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NMDA-Mediated Activation of the Medial Amygdala Initiates a Downstream Neuroendocrine Memory Responsible for Pseudopregnancy in the Female Rat

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In female rats, genitosensory stimulation received during mating initiates twice-daily prolactin (PRL) surges, a neuroendocrine response that is the hallmark of early pregnancy or pseudopregnancy (P/PSP). Nocturnal and diurnal PRL surges are expressed repeatedly for up to 2 weeks after copulation, suggesting that a neuroendocrine memory for vaginocervical stimulation (VCS) is established at the time of mating. These studies investigated whether the processing and retention of VCS involves acute glutamatergic activation or *de novo* protein synthesis within the medial nucleus of the amygdala (MEA), a VCS-responsive brain site that is implicated in P/PSP initiation. Pharmacological activation of the MEA with the glutamate agonist, NMDA, initiated nocturnal PRL surges, causing a PSP state in females that had not received VCS. P/PSP initiation by mating was prevented by intra-amygdalar infusion of the NMDA receptor antagonist, 2-amino-5-phosphonopentanoic acid (AP-5), provided that it was administered before mating. AP-5 treatment also disrupted mating-induced c-*fos* expression in the principle bed nucleus of the stria terminalis and the ventrolateral division of the ventromedial hypothalamic nucleus, but not in the medial or anteroventral periventricular preoptic nuclei. Neither P/PSP nor downstream cellular activation was prevented when a protein synthesis inhibitor, anisomycin, was administered to the MEA. The results indicate that MEA cells are critical to the early processing of VCS through NMDA channel activation, rapidly conveying information to downstream hypothalamic cell groups that modulate neuroendocrine function.

Key words: glutamate; medial amygdala; NMDA; VCS; sensory transduction; memory

Reproductive neuroendocrine systems are particularly amenable to investigation of the mechanisms through which sensory inputs induce long-term changes in brain function. These circuitries are markedly affected by experience, and persistent changes in neuroendocrine and behavioral function occur as a result of mating, parturition, and lactation (Keverne et al., 1993; Gies and Theodosis, 1994; Bridges et al., 1997). In female rats, the receipt of threshold amounts of vaginocervical stimulation (VCS) during mating triggers long-term changes in neuroendocrine function that are essential for pregnancy success. Bicircadian pituitary prolactin (PRL) surges are initiated in direct response to VCS, occurring repeatedly for 10–12 d and characterizing early pregnancy or pseudopregnancy (P/PSP) (Gunnet and Freeman, 1983; Erskine, 1995). Because PRL surges are expressed for up to 2 weeks without further genitosensory stimulation, it is postulated that a neuroendocrine memory, or mnemonic, is formed centrally by VCS (Freeman et al., 1974; Terkel et al., 1990).

The transduction mechanisms and CNS pathways responsible for the establishment and expression of the VCS mnemonic are poorly understood. However, there is substantial evidence that the posterodorsal medial amygdala (MEApd) is involved in initiating P/PSP. By measuring expression of the immediate-early gene, c-*fos*, we and others have identified an anatomically distinct group of MEApd cells that is activated selectively by VCS (Pfaus et al., 1993; Rowe and Erskine, 1993; Tetel et al., 1993; Polston and Erskine, 1995), and electrophysiological studies confirm that neuronal activity in the medial amygdala (MEA) is increased by VCS treatment (Kawakami and Kubo, 1971). Moreover, pharmacological excitation of the MEA by NMDA results in cessation of ovarian cyclicity for 10–12 d, as is typical of PSP (Numan et al., 1993; Polston and Erskine, 2001), and the incidence of P/PSP is reduced when the sodium channel antagonist, lidocaine, is infused into the MEA before mating (Coopersmith et al., 1996). Thus, although additional brain sites, including the medial preoptic area and the dorsomedial, ventromedial, and arcuate hypothalamic nuclei, may be involved in the expression of PRL surges (Erskine, 1995), activation of the MEApd appears to be particularly important to the initial transduction of VCS and to mnemonic acquisition.

The aim of the present experiments was to determine whether glutamate receptor activation or protein synthesis in the MEA is involved in the establishment of the mating mnemonic. We investigated whether daily PRL surges could be initiated through bilateral infusions of the glutamate agonist, NMDA, into the MEA of unmated animals and whether treatment of the MEA with glutamate antagonists or a protein synthesis inhibitor could block P/PSP in mated animals. Lastly, we examined the effects of NMDA receptor blockade or protein synthesis inhibition on mating-induced c-*fos* expression in the principle bed nucleus of the stria terminalis (BSTp), ventrolateral division of the ventro-

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medial hypothalamus (VMHvl), medial division of the medial preoptic nucleus (MPNm), and anteroventral periventricular preoptic nucleus (AVPV), areas that receive efferents from the MEApd and are involved in the regulation of PRL secretion (Krettek and Price, 1978; Canteras et al., 1995; Erskine, 1995).

