Cortical-Like Functional Organization of the Pheromone-Processing Circuits in the Medial Amygdala

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1Institute of Biophysics, Chinese Academy of Sciences; 2National Institute of Biological Sciences, Beijing, China; 3Department of Genetic and Behavioral Neuroscience, Gunma University Graduate School of Medicine and Solution Oriented Research for Science and Technology, Japan Science and Technology Corporation, Maebashi, Japan; and 4Department of Neurobiology, Yale University Medical Center, New Haven, Connecticut

Submitted 13 August 2007; accepted in final form 25 October 2007

Bian X, Yanagawa Y, Chen WR, Luo M. Cortical-like functional organization of the pheromone-processing circuits in the medial amygdala. J Neurophysiol 99: 77–86, 2008. First published October 31, 2007; doi:10.1152/jn.00902.2007. The medial amygdala (MeA) is a critical center for processing pheromonal signals that regulate social and reproductive behaviors, but the fundamental cellular mechanisms underlying signal processing in the MeA have remained largely unknown. Some studies suggest that the MeA belongs to the striatum and provides inhibitory output to hypothalamic areas including the ventromedial hypothalamus (VMH). By combining tract tracing, genetic labeling of GABAergic neurons, and immunostaining against VGLUT2, a marker of glutamatergic projection. We then performed de-termined whether the MeA

INTRODUCTION

Many mammals crucially rely on pheromones—a set of body odorants emanated by other individuals of the same species—for social communication (reviewed in Dulac and Torello 2003). Disrupting either the main olfactory system or the accessory olfactory system results in dramatic deficits in social and reproductive behavior (Del Punta et al. 2002; Ley-pold et al. 2002; Powers and Winans 1975; Stowers et al. 2002). The medial amygdala (MeA) is strategically positioned for processing pheromonal signals. It receives direct input from the accessory olfactory bulb (AOB) and indirect input from the main olfactory bulb (MOB) (Brennan and Zufall 2006; Dulac and Torello 2003; Krettek and Price 1978; Scalia and Winans 1975; von Campenhausen and Mori 2000). Its output targets several major hypothalamic nuclei essential for social and reproductive behavior (Keverter and Winans 1981), one of which is the ventromedial hypothalamus (VMH) (Canteras et al. 1995). Pheromonal stimuli activate MeA neurons (Fernandez-Fewell and Meredith 1994; Kollack and Newman 1992), and MeA inactivation results in deficits in mating and chemoinvestigative behavior (Kondo 1992; Lehman et al. 1980) as well as social memory formation (Ferguson et al. 2001).

Despite its importance, the cellular circuitry underlying the signal processing in the MeA remains largely unknown. Comparative neuroanatomical studies suggest that the MeA belongs to the ventral striatum (Swanson 2000; Swanson and Petrovich 1998). It is located near classical striatal areas including the caudate-putamen. Resembling the striatum, it is densely popu-lated by neurons synthesizing the neurotransmitter γ-aminobutyric acid (GABA) (Choi et al. 2005; Swanson and Petrovich 1998). Among primary projections out of the MeA, the MeA → VMH pathway is best characterized to date. The projection is roughly topographic; the dorsoposterior MeA (MePD) projects to the VMH ventrolateral part (VMHvl), a region important for mating, whereas the ventroposterior MeA (MePV) projects to both the VMHvl and VMH dorsomedial part (VMHdm), a region important for agonistic behavior (Canteras et al. 1995; Choi et al. 2005). Thus it is alternatively possible that one MeA subdivision, i.e., the MePD, uses striatal machinery, whereas other subdivisions do not.

In this study, we tested whether the circuit for processing pheromonal signals in the MeA possesses striatum-like characteristics. Striatal neurons have several key features that are conserved in both mammals and birds. First, medium spiny neurons (MSNs)—the principal neurons of the striatum—are GABAergic and inhibitory (Kita 1993; Luo and Perkel 1999). Second, MSNs have unique intrinsic physiological properties and highly spiny and stellate dendritic trees (Farries and Perkel 2002; Nisenbaum et al. 1994; Wilson et al. 1983). We examined whether the MeA → VMH projection is GABAergic or glutamatergic by combining tract tracing, genetic labeling of GABAergic neurons, and immunostaining against VGLUT2, a marker of glutamatergic projection. We then performed de-tailed analysis of the intrinsic physiological properties of VMH-projecting neurons in the MeA by whole cell patch-clamp recordings from brain slices of adult male mice. At the same time we filled recorded neurons with neurobiotin and

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examined the morphology of these neurons. Our data demonstrate that the VMH-projecting neurons in the MeA are distinct from striatal neurons but resemble a subset of pyramidal neurons in deep-layer piriform cortex in terms of neurotransmitter type, intrinsic physiological properties, and morphology. We suggest that, rather than being a striatal structure, the MeA processes pheromonal signals using mainly cortical circuitry.

