Effects of Repeated Cold Stress on Activity of Hypothalamic Neurons in Rats During Performance of Operant Licking Task

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INTRODUCTION

Repeated cold stress is a type of chronic cold stress in which environmental temperature changes rapidly and frequently several times within a day (Hata et al. 1984a). Animals exposed to chronic cold stress display various abnormalities including hyperphagia, decrease in weight gain, hyperalgesia, low blood pressure, decreased acetylcholine (ACh) response in the isolated duodenum, suppression of antibody formation, hypersensitivity in the immediate immunoreaction, and alteration in hypothalamo-pituitary-adrenal and sympathoadrenal systems (Akana and Dallman 1997; Bing et al. 1998; Fukuhara et al. 1996a,b; Hanson et al. 1996; Hata et al. 1984a,b, 1988a; Kawanishi et al. 1997; Kondoh et al. 1996; Kvetnansky et al. 1995; Leung and Horwitz 1976; Morrison 1981; Namimatsu et al. 1992; Okano et al. 1993; Snyder and Stricker 1985).

The hypothalamus, which is a center of motivational behaviors as well as autonomic, hormonal, and immune responses, is considered to be one of the most important brain areas in producing various symptoms under stressed conditions. In repeated cold stress, monoamine and ACh levels in the hypothalamus change more rapidly than in other brain areas (Hata et al. 1987a); interleukin-1β (IL-1β) gene expression is enhanced in the medial hypothalamic area (MHA) and suppressed in the lateral hypothalamic area (LHA) during repeated cold stress (Tagoh et al. 1995). Stress-induced changes in cytokine and neurotransmitter levels in the hypothalamus likely produce changes in neuronal activity in the hypothalamus. However, the effects of repeated cold stress on neuronal activity in the hypothalamus have not yet been examined in vivo.

When investigating the neuronal mechanism involved in motivational behaviors, we believe it is important to examine changes in neuronal activity recorded in awake, behavior performing animals. Therefore we have developed an experimental system for awake rats and several behavioral tasks including a cue-tone discrimination task (Nakamura and Ono 1986; Nishijo and Norgren 1990, 1991; Ono et al. 1985, 1986). The cue-tone discrimination task requires the rat to discriminate different cue-tone stimuli (CTSs) that indicate different sipid solutions and to lick a protruding spout. This method (the experimental system and task) has allowed us to link the activity of neurons in the hypothalamus and other brain regions to ongoing consummatory behaviors and to stimuli that predict the availability of gustatory rewards (Muramoto et al. 1993; Nakamura and Ono 1986; Nishijo et al. 1998; Ono et al. 1986; Oyoshi et al. 1996; Takenouchi et al. 1999; Uwano et al. 1995; Yonemori et al. 2000). By testing responses to a variety of different taste solutions, it is possible to determine whether neuronal responses to licking (and/or associated cue tones) are generalized across different taste stimuli, i.e., nondifferential responses to all the test stimuli, or are linked to a particular
class of tastant, i.e., differential (or specific) responses to one or some of the stimuli. Indeed, using this method, we have found both nondifferential neurons and differential neurons in the hypothalamic areas involved in the control of food intake (Tabuchi et al. 1991). Furthermore the method can also be used to identify effects of a particular pathological condition on neuronal activity (Tabuchi et al. 1991). A pathological condition could affect food intake in a generalized way such as hyperphagia or anorexia, in a specific way such as allotrophic-phy or selective taste aversion, or in a way that mixes the two. Such an effect of pathological condition on food intake may be reflected as changes in population of hypothalamic neurons. For example, deficiency of a requisite amino acid (lysine), leading to a specific preference for this amino acid (Torii 1987), increases the number of LHA neurons that respond specifically to lysine solution and/or its associated CTS in rats (Tabuchi et al. 1991).

Although some behavioral studies have reported that repeated cold stress produces abnormalities in food intake such as hyperphagia and a specific preference for histidine solution (Hata et al. 1988; Kita et al. 1979; Kondoh et al. 1996), little is known, at neuronal level, as to how repeated cold stress affects the CNS that is involved in the control of food intake (such as the LHA, MHA, and paraventricular nucleus). In the present study, therefore we addressed this issue as the principal purpose, using the method described in the preceding text. As an additional purpose, we also investigated the effect of repeated cold stress on plastic changes of hypothalamic neurons accompanied with learning process because repeated cold stress is known to produce impairment in learning (Yago et al. 1992; Yoneda et al. 1992). For these goals, rats were raised under a control or stress condition for 2 mo. Single neuronal activity was recorded from the LHA and MHA in these rats during the performance of the cue-tone discrimination task, and changes in neuronal activity were compared between these two conditions.

METHODS

Subjects

Thirty male albino Wistar rats (SLC, Hamamatsu, Japan), weighing 200–220 g at the beginning of the experiments, were used. The rats were divided into two groups: control (n = 15) and stressed (n = 15). Each rat was housed individually in a wire-meshed cage with free access to powdered food. The food, in which the main constituents were starch and wheat gluten, was made isonitrogenous and isocaloric to 20% purified whole-egg protein by adding a mixture of essential L-amino acids (Tabuchi et al. 1991; Torii et al. 1987). Tap water was also accessible ad libitum in the home cage except during the training and recording sessions. The rats were housed in a room where temperature (24 ± 1°C), relative humidity (60 ± 10%), and light (09:00–21:00) were automatically controlled except during the period of stress exposure.

Surgery

Twenty minutes after an injection of atropine (0.1 mg/kg ip), each rat was anesthetized with pentobarbital sodium (50 mg/kg ip) and mounted in a stereotaxic apparatus with its skull level between the bregma and lambda suture points. The cranium was exposed and five stainless steel screws (2-mm diam) were threaded into holes in the skull to serve as anchors for cranioplastic acrylic. Stainless steel wires were soldered onto two screws to serve as a ground. Two concentric bipolar electrodes (20–30 kΩ at 1,000 Hz) for intracranial self-stimulation (ICSS) reward were implanted in the lateral hypothalamic mediad forebrain bundle (A, −4.5 from bregma; L, ±1.4; V, 8.6) according to the atlas of Paxinos and Watson (1986). The concentric bipolar electrode consisted of an outer stainless steel tube (0.3-mm diam) and an inner wire (0.1-mm diam, enamel coated). The outer tube was insulated by polyurethane except for the tip area (about 0.1 mm long); the enamel insulation of the inner wire was removed at the tip (about 0.3 mm long). The cranioplastic acrylic was built up on the skull and molded around the conical ends of two sets of double stainless steel rods (fake ear bars) that had a single steel bar on one end and two bars on the other end. Once the cement had hardened, these bars were removed, leaving a negative impression of the double end on each side of the acrylic block. During subsequent surgery, training sessions and recording sessions, the double end of these fake ear bars was pressed into the indentations in the acrylic block while the single end was inserted into the normal ear bar slot in the stereotaxic apparatus and rigidly attached to it. Hence these artificial ear bars served the same purpose as regular ear bars but could be used in the unanesthetized animals because they did not involve painful insertion into the ear canal. A short length of 27-gauge stainless steel tubing was embedded in the cranioplastic acrylic near bregma to serve as a reference coordinate pin during chronic recording. After surgery, an antibiotic (gentamicine sulfate, Gentacin Injection, Schering-Plough, Osaka, Japan) was administered topically and systematically (8 mU im).