MATERIALS AND METHODS

Animals. Experimental animals were female Long–Evans rats (225–275 gm) obtained from Charles River Laboratories (Wilmington, MA). Mating stimulation was provided by sexually experienced males of the same strain (300–400 gm). Animals were housed singly in wire mesh cages with food and water available *ad libitum*. Because rats are a nocturnally active species, animals were housed in a room in which the light cycle was reversed from normal daylight hours (lights off from 8:00 A.M. to 8:00 P.M.), facilitating procedures that were conducted during the dark phase of the cycle. For presentation purposes, these times have been adjusted to correspond to natural daylight hours. Ovarian cyclicity of all females was monitored by daily vaginal lavage, and females exhibiting two consecutive estrous cycles of 4–5 d were used. After treatments, P/PSP was considered to have been induced if 8–13 d of consecutive diestrus smears, characterized by an absence of cornified epithelial cells associated with estrus and a preponderance of leukocytes, were observed (Cooper et al., 1993). All procedures were approved by the Laboratory Animal Use and Care Committee at Boston University (Boston, MA) in accordance with National Institutes of Health guidelines.

Administration of NMDA into the medial amygdala. To determine whether the sensory transduction mechanisms required for initiation of VCS-induced PRL surges may include activation of NMDA receptors within the MEA, cycling females were infused on proestrus with NMDA into the MEA, and repeated blood samples were obtained 6–7 d later at the time of the diurnal PRL surge, the intersurge period, and the nocturnal PRL surge as detailed below. For infusion of NMDA, females were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg, i.p.; J. A. Webster, Sterling, MA) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) with the toothbar set at −3.3 mm in accordance with the atlas of Paxinos and Watson (1986). Holes were drilled bilaterally in the skull, and a beveled 1 μl Hamilton syringe (model 7001) filled with NMDA (0.14 M; Sigma, St. Louis, MO) or PBS vehicle (VEH) was attached to a Kopf microinjector and lowered into the MEA [bregma −2.4 mm anteriorposterior (AP), ±3.75 mm mediolateral, dura −7.3 mm dorsoventral (DV)] with the bevel pointing medially. This directed the infusate toward the MEApd from the site of penetration at the lateral edge of the optic tract. NMDA or VEH was infused bilaterally into the MEA in a volume of 0.4 μl per side. Infusion occurred over 2 min, and syringes were left in place for an additional postinfusion period of 2 min to allow for drug diffusion away from the needle tip. After removal of the infusate needle, the holes in the skull were filled with bone wax, and the skin was sutured closed.

Blood sampling and PRL radioimmunoassay. Immediately after NMDA or VEH infusion, animals were fitted with intra-atrial catheters for repeated blood sampling as described previously (Kornberg and Erskine, 1994). Postoperatively, females were treated with atropine sulfate (0.05 mg per rat, s.c.; J. A. Webster) and daily injections of gentomycin sulfate (1.5 mg/d per rat, s.c.; Steris Laboratories, Phoenix, AZ). Catheters were kept patent by daily flushes with 100 U/ml sterile heparinized saline (Henry Schein Co., Port Washington, NY). Using previously published methods (Polston et al., 1998), we collected blood samples (0.3 ml) 6–7 d after drug treatment at 6:00 P.M., 12:00 A.M., and 6:00 A.M., times of day that correspond, respectively, to the diurnal PRL surge, the intersurge period, and the nocturnal PRL surge during P/PSP. Samples were centrifuged at 4°C for 20 min, after which plasma was collected and frozen at −20°C until radioimmunoassay was performed. Methods for radioimmunoassay of PRL were as previously reported (Polston et al., 1998). Briefly, plasma was diluted in 1% BSA (1:20) and incubated for 24 hr with anti-rat-PRL antibody [anti-r-PRL-S-9; National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Bethesda, MD] at a final tube concentration of 1:10,000. Radiolabeled PRL (^{125}I-PRL; 20,000 cpm/100 μl; Covance Laboratories, Vienna, VA) was added to all assay tubes, and incubation continued for an additional 24 hr. Antibody-bound isotope was precipitated by adding cold Protein A (IgGSorb; The Enzyme Center, Malden, MA), and pellets were counted in a gamma counter. Raw data were analyzed using Beckman EIARIA radioimmunoassay software. The within-assay coefficient of variation was 5.79%, and assay sensitivity was 30 pg of PRL.

Table 1. Effect of intra-amygdalar administration of AP-5 and ANI on FOS-IR labeling in the MEApd after mating

Treatment group	Number of FOS-IR cells[a]
Blank mated ($n = 6$)	216.7* ± 23.5
AP5 mated ($n = 4$)	80.2 ± 19.9
ANI mated ($n = 6$)	38.8 ± 18.8
Blank unmated ($n = 5$)	3.2 ± 0.5

Animals were killed 60–90 min after mating.