METHODS

Animals and tissue preparation

All procedures subsequently described followed institutional guidelines for animal care and use of the National Institute of Biological Sciences (Beijing, China). The GABAergic neurons were identified with green fluorescent protein (GFP)+ cells in heterozygous glutamate decarboxylase (GAD) 67–GFP (Δneo) mice, in which GFP is selectively expressed under the control of the endogenous GAD67 gene promoter (Tamamaki et al. 2003). These knock-in mice were referred to as GAD67–GFP mice for simplicity. Adult male mice (GAD67–GFP mice or C57BL/6J wild-type, 6–11 wk old) were deeply anesthetized with sodium pentobarbital (250 mg/kg) and decapitated. The brains were quickly removed and placed into ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM): 110 choline chloride, 2.5 KCl, 0.5 CaCl2, 7 MgCl2, 1.3 NaH2PO4, 25 NaHCO3, 25 glucose, 1.3 sodium L-ascorbate, and 0.6 sodium pyruvate, equilibrated with 95% O2-5% CO2. coronal slices (300 μm) were cut using a vibratome (Leica VT1000S) and incubated for ≥1 h at 34°C with aCSF containing (in mM) 125 NaCl, 2.5 KCl, 2.0 CaCl2, 1.3 MgCl2, 1.3 NaH2PO4, 25 NaHCO3, 10 glucose, 1.3 sodium L-ascorbate, and 0.6 sodium pyruvate. They were then transferred to a recording chamber on an upright fluorescent microscope equipped with differential interference contrast optics (DIC; Olympus BX51WI).

Slice recording

Slices were submerged and superfused with aCSF at 2 ml/min at 22–25°C. Whole cell patch recordings were routinely achieved from MeA neurons visualized either by DIC of fluorescence. Patch pipettes (3–5 MΩ) contained (in mM) 130 K-gluconate, 10 HEPES, 0.6 EGTA, 5 KCl, 3 Na2ATP, 0.3 Na3GTP, 4 MgCl2, and 10 Na2phosphocreatine (pH 7.2–7.4). For recording N-methyl-D-aspartate (NMDA) components, the pipette internal solution contained 125 CsOH, 8 CsCl, 125 gluconic acid, 10 HEPES, 0.2 EGTA, 10 tetraethylammonium chloride, 4 MgATP, 0.3 Na3GTP, and 10 Na2phosphocreatine (pH 7.2–7.3). Stock solutions of 6-cyano-7-nitroquinolxaline-2,3-dione (CNQX) were made in DMSO. All drugs were added to the superfusion medium by dilution of a stock solution. To evoke synaptic activity, afferents were activated with a single electric shock (100 μs; 50–200 μA) with a bipolar tungsten stimulating electrode (1 MΩ; Microprobes) placed within the AOB axonal pathway in the superficial layer of the MeA. Voltage-clamp and current-clamp recordings were carried out with a computer-controlled amplifier (Multiclamp 700B, Molecular Devices) and traces were low-pass filtered at 2.6 kHz and digitized at 10 kHz (DigiData 1322A, Molecular Devices). Data were collected and analyzed using Clampfit or Clampex 9.0 software (Molecular Devices). The values of membrane potential were reported after adjustment of the pipette junction potential. Summary data are presented as means ± SE. In some figures the stimulation artifacts were clipped.

Histology

Neurons were filled with neurobiotin (0.25%; Vector Laboratory) included in the internal solution. After recordings, slices were fixed with 4% paraformaldehyde. Filled cells were visualized using Cy3- or Cy2-conjugated streptavidin in 0.3% Triton in 0.1 M phosphate-buffered saline (PBS), and images were acquired using a laser-scanning confocal microscope (Zeiss 510 Meta).