After recovery (7–10 days) from the cranioplastic surgery mentioned in the preceding text and the following task training (2 wk; see next section), the rats were reanesthetized (pentobarbital sodium, 40 mg/kg ip) and mounted in the stereotaxic device using the fake ear bars. A hole (2–3 mm diam) for chronic recording was drilled through the cranioplastic cap and the underlying skull over the intended recording site (A, −1.5 to −3.0 from bregma; L, 1.5 to 2.5; V, 7.5 to 9.5 for the LHA, and A, −2.3 to −3.3 from bregma; L, 0.3 to 1.0; V, 8.0 to 10.0 for the MHA). The exposed dura matter was covered with hydrocortisone ointment (Rinderon-VG ointment, Shinonog, Osaka, Japan). The hole was covered with sterile cotton and sealed with epoxy glue.

The preceding surgical procedure was performed under aseptic conditions. All rats were treated in strict compliance with the “Guiding Principles for Research Involving Animals and Human Beings” recommended by The American Physiological Society, and with the “Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences” recommended by The Physiological Society of Japan.

Training and task paradigm

Prior to the cranioplastic surgery, the rats were acclimated by handling and accustomed to being placed into a small restraining cage that was constructed with stainless steel rods. Following recovery from the cranioplastic surgery, the rats were placed on a 20- to 22-h water-deprivation regime. During task training, the rat was placed in the restraining cage with the head fixed rigidly and painlessly in the stereotaxic devise by the fake ear bars. While restrained, the rat had access to a spout from which the rat learned to take fluids. The rat was also trained to lick the spout to get ICSS rewards. Licking was signaled by a photoelectric sensor triggered when the tongue crossed an infrared beam. The parameters for ICSS (0.5–s train of 100 Hz, 0.3-ms capacitor-coupled negative square wave pulses, 50–150 μA) were similar to those in our previous reports (Nakamura and Oto 1986; Nishijo et al. 1998; Oyoshi et al. 1996; Uwano et al. 1995).

In the initial training (1–2 h daily for 3–5 days), the rat was allowed to freely lick a spout placed close to the mouth. Later the spout was retracted, and the rat was trained to lick at the spout when it was extended to within close range of the mouth for 2.0 s. The positive
reinforcement was a drop of glucose solution or an ICSS reward to facilitate the licking behavior.

In the following training (2–4 h daily for 9–11 days), a 2.0-s cue tone preceded extension of the spout. The cue tone (about 80 dB), with a different frequency corresponding to each solution, was delivered by a mid range speaker 1 m above the rats; subsequently, the associated solution flowed from the tip of the spout for 2.0 s during spout extension if the rat licked the spout. The cue tones and their associated solutions were: 1,000 Hz, 0.2 M L-lysine HCl; 2,350 Hz, 0.15 M monosodium L-glutamate (MSG); 3,500 Hz, 0.05 M L-arginine; 5,400 Hz, 0.5 M glycine; 8,750 Hz, 0.15 M NaCl (saline); and 1,600 Hz, distilled water. The concentrations of the sapid solutions were determined according to our previous study in awake rats (Tabuchi et al. 1991). Training with each solution was carried out separately in one block of 10 trials. We did not use ICSS as rewards in the training period of this cue-tone association learning to minimize the interaction of ICSS with learning process as well as to avoid any interaction of ICSS with repeated cold stress. The rats were trained for progressively longer periods. After an adaptation period of several days, most rats accepted the restraint for up to 4 h per day without struggling. Finally, the total number of trials per day reached 400–500 in 4 h. Throughout the training and recording periods, the rat was permitted to ingest 30 ml of fluid per session in the restraining cage. If a rat failed to take a total volume of 30-ml fluid in the training, tap water was given when the rat was returned to its home cage so that the final volume of total intake was 30 ml.

**Stress exposure**

After the surgery and task training, the rats in the stressed group were housed individually for 2 min in a repeated cold-stress apparatus (modified M-9000 incubator made by Advantec Toyo, Tokyo) with a built-in heater and cooler that could be controlled by an adjustable self-timer except when neuronal activity was recorded. The environmental temperature in this apparatus alternated four times between 24 and −3°C at 1 cycle/2 h from 10:00 to 18:00; it was kept at −3°C from 18:00 until 10:00 the following morning. It took about 30 min to change the temperature from −3 to 24°C. The time constant (the time needed to go two-thirds of the remaining distance to the end point) was 7.1 min. It took about 1 h to change the temperature from 24 to −3°C. The time constant was 23.3 min. Recording sessions (for detail, see Electrophysiological recording) in the stressed group began 7–8 days after the onset of stress exposure; at this time point, several abnormalities reportedly reached a steady state (Hata et al. 1984a, 1986, 1987a; Hori et al. 1993). Electrophysiological recording was performed in each rat for 2–4 h every other day, and after the recording, the rat was returned to its cage in the repeated cold-tress apparatus.

**Electrophysiological recording**

After being placed in the restraining cage, the head was fixed rigidly and painlessly by the flare ear bars. The hydrocortisone ointment in the skull hole was removed, and the rat’s dura mater was incised with a fine needle for electrode insertion under local anesthesia (a drop of 2% lidocaine jelly).

Extracellular single neuronal activity was recorded from the LHA and MHA through glass microelectrodes (1–2 μm diam at tip) filled with 4 M NaCl (2–4 MΩ at 1,000 Hz). The recording electrode was stereotaxically inserted stepwise with a pulse motor-driven manipulator (SM-21, Narishige, Tokyo) into various parts of the LHA and MHA. During recording, one of the two ICSS electrodes served as an indifferent electrode. Since the rat usually stayed quietly in the restraining cage while receiving available rewards, movement artifacts were negligible. Furthermore the differential recording effectively eliminated, if any, the movement artifacts produced during licking; this allowed us to record neuronal activity stably for enough time to analyze data. Extracellular neuronal activity was passed through a high-input impedance preamplifier made of a dual-channel field-effect transistor (2SK18A, Toshiba, Japan), passed through a main amplifier (MEG-6100, Nihon Kohden, Japan), monitored on an oscilloscope, and recorded on a magnetic tape by a data recorder (XR-9000, TEAC, Japan). Lick signals and computer-generated synchronizing trigger signals that represented the onset of the trials were also stored on the same magnetic tape for later off-line analysis. Neuronal activity was processed through a window discriminator. The analog signal and discriminator output were monitored continuously on the oscilloscope during the analysis.

The cue tones and their associated solutions were the same as those used in the training. Some neurons were additionally tested with a 330-Hz tone associated with 0.05-M L-histidine and a 440-Hz tone associated with 0.3-M glucose. Inter-trial intervals were 25–50 s, and 5 to 10 successive trials were performed for each solution. To avoid interference of the preceding different kind of solution, data in initial two trials were discarded when solution was changed. If a neuron responded to CTSs, extinction test (the CTS was presented but the rewarding solution was not given to the rat) and re-association test (the CTS was again coupled with the rewarding solution) were carried out in additional 20–30 and 10–15 trials, respectively.

