Activin Antiserum Infused into the Lateral Hypothalamic Area Affects Operant Behavior of Rats Fed Lysine-Deficient Diet

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Abstract. **Rats were trained to maintain a high rate of bar pressing to receive 50-mg pellets of a complete diet when given a lysine-deficient (Lys-def) diet *ad libitum.* This bar-pressing behavior was significantly inhibited when rats were also allowed *ad libitum* access to 0.4 *M* Lys to drink. A brain activin system may modulate motivation to engage in bar-pressing behavior, since previous work has established that antagonism of activin by infusion of inhibin or follistatin, but not activin, into the lateral hypothalamic area (LHA) also inhibits bar-pressing behavior. The present study sought to clarify whether the effect of inhibin or follistatin might be mediated by antagonism of endogenous activin or by a separate direct effect of inhibin or follistatin. Thus, we infused an antiserum, which specifically inhibits activin A activity, into the LHA. Infusion of antiserum greatly inhibited bar-pressing behavior of rats fed a Lys-def diet and was additive with Lys consumption further to decrease bar pressing. *Ad libitum* Lys consumption was unchanged from control levels, indicating that it is likely that an endogenous activin system in the LHA mediates behavioral responsiveness when rats are fed a Lys-def diet but does not appear specifically to affect appetite for Lys.** [P.S.E.B.M. 1998, Vol 219]

Rats are able to detect when a single amino acid is deficient in a diet and to compensate specifically for the deficiency when given replacement drinking solutions of various amino acids (1, 2). Even a relatively small imbalance in amino acid ratios can remarkably affect dietary choice patterns (3, 4). Injection of an amino acid mixture into the dorsolateral perifornical hypothalamus inhibits subsequent feeding responses (5), and lateral hypothalamic neurons show changes in single unit discharge in response to chronic nutritional deprivation (6). We previously reported that an operant behavioral bar pressing task to receive complete diet pellets was maintained when rats were given a lysine-deficient (Lys-def) diet. When L-lysine HCl (Lys) was administered either as a voluntarily consumed drinking solution, by chronic intraperitoneal infusion, or by chronic infusion, into the lateral hypothalamic area (LHA), the bar-pressing behavior of rats given a Lys-def diet was greatly inhibited (7). The LHA has been shown to be highly sensitive to iontophoresis of essential amino acids (8) as well as to have altered neuronal activity in response to feeding a Lys-def diet (9) and a selective increase in single unit activity in response to iontophoresis of the deficient amino acid (10). Thus, the LHA appears to be a likely locus for regulation of amino acid homeostasis.

Activin or inhibin may also play a role in response to amino acid deficiency since rats fed either a Lys-def diet or a protein-free diet showed alterations in their serum levels of activin and inhibin (11). Although the LHA is generally considered to be inside the blood-brain barrier and hence should not be affected directly by serum activin or inhibin levels, there is strong evidence for an activin system intrin-

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sic to the LHA. The β_A subunit of activin and inhibin has been immuno-localized in a variety of brain regions including the LHA (11). Activin has been associated with the control of hypothalamic functions such as oxytocin or corticotropin releasing hormone secretion (12, 13, 14), and activin receptors are expressed in many brain regions including the hypothalamus and the LHA in particular (15, 16). We have previously examined whether activin, follistatin, or inhibin infusion into the LHA may play a role in alteration of behavioral responsiveness for compensation of deficiency of an amino acid (Lys) in the diet and found that inhibin or follistatin infusion led to a decrease in bar-pressing behavior to obtain complete diet pellets by rats fed a Lys-def diet (17). To clarify whether the effect of inhibin or follistatin might be mediated by antagonism of endogenous activin or by a separate direct effect of inhibin or follistatin *per se,* we infused into the LHA an antiserum that specifically inhibits activin A activity.