For tract tracing, mice were anesthetized with sodium pentobarbital (80 mg/kg) and then mounted in a stereotoxic apparatus. For retrograde tracing, tetramethylrhodamine dextran amines (TMR-DA, 5%, 3 kDa; Invitrogen) or biotinylated dextran amines (5%, 3 kDa; Invitrogen) were iontophoresically (5–15 μA, 7 s on/7 s off, 10–40 min) injected into hypothalamic nuclei according to predetermined stereotoxic coordinates. Texas Red–conjugated dextran amines (TR-DA, 5%, 10 kDa; Invitrogen) were used for anterograde tracing. After a survival time of 7–11 days, animals were overdosed with sodium pentobarbital (250 mg/kg) and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 M PB. Brains were cryoprotected with 30% sucrose in 0.1 M PB and sectioned in the coronal plane (20–60 μm thick) using a cryostat (Leica CM1900). For anti-VGLUT2 immunostaining, we used an anti-VGLUT2 primary antibody made in guinea pig (1:3,000; 60-h incubation at 4°C; Chemicon), followed by a biotinylated donkey anti-guinea pig IgG secondary antibody (1:500; overnight at 4°C; Jackson ImmunoResearch). We used Cy2-streptavidin for visualization (1:500; overnight at 4°C; Jackson ImmunoResearch). For anti-GAD immunostaining, the primary antibody was a rabbit anti-GAD65/67 (1:1,000; Chemicon), followed by Cy2-goat anti-rabbit secondary antibody (1:500; Jackson ImmunoResearch). Sections were then mounted in medium containing 50% glycerol and DAPI, coverslipped, and sealed with nail polish.

We delineated the borders of the MeA and VMH as well as their subnuclei by labeling of cell nuclei with DAPI and referring to an established mouse brain atlas (Franklin and Paxinos 1997).

RESULTS

A vast majority of the VMH-projecting neurons were glutamatergic.

Because the MeA has been hypothesized to be part of the striatum and the striatal projection neurons use GABA as a neurotransmitter, we first examined whether the VMH-projecting neurons in the MeA used GABA as a neurotransmitter. GABA is synthesized by two glutamate decarboxylase (GAD) isoforms, GAD67 and GAD65 (Kaufman et al. 1991), and both isoforms are present in most GABAergic neurons (Esclapez et al. 1993, 1994). We observed dense distribution of GFP+ neurons in all areas of the MeA of GAD67–GFP mice, although the density was higher in the MePD (Fig. 1A). Our immunostaining against GAD65/67 confirmed that GFP labeling in somata overlapped with GAD65/67 immunoreactivity in the MeA (data not shown). Henceforth, we describe the GFP+ neurons in the MeA of GAD67–GFP mice as GAD+.

We carried out tract tracing in the GAD67–GFP mice to test whether the VMH-projecting neurons in the MeA were GABAergic. To label the VMH-projecting neurons in the MeA, we deposited the neuronal tract tracer biotin-conjugated dextran amines (BDAs) into the VMH, one of the major targets of the MeA (Canteras et al. 1995; Kevetter and Winans 1981). After tracer deposit into the VMH, we examined with confocal microscopy whether the retrogradely labeled neurons in the MeA, including both MePV and MeD, were GAD+ (Fig. 1, D–J). Tracer deposits into the VMHdnn retrogradely labeled neurons mainly in the MePV (Fig. 1, B and C) (Canteras et al. 1995; Choi et al. 2005). GFP labeling was absent in all of the
VMH-projecting neurons in the MePV, indicating that the VMH-projecting neurons in the MePV are non-GABAergic (Fig. 1D; n = 181 cells examined in five mice). Tracer deposit into the VMHc and VMHvl retrogradely labeled neurons both in the MePD and the MePV (Fig. 1, E and F) (Canteras et al. 1995; Choi et al. 2005). All of these retrogradely labeled neurons in the MePV lacked GFP labeling, again suggesting that they were non-GABAergic (Fig. 1, G and H). In the MePD, we found that the majority of retrogradely labeled neurons in the medial part were not GAD+ (Fig. 1, I and J; n = 98/128 examined or 76.5% in six mice). In contrast, a vast majority of the VMH-projecting neurons in the more lateral part of the MePD were GAD+ (Fig. 1, I and J; n = 51/63 cells or 90% examined in six mice). In the anterior MeA, a vast majority of VMH-projecting neurons were non-GABAergic (n = 98/114 cells or ~86% examined in three mice).