Neuronal activity was monitored by a workstat (Masscomp 6300, Concurrent Nippon, Tokyo) on-line during recording. Peri-stimulus events (prestimulus, 3 s; poststimulus, 12 s) were stored to display rasters on each trial and accumulated to display peristimulus histograms by the workstat. Recording was performed from two rats a day around 18:00–24:00 (2–4 h/rat).

**Data analysis**

Both neuronal and licking data in each trial of the task were counted for three phases: a pretrial control phase (2 s), a CTS phase (2 s), and a licking phase (2 s). One-way ANOVA test was performed for discharge rates of these three phases to test main effect of phase. Excitatory or inhibitory neuronal responses were determined by a post hoc pairwise comparison (Tukey test) between the discharge rate in the pretrial control phase and that in the CTS or licking phase.

Neurons that responded during CTS or licking were labeled as cue-tone- or licking-related, respectively. Neurons that did not show a change in activity compared with the pretrial control phase were labeled as nonresponsive. Neurons that showed significantly different responses to different CTSs and/or to the licking of different solutions were labeled as differential. Neurons that showed similar responses to different CTSs and/or during licking of different solutions were labeled as nondifferential. Responsive (differential and nondifferential) neurons were further subclassified as neurons that responded to CTS only (CTS type), licking only (licking type), or both CTSs and licking (CTS and licking type). Percentages of categorized neurons were compared by Fisher’s exact probability test. Mean spontaneous firing rates were compared by Student’s t test.

All results are expressed as the means ± SE. Significance level employed for all tests was P < 0.05.

**Histology**

After the last recording session, each rat was reanesthetized with pentobarbital sodium (50 mg/kg ip). Several iron deposits were made stereotaxically around the recorded sites in the brain by passing a positive current (20 μA, 30 s) through a stainless steel electrode (0.2-mm diam, polyurethane insulated except for 0.1 mm at the tip). Rats were then given a further overdose of anesthetic and perfused transcardially with heparinized 0.9% saline followed by 10% buffered formalin containing 2% potassium ferrocyanide. The brain was removed, and cut into 50-μm frontal sections with a freezing microtome. Sections were stained with cresyl violet. All marking sites were verified microscopically. The location of each recording site was then calculated from the stereotaxic coordinates of the recording
electrode and the coordinates of the marking electrodes, and plotted on the atlas of Paxinos and Watson (1986).

RESULTS

Mortality, food intake, and body weight change during repeated cold stress

All the rats in the stressed group (n = 15) survived during the exposure to repeated cold stress. During the stress, food intake increased from the first day of stress exposure and reached a maximum on the second day. The food intake of the stressed group was about 50% higher than that of the control group throughout the rest of the stress period. In contrast to food intake, body weight decreased on the first day of stress exposure: It was about ∼200% of the change in the control group. However, it increased on the second day and was increased throughout the rest of the stress period although the rate of body weight gain in the stressed group was smaller than that in the control group. More specifically, the reduced body weight gain gradually recovered during the first week of the stress exposure and reached a steady state: during this steady state, the body weight gain in the stressed rats was one-third to half of that in the control rats.

LHA neurons

ANALYZED NEURONS. The activity of 421 neurons was recorded in the rat LHA (215 in the control rats; 206 in the stressed rats). Each neuron was tested with six standard liquids (distilled water and lysine, MSG, arginine, glycine, and NaCl solutions) and the associated CTSs. Eight neurons from the control rats and 55 neurons from the stressed rats were further tested with histidine solution and its associated CTS. Twenty-four neurons from the control rats and 132 neurons from the stressed rats were also tested with glucose solution and its associated CTS. As no neurons responded preferentially to histidine or glucose solution in the present study, all the neurons sampled were classified based on the responses to the six standard liquids and the associated CTSs. Table 1 summarizes responsiveness of the 421 LHA neurons.

RESPONSES OF NONDIFFERENTIAL NEURONS DURING CTS AND LICKING PHASES. Of 215 neurons recorded in the control rats and 206 neurons in the stressed rats, 117 (54.4%) and 139 (67.5%), respectively, were classified as nondifferential neurons. The percentage of nondifferential neurons in the stressed rats was significantly larger than that in the control rats (Fishér’s exact probability test, \( P < 0.01 \)) (Table 1). As described in METHODS, these nondifferential neurons were further divided into three subclasses (CTS, licking, and CTS and licking types). The percentage of CTS type was significantly larger in the stressed rats (18.0%, 37/206) than in the control rats (10.2%, 22/215; \( P < 0.05 \)). The percentage of licking type was significantly larger in the stressed rats (7.3%, 15/206) than in the control rats (3.0%, 28/215; \( P < 0.05 \)). The percentage of CTS and licking type was significantly larger in the stressed rats (42.2%, 87/206) than in the control rats (31.2%, 67/215; \( P < 0.05 \)).

Of the CTS-and-licking-type nondifferential neurons, 63 in the control rats and 75 in the stressed rats changed activity in the same response direction (excitatory or inhibitory) during the CTS and licking phases. The remaining 4 neurons in the control rats and 12 in the stressed rats changed activity in opposite response directions during these two phases. Figure 1 shows two examples of CTS-and-licking-type nondifferential neurons (recorded in the stressed rats) displaying activity changes in opposite response directions. Activity of the neuron shown in Fig. 1A decreased during the CTS phase and increased during the licking phase in a nondifferential manner [ANOVA, \( F(5, 39) = 2.05, P = 0.09 \), and \( F(5, 39) = 1.46, P = 0.22 \), respectively]. Activity of this neuron also decreased after the cessation of licking behavior. In contrast, activity of the neuron shown in Fig. 1B increased during the CTS phase and decreased during the licking phase in a nondifferential manner [\( F(4, 21) = 0.90, P = 0.48 \), and \( F(4, 21) = 2.42, P = 0.08 \), respectively]. The percentage of CTS-and-licking-type nondifferential neurons with activity changes in opposite response directions was significantly larger in the stressed rats (5.8%, 12/206) than in the control rats (1.9%, 4/215; \( P < 0.05 \)).

RESPONSES OF DIFFERENTIAL NEURONS DURING CTS AND LICKING PHASES. Eighteen (8.4%, 18/215) neurons in the control rats and 11 (5.3%, 11/206) in the stressed rats responded differentially during one or a few CTS and/or licking of one or a few sapid solutions (differential neurons; Table 1). These differential neurons were further classified into three subcategories (CTS, licking, and CTS and licking types) as were the nondifferential neurons. The proportion of differential neurons did not differ significantly between the two groups.

