mGluR2 activation of medial amygdala input impairs vomeronasal organ-mediated behavior

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Abstract
The accessory olfactory bulb normally receives chemosensory input from the vomeronasal organ. Input from accessory bulb to medial amygdala for natural pheromone-containing conspecific chemosignals activates both anterior and posterior medial amygdala and elicits or modulates reproductive and social behavior. Here, a non-specific activation of accessory olfactory bulb by infusion of mGluR2 agonist LCCG1 in male hamsters activates immediate-early gene (Fos) expression only in anterior and not posterior medial amygdala. mGluR2 stimulation concurrently with female chemosensory stimulation produces small changes in the normal chemosensory response in medial amygdala but impairs behavior normally driven by the chemosensory input. The distribution of Fos expression, with an increase in anterior but not posterior medial amygdala, is also seen with chemosensory stimulation by chemosignals from other species, socially non-relevant for hamsters, and by artificial electrical stimulation of the vomeronasal organ. We propose that the spatiotemporal pattern of amygdala input is important for eliciting normal species-specific behavior and that artificial and heterospecific stimulation fails to do so because it does not match the required pattern closely enough. Thus, modification of the pattern by addition of non-specific activation from mGluR2 agonist is sufficient to disrupt behavior normally driven by conspecific chemosensory stimulation.

1. Introduction
In male hamsters, natural chemosensory stimulation by biological substances of interest to the animal, such as pheromone-containing secretions from others of the same species, activates the accessory olfactory bulb (AOB) and both anterior (MeA) and posterior (MeP) medial amygdala [1–3]. These are the second and third order areas in the central vomeronasal pathway. Surprisingly, electrical stimulation of the VNO producing equivalent activation of anterior medial amygdala does not strongly activate the posterior medial amygdala [4]. One possible explanation is that electrical stimulation results in a generalized non-specific activation of all types of vomeronasal sensory neurons, producing a spatiotemporal pattern of input that is a “nonsense” pattern and is not recognized by information processing circuits in the AOB or amygdala. In the research reported here, we used a metabotropic glutamate-receptor agonist infused into the accessory olfactory bulb to produce a general activation of the AOB. The method should also provide a non-specific activation of the system but at a different hierarchical level (largely bypassing any selectivity in the AOB circuit) and by a totally different mechanism. Again the anterior but not posterior medial amygdala was activated, providing support for the hypothesis that the pattern of input to medial amygdala is not recognized as similar to that from conspecific chemosensory stimulation. Additional support comes from studies on selective activation of posterior medial amygdala by conspecific and not by heterospecific chemosensory stimuli in hamsters [5] and in mice [6]. Preliminary results from the study reported here were included in the paper by Meredith and Westberry ([5]: their Fig. 2b), and these are now updated with more extensive supporting data. By adding artificial AOB activation to a concurrent chemo-sensory stimulus we find small but significant changes in medial amygdala activation and a commensurate significant impairment of a behavioral response dependent on chemosensory input.

The accessory olfactory bulb has one of the highest densities of mGluR2 receptors in the rodent brain; much higher than in...
the main olfactory bulb [7]. The receptors are located on accessory bulb granule cells [8] and mitral cells [7]. In vitro work in mice [9,10] shows that activation of mGluR2 receptors reduces GABA inhibition from granule cells onto accessory-bulb mitral (output) cells, both recurrent inhibition (negative feedback) and lateral inhibition. In vivo, this action would be predicted to be disinhibitory and produce an increase in accessory bulb output, even in the absence of an increase in vomeronasal sensory input. We have confirmed this prediction in hamster and used it to explore the meaning of distributions of central vomeronasal pathway activation seen with natural chemosensory stimulation. Activation of AOB output by mGluR2 agonist increased immediate early gene expression in medial amygdala in the absence of overt VNO stimulation, with a distribution different from that due to activation by female chemosignals. The addition of mGluR2 activation resulted in only small changes in the level and distribution of amygdala activation produced by chemosensory stimulation of the same circuits by female chemosignals. However, the additional mGluR2 activation did significantly impair mating behavior induced by female chemosignals in a laboratory test. We suggest that normal mating behavior depends on a specific combinatorial pattern of input to medial amygdala from AOB output neurons and that degradation of the normal pattern by the addition of a non-specific disfacilitation of AOB output neurons results in failure of pattern recognition in MeA/MeP.