We next examined whether the VMH-projecting neurons within the MePD were glutamatergic by combining tracer injection into the MePD and immunostaining for VGLUT2, a marker of glutamatergic synapses (n = 8 mice) (Choi et al. 2005).
Deposit of TR-DA into the medial MePD of C57BL/6J mice anterogradely labeled terminals in the VMH (Fig. 2A), a majority of which were positive for VGLUT2 immunoreactivity in their boutons (Fig. 2B; 38/53 terminals examined). This suggests that a majority of the VMH-projecting neurons within the medial MePD are glutamatergic. Tracer injections into the central and lateral MePD (Fig. 2C) anterogradely labeled terminals in the VMH (Fig. 2D), some of which were GAD65/67+ as revealed by GAD65/67 immunostaining and some of which were VGLUT2+ (Fig. 2E). Together, our results indicate that all of the VMH-projecting neurons in the MePV and most of those in the medial MePD are non-GABAergic and mostly likely glutamatergic, whereas most in the lateral MePD are GABAergic. These results argue against the proposals that either the entire MeA or MePD belongs to striatum (Simerly 2002; Swanson 2000; Swanson and Petrovich 1998). They instead suggest that the pheromonal signals are sent from the MeA to the VMH mainly using glutamatergic but not GABAergic projections.

Physiological and morphological properties of the VMH-projecting neurons

Next, we tested whether VMH-projecting neurons in the MeA possess intrinsic membrane properties resembling those of the striatal MSNs, the principal neurons of the striatum that project to the globus pallidus. TMR-DA tracers were injected into the VMH of C57BL/6J mice to retrogradely label neurons in the MeA. We then prepared acute slices of the MeA and targeted TMR-DA–labeled neurons for whole cell patch recordings (Fig. 3, A and B). Recorded neurons were filled with neurobiotin and then stained with a different-color fluorophore to verify double labeling with the retrograde tracers (Fig. 3C). A total of 40 projection neurons were recorded, among which 12 were located in the MePV and 28 in the MePD (Fig. 3D).

The VMH-projecting neurons in both the MePD and the MePV were highly homogeneous in terms of both membrane properties and morphological features. These cells lacked spontaneous firing at resting membrane potential of $-74.6 \pm 0.7$ mV (means ± SE). Their input resistance at rest was $489 \pm 52$ MΩ.

Fig. 2. Combination of immunostaining against VGLUT2 and GAD65/67 and anterograde tracing indicates that a vast majority of VMH-projecting neurons in the MeA are glutamatergic, whereas a subset of VMH-projecting neurons in lateral MePD are GABAergic. A: tracer injection into the medial MePD (inset) anterogradely labeled terminals within the VMH. Arrows and numbers point to the boutons with high-power views shown in B1–B6. B: a vast majority of those terminals were VGLUT2+ in their boutons, indicating that the medial MePD → VMH projection was glutamatergic. Numbered pictures represent a zoom-in view of the anterogradely labeled boutons (red) and VGLUT2 immunoreactivity within the same sections (green) in A. C: tracer injection into the MePD. D: anterogradely labeled terminals in the VMH. E: dual labeling with tracing (red) and GAD immunostaining (green) revealed that only some of the axon boutons are GAD+ ($E_1$, but not $E_2$). Scale bars: A, D = 10 μm; B1–B6 and $E_1$, $E_2$ = 2 μm; C = 500 μm.
in Fig. 3, inward current when recorded in voltage-clamp mode (arrows) larizing sags when recorded in current-clamp mode and a slow dent inward rectifier was always activated, resulting in depo-
adaptation when membrane potentials were near (50 –100 pA) typically evoked regular firing patterns with clear
/G 3
hyperpolarization. (Note the time-dependent inward rectification during hyperpolarization (arrow) and postinhibitory rebound spikes after the termination of
injections (bottom).) The time-dependent inward rectification during hyperpolarization was mediated by low-threshold Ca2+
spikes. The threshold of these Ca2+
spikes was −65.9 ± 1.0 mV and the amplitude was about 16.8 ± 1.3 mV (range = 6.0–30.0; n = 29). Thus it is highly
likely that the broad and small action potentials were produced by the low-threshold T-type Ca currents (IT) (Deschenes et al. 1982; Llinás 1988; Luo and Perkel 1999). Unlike the fast action potentials, the broad action potentials were resistant to the application of tetrodo-
toxin (TTX, 1 μM, n = 8; Fig. 4B) but were eliminated in the presence of NiCl2 (200 μM, n = 5 of 5 tested; Fig. 4C), suggesting that they were Ca2+
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The time-dependent inward rectifier observed in the VMH-projection neurons shared many properties of hyperpolarization-activated cation current (Ih) (McCormick and Pape 1990;Pape 1996). In voltage-clamp mode, hyperpolarizing potentials activated slow inward currents with median activation time constant of 568.9 ms (range = 151–1,351) at −100 mV (Fig. 4D). Application of cesium (1 mM CsCl) blocked this slow inward current, and the blockade was reversed by washing in