Figure 2 shows two examples of differential neurons with responses to MSG in the control (A) and stressed (B) rats. The neuron shown in Fig. 2A was a CTS and licking type. It showed increased activity during the CTS associated with MSG solution and licking of MSG solution (Fig. 2Aa; Tukey test after ANOVA, \( P < 0.01 \)) but did not respond during any phase of lysine or water trials (Fig. 2Aa, c and e). Neuronal activation during licking of MSG solution was also observed when CTS was absent, as shown in Fig. 2Ab (\( P < 0.01 \)). This suggests that the response during licking was not simply due to a prolonged

### Table 1. Classification of LHA neurons in control and stressed rats

<table>
<thead>
<tr>
<th>Type of Neurons</th>
<th>Control</th>
<th>Stressed</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>215 (100)</td>
<td>206 (100)</td>
<td></td>
</tr>
<tr>
<td>Responsive</td>
<td>135 (62.8)</td>
<td>150 (72.8)</td>
<td>( P &lt; 0.01 )</td>
</tr>
<tr>
<td>Nondifferential</td>
<td>117 (54.4)</td>
<td>139 (67.5)</td>
<td>( P &lt; 0.01 )</td>
</tr>
<tr>
<td>CTS</td>
<td>22 (10.2)</td>
<td>37 (18.0)</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td>Licking</td>
<td>28 (13.0)</td>
<td>15 (7.3)</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td>CTS and licking</td>
<td>67 (31.2)</td>
<td>87 (42.2)</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td>Differential</td>
<td>18 (8.4)</td>
<td>11 (5.3)</td>
<td>NS</td>
</tr>
<tr>
<td>CTS</td>
<td>6 (2.8)</td>
<td>3 (1.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Licking</td>
<td>10 (4.7)</td>
<td>6 (2.9)</td>
<td>NS</td>
</tr>
<tr>
<td>CTS and licking</td>
<td>2 (0.9)</td>
<td>2 (1.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Nonresponsive</td>
<td>80 (37.2)</td>
<td>56 (27.2)</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td>Cue-tone related</td>
<td>99 (46.0)</td>
<td>133 (64.6)</td>
<td>( P &lt; 0.01 )</td>
</tr>
<tr>
<td>Increased activity</td>
<td>50 (23.3)</td>
<td>53 (25.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Decreased activity</td>
<td>49 (22.8)</td>
<td>80 (38.8)</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td>Licking related</td>
<td>111 (51.6)</td>
<td>113 (54.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Increased activity</td>
<td>53 (24.7)</td>
<td>51 (24.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Decreased activity</td>
<td>58 (27.0)</td>
<td>62 (30.1)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Numbers of each type of neurons were presented. The percentages (in parentheses) of each type of neurons were calculated in 215 neurons in control and 206 neurons during stress and were compared by Fisher’s exact probability test. CTS, cue tone stimuli; NS, not significant; LHA, lateral hypothalamic area.
response or afterdischarge to CTS but was rather due to the licking of the MSG solution itself. Although MSG is a sodium salt, the neuronal activity was suppressed both during the CTS associated with saline and licking of saline (Fig. 2Ad; $P_s < 0.01$). This suggests that the neuronal activation by ingestion of MSG solution was not due to the presence of sodium but rather glutamate or the complex taste of sodium plus glutamate.

The neuron in Fig. 2B recorded from a stressed rat was the licking type. It showed increased activity during the ingestion of MSG solution (Fig. 2Ba; $P < 0.01$) but not during the ingestion of other sapid solutions (Fig. 2B, b–f).

CUE-TONE-RELATED AND LICKING-RELATED NEURONS. Ninety-nine neurons (46.0%, 99/215) in the control rats and 133 neurons (64.6%, 133/206) in the stressed rats responded to one or more CTSs (cue-tone-related neurons; Table 1). The percentage of the cue-tone-related neurons was significantly larger in the stressed rats than in the control rats ($P < 0.01$). Of these cue-tone-related neurons, 50 (23.3%, 50/215) in the control rats and 53 (25.7%, 53/206) in the stressed rats showed an increase in activity during CTSs, whereas the remaining 49 (22.8%, 49/215) and 80 (38.8%, 80/206), respectively, showed a decrease in activity during the CTS phase. The percentage of the cue-tone-related neurons with decreased activity was significantly larger in the stressed rats than in the control rats ($P < 0.05$). In contrast, the percentage of the cue-tone-related neurons with increased activity did not differ statistically between the two groups. In the control rats, the percentage of the cue-tone-related neurons with increased activity (50.5%, 50/99) was similar to that of the neurons with decreased activity (49.5%, 49/99). In the stressed rats, the percentage of the cue-tone-related neurons with decreased activity (60.2%, 80/133) was significantly larger than that of the neurons with increased activity (39.9%, 53/133; $P < 0.05$). One hundred and eleven neurons (51.6%, 111/215) in the control rats and 113 neurons (54.9%, 113/206) in the stressed rats responded during licking of one or more sapid solutions (licking-related neurons). The percentage of the licking-related neurons in the stressed rats was similar to that in the control rats. Of the licking-related neurons, 53 (24.7%, 53/215) in the control rats and 51 (24.8%, 51/206) in the stressed rats showed increased activity during licking, whereas the remaining 58 (27.0%, 58/215) in the control rats and 62 (30.1%, 62/206) in the stressed rats showed decreased activity during licking. In the control rats, the percentage of the licking-related neurons with increased activity (47.7%, 53/111) was similar to that of the neurons with decreased activity (52.3%, 58/111). In the stressed rats, the percentage of the licking-related neurons with increased activity (45.1%, 51/113) was also similar to that of the neurons with decreased activity (54.9%, 62/113). Thus the results indicated that the increase in the number of responsive
neurons in the stressed rats was due to a selective increase in the number of neurons that responded to CTSs with decreased activity.

To analyze the effect of stress stage on neuronal activity, we compared the relative number of cue-tone-related neurons and licking-related neurons during earlier recording sessions (days 8–29 in the stress exposure) with these values during later sessions (days 30–60). The percentage of cue-tone-related neurons recorded in the stressed rats during the earlier sessions (62.6%, 67/107) was similar to that during the later sessions (66.7%, 66/99). The percentage of licking-related neurons during the earlier sessions (49.5%, 53/107) did not differ significantly from that during the later sessions (60.6%, 60/99; P > 0.05).

SPONTANEOUS FIRING RATE. Spontaneous firing rates of LHA neurons in the two groups are shown as histograms in Fig. 3. The spontaneous firing rates ranged from 0.7 to 62.9 spikes/s (n = 215) in the control rats and from 2.8 to 53.3 spikes/s (n = 206) in the stressed rats. In the stressed rats, distribution of spontaneous firing rates was clearly shifted to the right relative to the control rats. The peak of the histogram was 4–6 spikes/s in the control rats, and 10–12 spikes/s in the stressed rats. The mean spontaneous firing rate in the stressed rats (17.0 ± 0.71) was 34% higher than that in the control rats (12.6 ± 0.66; Student’s t test, P < 0.01). In the stressed rats, the mean spontaneous firing rate in the later recording sessions (15.9 ± 1.02, n = 99) did not differ significantly from that in the earlier sessions (17.9 ± 1.02, n = 107).