Materials and Methods

Animals. Male Sprague-Dawley rats (Charles River, Japan) were individually housed in hanging wire mesh cages at 20.4°–25.3°C. Animals weighed 185–205 g at the beginning of training. Tap water was available *ad libitum* except when amino acid solutions were made available, in which case distilled water was also made available *ad libitum.* Lights were on from 0700 for 12 hr daily. Behavioral testing always occurred between 1230 and 1700. Initially, complete diet food pellets (CRF-1, Charles River, Japan), which contained 1.32% Lys, were available, but daily food consumption was restricted to maintain the animals at 80% of initial body weight during operant training. Subsequently, animals were given a Lysine-deficient diet (Lys-def) *ad libitum* (see Ref. 9 for detailed composition of the diet), which contains 0.27% Lys by weight. *Ad libitum* consumption of the Lys-def diet was such that body weight increased to 275–325 g at the time of implantation of cannulae into the lateral hypothalamus. When diets or solutions are noted as being available *ad libitum,* this means they were continuously available in the home cage, but not during the daily 1-hr operant test sessions. Use of animals was in accordance with and approved by the host institution's Animal Care and Use Committee.

Behavioral Testing. For the behavioral task, four operant chambers were used, which were located in individual sound attenuated boxes with ventilation fans providing masking noise. A microcomputer (PC-9801, NEC, Japan) was used to program session conditions and record responses. Animals were trained to press a lever on a fixed-ratio 30 (FR30) schedule to receive a 50-mg complete diet pellet (CE-2, Muromachi Kikai, Japan) which contained 1.34% Lys. Test sessions lasted for 1 hr/day, and the number of bar presses was recorded over 5-min intervals. Data from 4–5 days of testing were averaged together. When diets or test conditions (i.e., availability of Lys or other amino acid solutions, or surgical implantation) were changed, animals were allowed 3–4 days to adapt to the new conditions before behavioral testing. This not only gave time to establish a steady state with regard to lysine deficiency and repletion but also to avoid nonspecific depression of lever-pressing response rates following surgery, since, in pilot experiments, the response rate was depressed in the first 2 days after surgical manipulation, but recovered thereafter.

Experiment 1. After initial training for 4 weeks to a stable baseline response rate when fed a restricted complete diet, the rats ($n = 9$) were switched to *ad libitum* Lys-def diet and also given *ad libitum* access in their home cages to either a 0.4-*M* Lys HCl or a 0.4-*M* L-threonine (Thr) drinking solution (all amino acids were obtained from Ajinomoto Co., Japan) in addition to *ad libitum* access to distilled water. Daily operant behavioral testing for bar pressing to receive complete diet pellets was continued. Half the animals were given either Lys or Thr the first week of testing. During the second week, Lys or Thr solutions were switched so that each animal served as its own control. Data from the 2 weeks were averaged together since there was no significant effect observed for the order of treatment. Following bilateral cannula implantation into the LHA, the animals were given *ad libitum* complete diet for a 1-week recovery period. Then, the rats were again given a Lys-def diet and were behaviorally tested for 1 week to establish a baseline control response rate. Either artificial cerebrospinal fluid (CSF) (18) mixed with dialyzed glycine eluent solution (0.87:1, vol/vol) as the vehicle or an IgY solution prepared from chickens immunized with recombinant activin A and affinity purified against bound activin A, eluted with 0.17 *M* glycine, pH 2.3, neutralized with NaOH to pH 7.0, and dialyzed against physiological saline (19) was continuously infused *via* osmotic minipumps (Alzet #2001, Alza Corp., Palo Alto, CA) that deliver 1 μl/hr of fluid. The concentration of antibody was sufficient to neutralize 75 pg activin/hr as measured by the Friend cell erythrocyte differentiation assay and was specific for activin A but did not block inhibin activity (19). After 1 week, minipumps were changed with fresh pumps such that each animal served as its own control by receiving the opposite solution to that which it had received the previous week.

Experiment 2. Another group of rats ($n = 10$) was trained, and cannulae were implanted bilaterally into the LHA using the same conditions as in Experiment 1, such that a high rate of bar pressing behavior was maintained while fed Lys-def diet. Infusions were also carried out as done previously with either control solution or anti-activin IgY solution continuously delivered by implanted minipumps (1 μl/hr). However, in this case half the animals in each infusion group also had a drinking solution of 0.4-*M* L-lysine HCl available *ad libitum,* whereas the other half of the animals in each group had 0.4 *M* L-threonine available in addition to *ad libitum* distilled water. Thus, there were four test conditions: Control infusion/Lys, Control infusion/Thr, Anti-activin/Lys, or Anti-activin/Thr. In a 4 × 4 latin

square design, after 1 week, minipumps were changed so that, in each of 4 successive weeks, groups of two to three animals were exposed to one of the four conditions such that each animal was behaviorally tested for bar-pressing behavior once for each test condition. As in the first experiment, results from these 4 weeks of testing were averaged together since there was no effect of treatment order observed.

