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ACTIVATION OF PROJECTIVE NEURONS FROM THE NUCLEUS ACCUMBENS TO VENTRAL PALLIDUM BY A LEARNED AVERSIVE TASTE STIMULUS IN RATS: A MANGANESE-ENHANCED MAGNETIC RESONANCE IMAGING STUDY

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Abstract—Conditioned taste aversion (CTA) causes a palatability shift of a taste stimulus (conditioned stimulus, CS) from ingestive to aversive. We previously found that the ventral pallidum (VP) mediates the palatability shift in CTA. Because the VP receives major projections from the nucleus accumbens (NAc), we examined whether the presentation of CS activates the NAc–VP projective neurons after the establishment of CTA, using a manganese-enhanced magnetic resonance imaging technique. Rats were implanted with a guide cannula in the NAc and an intraoral cannula. After the surgery, they received a pairing of 5 mM saccharin solution with an i.p. injection of 0.15 M lithium chloride (CTA group) or saline (sham group). Two days after the conditioning, rats were microinjected with manganese chloride ($MnCl_2$) into the NAc. Thirty minutes later, the rats were presented with saccharin (CTA-CS and sham-CS groups) or water (CTA-DW and sham-DW groups) via the intraoral cannula. Only the CTA-CS group showed a robust aversion to the CS. At 1 and 2 h after the $MnCl_2$ injection, T1-weighted MR images were acquired using an 11.7 T MRI. Imaging analysis showed that significantly more manganese moved toward the VP in the CTA-CS group than in the other groups. These results indicate that the conditioned aversive taste enhanced the activities of the projective NAc–VP neurons and suggest specific involvement of the NAc–VP pathway in the rejection of CS in retrieval of CTA.

Key words: conditioned taste aversion, palatability, nucleus accumbens, ventral pallidum, projective neuron, manganese-enhanced MRI.

The tastes of foods signal the existence of nutrients and irritant materials (Myers and Hall, 1998; Scott and Verhagen, 2000). Palatability of food tastes is critical for ingestive behavior because greater palatability evokes greater food intake. Many species, including humans, generally prefer sweetness but reject bitterness. Although these palatability preferences are evident from birth and probably innate (Steiner et al., 2001), acquisition of taste memory can alter them. For example, when animals drink a novel taste solution and experience a subsequent malaise, they learn to avoid the taste solution thereafter. This phenomenon is referred to as conditioned taste aversion (CTA) (Garcia et al., 1955; Bures et al., 1998). Previous behavioral research has shown that the establishment of CTA causes a shift in the palatability of a taste solution from ingestive to aversive (Spector et al., 1988), but the underlying neural mechanisms of this shift remain unclear.

The ventral pallidum (VP), a part of brain reward circuit, is a critical region in taste palatability; for example, microinjections of the $GABA_A$ receptor antagonist bicuculline into the VP increase the intake of a saccharin solution (Shimura et al., 2006) and the mu-opioid receptor agonist [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin elevate the palatability of a sucrose solution (Smith and Berridge, 2005). In a CTA experiment, we have shown that bicuculline injections into the VP change the palatability of a learned aversive saccharin solution to ingestive and increase intake of the saccharin solution (Inui et al., 2007). Furthermore, in CTA-acquired rats, the presentation of a learned aversive saccharin solution causes a significant increase in the extracellular release of GABA in the VP (Inui et al., 2009). Because it is well known that the VP receives dense GABAergic projections from the nucleus accumbens (NAc) (Groenewegen and Russchen, 1984; Zahm and Heimer, 1990, 1993; Hakan et al., 1992; Usuda et al., 1998; Schwarzer et al., 2001; Zhou et al., 2003; Tripathi et al., 2010), the NAc are likely to have influenced changes in GABA release in the VP.

The aim of the current study was to use manganese-enhanced magnetic resonance imaging (MEMRI) to determine whether, after establishment of CTA, a learned aversive taste solution activates projective neurons from the NAc to VP. The MEMRI is a newly developed imaging

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Abbreviations: CS, conditioned stimulus; CTA, conditioned taste aversion; MEMRI, manganese-enhanced magnetic resonance imaging; $MnCl_2$, manganese chloride; NAc, nucleus accumbens; VP, ventral pallidum.

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technique that allows depiction of excited projective neurons in the brain. Manganese enters active cells via voltage-gated calcium channels and is transported through the axons (Anderson, 1983; Merritt et al., 1989; Narita et al., 1990). Manganese also changes the spin lattice relaxation, producing differences in the signal intensity in the MR images. These features make MEMRI a useful tool for investigating the projection patterns of activated neurons (Chuang et al., 2009), and the amount of transported manganese may reflect the strength of neural activity (Lin and Koretsky, 1997). Thus, we investigated whether the NAc-VP pathway is excited under learned aversive taste stimulation by visualizing the activity of the projective neurons from the NAc to the VP, using the MEMRI technique.

EXPERIMENTAL PROCEDURES

Animals

This study involved 28 male Wistar rats (CLEA, Osaka, Japan) with an average weight of 190–210 g on the day of surgery. All rats were individually housed in transparent plastic cages ($20\times30\times20$ cm^3). The temperature and humidity of the animal room were maintained within a stable range (23 ± 1 °C, 65–70%), and food and water were available *ad libitum* except where noted. All animals were handled in accordance with the procedures outlined in the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health Guide), and the institutional committee on animal research approved the study.