To test whether the stress condition differentially affected the spontaneous activity in different neuron types, we calculated mean spontaneous firing rates of each neuron type in each group and compared them among groups (Table 2). The mean spontaneous firing rates of both nondifferential and nonresponsive neurons were significantly higher in the stressed rats (17.5 ± 0.85 and 16.1 ± 1.54, respectively) than in the control rats (13.1 ± 0.92 and 11.2 ± 1.04, respectively; Ps < 0.01). In contrast, the mean spontaneous firing rates of the differential neurons did not differ significantly between the two groups. The mean spontaneous firing rates of the CTS-type and CTS-and-licking-type nondifferential neurons were significantly higher in the stressed rats (19.9 ± 1.84) than in the control rats (19.9 ± 1.84). The mean spontaneous firing rate of the CTS-type nondifferential neurons recorded in the stressed rats (19.9 ± 1.84) was the highest of all the neuron types. The spontaneous firing rate of licking type nondifferential neurons did not differ significantly between the two groups, although it was greater by 30% in the stressed rats than in the control rats.

The spontaneous firing rates of the cue-tone-related and licking-related neurons were also compared between the two groups (Table 2). The mean spontaneous firing rate of cue-
The present results indicated that the stress-induced increase in spontaneous firing rates in the LHA was most obvious in the control rats (13.8 ± 1.01; P < 0.01); that with either increased activity or decreased activity was also significantly higher than that in control rats (Ps < 0.05). The mean spontaneous firing rate of the licking-related neurons was significantly higher in the stressed rats (16.4 ± 0.88) than in the control rats (13.4 ± 0.94) (P < 0.05). However, the difference of the spontaneous firing rates for the neurons with either increased or decreased activity between the two groups did not reach the statistically significant level.

The present results indicated that the stress-induced increase in spontaneous firing rates in the LHA was most obvious in the neurons that responded to CTS and in the nonresponsive neurons.

**NEURONAL RESPONSES TO CTS IN EXTINCTION AND RE-ASSOCIATION TESTS.** Of the 99 cue-tone-related neurons in the control rats and 133 in the stressed rats, 19 and 47, respectively, were further tested in the extinction and re-association trials. Of the 19 neurons recorded in the control rats, 9 (47%), 9/19) showed a decrease in response to CTS during the course of the extinction test, and recovered rapidly during the re-association test. The remaining 10 neurons showed no significant change in response to CTS in either the extinction or re-association test. Figure 4 shows representative plastic changes in neuronal and behavioral responses of the control rats during the pre-extinction, extinction, and re-association trials. In the pre-extinction trials (Fig. 4Aa and Ab), the neuron showed decreased activity (inhibitory responses) both during the CTS associated with MSG solution and during ingestion of the MSG solution. In the early extinction trials (Fig. 4, Ab and Bb), the inhibitory responses during the CTS and licking phases abated. The licking behavior also gradually decreased in frequency until it disappeared on the second trial of the extinction test (trial 12). In the late extinction trials (Fig. 4, Ac and Bc), neuronal activity during the CTS and licking phases was similar to that during the prettrial control phase (no inhibitory responses). In the first trial of the re-association test (Fig. 4Ad, trial 31), the neuron did not show decreased activity during the CTS although the rat did lick the spout. However, from the 2nd to the 10th trials of the re-association test (trials 32–40), the neuron again showed decreased activity during the CTS as in the pre-extinction trials. The inhibitory neuronal response during the licking phase also recovered in the first trial of the re-association test, and this was observed throughout the remaining re-association trials. These changes in neuronal responses are shown in Fig. 4Ca quantitatively as line graphs. The magnitude of the inhibitory response during the CTS and licking phases gradually decreased over the course of the extinction test (trials 11–18) and reached a level close to the spontaneous firing rate (trials 19–30). In the re-association test, the inhibitory responses recovered rapidly to almost the same level as that in the pre-extinction trials. Mean response magnitudes during the CTS and licking phases were statistically compared between the four trial blocks (each block consisted of 10 trials, Fig. 4Cb): one pre-extinction block (block 1), two extinction blocks (blocks 2 and 3) and one re-association block (block 4). Mean response magnitude during the CTS phase was significantly smaller in block 3 than in block 1 (pre-extinction; Tukey test after 1-way ANOVA, P < 0.05). Mean response magnitude during the licking phase was significantly smaller in blocks 2 and 3 than in block 1 (Ps < 0.01). Mean response magnitudes during the CTS and licking

<table>
<thead>
<tr>
<th>Type of Neurons</th>
<th>Control</th>
<th>Stressed</th>
<th>Mean Difference</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>12.6 ± 0.66 (215)</td>
<td>17.0 ± 0.71 (206)</td>
<td>4.34 (+3)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Nondifferential</td>
<td>13.1 ± 0.92 (117)</td>
<td>17.5 ± 0.85 (139)</td>
<td>4.46 (+3)</td>
<td>P &lt; 0.01</td>
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<tr>
<td>CTS</td>
<td>13.6 ± 2.03 (22)</td>
<td>19.9 ± 1.84 (37)</td>
<td>6.36 (+7)</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Licking</td>
<td>10.9 ± 1.70 (28)</td>
<td>14.2 ± 1.95 (15)</td>
<td>3.27 (+3)</td>
<td>NS</td>
</tr>
<tr>
<td>CTS and Licking</td>
<td>13.8 ± 1.28 (67)</td>
<td>17.0 ± 1.04 (87)</td>
<td>3.23 (+3)</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Differential</td>
<td>16.0 ± 1.82 (18)</td>
<td>14.7 ± 2.50 (11)</td>
<td>−1.26 (−8)</td>
<td>NS</td>
</tr>
<tr>
<td>Nonresponsive</td>
<td>11.2 ± 1.04 (80)</td>
<td>16.1 ± 1.54 (56)</td>
<td>4.86 (+43)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Cue-tone related</td>
<td>13.8 ± 1.01 (99)</td>
<td>17.7 ± 0.88 (133)</td>
<td>3.95 (+29)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Increased activity</td>
<td>13.1 ± 1.46 (50)</td>
<td>17.5 ± 1.55 (53)</td>
<td>4.40 (+3)</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Decreased activity</td>
<td>14.5 ± 1.40 (49)</td>
<td>17.9 ± 1.06 (80)</td>
<td>3.41 (+24)</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Licking related</td>
<td>13.4 ± 0.94 (111)</td>
<td>16.4 ± 0.88 (113)</td>
<td>3.04 (+23)</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Increased activity</td>
<td>11.9 ± 1.41 (53)</td>
<td>15.8 ± 1.49 (51)</td>
<td>3.89 (+33)</td>
<td>NS</td>
</tr>
<tr>
<td>Decreased activity</td>
<td>14.8 ± 1.23 (58)</td>
<td>17.0 ± 1.04 (62)</td>
<td>2.20 (+15)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values expressed as means ± SE in spikes/s; n values in parentheses for control and stressed columns and percentages in mean difference column. CTS, cue tone stimuli; NS, not significant.
phases in block 4 did not differ significantly from those in block 1 (Ps > 0.05).