[a]Mean ± SEM.

*Significantly higher than all other groups ($p \leq 0.002$).

Administration of antagonist drugs. The NMDA receptor antagonist, 2-amino-5-phosphonopentanoic acid (AP-5), and the protein synthesis inhibitor, anisomycin (ANI), were administered into the MEA at several times around the time of mating to determine the efficacy of these drugs on the inhibition of mating-induced P/PSP. To allow treatment of awake animals with AP-5 and ANI, steel guide cannulas were constructed from wide-bore 23 ga needles (Becton Dickinson, Franklin Lakes, NJ) and cut to a length of 15 mm. Cannulas were implanted and affixed to the skull with dental cement using standard stereotaxic techniques. They were lowered until the ventral tips resided 2.0 mm dorsal to the MEApd (DV −5.3 mm from dura). Cannulas were cleared daily with size 00 insect pins cut to the same length. After establishing that normal estrous cycles continued postoperatively, AP-5 (0.05 M, 1.0 μl per side; Sigma) or VEH (1.0 μl per side) was administered into the MEA on the evening of the proestrus vaginal smear (11:00 P.M.) using a beveled 30 ga infusion needle that extended 2.0 mm beyond the ventral tip of the guide cannula (Coopersmith et al., 1996). The infusion needle was connected by polyethylene tubing (PE20; Clay Adams, Parsippany, NJ) to a 1 μl syringe was placed in a Kopf microinjector. Animals were held gently by the investigator, and infusates were delivered to unanesthetized females over the course of 1–2 min per side. An additional 1–2 min was allowed before withdrawal of the infusion needle.

Anisomycin (Sigma) was administered in crystalline form. Insert cannulas were constructed of 28 ga stainless steel hypodermic tubing. They were filled to a depth of 2 mm with ANI by tamping the end of the insert cannula into the powdered drug and measuring the depth by means of a thin wire inserted from the top. The outside of each insert was cleaned with ethanol; the inserts were lowered through the guide cannulas such that they extended 2 mm beyond the tip of the guide cannula. Inserts were held in place by a piece of polyvinyl tubing attaching them to the guide cannulas. ANI-filled implants were lowered into the MEApd either 1.5 hr before mating or 1, 2, or 4 hr after mating and left in place for a total of 3 hr. Control groups were given empty implants 1.5 hr before mating or ANI implants for 3 hr without mating. At the conclusion of the treatment period, insert cannulas were removed, and animals were returned to their home cages. Examination of the insert cannulas showed that ANI had not diffused to the point of depletion after the 3 hr treatment period.

Verification of drug efficacy and implant–infusion sites. To verify that AP-5 blocked neuronal activation and that ANI prevented protein synthesis within the MEA, separate groups of animals were treated with AP-5 or ANI as above, and the ability of mating stimulation to induce c-*fos* expression within the MEApd was measured as indicated below. Table 1 shows the efficacy of AP-5 and ANI treatments within the MEApd as measured by the number of FOS-immunoreactive (FOS-IR) cells induced by mating. Animals were treated with either a single infusion of AP-5 15 min before mating ($n = 4$) or an implant of ANI 1.5 hr before mating ($n = 6$); these times corresponded to the premating treatment times in the experimental groups. Mated ($n = 6$) and unmated ($n = 5$) control females received blank implants. Local treatment with AP-5 and ANI prevented the mating-induced FOS response seen in the mated controls ($F_{(3,17)} = 26.64$; $p \leq 0.001$). Both AP-5 and ANI treatments eliminated the predominant streak of FOS-IR cells that are observed normally after mating (Polston and Erskine, 1995), significantly reducing the number of FOS-IR cells within the treated area compared with that of untreated, mated animals ($p \leq 0.002$). The suppressive effects of both treatments were comparable, and the numbers of FOS-IR cells in the treated groups were not significantly higher than those that were observed in the unmated females. Thus, AP-5 treatment success-

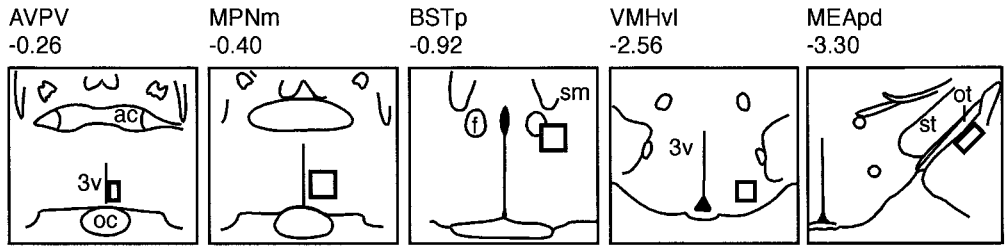

Figure 1. Schematic representation of the five brain areas in which FOS-immunoreactivity was quantified. *Outlined boxes* represent the region of interest templates within which the numbers of FOS-IR cells were counted. *Numbers* represent the distance from bregma in the AP plane. *3v*, Third ventricle; *ac*, anterior commissure; *AVPV*, anteroventral periventricular preoptic nucleus; *BSTp*, principle bed nucleus of the stria terminalis; *f*, fornix; *MEApd*, posterodorsal division of the medial amygdala; *MPNm*, medial division of the medial preoptic nucleus; *oc*, optic chiasm; *ot*, optic tract; *sm*, stria medularis; *st*, stria terminalis; *VMHvl*, ventrolateral division of the ventromedial hypothalamic nucleus.