28 MΩ (means ± SE, range = 247–780) at room temperature (22–25°C). When hyperpolarized to −100 mV, a time-depen-
dent inward rectifier was always activated, resulting in depo-
larizing sags when recorded in current-clamp mode and a slow inward current when recorded in voltage-clamp mode (arrows in Fig. 3, E and F). A small amount of depolarizing current (50–100 pA) typically evoked regular firing patterns with clear adaptation when membrane potentials were near −70 mV (Fig. 3G; half-spike duration for initial spikes = 1.9 ± 0.1 ms). When their membrane potentials were hyperpolarized by nega-
tive holding currents, depolarizing current of same amplitudes evoked a burst of two to four fast action potentials at the frequency of about 100 Hz (Fig. 3H). This burst always rode on a slow-depolarizing envelope with a duration at half-maximal amplitude of ≈25 ms.

We examined pharmacologically whether the broad depolarizing envelope was mediated by low-threshold Ca2+
currents (Fig. 4, A–C) (Deschenes et al. 1982; Llinás 1988; Luo and Perkel 1999). Unlike the fast action potentials, the broad action potentials were resistant to the application of tetrodo-
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FIG. 3. Intrinsic properties of the VMH-projecting neurons in the MeA. A: schematic diagram illustrating our method of recording from the MeA neurons that projected to the VMH. MeA projection neurons were retrogradely labeled from tetramethylrhodamine dextran amine (TMR-DA) injection into the VMH. They were then recorded with whole cell patch clamp in a brain slice preparation. B: retrogradely labeled somata in the MePV after tracer injection into the VMH. C: a cell was visualized by retrograde labeling (left) and filled with neurobiotin (right), confirming recordings from the projection neurons. D: spatial distribution of VMH-projecting neurons recorded from the MeA containing both the MePV and the MePD. E: a recorded cell's voltage response to a family of current injections (bottom). Note the time-dependent inward rectification during hyperpolarization (arrow) and postinhibitory rebound spikes after the termination of hyperpolarization. F: whole cell currents recorded in voltage-clamp mode. Bottom shows voltage commands. G: a cell exhibited tonic activity patterns with adaptation in response to the depolarizing current injection of 0.1 nA when its membrane potential was −70 mV. H: when hyperpolarized to −90 mV, the same cell as in G showed initial bursting behavior in response to the same amount of depolarizing current injection. Scale bars: B = 200 μm; C = 10 μm; horizontal bar in E–H: 500 ms; vertical bars in E = 20 mV and 80 pA; F = 40 pA and 20 mV; G, H = 20 mV.
regular aCSF (cesium 7.0 ± 3.0%, n = 8; wash 51.5 ± 18.9%, n = 4; Fig. 4, E–G). Thus the inward rectification shown in the current-clamp mode resulted from the action of $I_h$.

These two prominent features, the $I_T$ and $I_h$, distinguished the VMH-projecting neurons from striatal MSNs, which lack $I_T$ or $I_h$. Striatal MSNs exhibit delayed firing when injected with depolarizing current and respond with fast inward rectification in response to hyperpolarizing current pulses (Bargas et al. 1989; Farries and Perkel 2002; Gabel and Nisenbaum 1998; Nisenbaum et al. 1994). The presence of $I_T$ and $I_h$ suggests that MeA projection neurons resemble the pyramidal cells from the deep layer of piriform cortex (Tseng and Haberly 1989b) and low-threshold bursting-type pyramidal cells from layer IV of neocortex (Schubert et al. 2003). They were also reminiscent of thalamocortical (TC) neurons. In the TC neurons, $I_T$ and $I_h$ interact so that cells burst spontaneously at the delta frequency (0.5–4 Hz) when hyperpolarized (Luthi and McCormick 1998; McCormick and Pape 1990). None of the MeA neurons exhibited spontaneous bursting at delta frequency at the membrane potentials between −70 and −90 mV, likely because of the smaller amplitudes of their $I_h$ and $I_T$.