Of the 47 neurons recorded in the stressed rats, 26 (55%, 26/47) showed a decrease in response to CTS(s) in the extinction test, which then recovered in the re-association test, a trend in the control rats. However, we should emphasize that in the stressed rats, different patterns of response change were observed in nine neurons (19%, 9/47). Of these nine neurons, five (3 with excitatory response and 2 with inhibitory response) significantly enhanced their responses to CTS(s) in the extinction test. The remaining four neurons reversed the direction of the response to CTS(s) in the extinction test: three neurons reversed the response from inhibitory to excitatory and one neuron did so from excitatory to inhibitory. In the control rats, neither an enhanced nor a reversed response change was observed in the extinction test. Figure 5 shows a representative LHA neuron with response enhancement in the extinction test. Figure 4 shows a representative LHA neuron in extinction and re-association trials in control rat. All records are from the same neuron. A and B: raster displays and histograms, respectively, of neuronal responses to CTS associated with MSG solution and ingestion of MSG solution in pre-extinction trials (a, trials 1–10), early extinction trials (b, trials 11–20), late extinction trials (c, trials 21–30), and re-association trials (d, trials 31–40). Numbers at left in A are sequential trial numbers. and , duration of CTS phase and licking phase, respectively. Each above raster display indicates 1 lick. B: top, accumulated neuronal activity; bottom, accumulated licks. C: neuronal response magnitudes in CTS and licking phases in each trial (a) and in each block consisting of 10 trials (b). Response magnitude in CTS phase was defined as the mean firing rate during CTS phase (0.0–2.0 s) minus the mean spontaneous firing rate during pretrial control phase (~2.0–0.0 s). Response magnitude in licking phase was defined as the mean firing rate during licking phase (2.0–4.0 s) minus the mean spontaneous firing rate. * and **, changes in response magnitudes were significantly greater than that of respective pre-extinction trials (block 1).
tional 4 s beyond the licking phase despite the absence of licking behavior. These enhanced responses were maintained throughout the later extinction trials (Fig. 5, Ac and Bc). In the subsequent re-association test (Fig. 5, Ad and Bd), however, activity of this neuron during the CTS phase returned to the pre-extinction level. These changes in neuronal responses are shown in Fig. 5Ca quantitatively as line graphs. Furthermore mean response magnitudes in each block of 15 trials during these tests were statistically compared (Fig. 5Cb). In the CTS and licking phases, mean response magnitudes in blocks 2 and 3 were significantly greater than their respective mean response magnitudes in block 1 (Ps < 0.01). Mean response magnitudes during the CTS and licking phases in block 4 did not differ significantly from those in block 1 (Ps > 0.05). Similar results were observed in this neuron when MSG was used as the test solution (data not shown).

Figure 6 shows a representative LHA neuron that reversed responses to the CTS in the extinction test. In the pre-extinction trials (Fig. 6, Aa and Ba), the neuron showed biphasic response to the CTS associated with MSG, i.e., during the 2.0-s CTS phase the neuron showed a transient increase in activity for the initial 0.3 s and a subsequent decrease in activity for the following 1.7 s. In the extinction test, the neuron reversed the late response component rapidly from inhibition to excitation in trials 11 and 12. This reversed responsiveness was maintained during the remaining extinction trials (Fig. 6, Ab, Ac, Ad, and Ae).
In the re-association test, the neuron recovered the original response property (Fig. 6, Ad and Bd). The early response component was not affected by the extinction and re-association tests. These changes in neuronal responses are shown in Fig. 6Ca quantitatively as line graphs. Mean response magnitudes in each block of 10 trials during these tests were statistically compared (Fig. 6Cb). The mean magnitudes of the late response component in blocks 2–4 were significantly higher than that in block 1 (Ps < 0.01). In blocks 3 and 4, the mean response magnitudes of the late response component were also greater than those in the pretrial control phase (block 3, P < 0.01; block 4, P < 0.05). The mean response magnitude in block 5 did not differ significantly from that in block 1 (P > 0.05). There were no significant differences in the mean response magnitudes of the early response component between these five blocks [F(4, 45) = 0.25; P > 0.05].

In the pre-extinction, extinction, and re-association trials, the change in licking behavior observed in the stressed rats was similar to that in the control rats (e.g., lick responses in Fig. 4Aa vs. Figs. 5 and 6, Aa). Therefore the unusual (enhanced or reversed) changes in neuronal responses observed in the extinction test for the stressed rats could not be attributed to changes in licking behavior.

**MHA neurons**

**ANALYZED NEURONS.** The activity of 127 neurons was recorded in the MHA (67 in the control rats; 60 in the stressed rats). Each neuron was tested with the six standard liquids and the associated CTSs. Forty-two neurons from the control rats and 37 neurons from the stressed rats were further tested with histidine solution and its associated CTS. Fifty-eight neurons from the control rats and 43 neurons from the stressed rats were also tested with glucose solution and its associated CTS. In contrast to the LHA neurons mentioned
in the preceding text, the percentage of MHA neurons that responded during the CTS and/or licking phase was relatively low. Of the 67 neurons recorded in the control rats and 60 recorded in the stressed rats, 9 (13.4%) and 3 (5.0%), respectively, were classified as nondifferential neurons. The remaining 58 (86.6%) and 57 (95.0%), respectively, were classified as nonresponsive neurons. No differential MHA neurons were recorded in the present study. Of the nine nondifferential neurons recorded in the control rats, one was CTS type, one was licking type, and the remaining seven were CTS and licking type. Of the three nondifferential neurons recorded in the stressed rats, one was licking type, and the other two were CTS and licking type.

SPONTANEOUS FIRING RATE. Spontaneous firing rates of MHA neurons in the two groups are shown as histograms in Fig. 7. Spontaneous firing rates ranged from 1.3 to 19.1 spikes/s (n = 67) in the control rats and from 1.1 to 8.9 spikes/s (n = 60) in the stressed rats. The mean spontaneous firing rate in the stressed rats (3.12 ± 0.22) was 36% lower than that in the control rats (4.87 ± 0.42; Student’s t-test, P < 0.01). Although the histogram peak appeared in the range of 2–4 spikes/s both in the control and stressed groups, the histogram distribution in the stressed group was shifted to the left relative to the control group.

Recording sites

The recording sites for all the neurons sampled are shown in Fig. 8. Given that the total number of differential neurons was small, this type of neuron was included in the cue-tone-related and/or licking-related neurons. The recording sites in the stressed rats were almost identical to those in the control rats. The neurons that responded to CTSs and/or during licking with either increased or decreased activity were diffusely distributed in the LHA both in the control and stressed groups. In each atlas plane, the number of cue-tone-related LHA neurons showing inhibitory responses was larger in the stressed group than in the control group. The percentage of neurons showing inhibitory responses to CTS in the most anterior portion of the LHA (Fig. 8A, 7,200 μm) was significantly larger in the stressed rats (48.1%, 25/52) than in the control rats (13.2%, 7/53; Fisher’s exact probability test, P < 0.01).

The LHA neurons sampled in each group were divided into four populations according to the anterior-posterior (A-P) levels of their recording sites, and mean spontaneous firing rate was calculated for each population (Table 3). The mean spontaneous firing rates were higher in the control rats than in the control rats in all the A-P ranges except for the most anterior one (7,500–7,100 μm). The difference in the mean spontaneous firing rates between the control and stressed rats tended to be larger in the posterior ranges than in the anterior ranges.