fully inhibited activation of cells known to be responsive to VCS, and ANI treatment effectively suppressed protein synthesis within the same site.

For histological analysis of placement sites, animals were deeply anesthetized with sodium pentobarbital 12–16 d after NMDA, receptor antagonist, or ANI treatment and perfused transcardially using 10% formalin as previously described (Coopersmith et al., 1996). Brains were removed and stored in cryoprotectant (25% sucrose in 10% formalin). Coronal 40 μm sections through the amygdala were cut on a cryostat (model 1800; Leica, Holliston, MA), mounted onto gelatin-coated slides, stained with cresyl violet acetate, and coverslipped using Permount mounting medium (Sigma). Sites of NMDA, AP-5, and control infusions and ANI implantation were traced onto coronal diagrams from the atlas of Paxinos and Watson (1986) by an investigator blind to the condition of the animals. Only data from animals with bilateral infusion–implant sites that were within the posterior portion of the MEA from the AP plane −3.14 to −3.80 were included in the analyses. Because NMDA infusions caused eventual cytotoxic damage to the area (Dusart et al., 1991), the presence of gliosis was used as an additional verification of needle placement in that group.

Administration of mating stimulation. Behavioral treatments were administered in a dimly illuminated room between 10:00 P.M. and 2:00 A.M. on the night of the proestrus vaginal smear. Females showing a positive lordosis response to at least two of three manual palpations were placed alone into glass testing chambers (30 × 26 × 50 cm) for a 10 min habituation period, and then stimulus males were introduced into the chambers. Behavioral tests continued until males had achieved 15 intromissions, including ejaculations when they occurred. This number of intromissions has induced P/PSP in 91% of females in this laboratory (Kornberg and Erskine, 1994; Coopersmith et al., 1996; Polston and Erskine, 2001). Behavioral records of the mating stimulation received by each female included the number of mounts, intromissions, and ejaculations received and the proportion of mounts that included intromission and ejaculation (mating efficacy). Measures obtained of the level of sexual receptivity shown by each female were the percentage occurrence of lordosis in response to male mounts [lordosis quotient (LQ)] and the mean intensity of lordosis responses based on a four-point rating scale [lordosis rating (LR)] (Hardy and DeBold, 1971). At the end of the behavioral treatments, females were returned to their home cages for the remainder of the experiment.

Hypothalamic and preoptic areas responsive to VCS-induced activity within the MEA. To determine whether the effects of AP-5 infusion in the MEA on P/PSP induction could be demonstrated to include disruption of mating-induced activity within downstream brain sites involved in PRL regulation, we examined mating-induced c-*fos* expression in separate groups of animals in four preoptic and hypothalamic target areas, the BSTp, the VMHvl, the MPNm, and the AVPV, after unilateral infusion–implantation of AP-5 or ANI into the MEA. AP-5 was infused 15 min before mating, ANI was implanted 1.5 hr before mating as above, and saline infusions or blank implants were administered to the contralateral side of the brain. A group of unmated control females received blank implants on both sides. For determination of neuronal activation, brain areas were labeled immunocytochemically for nuclear FOS protein, and the number of FOS-IR cells within the ipsilateral and contralateral sides were compared to determine the effects of treatment within individual animals.

Females were anesthetized 1.0–1.5 hr after mating and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde. Brains were notched unilaterally on the cortical surface to identify the sides ipsilateral and contralateral to treatment and were sectioned at 60 μm on a vibratome in the coronal plane (Paxinos and Watson, 1986). Free-floating sections through the AVPV, MPNm, BSTp, and VMHvl were labeled for FOS as previously reported (Polston and Erskine, 1995). Briefly, sections were preincubated in 1% normal goat serum/1% H_2O_2 in PBS for 20 min, and, after two 10 min rinses in PBS, sections were incubated for 24 hr at room temperature in primary anti-FOS antibody (1:2000 in 0.4% Triton X-100 in PBS solution; sc52; Santa Cruz Biotechnology, Santa Cruz, CA). Visualization was performed using biotinylated anti-rabbit IgG antibody generated in goat (1:200 in 0.4% Triton X-100 in PBS) followed by Vectastain Elite avidin–biotin complex and nickel-enhanced DAB peroxidase substrate (Vector Laboratories, Burlingame, CA). Stained sections were mounted onto gelatin-coated slides, dehydrated in ethanol, cleared in xylenes and coverslipped as above. Verification of specificity of the primary antibody has been demonstrated previously (Erskine and Hanrahan, 1997). FOS-IR cells were identified by their brown–black nuclear staining and were counted if they had distinct nuclear boundaries. Cell counts were taken on both sides of the brain within each area of interest using a CCD camera and the NIH Image analysis program or a camera lucida (Polston and Erskine, 1995). Standard templates delineating an area 410 × 420 μm for the MPNm and BSTp, 460 × 210 μm for the MEApd, 300 × 310 μm for the VMHvl, and 305 × 140 μm for the AVPV were superimposed over the nuclei at a magnification of 200×, as depicted in Figure 1, and labeled cells within these templates were quantified by an investigator blind to the treatment groups of the animals.