We then examined whether the morphology of VMH-projecting neurons in the MeA resembled that of striatal projection neurons. Specifically we examined whether they possess a stellate dendritic tree and highly spiny dendrites. In contrast to the striatal MSNs (Wilson et al. 1983), the VMH-projecting neurons in the MeA had simpler dendritic trees (Fig. 5; n = 15 cells). Resembling the deep-layer pyramidal cells in the piriform cortex, they typically had one to three major dendrites extending toward the superficial layer of the MeA (Tseng and Haberly 1989a). The dendrites bifurcated multiple times in the external layer of the MeA, where the axons of AOB mitral cells terminated (dashed line in Fig. 5A). Unlike the pyramidal cells in the piriform cortex, they tended to have one to three primary dendrites extending toward opposite directions (Fig. 5A). Many of these projection neurons (n = 9/15 cells) extended their dendrites into the basomedial amygdala or the central amygdala. Spines with various shapes were frequently observed on the dendrites (Fig. 5B). Axon collaterals with boutons were also observed surrounding somata, suggesting that projection neurons could contribute to local signal processing (arrows in Fig. 5A).

![Image](http://jn.physiology.org/)

**Fig. 4.** VMH-projecting neurons in the MeA contained both low-threshold T-type Ca current ($I_T$) and hyperpolarization-activated cation current ($I_h$). A: when a projection neuron was hyperpolarized to −90 mV, depolarizing current injection of 60 pA evoked a broad action potential with a burst of fast spikes riding on top. $B$: broad action potential was resistant to tetrodotoxin (TTX) application. C: TTX-resistant action potential was blocked by Ni$^{2+}$, suggesting that the broad action potential was a low-threshold calcium spike. $D$: hyperpolarization activated a slow inward current. $E$ and $F$: slow inward rectification was blocked by cesium (E) and the blockade was reversed by wash (F), indicating $I_h$. $G$: quantification of the effect of cesium application on $I_h$ amplitudes. Horizontal scale bars in $A$–$F$ = 500 ms; vertical bar in $A$–$C$ = 20 mV, $D$–$F$ = 20 pA and 40 mV.

**Fig. 5.** Morphology of VMH-projecting neurons in the MeA resembled that of the pyramidal cells in the piriform cortex but not that of the medium spiny neurons (MSNs). $A$: morphology of the same cell as in Fig. 1C. Arrows indicate a few axon collaterals with boutons near the soma. Dashed line indicates the border of the MeA external layer. $B$: high-power view showing modest number of spines on the dendrites within the dashed box in $A$. Scale bars: $A$ = 100 μm; $B$ = 5 μm.
Because a subset of VMH-projecting neurons in the lateral MePD are GABAergic, we tested whether these neurons might have intrinsic properties resembling those of striatal MSNs. We recorded six neurons in the lateral MePD that were both GAD\^+ and retrogradely labeled after TMR-DA injection into the VMH of GAD67–GFP mice (Fig. 6A). Interestingly, their intrinsic properties resembled those of non-GABAergic projection neurons in other areas of the MeA. Their input resistance was 496 ± 64 M\(\Omega\), resting membrane potential was 71.4 ± 2.6 mV, and spike duration at half-maximal amplitude was 2.2 ± 0.3 ms. They exhibited time-dependent inward rectification when hyperpolarized (Fig. 6, B and C), indicating the presence of \(I_h\). After the termination of hyperpolarizing-current injection, these neurons exhibited postinhibitory rebound composed of fast action potentials riding on a slow-depolarizing envelope (Fig. 6B), suggesting the presence of \(I_F\). Their morphology also resembled that of the non-GABAergic MeA projection neurons. They typically had one to two proximal dendrites extending into the superficial layer of the MeA and another one to two proximal dendrites extending into the lateral and dorsal part of the MeA as well as the central amygdala (Fig. 6D\(_1\); \(n = 3\) cells). Their proximal dendrites tended to have more spines than distal dendrites (Fig. 6, \(D_2\) and \(D_3\)).

**Synaptic inputs to VMH-projecting neurons**

Because a majority of pheromonal signals are propagated to the MeA from the AOB, we examined the properties of synapses between these two structures. We recorded the synaptic responses of MeA neurons after the stimulation of afferent fibers within the MeA external layer because AOB mitral cells terminate their axons in this layer (Fig. 7A) (von Campenhagen and Mori 2000). At rest, single-shock stimulation of the afferent fibers typically led to inward currents of short latency (4.06 ± 0.14 ms, \(n = 26\); Fig. 7). In many cases, the postsynaptic currents consisted of initial inward currents followed by outward currents when the cells were held at about −60 mV (\(n = 9/26\)). The late components reversed sign with voltage command at around the reversal potential of GABA\(_A\)-receptor–mediated responses (−67.4 ± 2.7 mV; \(n = 8\); Fig. 7B\(_1\)). The GABA\(_A\) contribution to the late component was confirmed by the blockade of outward currents with bicuculline methiodide.