DISCUSSION

Effect of repeated cold stress on spontaneous firing rates of LHA and MHA neurons

In the present study, repeated cold stress increased the spontaneous firing rates of LHA neurons and decreased those in the ventromedial hypothalamic (VMH) neurons sampled in the control or stressed group x 100. Inset: mean (±SE) spontaneous firing rate. **P < 0.01, significance between control and stressed rats by Student’s t-test.

FIG. 7. Histograms of spontaneous firing rate of MHA neurons in control (□) and stressed rats (●). Mean spontaneous firing rate in stressed rats was lower than that in control rats. Bin width, 2 spikes/s. Ordinate, percentage of neurons [the number of neurons in each bin divided by the total number of ventromedial hypothalamic (VMH) neurons sampled in the control or stressed group x 100]. Inset: mean (±SE) spontaneous firing rate. **P < 0.01, significance between control and stressed rats by Student’s t-test.

FIG. 8. Recording sites of neurons responding to CTSs (A) and licking of various solutions (B) recorded in control and stressed rats. Numbers under each plane, anterior-posterior stereotaxic coordinates from the interaural line according to the atlas of Paxinos and Watson (1986); A, anterior; ○, neurons with increased activity (excitatory response); ●, neurons with decreased activity (inhibitory response); - , no response. AH, the anterior hypothalamus; VMH, the ventromedial hypothalamic nucleus; DMH, the dorsomedial hypothalamic nucleus; F, the fornix; CI, the internal capsule.

FIG. 7. Histograms of spontaneous firing rate of MHA neurons in control (□) and stressed rats (●). Mean spontaneous firing rate in stressed rats was lower than that in control rats. Bin width, 2 spikes/s. Ordinate, percentage of neurons [the number of neurons in each bin divided by the total number of ventromedial hypothalamic (VMH) neurons sampled in the control or stressed group x 100]. Inset: mean (±SE) spontaneous firing rate. **P < 0.01, significance between control and stressed rats by Student’s t-test.

FIG. 8. Recording sites of neurons responding to CTSs (A) and licking of various solutions (B) recorded in control and stressed rats. Numbers under each plane, anterior-posterior stereotaxic coordinates from the interaural line according to the atlas of Paxinos and Watson (1986); A, anterior; ○, neurons with increased activity (excitatory response); ●, neurons with decreased activity (inhibitory response); - , no response. AH, the anterior hypothalamus; VMH, the ventromedial hypothalamic nucleus; DMH, the dorsomedial hypothalamic nucleus; F, the fornix; CI, the internal capsule.
of MHA neurons. These changes can be interpreted to indicate that basal activity level of the LHA was increased and that of MHA was decreased under repeated cold stress. This interpretation is of interest when considering the functional implication of these two brain areas. For instance, hyperphagia is a unique characteristic of chronic cold stress including repeated cold stress (Bing et al. 1998; Hata et al. 1984a, b, 1988a; Kondoh et al. 1996; Leung and Horwitz 1976; Morrison 1981; Okano et al. 1993; Snyder and Stricker 1985), which can be clearly distinguished from the anorexia induced by severe acute stress conditions (Morley and Levine 1982; Shimizu et al. 1989). Consistent with the previous studies (Hata et al. 1984a, b, 1988a; Kondoh et al. 1996; Okano et al. 1993), in the present study we also observed an increase in food intake in the rats exposed to repeated cold stress. Previous behavioral and neurophysiological studies have suggested that the LHA and ventromedial hypothalamic nucleus (VMH, a part of the MHA defined in the present study) control feeding behavior in opposing manners; the LHA is thought to be a feeding center and the VMH together with the PVN are satiety centers (Delgado and Anand 1953; Leibowitz 1986; Oomura et al. 1967, 1969; Shimizu et al. 1987; Smith 1956; Winn et al. 1984). Thus the present finding of increased spontaneous activity in the LHA (feeding center) and decreased spontaneous activity in the VMH (satiety center) could be involved in the increase in food intake observed in the stressed rats.

A variety of substances (nutrients and their metabolites, neurotransmitters, hormones, etc.) are known to affect activity of LHA and/or MHA neurons so as to control feeding behavior (for review, see Leibowitz 1986; Oomura 1989). Although it is difficult to determine based on the present results what substances are responsible for the activity changes in the hypothalamic neurons during repeated cold stress, several possibilities were suggested. When rats are chronically exposed to a cold environment, the sympahto-adrenal system and hypothalamo-pituitary-thyroid axis are activated to enable adaptation to the cold environment (Fukuhara et al. 1996a, b), resulting in thermogenesis (energy expenditure) and compensatory hyperphagia. Oomura et al. demonstrated the presence of LHA neurons (glucose-sensitive neurons) that decrease activity and the presence of VMH neurons (glucose receptor neurons) that increase activity when glucose is directly applied to the membrane of these neurons (Oomura et al. 1969, 1974). Furthermore free fatty acids stimulate glucose-sensitive neurons while suppressing glucose receptor neurons (Oomura et al. 1976). Therefore a decrease in blood glucose levels and an increase in blood free fatty acid levels accompanied by increased energy expenditure during cold exposure could increase the mean spontaneous firing rate of LHA neurons and decrease that of MHA neurons. Bing et al. (1998) reported that chronic cold exposure decreases plasma leptin and insulin levels but does not significantly change hypothalamic neuropeptide Y and plasma cortisol levels. Leptin and insulin are transported from plasma to the hypothalamus through the blood-brain barrier (Banks et al. 1996; King and Johnson 1985; Pardridge 1986; Schwart et al. 1991), and these peptides in turn suppress food intake when centrally administered (Campfield et al. 1995; Woods et al. 1979). In the LHA and MHA, many neurons have leptin receptors (Elmquist et al. 1998; Funahashi et al. 1999; Hakansson et al. 1998); LHA and MHA neurons also have insulin receptors (Havrankova et al. 1979; Unger et al. 1991). These peptides inhibit activity of LHA glucose-sensitive neurons and stimulate VMH glucose receptor (or glucose-responsive) neurons (Funahashi et al. 1999; Oomura et al. 1976). Therefore the reported decrease in these peptides in plasma is consistent with the changes in spontaneous activity of LHA and MHA neurons observed in the present study. Furthermore we recently found that mRNA level of IL-1β decreased in the LHA while increasing in the MHA in mice exposed to repeated cold stress (Tagoh et al. 1995). IL-1β, which has an inhibitory effect on food intake, suppresses the activity of neurons in the LHA and VMH (Kuriyama et al. 1990; Plata-Salaman et al. 1988). Therefore the site-specific change in IL-1β level (Tagoh et al. 1995) is also consistent with the present finding of changes in spontaneous activity of LHA and MHA neurons.