1.1. Background: modulation of feedback mechanisms in AOB

The disinhibition mechanism, which is thought to be normally triggered by norepinephrine (NE), has been shown to be involved in memory formation in the “Bruce Effect”, the pregnancy-block in female mice due to pheromone stimulation from a male of an unfamiliar strain [11,12]. Although we are not concerned primarily with this effect in our work with hamsters, the proposed mechanism, which can also be activated by mGluR2 agonist [9,10], is pertinent to interpretation of our results and their place in an integrated understanding of accessory bulb function. Norepinephrine released during mating produces a general interruption of the feedback inhibitory circuits involving the AOB mitral (output) cells, leading to an enhanced level of activity in mitral cells responding to the pheromone stimulus [13,11]. The memory arises because this increased activity leads to selective upregulation of inhibitory feedback circuits from granule cells to those mitral cells. Subsequently, the selective inhibition prevents the same male’s pheromone stimulus producing a strong activation of AOB; and there is no pregnancy block. Other male’s pheromone signatures activate a different combination (pattern) of mitral cells in the AOB, leading to an output strong enough to produce hormonal changes and pregnancy block [10,14]. A memory for a pheromone stimulus can be produced by artificial disinhibition of AOB mitral cells by exogenous adrenergic, or mGluR2, agonists during exposure to that pheromone [11]. These studies provide evidence that natural stimuli produce patterned activation in the accessory bulb (not unexpectedly) and provide a method for a general (unpatterned) enhancement of AOB output. We are not concerned here with the AOB memory mechanism but with the fate of signals that do emerge from the AOB circuits and must be analyzed downstream. We use mGluR2 agonist to change the output pattern of the AOB and study the consequences for behavior and activation of more central parts of the vomeronasal sensory pathway, in the medial amygdala.

2. Materials and methods

Animals were sexually-naïve male Syrian golden hamsters (Mesocricetus auratus) 2–3 months old, bred in the laboratory, all on a long-day light cycle (14 h light/10 h dark) with ad lib food and water. All drug injection and behavioral tests were begun approximately two hours into the dark phase, in a room dimly illuminated with a red light. The Florida State University Institutional Animal Care and Use Committee approved all animal procedures.

2.1. Implantation of guide tubes and injection of drug

Bilateral guide tubes (26 ga.; Plastics One) were implanted, one into each olfactory bulb just rostral to each accessory olfactory bulb (5.958 mm rostral to Bregma), under sodium pentobarbital anesthesia (80 mg/kg, IP). The placement maximized the access of mGluR2 agonist to the AOB external plexiform layer while avoiding damage to the AOB itself. Guide tubes were secured with dental acrylic and two 0–80 SS skull-screws above the caudal olfactory bulb. Before and after use, the guide tubes were sealed with a cap and styllet (dummy cannula) that extended 0.50 mm beyond the end of the tube. Animals were individually housed and allowed to recover for at least one week prior to injection. For injection of the mGluR2/3 agonist, (2S,1S)-2-carboxycyclopropyl glycine (LCCG1; Tocris-Cookson; 1 uL) or saline (1 uL), animals in their home cages were momentarily restrained by hand, the dummy cannula was replaced by a 33-gauge cannula and the sterile solution injected by two brief pressure pulses 30 s apart from a Picospritzer (General Valve Co.). The dummy cannula was replaced after a further 90 s. Cannulae were individually calibrated by weighing water ejected onto filter paper before sterilization [15].

2.2. Immunocytochemistry

Animals were deeply anesthetized with sodium pentobarbital and perfused through the heart with fresh 4% paraformaldehyde at 4 °C. Brains were embedded in gelatin and postfixed in the same fixative at 4 °C overnight. Coronal 50 um Vibratome sections from all experimental groups were processed together to minimize variations in background staining between groups.

2.2.1. mGluR2/3 immunocytochemistry

Free-floating sections were washed for 1 h at room temperature then incubated for 24 h at room temperature with a polyclonal rabbit anti-mGluR2/3 antiserum (Chemicon
(ACN) areas were selected using neuroanatomical criteria [2].

respectively), and the anterior cortical nucleus of the amygdala and caudal–posterodorsal medial amygdala (MeA and MeP, bed nucleus of the stria terminalis (BNSTr,c), rostral–anterior sections through the medial preoptic area (MPOA), rostral

assisted image analysis (Image-Pro plus), calibrated by hand

2.2.4. Counting Fos-positive nuclei

Fos-immunoreactive nuclei were counted using computer assisted image analysis (Image-Pro plus), calibrated by hand counting on representative sections. Single representative sections through the medial preoptic area (MPOA), rostral and caudal portions of the dorsal-encapsulated posteromedial bed nucleus of the stria terminalis (BNSTr,c), rostral—anterior and caudal—posterodorsal medial amygdala (MeA and MeP, respectively), and the anterior cortical nucleus of the amygdala (ACN) areas were selected using neuroanatomical criteria [2]. The number of Fos-positive cell-nuclei within the boundary of each neuroanatomical nucleus of interest was counted and averaged across all animals in a group. Data are presented as means and standard errors for each group.