Statistical analyses. Effects of drug treatments on the number of subsequent days of diestrous smears, prolactin levels measured 6–7 d after NMDA or VEH infusion, and the number of FOS-IR cells within the MEApd after local treatment with AP-5 or ANI were compared by ANOVA followed by Scheffé's *post hoc* tests. Numbers of FOS-IR cells in ipsilateral and contralateral sides downstream to the unilateral MEA treatments were analyzed using paired *t* tests within each brain area. All effects were considered to be statistically significant when $p \leq 0.05$.

RESULTS

Effect of NMDA infusion into the MEA on induction of PRL surges and P/PSP

Intra-amygdalar infusion of NMDA resulted in prolonged ovarian acyclicity characteristic of PSP in 100% of the animals, compared with 28% in the VEH-infused group ($F_{(2,14)} = 9.87$; $p \leq 0.002$). To determine whether this effect reflected the induction of PRL surges by NMDA treatment, plasma PRL concentrations at the times of the diurnal surge (6:00 P.M.), intersurge period (12:00 A.M.), and nocturnal surge (6:00 A.M.) were compared between NMDA-infused PSP females ($n = 7$) and VEH-infused cycling females ($n = 5$). As shown in Figure 2, there were significant effects of drug treatment ($F_{(1,10)} = 5.46$; $p \leq 0.05$) and sample time ($F_{(2,20)} = 4.01$; $p \leq 0.05$) and a drug × sample time

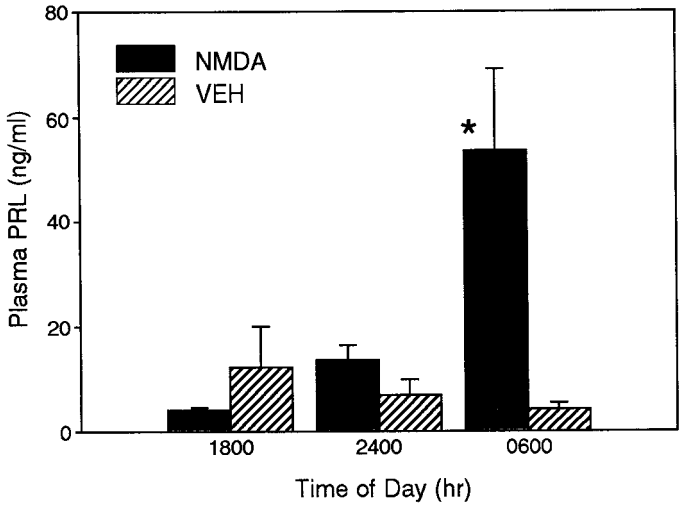

Figure 2. Plasma PRL (in nanograms per milliliter) concentrations at the times of the diurnal surge (6:00 P.M.), the intersurge period (12:00 A.M.), and the nocturnal surge (6:00 A.M.) in animals receiving bilateral infusions of NMDA (*filled bars*; $n = 7$) or VEH (*hatched bars*; $n = 5$) into the MEA 6–7 d previously. Values are mean ± SEM. * indicates significantly higher levels compared with VEH controls ($p \leq 0.03$).

