Research report

Effects of threonine injections in the lateral hypothalamus on intake of amino acid imbalanced diets in rats

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Abstract

Previous work from this laboratory suggests that animals decrease their intake of an amino acid imbalanced diet (IMB), due in part to a drop in the concentration of the dietary limiting amino (DLAA) in the anterior piriform cortex (APC). Administration of the DLAA, but not of a non-limiting amino acid into the APC, blocks the anorectic response to IMB. To our knowledge, the effects of DLAA injections on intake of a diet devoid of the DLAA (DEV), have not been examined in areas outside the APC. We hypothesized that the LH is a potential chemosensory area for DLAA. Our objectives were: (1) to determine whether injections of the DLAA threonine into the lateral hypothalamus (LH) alter intake of a threonine-devoid diet (DEV); and (2) to examine the dose–response effects of threonine injections into the LH on intake of threonine-corrected diet (COR). Administration of threonine into the LH stimulated DEV intake during the first 6 h at the 0.25 and 1-nmol doses by approximately 26 and 24%, respectively. Threonine (0.25, 2.5 nmol) did not alter COR intake at any time during the first 12 h. Our results suggest that: (1) the LH, along with the APC, likely acts as a chemosensory brain area for indispensable amino acids; and (2) both the APC and LH are part of a circuit that is involved in the short term anorectic response to amino acid imbalanced diets. © 2000 Elsevier Science BV. All rights reserved.

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1. Introduction

Rats have the ability to detect a deficiency of dietary limiting amino acids (DLAA) within minutes after ingestion of a meal [16]. Evidence suggests this recognition occurs in the central nervous system, specifically in the anterior piriform cortex (APC). Ingestion of diet with imbalanced DLAA (IMB) results in anorexia and if prolonged, may result in increased susceptibility to epileptogenesis [14]. However, the APC may not be the only brain area involved in detection of amino acid levels. Experiments show that the lateral hypothalamus (LH) may also be involved in detection and processing of information about DLAA levels. We hypothesized that if the LH acts as a chemosensory area, injection of DLAA into this region should block or diminish IMB anorexia. If the LH is merely involved in processing, little effect should be observed.

An amino acid imbalanced (IMB) or devoid diet (DEV) results in a reduced concentration of the DLAA in the brain. In response to IMB, the DLAA concentration is reduced much more in the brain relative to plasma [16,20,37,38,48,49]. A drop in tissue levels in the brain may initiate mechanisms which signal deficiency while normal levels appear to signal balanced intake. Studies have shown that the DLAA transport into brain slices [32,47] is inhibited following introduction of the IMB diets. IMB consumption returns to control levels if the dietary limiting amino acid (DLAA) is infused into the carotid artery, whereas infusion of the same concentration of the DLAA into the jugular vein does not prevent anorexia [28,50].

The anterior piriform cortex (APC) serves an integral role in the recognition of amino acid deficiencies. Rats with bilateral lesions of the APC fail to reduce their food intake when fed IMB while lesions of many other brain areas involved in food intake do not produce this effect.
(reviewed in Refs. [13,30,33]). The APC also appears to have a direct role in the detection of amino acid deficiencies. Rats fed IMB have reduced threonine levels in the APC [15], anterior cingulate cortex, locus ceruleus and nucleus tractus solitarius (NTS), but not in the paraventricular nucleus (PVN), lateral hypothalamus (LH), or ventromedial hypothalamus (VMH) [17], areas implicated in the control of food intake [2,21,27]. This suggests that ingestion of a diet deficient in the DLAA will result in a low DLAA concentration in specific regions of the brain involved with DLAA chemoreception. Furthermore, injection of threonine, but not other essential AA (isoleucine), into the APC stimulates intake of a threonine IMB but not of a low protein diet [5]. Likewise, threonine injections into the APC stimulate intake of DEV, but not of a standard diet [34]. Expression of the immediate-early gene, c-fos, also provides evidence that neurons in the APC are involved in DLAA chemoreception. Increased Fos-like immunoreactivity is seen in the APC 1 h after introduction of an IMB. By 2 h this Fos-like immunoreactivity is significantly greater than for rats fed an amino acid corrected diet (COR) [53].