Surgery. For intra-hypothalamic infusion, rats were first stereotaxically implanted with bilateral 23-gauge guide stainless steel cannulae under pentobarbital sodium (50 mg/kg, ip) anesthesia. Three stainless steel screws were affixed to the skull; a hole was drilled through the skull; and the guide cannula was lowered into place and held there with dental acrylic resin. The stereotaxic coordinates used were: lateral: ±2 mm; vertical: −8.0 mm from the skull surface; anterior-posterior: −2.7 mm from the Bregma, according to the atlas of Paxinos and Watson (20). The animals were allowed 1 week for recovery, then a stainless steel infusion needle (26-gauge) was inserted through the guide cannula to extend 1 mm beyond the tip of the guide cannula, fixed in place with more acrylic resin, and connected by PE-10 and PE-60 tubing to minipumps placed subcutaneously in the scapular region, under diethyl ether inhalation anesthesia.

Histology. To verify LHA cannula placement, animals were anesthetized with ether, and the brains were fixed with 3.7% formalin solution. Brains were then kept at 4°C in several changes of 15% sucrose/0.1 *M* phosphate buffer for several days. Then 40-μm thick sections were cut on a cryotome and stained with toluidine blue. All animals were found to have cannulae placed into the lateral hypothalamic area, generally between the fornix and optic tract.

Statistics. Statistics were performed using the StatView II software package (Abacus Concepts, Berkeley, CA) for ANOVA and the Scheffe' F-test for comparison between means. A $P < 0.05$ level was used as the threshold for significance. All data are expressed as means ± SEM.

Results

Experiment 1. As reported previously (7, 17), rats maintained on a Lys-def diet with *ad libitum* access to Lys drinking solution displayed greatly decreased bar-pressing behavior during the 1-hr test session; whereas, animals given *ad libitum* Lys-def diet with *ad libitum* access to a Thr drinking solution pressed at the same high rate as when given a Lys-def diet alone (not shown). After implantation with cannulae into the LHA, rats were infused continuously *via* implanted osmotic minipumps with either an antiactivin IgY solution or vehicle control. Infusion with control solution did not affect the bar-pressing rate significantly compared to the pre-infusion baseline condition, but infusion with the antiserum solution greatly inhibited ($F_{2,18}$ = 91.719, $P < 0.0001$) bar-pressing behavior (Fig. 1). There was no difference in the daily amount of *ad libitum* Lys-def diet consumed between the test conditions (baseline: 15.4 ± 0.9 g/d, control: 14.7 ± 0.9 g/d, antiserum: 16.3 ± 0.7 g/d; $F_{2,18}$ = 0.741, P = 0.4908), so the effect of the antiserum

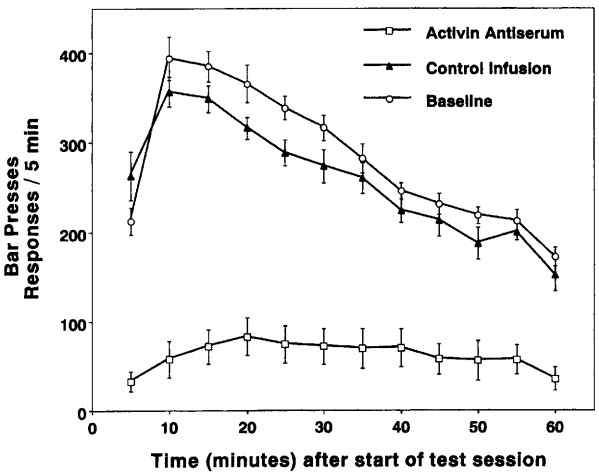

Figure 1. Bar-pressing responses of rats to receive complete diet averaged every 5 min for a 1-hr test session. Animals were bilaterally implanted with cannulae into the LHA and fed Lys-def diet *ad libitum* (baseline). Osmotic minipumps were then attached, and 1 μl/hr of either control vehicle or activin antiserum was infused continuously for 1 week. Baseline or control and antiserum infusion conditions were significantly different ($F_{2,18}$ = 91.719, $P < 0.0001$). Each point is mean ± SEM.

was probably not the result of a general change in appetite resulting in overfeeding or underfeeding of the home-cage Lys-def diet. Thus, the effect of antiserum infusion appears to be on the motivation to bar press for a complete diet.