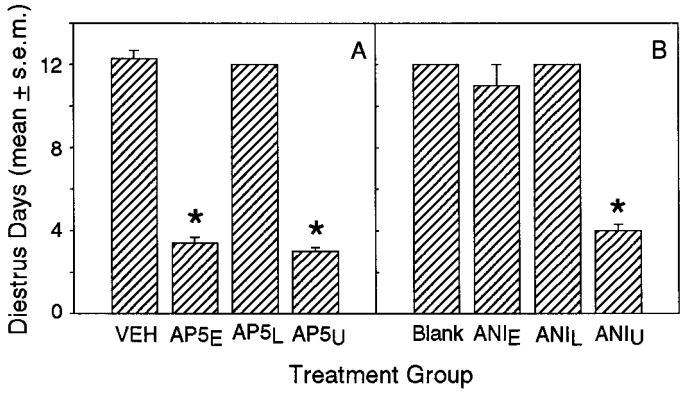

Figure 3. Number of diestrus days (mean ± SEM) observed after mating in groups receiving bilateral infusions of AP-5 (*A*) beginning either before ($AP\text{-}5_E$; $n = 8$) or after ($AP\text{-}5_L$; $n = 4$) mating, or bilateral implants of ANI (*B*) beginning before (ANI_E; $n = 9$) or after (ANI_L; $n = 7$) mating. Control groups received either VEH infusions ($n = 6$) or blank implants ($n = 7$) before mating or received AP-5 ($n = 7$) or ANI ($n = 5$) without mating. Animals that were pregnant rather than PSP were assigned a diestrus duration of 12 d. * indicates significant difference from VEH or blank controls ($p \leq 0.001$).

interaction ($F_{(2,20)} = 6.85$; $p \leq 0.005$). A statistically significant elevation of PRL levels occurred in the NMDA-infused animals compared with the VEH-infused animals at 6:00 A.M., indicating the induction of nocturnal PRL surge release by this glutamate agonist. Diurnal surges were not observed, as is consistent with the reported inhibition of this surge in animals bearing intraatrial catheters (Freeman et al., 1974; Polston and Erskine, 2001). Because the nocturnal PRL surge has been demonstrated to be primarily responsible for maintenance of P/PSP (Gala and Haisenleder, 1984) and is observed physiologically only in females that have received pelvic nerve stimulation through VCS (Spies and Niswender, 1971; Kornberg and Erskine, 1994), the presence of the nocturnal surge 6–7 d subsequent to NMDA infusion confirms that pharmacological stimulation of the MEA initiated central events that are associated specifically with the processing of the VCS stimulus.

Effect of NMDA receptor blockade and protein synthesis inhibition within the MEA on initiation of P/PSP by mating

The effects of local treatment with AP-5 and ANI on the induction of P/PSP are presented in Figure 3. Bilateral infusion of AP-5 15 min before mating and 75 min after mating [early group ($AP\text{-}5_E$); $n = 8$] completely blocked the induction of P/PSP by mating in all animals, whereas AP-5 given at +0 and +90 min after mating onset [late group ($AP\text{-}5_L$); $n = 4$] failed to prevent P/PSP induction. All mated control females infused with VEH and none of the unmated animals treated with AP-5 ($AP\text{-}5_U$) became P/PSP. As shown in Figure 3*A*, significant group differences were observed in the number of days of vaginal diestrus observed after treatment ($F_{(4,24)} = 182.51$; $p \leq 0.001$). The mean number of consecutive daily diestrous smears was significantly higher among the $AP\text{-}5_L$ and VEH animals than among the $AP\text{-}5_E$ and $AP\text{-}5_U$ animals ($p \leq 0.001$).

A high proportion (88.9%) of animals treated with ANI bilaterally for 3 hr beginning 1.5 hr before mating [early group (ANI_E); $n = 9$] and 100% of animals given ANI beginning 1–4 hr after mating [late group (ANI_L); $n = 7$] became P/PSP. Control animals given 3 hr exposure to ANI without mating (ANI_U; $n = 5$) did not become P/PSP. As shown in Figure 3*B*, significantly lengthened periods of acyclicity were observed after mating in the ANI_E and ANI_L groups but not in the ANI_U group ($F_{(3,24)} = 26.46$; $p \leq 0.001$).

Analysis of the behavioral measures obtained showed that there were no significant differences between drug-treated and control groups in the quantity of VCS received or in the level of sexual receptivity (LQ and LR) exhibited (data not shown). The mating efficacy was equally unaffected as a function of treatment, suggesting that there were no drug-related behavioral effects that might have prevented the female from receiving normal intromissive stimulation.

Effect of NMDA receptor blockade and protein synthesis inhibition on mating-induced cellular activation in sites downstream to the MEApd

Unilateral infusion of AP-5 into the MEA 15 min before mating resulted in significantly lower numbers of FOS-IR cells in the ipsilateral than in the contralateral BSTp ($t = 5.52$; df = 5; $p \leq 0.003$) and VMHvl ($t = 7.79$; df = 4; $p \leq 0.001$) 1.0–1.5 hr after mating (Fig. 4*A*). There was no effect of this treatment on c-*fos* expression within the ipsilateral compared with contralateral MPNm or AVPV. In contrast, local unilateral treatment of the MEA with ANI 1.5 hr before mating did not influence mating-induced FOS expression in any of the four downstream sites examined (Fig. 4*B*), and numbers of FOS-IR cells were comparable on the ipsilateral and contralateral sides in these areas.