The medial and lateral hypothalamic nuclei, along with the APC, are also involved in mediating the anorexic response to IMB or DEV diets. Electrocolytic lesions of the DMH increase 3-h IMB intake [4]. VMH lesioned rats eat more of a mild ILE-IMB [29] and a histidine IMB diet [35] but not of a severe ILE IMB [29] or of a threonine IMB diet [40]. Levels of the DLAA are decreased in the DMH in response to IMB [15]. Onset of c-fos expression in the DMH, infralimbic cortex, claustrum, and dorsal endopiriform nucleus (deep piriform cortex [39]) was similar to the APC suggesting that these areas may also be involved in the early recognition of AA deficiency [53].

Evidences suggests that the LH, along with the APC, may have a role in mediating the anorexic response to IMB or DEV diets. Differential activation of neurons was seen in the dorsal LH in response to cues associated with lysine in rats deficient in lysine [17]. Neuronal activation is observed in the LH following injections of threonine into the APC of animals fed DEV [34], but little change is seen in animals fed a standard diet or those receiving saline injections. However, c-fos expression in the LH of rats fed IMB was not significantly different from rats eating a threonine basal or COR diets [53]. There is evidence that suggests a chemosensory function for neurons in the LH. Electrophoretic application of essential amino acids activates neurons in the medial forebrain bundle region of the LH [31]. Detection of brain oxygenation using magnetic resonance imaging revealed that lysine deficiency induces changes in the LH and VMH. Microinjection of lysine, but not other amino acids into the LH decreased bar pressing for a balanced diet following overnight exposure to a lysine deficient diet [51]. As noted, threonine concentrations in the LH do not appear to be reduced with an IMB diet [17]. Likely, this result may depend on the specific area of the LH sampled. Microinjection of balanced amino acid solutions directly into the perifornical hypothalamus depressed eating [18], suggesting that an area adjacent to the medial/dorsal LH is sensitive to the effects of amino acids on feeding behavior. Tract tracing studies from the area of the APC that we have found sensitive to threonine injections show projections to the LH, medial and reticular thalamic nuclei [1], suggesting that repletion of DLAA is recognized by a pathway that includes the APC and the LH.

Sufficient evidence supports the hypothesis that the LH may have a role in the detection of DLAA. If the LH acts as a chemosensory area for DLAA, then injections of threonine into the lateral hypothalamus should increase intake of a threonine devoid diet, while injections into the LH should have no effect on COR intake.

2. Methods

2.1. Subjects

Adult, male Sprague Dawley rats from Simonsen Laboratories, Gilroy, CA, USA weighing between 196 and 244 g were used. Animal care was according to the National Institutes of Health guidelines. Animal protocols were approved by the University of California, Davis Animal Use and Care Committee. The animals were housed individually in hanging wire-mesh cages in a temperature-controlled room (22±2°C) under a 12/12 h light–dark cycle (lights off 1200 h) with deionized water and food freely available.

2.2. Implantation of guide cannulas into the LH

Rats were surgically implanted with hypothalamic cannuulas according to methods previously described [42]. Each rat was anesthetized as previously described [8] with a ketamine cocktail (1 ml/kg body weight, i.p., containing 5 ml ketamine hydrochloride (100 mg/ml, Fort Dodge, IA, USA), 1.25 ml xylazine (20 mg/ml, Mobay, Shawnee, KA, USA), 0.75 ml acepromazine maleate (10 mg/ml, Avco, Fort Dodge, IA, USA). The animals were placed in a stereotoxic apparatus with the incisor bar positioned 3.3 mm below the ear bar. Bilateral stainless steel guide cannulas (18±0.05 mm tolerance, 26 gauge, Small Parts, Miami Lakes, FL, USA) were stereotaxically directed to a position 1 mm dorsal to the intended area, ventral LH [coordinates (mm): anteroposterior to interaural line (A/P)=A5.9, lateral (L)=1.9, dorsal (D)=8.5 (9.5)] and dorsal LH [A5.9, L=1.9, D=8.4 (9.4)] using the coordinates of Paxinos and Watson [36]. The cannulas were secured to the surface of the skull with dental acrylic (Hygenic, Akron, OH, USA) anchored by stainless steel screws. A 33-gauge obturator was inserted into the guide cannulas in order to maintain patency. An antibiotic
[penicillin (Aquacillin, Vedco, St. Joseph, MO, USA 300 000 units/ml); 60 000 units IM] was administered at the completion of surgery.