Experiment 2. The results of this experiment were similar to those from Experiment 1 in that infusion with antiactivin antiserum solution strongly inhibited bar-pressing behavior. A similar result occurred in the group with voluntary access to the lysine drinking solution. The lysine consumption or antiserum infusion groups had a very similar inhibition of response rate (Fig. 2). However, the group that had combined access to lysine solution and antiserum infusion showed a further, statistically significant, inhibition of bar-pressing behavior to receive a complete diet (antiserum/Lys vs. Lys alone: $F_{1,28}$ = 34.461, $P <$ 0.0001; antiserum/Lys vs. antiserum alone: $F_{1,28}$ = 21.37, $P < 0.0001$). There was no significant difference in consumption of either amino acid drinking solutions or water within the Lys or Thr groups. However, a greater volume of the Thr solution was consumed compared to the lysine solution so there was a difference in daily distilled water consumed between the antiserum/Thr group and the antiserum/Lys or control/Lys groups, although total daily fluid consumption was not different ($F_{3,54}$ = 0.972, P = 0.4126) among the four groups. Lys-def diet food consumption was not significantly different among the groups (antiserum/Lys: 15.4 ± 0.4 g/d; control/Lys: 16.1 ± 0.6 g/d; antiserum/Thr: 14.6 ± 0.8 g/d; control/Thr: 15.9 ± 0.8 g/d; $F_{3,54}$ = 1.547, P = 0.2128).

Discussion

The present results extend our previous reports (7, 17) that infusion of Lys into the LHA alters operant behavioral

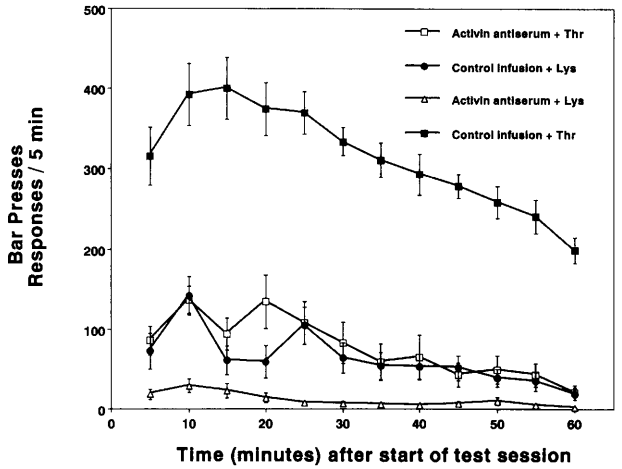

Figure 2. Bar-pressing responses of rats implanted with cannulae into the LHA and fed Lys-def diet *ad libitum*. Lysine or threonine solution was then made available *ad libitum*, and vehicle or activin antiserum was infused continuously into the LHA by minipump. Activin antiserum/Thr and Control infusion/Lys groups were not significantly different from each other but were significantly different from either Control infusion/Thr or Activin antiserum/Lys groups (Two-factor repeated measures ANOVA: antiserum/Lys vs. control/Lys: $F_{1,28} = 34.461$, $P < 0.0001$; antiserum/Lys vs. control/Thr: $F_{1,28} = 227.787$, $P < 0.0001$; antiserum/Lys vs. antiserum/Thr: $F_{1,28} = 21.37$, $P < 0.0001$; antiserum/Thr vs. control/Thr: $F_{1,28} = 94.58$, $P < 0.0001$; control/Lys vs. control/Thr: $F_{1,28} = 133.538$, $P < 0.0001$; antiserum/Thr vs. control/Lys: $F_{1,28} = 0.706$, $P = 0.4079$).