Surgery

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.; Nembutal, Abbott, North Chicago, IL, USA) and placed in a stereotaxic apparatus (SR-8, Narishige Scientific Instrument LAB., Tokyo, Japan). The skull was leveled between bregma and lambda by adjustment of the bite bar. A small hole was drilled in the skull, and a non-metallic guide cannula (C315G/PK, Plastics One Inc., Roanoke, VA, USA) was implanted above the NAc. The stereotaxic coordinates were selected according to Paxinos and Watson's atlas (1997). Coordinates for the NAc were as follows (in mm): +1.5 (A–P), −1.6 (L–M), and +4.8 (D–V). The cannula was secured to the skull with dental cement. A 33-gauge non-metallic dummy cannula (C315DC/PK, Plastics One Inc., Roanoke, VA, USA) was inserted into each guide cannula to prevent clogging. In the same surgery, all rats were implanted with a unilateral intraoral cannula (PE-50) for oral fluid infusion. The intraoral cannula was inserted through the temporalis muscle and opened into the oral cavity just lateral to the first molar (Grill and Norgren, 1978). After the surgery, the rats were allowed to recover for 3 days before the start of behavioral testing.

Behavioral procedure and analysis

On training days, rats were placed in a Plexiglas® cylindrical chamber (30-cm diameter, 40-cm height) in a testing room. The intraoral cannula was connected via polyethylene tubing to an infusion pump that delivered fluid at a rate of 0.5 ml/min. The animals drank 5 ml of distilled water. After undergoing this training for 2 days, on the conditioning day (day 3), all animals were presented with 5 ml of a 5-mM saccharin solution as a conditioned stimulus (CS), followed by an i.p. injection (2% of body weight) of 0.15 M lithium chloride (LiCl) (CTA) or saline (sham). Two days after the conditioning, animals received a 10-min oral infusion (0.5 ml/min) of the CS (CTA-CS and sham-CS groups) or water (CTA-DW and sham-DW group) 30 min after a microinjection of 40 mM manganese chloride ($MnCl_2$) into the NAc. The rat's orofacial and somatic responses to the fluids were videotaped through a mirror placed at a 45° angle beneath the transparent Plexiglas® floor of the Plexiglas® chamber.

Ingestive and aversive response patterns were scored off-line in slow motion (1/30 s frame by frame) using established behavioral classifications and time bin scoring procedures developed to assess taste palatability (Berridge and Grill, 1984; Berridge, 2000). Ingestive responses are typical orofacial and somatic responses, including tongue protrusions, lateral tongue protrusions, and paw licking, that a preferred taste solution evokes. Aversive responses include gaping, head shaking, forelimb flailing, chin rubbing, and paw wiping (Spector et al., 1988). The data were statistically analyzed using STATISTICA (Ver 5.5, StatSoft, Tulsa, OK, USA) software. We used the Kruskal–Wallis test and the Mann–Whitney *U*-test with a significance level of $P<0.05$.

Manganese microinjection

Microinjection of $MnCl_2$ into the NAc was performed through a non-metallic 30-gauge injector cannula (C315I/PK, Plastics One Inc., Roanoke, VA, USA) connected to a 10-μl Hamilton gastight microsyringe via a polyethylene tube (PE 10). The injector cannula extended 2.0 mm beyond the tip of the guide cannula. A total of 0.05 μl of 40-mM $MnCl_2$ was slowly injected (0.05 μl/min) with a microinfusion pump (CMA 11, CMA Microdialysis AB, Solna, Sweden). The injector cannula was left in place for an additional 2 min to allow diffusion of the $MnCl_2$ away from the cannula tip and to avoid backflow.

MRI measurements

MRI measurements were performed with an 11.7 T Bruker BioSpec scanner (AVANCE 500WB, Bruker Biospin MRI Gmbh, Ettlingen, Germany) with animals under anesthesia (50 mg/kg, i.p.; Nembutal, Abbott, North Chicago, IL, USA). Physiological conditions were kept stable by monitoring of respiration and adjustment of the anesthesia level with isoflurane (2%). T1-weighted MR images were acquired with a spin echo technique (repetition time=400 ms; echo time=11 ms; matrix=256×256; field of view=32×32 mm in axial orientation; pixel color depth=16 bits, gray scale). Slice thickness was 0.5 mm, and scanning time was about 14 min. For the analysis of changes in the signal intensity in the VP area, two MR images of the horizontal plane of each brain were acquired 1 and 2 h after the manganese injection. Section levels were determined by referencing of the bottom surfaces of the olfactory bulb and brainstem as landmarks according to the brain map (Paxinos and Watson, 1997). In addition, to observe the direction of manganese movements, the MR images of the coronal and sagittal planes were acquired after the 2-h horizontal MR image.

Analysis of manganese movement

For assessment of the strength of activity of the projective neurons from the NAc to VP, the amounts of manganese transported toward the VP were measured by determination of signal intensity in the VP area on the horizontal plane of each brain (Fig. 1A), using the image processing application ImageJ (ver. 1.37v). Initially, the signal intensities of a square (1 mm^2) at four corners in each image were averaged as the background. All background values were less than 1000, while the maximum value of 16-bit gray-scale images was 65,536. To adjust for differences in image acquisition conditions, the signal intensities of the raw images were normalized by equalization of the background value (1000). The signal intensity of the VP area was quantified as the sum of all pixel values within three circular areas (0.188 mm^2, respectively), which were located 1.5, 1.75, and 2.0 mm posterior to the manganese injection site and 2.0, 2.25, and 2.5 mm lateral to the midline (Fig. 1A). The minimum and maximum of the pixel values