**FIG. 6.** Intrinsic properties and morphology of the GABAergic VMH-projecting neurons in the lateral MePD. \(A_1\): a retrogradely labeled soma (left) is GAD–GFP+ (right) in lateral MePD. \(A_2\): distribution of whole cell recorded MeA neurons projecting to the VMH. \(B\): change of membrane potentials after current injections. Note the presence of time-dependent inward rectification during hyperpolarization. \(C\): whole cell currents recorded in voltage-clamp mode. \(D\): morphology of the cell shown in \(B\) and \(C\). \(D_2\): high-power view of a proximal dendrite. \(D_3\): distal dendrite in the MeA external layer. Scale bars: \(B = 40\) mV, 0.5 s; \(C = 50\) pA; \(D_1 = 100\) \(\mu\)m; \(D_2\) and \(D_3 = 10\) \(\mu\)m.
Application of the \(-\)amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)–type glutamate receptor antagonist CNQX (10 \(\mu\)M) completely blocked the inward currents isolated by BMI (Fig. 7B2). This suggested that the VMH-projecting neurons received a strong and likely monosynaptic glutamatergic input from the AOB, and this input was mainly mediated by AMPA-type glutamate receptors. In many cases of synaptic currents of initial inward and later outward components (Fig. 7B3), application of CNQX (10 \(\mu\)M) largely eliminated the evoked currents (percentage blockade 80 \pm 6\%, \(n = 8\); holding voltage = –100 mV), suggesting that the VMH-projecting neurons in the MeA received a polysynaptic inhibition from the AOB.

In addition to AMPA-receptor–mediated excitatory responses, slow excitatory postsynaptic currents (EPSCs) were also observed when cells were held at +50 mV (Fig. 7C; \(n = 5\) cells). These slow EPSCs were resistant to the blockade of GABA\(_A\) components with BMI application but were reversibly blocked by NMDA-receptor antagonist APV (50 \(\mu\)M; Fig. 7C), suggesting that the VMH-projecting neurons in the MeA use both AMPA- and NMDA-type glutamate receptors for propagation of pheromonal signals from the AOB.

Because VMH-projecting neurons in the MeA are largely non-GABAergic but dense GAD neurons were present in the MeA, GABAergic neurons likely play an important role in local processing. To test whether the AOB could excite local GABAergic neurons, we carried out targeted recording of 10 GAD neurons from the slice preparations of the GAD–GFP mice in both the MePV and the MePD. In these neurons, a single-shock stimulation of the afferent fibers in the MeA external layer evoked excitatory responses from GABAergic neurons in the MeA. Evoked EPSCs were reversibly blocked by CNQX application (Fig. 7D). Thus the AOB input could excite GABAergic neurons in the MeA and in turn produce polysynaptic inhibition on projection neurons (Fig. 7B1).

**DISCUSSION**

The MeA is situated strategically for processing pheromonal signals and regulating social/sexual behavior in mammals. This
study was undertaken to examine the cellular properties in the MeA that are fundamental to the processing of pheromonal signals and thus critical for regulating social behavior. We find that a vast majority of VMH-projecting neurons in the MeA is glutamatergic, although a minor population of VMH-projecting neurons in the lateral MePD is GABAergic. Moreover, all of the VMH-projecting neurons have characteristic intrinsic properties resembling those of deep-layer projection neurons in the piriform cortex. We also find that VMH-projecting neurons in the MeA receive glutamatergic and excitatory input from the AOB and inhibitory input likely from local GABAergic neurons. Based on our results we conclude that the MeA processes pheromonal signals using a unique cortical mechanism, but not striatal mechanism, as suggested by some earlier studies.