2.3. Diet composition

Experimental diets, which have been described previously [6] (Table 1), and water were available ad libitum. These diets included free L-AAs as the protein source, with cornstarch and sucrose (2:1) as the carbohydrate source, 5% corn oil as the fat source, 1% vitamin mix, and 5% salt mix. The threonine basal diet was a low protein diet, providing approximately 12% crude protein equivalent, with threonine as the growth-limiting AA, present at about 40% of its requirement for the growing rat [23]; all other essential amino acids were added to account for approximately 70% of their requirement for the growing rat. The threonine corrected diet contained a balanced pattern of amino acids at approximately 100% of the requirement. The DEV contained an excess of essential AAs other than threonine and was completely lacking in threonine. For the addition of the AAs, a proportionate amount of the carbohydrate fraction was removed. Animals were placed on the threonine basal diet for 7–16 days post surgery.

Following implantation of the hypothalamic cannulas, rats were adapted to handling and a 2-h fast prior to the start of the dark cycle. Food intake was determined by weighing food bowls to the nearest 0.1 g and correcting for spillage.

2.4. Injection procedure

Injections were made by lowering the 33-gauge injector with its tip extending 1 mm beyond the guide cannula into the brain site of interest. Each injection needle was connected via PE-20 tubing (O.D. (0.043” I.D. (0.015”)) Plastics One, Roanoke, VA, USA) to a 10-µl syringe fitted into a microinjection pump (CMA/100, Bioanalytical Systems, West lafayette, IN, USA). Bilateral injections (0.3 µl per side) of either saline or l-threonine (Fluka, Ronkonkoma, NY, USA) were delivered simultaneously at a constant rate of 0.06 µl/min within 2 h prior to the start of the dark cycle. The volume of injectate was verified by forward movement of an air bubble to a pre-calibrated distance (3 mm), corresponding to the appropriate volume. Injection needles were left in place for approximately 60 s after the injection was completed before being removed slowly. Following each injection, obturators were replaced and each animal was returned to its cage. Food bowls were returned to the respective cages at the completion of all injections.

2.4.1. Trial 1: Effects of threonine injections into the ventral LH in rats fed a DEV diet

Rats (n=24, 210–244 g) fed moistened DEV diet (20% H2O v:w) received bilateral injections of threonine (0, 0.25, 0.5, 1, 2, 4 nmol) in saline into the LH. Food intake was measured at 3, 6, 9, 12, and 24 h post-injection.

2.4.2. Trial 2: Effects of threonine injections into the dorsal LH in rats fed a DEV diet

Due to the ineffectiveness following THR injections into cannulas positioned within the ventral LH, in a separate group of animals (n=20, 200–217 g) threonine was administered in an identical manner via cannulas positioned 0.1 mm dorsal and intake of dry powdered DEV diet was measured. No differences in meal patterns have been observed between moistened and powdered IMB diets (unpublished data). Therefore, both diets were examined in this study. Although evaporative loss from the moistened diet was negligible, the powdered diet was used in the trial 2 and in the COR diet study due to the possibility of error associated with animals getting the diet on their fur over time.

2.5. Effects of threonine injections into the dorsal LH in rats fed a COR diet

Rats (n=19, 196–212 g) fed a COR diet received bilateral injections of threonine (0, 0.25, 2.5 nmol) in saline into the dorsal LH, the same site where THR stimulated intake in both moistened and powdered DEV. These doses covered the range of effective doses that stimulated intake of DEV (0.25, 2 nmol) and tested whether the stimulatory effect of THR on DEV was specific to a DEV situation. Food intake was measured as described above.

2.6. Histological verification of injection sites

Upon completion of the experiments, animals were sacrificed by ether inhalation and immediately decapitated. This was followed by subsequent removal of the guide cannula, cannula crown, and the brain, respectively. Brains
were stored in 10% formalin for 24 h followed by 10% sucrose for at least 48 h prior to freezing and sectioning. Coronal sections (40 μm) were taken through the most ventral portion of the cannula track. Sections were mounted on microscope slides and allowed to dry for 24 h prior to staining with Cresyl violet. A projection microscope was used to project the image of the brain section showing the most ventral portion of the injectate (Fig. 1), defined as the injection site, onto the corresponding section from the rat brain atlas of Paxinos and Watson [36]. In trial 1, data were grouped based on cannula placements either symmetrically within the ventral LH or animals having one injection site within the ventral LH and the other at the level of the fornix. Similarly, in trial 2, data were grouped based on symmetrical injection sites within the dorsal LH or had one injection site within the dorsal LH and the other at the level of the fornix.