DISCUSSION

The present experiments indicate that a specific viscerosensory stimulus, VCS, can cause long-term alterations in brain function through a temporally limited action of glutamate within the MEA. Direct application of NMDA into the MEA mimicked the sensory stimulation received by an estrous female during natural mating, initiating PRL secretory patterns that are seen normally only after receipt of VCS. Moreover, application of the selective NMDA receptor antagonist, AP-5, to this same area before

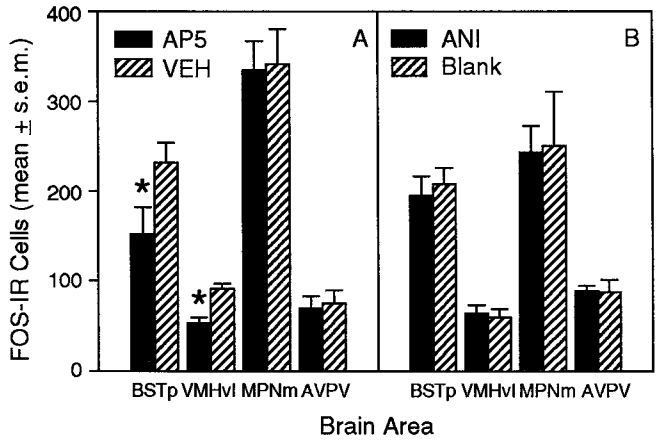

Figure 4. Number of FOS-immunoreactive cells 1.0–1.5 hr after mating in sites downstream to the MEApd in groups of animals receiving unilateral treatment of the MEA with AP-5 (*A, filled bars*) or ANI (*B, filled bars*). Contralateral areas were counted for each animal as a control. Values are mean ± SEM. * indicates significantly lower ($p \leq 0.05$) numbers compared with the untreated side (*hatched bars*). There were three to six animals per group.

mating prevented PRL surges and P/PSP. Previous studies support the hypothesis that activation of the MEA contributes to P/PSP initiation. The MEApd exhibits substantial immediate-early gene responses to both natural and artificial mechanical VCS, and is the single area out of several mating-responsive brain sites in which FOS responses reflect sensitivity to subtle changes in the quantity (Polston and Erskine, 1995) and quality (Erskine and Hanrahan, 1997) of VCS received. Infusion of lidocaine into this area significantly reduced the incidence of P/PSP in mated females (Coopersmith et al., 1996), further verifying that neuronal excitation within the MEA is necessary for P/PSP induction.

The question of whether NMDA acted by agonistic stimulation of excitatory responses or by inducing excitotoxic effects that led to eventual cell death in the MEA is particularly germane to interpretation of these results. Necrotic responses to excitatory amino acid injections are well described, comprising an early phase lasting 1–14 d after infusion and a later stage that is characterized by an infiltration of astrocytic glia cells (Dusart et al., 1991; Marty et al., 1991). In the present experiment, glial coverage was observed in brains taken 16 d after NMDA infusion, and nocturnal PRL surges observed 6–7 d after NMDA infusion were diminished in magnitude, as is characteristic for MEA-lesioned females (Clark and Gala, 1985; Polston and Erskine, 2001). Nevertheless, several lines of evidence suggest that it is the initial excitatory effect of NMDA on MEA neurons that is responsible for the present effects. First, loss of function of glutamate-sensitive cells would be predicted to cause effects similar to those of glutamate antagonists. Contrary to this, NMDA channel agonist and antagonist treatments resulted in opposite neuroendocrine profiles in these studies, with agonist treatments inducing PSP and antagonists preventing P/PSP induction. Second, because $AP\text{-}5_L$ treatment was ineffective in preventing P/PSP, critical NMDA-mediated events in the MEA must be extremely rapid, occurring within the course of the mating session, and thus are unlikely to reflect the signaling transduction processes by which neuronal overstimulation leads to cell death. Lastly, the influences of NMDA on neuroendocrine profile are temporary, lasting approximately as long as a naturally induced PSP (Numan et al., 1993; Polston and Erskine, 2001). Although recent results have demonstrated that the ability of mating to induce P/PSP is regained after long-term MEA lesion (Polston and Erskine 2001), the cessation of estrous cyclicity seen in the present study is unlikely to reflect a temporary loss of function. Rather, the extremely specific nature of nocturnal PRL surge secretion suggests that the observed effects are caused by a temporally isolated, excitatory response to NMDA treatment.