responsiveness of animals maintained on a Lys-def diet and indicate that a central activin system may modulate appetitive behavior in response to Lys deficiency. As was found previously (17 and present results, not shown) rats consuming a Lys-def diet receive approximately 50 mg/day of Lys from the diet and can receive up to double that amount from complete diet pellets at maximal bar-pressing rates. However, the animals still display a strong appetite for Lys since they will consume an additional 500–700 mg/day of Lys from an *ad libitum* Lys solution both when given a Lys-def diet alone or when earning supplemental complete diet pellets. Compared to a Lys-def diet, animals receiving *ad libitum* complete diet consume about 300 mg/day more Lys from the diet and will consume an additional 300–400 mg/day of Lys from an *ad libitum* Lys solution (5). Thus, total daily Lys intake from all available sources was similar at about 700–800 mg/day.

The present results, using a specific antiserum to activin, indicate that the behavioral effects previously observed for inhibin or follistatin infusion (17) are likely to be mediated by antagonism of activin function, rather than by some direct effect of inhibin or follistatin independently of activin. Thus, since infusion of inhibin, follistatin, or antiserum to activin into the LHA greatly decreased bar pressing for a complete diet by rats maintained on a Lys-def diet, we hypothesize that there is a hypothalamic activin system that can regulate behavioral responsiveness to dietary deficiency. However, since consumption of *ad libitum* Lys solution was not changed by antagonism of activin (Lys infusion into the LHA did reduce *ad libitum* Lys consumption), it seems that the behavioral effect of activin antagonism may not be directly to alter LHA-mediated appetite for Lys, but rather may act to modulate a more general homeostatic response mechanism to motivate behavior to alleviate dietary deficiency. This is indicated by the additivity of Lys consumption and activin antiserum infusion almost completely to suppress bar pressing for a complete diet below the response rates for either treatment alone. Thus, rats voluntarily consuming Lys solution in addition to a Lys-def diet will continue a low rate of bar-pressing behavior, indicating some motivational preference for consumption of the bar-pressing reward pellets. But inhibition of LHA activin is additive with Lys replacement almost completely to inhibit bar-pressing behavior.

While further experiments would be necessary to rule out an effect of activin antiserum to produce a state of illness or some general locomotor effects, the lack of any effect on basal food consumption argues against a general malaise. Although not quantitative, over the course of several weeks of direct observation for several hours daily, the animals voluntarily consuming lysine solution and the animals receiving activin antiserum or control infusions had no grossly observable differences in general behavioral and locomotor activity, only the usual pattern of exploratory activity and sleeping being observed. The fact that LHA infusion of the activin antagonists, inhibin, follistatin, and activin antiserum, all had similar effects on bar-pressing behavior, without altering basal food or fluid consumption, strongly suggests an effect on an endogenous activin system as modulating motivation to engage in bar-pressing behavior.

Regulation of behavioral motivation in response to a deficiency of dietary intake could be the result of the reported effect of activin to induce mRNAs for several peptides, such as cholecystokinin (CCK), neuropeptide Y, calcitonin gene-related peptide, and dynorphin (21), which are thought to control appetite for specific macronutrients such as protein, fat, or carbohydrates (22). In particular, the perifornical region of the lateral hypothalamus, implicated in control of hunger for protein (22), is a site of action for regulation of food intake by several peptides, including CCK (23, 24). That a complex behavioral repertoire could be adequately adjusted by activin/inhibin regulation is suggested by the report that neuronal depolarization can change further the expression pattern of peptides induced by activin administration (25). In preliminary experiments, we have not observed any change in bar pressing behavior of rats fed a Lys-def diet following LHA infusion of CCK, galanin, or neuropeptide Y. This lack of effect of infusion of other peptides argues against a general nonspecific effect of infusion of protein into the LHA as mediating the observed effect of inhibin or activin antiserum. Further work is necessary to establish dose/response relationships among inhibin, follistatin, and activin antiserum to determine a threshold level of effect and to examine for effects on behaviors maintained by mechanisms other than amino acid

deficiency such as in the case of animals given a complete diet but restricted to 80% of *ad libitum* amounts to produce a general caloric or nutritional deficit.

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