2.7. Statistical analyses

Data are presented as means±S.E.M. Results from each study were analyzed separately by one-way ANOVA. Results were considered significant if P<0.05. Each animal served as its own control in a crossover design with the exception of the effects of threonine into the dorsal LH on intake of corrected diet. All analyses were conducted using version 7.0 of PC-SAS (SAS, Cary, NC, USA).

3. Results

3.1. Effects of threonine injections into the ventral LH on intake of DEV diet

Threonine injections into animals (n=8) that had cannulas placed within the ventral LH as described in Section 2 did not affect intake of the moistened DEV diet at any time measured (data not shown).

3.1.1. Trial 1. Moistened DEV diet (dorsal LH)

Threonine injections into cannulas placed stimulated moistened DEV intake (n=4) at 0.25 nmol through the first 9 (P=0.056, trend) and 12 h (P=0.046) by 35 and 30%, respectively (data not shown). A similar response was seen in an animal whose placement was slightly ventral to the fornix on one side and dorsal to the fornix, ventral to the internal capsule on the other, 121 and 39% stimulation at 6 h and 29 and 62% stimulation at 9 h at 0.25 and 1 nmol respectively. Food intake over 24 h following saline injections was 12.64±1.18 g, which was not significantly different from intake at this time following threonine injections at any dose.

3.1.2. Trial 2. Powdered DEV diet (dorsal LH)

In a separate group of animals, threonine administered into sites dorsal to the fornix (Fig. 2) tended to stimulate

Fig. 1. Representative photomicrograph taken of injection sites within the dorsal LH.
Fig. 2. Effects of threonine injections into the dorsal LH on intake of DEV diet. Significant increases are indicated by $P$ values for the 0.25 and 1 nmol doses for the 0–6 h period. Data are presented as mean±S.E.M and were analyzed by a one-factor ANOVA.

powdered DEV intake by 28 and 26% ($n=12–14$) during the first 3 h at 0.25-nmol ($P=0.059$) and 2-nmol doses ($P=0.071$). After 6 h, threonine had significantly increased DEV intake by approximately 26 and 24% at the 0.25 ($P=0.037$) and 1-nmol ($P=0.047$) doses, respectively. Fig. 3 shows the distribution of injection sites within this area of the LH. Food intake at 24 h following saline injections was 12.14±1.27 g, which was not significantly different from intake at this time following threonine injections at any dose.

3.2. Dorsal LH (corrected diet)

Administration of threonine at 0.25- and 2.5-nmol doses into sites in the LH dorsal to the fornix did not stimulate COR intake at any time during the first 12 h compared to the same animals that received saline ($n=5–7$, data not shown).

Fig. 3. Schematic representation from Paxinos and Watson showing the injection sites corresponding to the dorsal LH in animals exposed to the DEV diet. The symbol ■ is used to designate injection sites included in the data analysis.

4. Discussion

Accumulating evidence suggests that the LH (and/or zona incerta) may be part of a relay initiated in the APC that is involved in early detection of amino acid imbalance [1,4,17,18,33,53]. Neuronal activity increases in the LH following APC injection of threonine, at a dose that stimulates DEV intake [34], but little change was seen in animals fed a standard diet or those receiving saline injections. Tract tracing studies from the area of the APC that we have found sensitive to threonine injections show projections to the LH, medial and reticular thalamic nuclei [1]. Differential activation of neurons was seen in the dorsal LH in response to cues associated with lysine in rats deficient in lysine, with nondifferential activation being evident when balanced amino acid solutions were offered [17]. Microinjection of balanced amino acid solutions directly into the perifornical hypothalamus decrease food intake [18]. Taken together, these data suggest that repletion of DLAA is recognized by a pathway that includes the APC and the LH.

