tested again after i.c.v. injections of dipsogens as described below. However, if a neuron did not respond to the sight or approach of food or water its position was noted but it was not selected for further study and the electrode was moved down to find another neuron to test. Some individual neurons were studied for more than 3 h if their responses were of interest. For a neuron to be classified as responding to the visual presentation of substances during either static or visual approach, it was required to display more than 50% change in firing rate compared with baseline control and a statistically significant increase from baseline as determined by a paired t test.

2.5. Intracerebroventricular injections

The permanent indwelling cannula described in the surgical section acted as a guide tube for access to the lateral cerebral ventricle. Single pulse injections, taking...
about 30 s to deliver 1 ml, were made by inserting a 22-G injection needle down the guide tube. Entry into the ventricle was confirmed by saline running in under gravity or by tapping CSF. Once the depth of the ventricle was established subsequent injections could be made with the 22-G needle set to that depth. At least 1 min was allowed for any i.c.v. injection to take effect before any tests were made. The substances injected were hypertonic saline (0.85 M NaCl), 200 ng ANG II in distilled water and 0.85 M NaCl containing 200 ng ANG II. They were used to provide strong dipsogenic stimuli. A volume of 1 ml was injected over a 30-s period and the sheep showed no signs of discomfort during the injections.

2.6. Localization of recording sites

At the end of the series of recording sessions the sheep were anesthetized with an i.v. injection of Nembutal and a stainless steel marker electrode lowered into the brain to selected coordinates where interesting neuronal activity had been found. The stainless steel electrode was insulated except for a small region at the tip and on anodal DC current (100 μA for 10 s) was passed through the electrode to make a small lesion and also deposit ferrous ions in the tissue (Lesion Maker, Grass® Model LM 4, Grass Medical Quincy, MA, USA). After the sites at which neuronal responses to dipsogenic stimuli had been lesioned, the animal was given a lethal dose of barbiturate and the brain was perfused via the carotid arteries with normal saline followed by 10% formol saline to which had been added 3% (w/v) of potassium ferrocyanide, 3% of potassium ferricyanide and 0.5% of trichloroacetic acid had been added. Histological sections were cut at 100 μm on a freezing microtome and stained with crystal violet and neutral red. Recording sites were localized using a combination of histological evidence from the deposition of ferrous ions from the stainless marker electrode, revealed by Prussian Blue spots and also from lateral X-rays which were taken at the end of some of the recording sessions. The anatomical location of recording sites was made with reference to the stereotaxic atlas of the Préalpes de Sud breed of sheep [27].

3. Results

A total of 83 neurons were recorded from and 12 were selected for detailed study as they were stable and initially responded only to food.

3.1. Effect of intracerebroventricular injection of 0.85 M sodium chloride

The i.c.v. injection of 0.85 M NaCl induced, with a short latency, copious drinking when water was offered to the sheep. When the sheep was given clinical tests to the static sight or visual approach of water the LH or ZI neurons, which had previously only responded to the sight of food, now exhibited a large and significant response to the sight or approach of water.

A representative response of a single neuron before and after treatment with dipsogenic stimuli is illustrated in Fig. 1. As can be seen in Fig. 1A, before the i.c.v. injection of 0.85 M NaCl the neuron only responded to the sight or visual approach of food and did not respond to the sight of or visual approach of water (Fig. 1B). Following i.c.v. injection of 0.85 M NaCl the neuron displayed a marked response to the sight or visual approach of water (Fig. 1C). The mean results from 12 neurons tested are shown in Fig. 2A–D. After the i.c.v. injection of 0.85 M saline the sheep was allowed to drink water and the dipsogenic effect of the hypertonic saline soon disappeared and, after 30 min, it was possible to test the same neuron with i.c.v. injection of ANG II.

3.2. Effect of intracerebroventricular injection of 200 ng angiotensin II

When the effects of injection of 0.85 M NaCl had dissipated (30 min after injection was allowed and the response to sight of water was not present), the i.c.v. injection of 200 ng ANG II dissolved in 1 ml of water resulted in drinking when water was offered and elicited a strong neuronal response to the sight or visual approach of water. Fig. 1D shows the response of a single neuron. The mean results from 12 neurons which were tested are shown in Fig. 2E.