A comparative view of the organization of the MeA

Although the MeA or its subnucleus MePD has been suggested to belong to the striatum (Simerly 2002; Swanson 2000; Swanson and Petrovich 1998), our data suggest mainly cortical, rather than striatal, mechanisms for processing pheromonal signals in the MeA. The MeA neurons projecting to VMH have \( I_T \) and \( I_h \). They lack delayed firing, fast inward rectification, and stellate dendritic morphology, which are the hallmarks of striatal MSNs (Bargas et al. 1989; Farries and Perkel 2002; Gabel and Nisenbaum 1998; Nisenbaum et al. 1994; Wilson et al. 1983). Furthermore, a majority of MeA projection neurons are most likely glutamatergic. Thus the VMH-projecting neurons are distinct from striatal neurons in membrane properties, morphology, and neurotransmitter types. In contrast, they resemble neurons in the piriform cortex and a subset of deep-layer neocortical cells in their intrinsic properties (\( I_h \) and \( I_T \)), dendritic morphology, and neurotransmitter phenotype (Schubert et al. 2003; Tseng and Haberly 1989a,b). Both physiological properties and anatomical organization of VMH-projecting neurons in the MeA are compatible with the suggestion that the MeA is organized as an extension of nearby piriform cortex. The MeA is located near the piriform cortex and cortical amygdala. It has been named the “accessory olfactory cortical” region in a tract-tracing study (von Campenhagen and Mori 2000).

Comparative neuroanatomy has contributed substantially to our understanding of neural circuit in the amygdalar nuclei (Swanson 2000; Swanson and Petrovich 1998) as well as other brain structures (Farries and Perkel 2002; Luo and Perkel 1999). This approach leads to the hypothesis that the neural circuit in the central amygdala, a nucleus essential for fear (Hitchcock et al. 1989), mainly contains striatal components (Swanson 2000; Swanson and Petrovich 1998). This hypothesis has been verified by studies of tract tracing, GAD immunostaining, and electrophysiology (Martina et al. 1999). At this moment, our study does not rule out a striatal component within the MeA. In addition to the VMH, the MeA projects to additional brain areas, including the bed nucleus of stria terminalis, frontal cortex, other amygdalar nuclei, and hypothalamic regions other than the VMH (Canteras et al. 1995; Kevetter and Winans 1981). Combined tract-tracing and physiological recordings can be used to test whether projections from the MeA to these target stations are “striatum-like.”

Working hypothesis of MeA signal processing

All of the VMH-projecting neurons in the posterior MeA shared comparable membrane properties and morphology, suggesting that they belong to one homogeneous group. Most of the GABAergic neurons are likely interneurons (X. Bian and M. Luo, unpublished observation). Stimulation of the afferent fibers in MeA external layer evokes CNQX-sensitive EPSCs in the VMH-projecting neurons, suggesting that these neurons receive a direct glutamatergic input, most likely from the AOB. Additionally they receive input from GABAergic interneurons that provide an indirect inhibition by the sensory signals from the AOB and other known MeA input.

When the projection neurons are excited by pheromonal signals, the low-threshold Ca\(^{2+}\) currents and the inward rectifier in these cells can combine to activate the cell and generate bursts of multiple sodium spikes. The bursting activity can ideally serve as an indicator of the presence of other conspecific individuals, triggering either aggression or mating behavior. Neuropeptides may function as additional neurotransmitters in these cells (Smock et al. 1998). As in the gonadotropin-releasing hormone neurons (Kuehl-Kovarik et al. 2002), the bursting activity may facilitate the release of neuropeptides in the axon terminal of the VMH-projecting neurons.

Studies using comparative anatomical approaches suggest that the MeA \( \rightarrow \) hypothalamus projections may be GABAergic and inhibitory. The VMH-projecting neurons in the MePV are glutamatergic and non-GABAergic. A majority of VMH-projecting neurons in the medial MePD were GFP-negative in the GAD67–GFP mice, and tracer deposit into the medio MePD anterogradely labels terminals in the VMH that are VGLUT2+. Therefore instead of being GABAergic, the majority of VMH-projecting neurons in the MeA are glutamatergic. Many of the lateral MePD projection neurons are GFP + in the GAD67–GFP mice. An early study indicates that some GABAergic neurons in the MePD receive chemosensory input and project to the VMH (Choi et al. 2005). Although the intrinsic physiological properties of the VMH-projecting neurons appear to be homogeneous, the MePD projection neurons may contain heterogeneous populations in terms of their neurotransmitter phenotype.

Acknowledgments

We thank T. L. Xu for technical advice and assistance and A. Person for critical reading of the manuscript.

Grants

This study was supported by grants from China Ministry of Science and Technology, Natural Science Foundation of China, and Human Frontier Science Program to M. Luo; National Institutes of Health grant to W. R. Chen; and Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labor and Welfare, Japan to Y. Yanagawa.

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