Absence of specific effect of repeated cold stress on responses of hypothalamic neurons to tastants

Pathological conditions may affect hypothalamic neuronal responses in a tastant (or nutrient) specific manner. We have previously found that deficiency of a requisite amino acid (lysine), leading to a specific preference for this amino acid (Tori 1987), increased the number of LHA neurons that responded specifically to lysine solution and its associated CTS in rats (Tabuchi et al. 1991). The levels of histamine and histamine turnover in the hypothalamus change during cold stress (Taylor and Snyder 1971). We recently found that repeated cold stress induces a specific preference for histidine (Kondoh et al. 1996). Therefore we originally predicted that repeated cold stress would change the proportion of differential neurons or increase the number of neurons that specifically respond to histidine solution and its associated CTS. We also thought that repeated cold stress might preferentially affect responsiveness of hypothalamic neurons to glucose solution and its associated CTS since cold exposure increases energy expenditure and results in hyperphagia. However, the present results were contrary to our prediction, at least for LHA neurons: the repeated cold stress did not change the relative

<table>
<thead>
<tr>
<th>Planes, μm</th>
<th>Control</th>
<th>Stressed</th>
<th>Mean Difference</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, 7500–7100</td>
<td>14.24 ± 1.80 (53)</td>
<td>17.87 ± 1.46 (52)</td>
<td>3.63 (+25)</td>
<td>NS</td>
</tr>
<tr>
<td>A, 7000–6700</td>
<td>12.18 ± 0.93 (72)</td>
<td>15.79 ± 1.58 (46)</td>
<td>3.61 (+30)</td>
<td>P &lt; 0.05</td>
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<tr>
<td>A, 6600–6400</td>
<td>13.42 ± 1.22 (50)</td>
<td>17.51 ± 1.41 (61)</td>
<td>4.09 (+30)</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>A, 6300–6000</td>
<td>10.39 ± 1.27 (40)</td>
<td>16.46 ± 1.33 (47)</td>
<td>6.07 (+58)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Total</td>
<td>12.63 ± 0.66 (215)</td>
<td>16.97 ± 0.71 (206)</td>
<td>4.34 (+34)</td>
<td>P &lt; 0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE in spikes/s. Parentheses enclose n values in stressed and control columns and percentages in mean difference column. A, anterior; NS, not significant.
percentage of differential neurons nor did it increase the number of LHA neurons that specifically responded to the glucose or histidine solution and its associated CTS. Indeed no neurons displayed preferential responses to glucose or histidine. Although to date we have no data to explain this dissociation between our original prediction and the present results, repeated cold stress may have affected the hypothalamus in a more “general” way than the lysine deficiency. Animals can use not only carbohydrate but also fat and protein as energy sources, and thus LHA neurons might not necessarily differentiate the gustatory stimuli tested in the present study, while the specific deficiency of lysine could affect the gustatory responses of LHA neurons in a more specific way because of its specificity for nutrient deficiency. The water deprivation regimen might also have abolished or masked specific changes in neuronal activity related to hyperphagia since water restriction reduces food intake; it could also affect any specific preference for histidine, although we did not test this possibility in the present study. Further studies are necessary to elucidate these points.

Effect of repeated cold stress on nondifferential neurons

In the present study, the relative percentage of nondifferential LHA neurons was increased and that of nondifferential MHA neurons decreased in stressed rats. Enhanced awareness of and responsiveness to environmental stimuli by repeated cold stress may have played a role in these changes. Rats exposed to repeated cold stress exhibit hypersensitivity to external stimuli in galvanic skin response, resting-arousal electrocorticograms with low-voltage fast waves, and hyperactivity accompanied by increased decation in an open-field test (Hata et al. 1987b, 1988b). Furthermore hyperalgesia is a unique characteristic of repeated cold stress (Hata et al. 1988a; Kawanishi et al. 1997; Kita et al. 1979) in contrast to the stress-induced analgesia caused by acute stress (Bondnar et al. 1980). These results suggest that animals exposed to repeated cold stress are in a state of hyperreactivity to external stimuli. Previous anatomical and neurophysiological studies have suggested that the LHA is at the rostral end of the ascending brain stem reticular formation (Nieuwenhuys et al. 1982), and that LHA neurons are involved in information processing concerning not only internal but also external environments to produce appropriate autonomic and behavioral responses (Iwata et al. 1986; Ono et al. 1981). Furthermore electrical stimulation of VMH and DMH induced pain inhibition (Rhodes and Liebeskind 1978), while lesions of the VMH caused hyperalgesia (Hata et al. 1988a). The decreased nociceptor threshold in repeated cold stress is increased by LHA lesions in rats (Hata et al. 1988b). Furthermore hyperalgesia is a unique characteristic of repeated cold stress (Hata et al. 1987a; Kita et al. 1986; Myers 1980; Yoneda et al. 1992). The results of the present study together with those of the previous studies suggest the possibility that the stress-induced unusual (i.e., enhanced or reversed) changes in LHA neuron responses are involved in the learning deficits observed in the rat exposed to repeated cold stress. Repeated cold stress, which produced changes in the levels of DA and ACh in the hypothalamus, might have affected LHA neuron activity to abolish normal plasticity in the formation and extinction of stimulus-reinforcement association.

Effect of repeated cold stress on changes in responsiveness of LHA neurons during extinction test

In the present study, some LHA neurons in the stressed rats showed enhanced or reversed responses to CTSs in extinction trials. No such response changes were ever observed in the control rats, a finding that was consistent with the results of our previous studies on response properties of LHA and VMH neurons (Nakamura and Ono 1986; Nishino et al. 1988; Ono et al. 1986, 1992). In these studies, we regarded plastic changes in neuronal responses to CTSs associated with rewarding or aversive stimuli during the course of extinction and re-association tests as CTS learning of positive and negative reinforcement.

Repeated cold stress is also known to produce impairment in passive avoidance learning during consolidation phase in rats and mice (Yago et al. 1992; Yoneda et al. 1992). A previous study from our laboratory demonstrated that dopamine (DA) is involved in activity change of LHA neurons accompanied with CTS learning of positive reinforcement, whereas ACh is involved in that of negative reinforcement (Ono et al. 1992). Exposure to a cold environment, including repeated cold stress condition significantly affects the levels of hypothalamic DA and ACh (Hata et al. 1987a; Kita et al. 1986; Myers 1980; Yoneda et al. 1992). Furthermore physostigmine, an acetylcholinesterase inhibitor, improves impaired passive avoidance learning in rats exposed to repeated cold stress (Yago et al. 1992). The results of the present study together with those of the previous studies suggest the possibility that the stress-induced unusual (i.e., enhanced or reversed) changes in LHA neuron responses are involved in the learning deficits observed in the rat exposed to repeated cold stress. Repeated cold stress, which produced changes in the levels of DA and ACh in the hypothalamus, might have affected LHA neuron activity to abolish normal plasticity in the formation and extinction of stimulus-reinforcement association.

In the present study, we did not observe any differences in the behavioral (licking) changes between the control and stressed groups during the course of the extinction and re-association tests in most of the recording sessions. This was likely due to the fact that the behavioral requirement in the extinction and re-association tests was very simple so that the rats, even under the stressed condition, readily learned how to behave in these tests; this would make it difficult to detect behavioral differences between the control and stressed rats during the course of these tests. It may be necessary to use different tasks with more complex learning requirements or naive rats to establish any relationship between learning-related changes in behavior and neuronal activity during repeated cold stress.

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HYPOTHALAMIC NEURON ACTIVITY IN CHRONIC COLD STRESS