3.3. Effects of intracerebroventricular injection of 0.85 M sodium chloride plus 200 ng angiotensin II

About 30 min after the injection of 200 ng ANG II the dipsogenic effects had dissipated and the same neuron was tested with the combined dipsogenic stimuli of 0.85 M NaCl plus 200 ng ANG II. This combined stimulus resulted in avid drinking when water was offered and also elicited a marked neuronal response to the sight or visual approach of water (Fig. 1E). The mean results from 12 neurons tested are shown in Fig. 2F. After 30 min the effect of the combined stimuli had dissipated and as can be seen in Fig. 1F and G the single unit did not respond to the sight or approach of water but did respond to the sight or approach of food. The mean results from 12 neurons tested with water or food are displayed in Fig. 2G and H.

3.4. Recording sites

Analysis of the results of mapping the active sites with the Prussian Blue reaction and small lesions revealed the neurons tested were all in the ZI and LH (Fig. 3) as these structures are displayed in the sheep brain atlas [27]. This finding replicates previous studies on neurons in sheep responsive to ingestive stimuli [16,17,20].
Fig. 1. Recordings from a single neuron in the zona incerta. The neuron was tested with visual presentation of food and water and initially only responded to the sight or visual approach of food (A) but not to water (B). After i.c.v. injection of dipsogens (C–E) the neuron responded strongly to the sight and visual approach of water. The histogram illustrated changes in firing rate during each 5-s period of the trace. C, control baseline; S, static sight of food or water; A, approach of food or water; E, eating food; D, drinking water; T/L, touch with lips.
4. Discussion

The experiments demonstrated clearly that individual neurons in the LH or ZI, which initially responded only to the sight or approach of food, but did not respond to the sight or approach of water, could be induced to respond to water by the i.c.v. injection of 0.85 M saline or 200 ng ANG II or a mixture of the two dipsogens (Fig. 1A–E). The mean responses from 12 neurons which were tested with the various stimuli are displayed in Fig. 2.

Hypertonic saline provides a strong dipsogenic stimulus by acting upon osmoreceptors which are present in the hypothalamus [1,12,31] and in addition the high concentration of sodium ions [Na+] may act on sodium receptors which have been postulated to exist in the hypothalamus [1]. Osmoreceptors are present in the preoptic area (POA), the organ vasculosum of the lamina terminalis (OLVT), the subfornical organ (SFO) and the supraoptic nucleus (SON) [6]. It is probable that osmoreceptors in these regions would be stimulated by i.c.v. injection of hypertonic saline. The POA is of particular importance in relation to the present study as it has been shown in anesthetized rats that injections of hypertonic saline in the third ventricle or the POA can alter the firing rates of neurons in the ZI [22]. It is known that direct injection of hypertonic saline into the POA elicits drinking [25] while lesions in this area inhibit drinking in response to hypertonic stimuli [4]. The POA has extensive connections with the LH via the medial forebrain bundle [37] and also projections to the ZI [36]. Experiments on sheep [21] indicate that both sodium receptor and osmoreceptor mechanisms may be involved in water intake in this species and there is evidence that, in sheep, the thirst induced by increasing brain [Na+] is mediated by brain angiotensin [3,41]. In sheep, hypertonic sucrose given i.c.v. elicits drinking when it is dissolved in artificial CSF but the response is less than that seen following injection of equimolar solutions of sodium chloride [21].