The present experiments required infusion volumes that would influence the entire MEA. However, the selective effects of AP-5 administration to the MEA on FOS expression within the BSTp and VMHvl support the hypothesis that cells within the MEApd, specifically, are responsible for the reported observations. Neuroanatomical studies demonstrate that MEApd efferents alone project directly to the neuroendocrine hypothalamus, innervating the BSTp and the VMHvl as well as the MPNm and AVPV (Krettek and Price, 1978; Canteras et al., 1995; Coolen and Wood, 1998; Wang and Swann, 2000). Although the MEApd is typically associated with pheromonal modulation of reproductive function, a subpopulation of MEApd cells has been described that is sensitive to VCS but not vomeronasal cues (Rowe and Erskine, 1993). On the basis of the ability of AP-5 infusions in the MEA to block the mating-induced FOS responses seen normally within the BSTp and VMHvl, we propose that excitation of these MEApd cells by glutamate initiates a series of events within a larger neuroendocrine circuit (Simerly, 1995). The BSTp appears to play an important role in the amygdalar–hypothalamic communication of VCS, and BSTp neurons that are stimulated by VCS-responsive cells in the MEApd may act as a site of convergence for pheromonal and VCS inputs. In turn, both the MEApd and the BSTp send projections to the MPNm and AVPV (Simerly, 1995). However, MEA treatments that suppressed FOS expression in the BSTp did not influence mating-induced FOS expression in the MPNm or AVPV, areas thought to be involved in regulating PRL secretory responses (Erskine, 1995; Gu and Simerly, 1997). The role of the VMHvl in the processing of VCS-derived information is less clear, but results from early studies using lesions of the dorsomedial and ventromedial hypothalamic nuclei suggest that the VMH may be involved in the expression of PRL surges and PSP (Gunnet et al., 1981).

The expression of daily PRL surges after mating requires that long-term changes occur in brain, retaining the imprint of VCS for many days. Therefore, it is possible that cellular mechanisms similar to those involved in the retention of explicit memory may play a role in this model. NMDA channel function has been implicated consistently in amygdalar long-term potentiation (LTP), as well as in amygdala-mediated learning paradigms (Yamamoto et al., 1998; Maren, 1999), and NMDA-dependent LTP has been demonstrated electrophysiologically within medial amygdalar circuits (Gean et al., 1993; Shindou et al., 1993). In the MEA, these processes of potentiation may be facilitated further by high levels of circulating estrogens, such as are seen at the time of mating (Schiess et al., 1988). However, the failure of crystalline ANI implants to prevent PRL surges in the present study suggests that the establishment of LTP in the amygdala is not required for the expression of the long-term VCS mnemonic, because LTP has been shown consistently to require *de novo* protein synthesis (Huang et al., 2000). Rather, the data suggest that the amygdala is involved in the initial transduction of VCS, rapidly processing sensory information during the course of the mating session. Because protein synthesis in the MEA does not influence P/PSP induction, other downstream brain sites, such as the BSTp, are

likely to be involved in the long-term storage and subsequent expression of the mating mnemonic. However, whether the amygdala acts solely to relay VCS to the neuroendocrine hypothalamus or is directly involved in initial stages of mnemonic acquisition remains undetermined.

Central to the question of how VCS is processed in the brain are the substantial data which demonstrate that a threshold amount of VCS is required for P/PSP induction. Approximately 10–15 intromissions from males are required to initiate PRL surges (Terkel and Sawyer, 1978; Polston and Erskine, 2001). The inability of lower numbers of intromissions to induce P/PSP suggests that multiple VCS must be processed additively in the CNS, and we have hypothesized that a second, short-term mnemonic process is responsible for accumulating information from a series of VCS toward the threshold for PSP induction. The MEApd may be involved in this summation process, because FOS-IR in the MEApd increases in a graded fashion with increasing amounts of VCS (Polston and Erskine, 1995) and, when females self-regulate the rate at which VCS is received, a treatment facilitatory to PSP induction (Erskine et al., 1989), c-*fos* expression is enhanced only in this area (Erskine and Hanrahan, 1997). Although the single, bolus NMDA infusion used in the present study was likely to be sufficiently strong and/or persistent to excite MEA neurons to a threshold comparable with that of multiple intromissive stimuli, the effects of AP-5_E treatments are consistent with the possibility that summation of multiple VCS occurs within the MEA. This hypothesis is strengthened further by our recent findings that three temporally spaced intra-amygdalar infusions of NMDA at a subthreshold dose can initiate P/PSP (Lehmann and Erskine, 2000).

The central mechanisms by which somatosensory stimuli modulate the formation of memories are not well understood. Typically, these questions are investigated using classical associative and behavioral methodologies, which are confounded by multimodal sensory inputs and complex cognitive responses. The mating-induced neuroendocrine mnemonic in the rat offers a novel model in which a defined sensory input leads to long-term, measurable changes in brain function. Using this model, these studies demonstrate that activation of NMDA channels within the MEA is both necessary and sufficient for the establishment of long-term, VCS-mediated neuroendocrine responses, and moreover, that the MEA is involved specifically in the early sensory transduction processes that contribute to mnemonic acquisition. Furthermore, we demonstrate that mnemonic formation may be dependent selectively on activation of the BSTp and/or VMHvl after glutamatergic excitation of the MEApd. Because PSP induction requires only pelvic nerve stimulation and is expressed as a unique neuroendocrine profile, the multiple-intromission mating paradigm in the rat provides a simple, neuroethological approach to the study of memory in mammalian brain.

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