An indirect pathway from the APC to the LH directed through the DMH might also be important in mediating the recognition and/or learned aversive response to IMB. The DMH has direct connections with the LH [46]. Knife cuts extending laterally, ventrally or dorsally from the DMH produced similar stimulatory effects on IMB intakes did electrolytic lesions within the DMH, suggesting that fibers...
coursing in and/or around the DMH are important in mediating the anorectic response to amino acid deficiency [4]. Similar to the APC, changes in the levels of the DLAA [15] and c-FOS [53] in the DMH occur after eating IMB, and microinjections of the DLAA into the DMH were shown to increase DEV intake [7].

NPY and somatostatin have both been found to alter intake of IMB following injections into the APC. NPY mRNA in the APC increases in animals fed a threonine deficient diet and NPY decreases IMB intake following APC injection (1–1.5 nmol in 0.5 ml) up to 6 h postinjection [9]. These results contrast to the well known stimulatory effects of NPY following injections into the perifornical area and the LH on food intake [43], but are consistent with data showing a stimulatory effect on food intake following injections of antisense oligonucleotides to the NPY-Y1 into the amygdala, an area which also sends and receives neuronal projections to the APC [18]. Therefore, one potential mechanism that may act, in part, to initiate the stimulatory response on intake of DEV and neuronal activity within the LH following THR injections into the APC [34], may involve an increase in NPY action in the LH with a corresponding decrease in the amygdala. More experiments will need to be completed in order to clarify this issue.

The effects of somatostatin on intake of IMB or of food intake in general vary according to injection site, dose of administration, and feeding paradigm. APC injections of somatostatin-28 produced a biphasic response to IMB, with a stimulation of IMB at 1 pmol and an inhibition of IMB at 2 pmol [9]. Other studies found SOM-14 to inhibit food intake following ICV [(3) (10 000 pmol) [11,12], (3 pmol)] or LH administration (900 pmol [22]), the LH being the same site where increases in somatostatin were found in satiated relative to hungry rats [22]. Alternatively, previous studies report increases in food intake following ICV administration of either a somatostatin analogue (SMS-201-995 [10]) or SOM-14 [3] (10 000 pmol) [11,12], (1 pmol) with a corresponding decrease following ICV injection of somatostatin antiseraum [10]. Recent data shows that a somatostatin-like peptide found within the hypothalamus contains the CART fragment 82-103 (cocaine and amphetamine related transcript [25]), a peptide found to inhibit feeding when administered ICV and also found to reduce NPY-induced feeding [26]. NPY varicosities were also observed around cell bodies containing CART, suggesting possible interactions between the two systems in the control of food intake [26]. The variations between the behavioral studies utilizing somatostatin and/or somatostatin antiseraum do not provide a clear understanding of the role somatostatin may have within the LH in mediating the anorectic response to DEV, therefore further studies are necessary.

The glutamatergic system appears to have an integral role in the recognition of amino acid imbalance within the APC, but the role of glutamate in the LH in mediating the feeding response to IMB is not clear. The pyramidal cells, which contain the excitatory neurotransmitter glutamate, are the primary output neurons of the APC [19], which project to the LH [39]. Blockade of the NMDA and the AMPA receptors within the APC following bilateral injections of the NMDA and AMPA receptor antagonists AP5 and NBQX respectively result in increased IMB intake [52]. Recent studies by Stanley et al. provide evidence that the LH is the most sensitive site to glutamate [45] and that different feeding mechanisms via the kainate, AMPA, and NMDA receptors [41,44] are involved in the control of food intake within the LH. AP5 stimulates feeding at doses that do not alter the decrease in feeding following LH injections of kainate or AMPA. These injections provide evidence implicating endogenous glutamatergic activity within the LH at the NMDA receptor in the regulation of food intake [44]. Furthermore, c-FOS activity was increased in the piriform cortex in response to NMDA administration into the LH by reverse-dialysis, suggesting a common pathway including the APC and the LH perhaps mediates the glutamatergic responses to feeding behavior [24]. Additional studies will need to be done in order to determine how the glutamatergic system in the LH may be involved in the recognition of amino acid imbalance.

In summary, our results suggest that the LH/zona incerta may be part of a circuit involved in the short term anorectic response to the severe amino acid deficiency. Further studies examining the use of reverse-phase dialysis of threonine into the LH on both the intake of DEV and the release of neurotransmitters in this area will help to clarify the mechanism(s) underlying this response to amino acid deficiency.

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