Drinking elicited by ANG II in the sheep has been extensively investigated [3,39–41]. ANG II injected i.c.v. elicits drinking by acting upon sensitive neurons which are present in several brain regions [see reviews 11,26]. Brain regions containing sensitive neurons include the SFO and OLVT which are circumventricular organs considered to be outside the blood–brain barrier. Sensitive regions in the brain include the median preoptic nucleus (MnPO), POA, paraventricular nucleus (PVN), SON, median eminence (ME) and nucleus tractus solitarius (NTS). ANG II elicits drinking by acting upon specific ANG II type I receptors [11] which are present in high density in the SFO, OLVT and MnPO nucleus and medial POA which are located near the third ventricle in the anterior hypothalamus. Obviously with i.c.v. injection it is not possible to locate the site of action of the injected ANG II, but it is likely that periventricular structures are strongly stimulated. It has been shown that injection of ANG II into the SFO altered the firing rate of neurons in the ZI and LH [23]. In the same series of experiments it was demonstrated that direct injection of hypertonic saline into the POA influenced the firing of neurons in the ZI and LH. Recently,
it has been shown that the i.c.v. injection of ANG II resulted in the expression of c-fos protein in several brain regions including the medial POA and the SFO [42]. Thus, i.c.v. injection of ANG II stimulates c-fos production in regions capable of influencing unit neuronal activity in the ZI and LH [13].

The results in Figs. 1 and 2 demonstrate that neurons which initially only responded to the sight or approach of food, but not to water, returned to this pattern of response when the thirst-inducing stimuli had dissipated. In a previous study in which sheep depleted of sodium were examined [16] it was shown that some neurons would respond to the sight of food as well as sodium bicarbonate solution. However, if over a 3-h period the sheep was allowed to drink some of the solution every 15 min, the response to the sodium bicarbonate gradually declined, while the response to food increased. The general conclusion from this study was that, while some neurons may exist which respond exclusively to the sight of food, even if the animal is thirsty or depleted of salt, the majority of neurons vary their response in accordance with the drive state which is dominant at the time of testing [16].

There is considerable experimental evidence supporting the concept that the neurons in the LH or substantia innominata, which respond to the sight of food when the monkey sees food, are involved in motivational processes which result in feeding behavior. Rolls [28] considers that these neurons are reward cells in that their activity is related to the reward value that food has for a hungry animal. The response of these neurons to the sight of food is modulated by satiety, which can be sensory specific in that they will not respond to the sight of a food that the animal has eaten to satiety, but will respond to a different food. The neurons can also 'learn' to respond to the sight of stimuli paired with food [17,24].

Some of the neurons activated by food when the monkey is hungry are also activated by electrical brain stimulation-reward in other parts of the brain [34]. It was
also demonstrated that self-stimulation could be elicited through the unit-recording electrode if it was near a site where hypothalamic neurons responded to food and that this self-stimulation rate was reduced when the monkey was satiated [34].

In experiments in which the latency to respond to the sight of food of neurons in the LH and substantia in-nominata of monkeys was recorded, they responded with a short latency of 150±200 ms and their activity preceded and predicted the motor response of the animal to obtain food [35]. This suggests that these reward neurons have a role in the motivational process involved in feeding. Some neurons in the LH project directly to the cerebral cortex [18]. Rolls [28] mentions experiments, using antidromic stimulation techniques, that demonstrate that some feeding-related neurons in the LH and SI project directly to the prefrontal cortex and supplementary motor cortex. These connections could provide a route for information from the reward neurons to be involved in the process which initiates feeding behavior.

In the present study the rapid change of drive state from hunger to thirst was accompanied by changes in the neuronal response to the sight of water and this suggests that the neurons respond to stimuli associated with whatever drive state is dominant at the time of testing. It would be of interest in future to determine whether eliciting food intake by i.c.v. injection of peptides such as NPY, which stimulate feeding, or peptides such as CCK and α-MSH, which reduce food intake, would change the response of these neurons to food stimuli. The combination of unit recording in conscious animals together with altering the external neuronal environment by i.c.v. injection of neuro-peptides promises to be a useful method for the study of motivational systems.

Acknowledgements

This work was supported by a Grant from the Neuro-science Graduate Program at Neuro-Behavioral Biology Center, Institute of Science and Technology for Research and Development, Faculty of Graduate Studies, Mahidol University at Salaya, Nakorn Pathom 73170, Thailand, and a Wellcome Trust Travel Grant to Dr. B.A. Baldwin. The authors appreciate the courtesy of Dr. J.A. Rawson, Department of Physiology, Monash University, Clayton, Victoria, Australia, for the ANG II. They also thank Mrs. K. Putpongpruk for animal care and Miss A. Narkthip for histological preparations.

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