To determine whether counts from single sections were representative of the activity within a brain region in animals used in the experiment with 4 μg/mL LCCG1 injection and no overt chemosensory stimulation (see below), we also counted multiple sections. Five non-adjacent sections through MeA (spaced 50 μm apart from one another) and five through MeP were counted and the results compared to those from counting

a single representative section of each brain region. With each section 50 μm thick, the multisection counts for MeA and for MeP comprise the mean number of Fos-positive nuclei within 50 μm sections sampled at 100 μm intervals across a total of 450 μm in the rostral-to-caudal direction.

2.3. Experimental design

2.3.1. mGluR2-ir distribution

Adult (2–3 months of age) and juvenile (6–7 days of age) male and female hamsters taken from their home cages and given no other treatment, were used to determine the distribution of mGluR2 immunoreactivity (-ir). Sexually-naive adult males were used for all other experiments. Adult hamsters were deeply anesthetized and perfused as above; juveniles were decapitated after anesthesia and their brains fixed by immersion.

2.3.2. LCCG1 dilution series

In order to find a dose of the mGluR2 agonist that would activate accessory olfactory bulb (AOB) output cells with minimal activation of those in the main olfactory bulb (MOB), we compared Fos activation in the downstream targets of these two regions. These are the anterior medial amygdala (MeA; of the “vomeronasal amygdala”) receiving output from the AOB, and the anterior cortical nucleus (ACN; of the “olfactory amygdala”) receiving output from the MOB. One microliter of one of four dilutions of LCCG1 (2, 4, 8, and 32 μg/ml) was delivered unilaterally, as above, to animals in their homecages. Animals were perfused 90-min later and Fos-immunoreactive nuclei in MeA and ACN were counted on both sides of LCCG1-injected animals, in control animals receiving unilateral injection of 0.9% saline and in non-injected controls with no implanted guide tubes. Fos expression there reflects AOB and MOB activation in response to increasing concentrations of LCCG1.

2.3.3. LCCG1 without overt chemosensory stimulation

The distribution of Fos expression in the corticomedial amygdala and associated areas was mapped for the dose of mGluR2 agonist (1 μL of 4 μg/mL LCCG1, or 4 ng/μL) that produced the maximal activation of vomeronasal projection areas and the minimal activation of main olfactory projection areas.

2.3.4. LCCG1 and HVF exposure

To determine whether mGluR2 stimulation of the AOB sufficient to activate the amygdala would disrupt the normal distribution of amygdala activation with female stimuli, males were presented with female hamster vaginal fluid (HVF) following injection of LCCG1. Freely moving adult male hamsters received half the standard dose, (2 ng/0.5 ul) of LCCG1 (or saline) unilaterally in their homecage, 5 min before transfer to a clean test cage in which HVF was presented, and again 10 min later during the HVF exposure. Approximately 0.2 ml of HVF, collected from several behaviorally-receptive naturally-cycling females and diluted 1:2 with distilled water,
was placed on an overturned glass Petri dish where it was accessible to the male to investigate and ingest, and was replenished four or five times during the 45-min test; after which the animal was left alone for 45 min before perfusion.

2.3.5. LCCG1 and pheromone-mediated reproductive behavior
In order to determine whether mGluR2 activation of the AOB affected sexual behavior mediated by chemosensory stimulation, and/or the Fos expression associated with such behavior, test males received bilateral injections of 4 ng/uL LCCG1 in their homecages 10 min prior to behavioral testing. All animals underwent two behavioral tests in sequence. In the first of two tests (scented male test), the test male was given a 15-min exposure to a “surrogate female”, an anesthetized adult male hamster (>5 months of age) scented on the hindquarters with undiluted HVF (freshly collected from a naturally-cycling, behaviorally receptive female). The scented male was placed in a simulated lordosis-posture on a small mound of clean bedding in a clean 44-× 21-×18-cm plastic cage. The test male was then placed into the cage and behavior was recorded for 15 min using a keypad with keys coded for sexual and nonsexual behaviors [2]. Behavior recorded included mounts, where the male straddles the mating partner with his forelimbs; pelvic thrusts, which are rapid probing movements of the males’ hindquarters; and intromission-like pelvic flexures. These intromission-thrusts are easily distinguished from the probing thrusts that precede them. Each behavior was recorded as correct or incorrect to distinguish behaviors in which the male was correctly oriented over the surrogate female’s hindquarters as if for intromission. The test male was returned to its homecage for one hour. Then (at 85 min after injection), the test male was given a 5-min mating test with the female from which HVF used in the scented male test had been obtained and in the same test cage as the scented male test. Reproductive and other behaviors were recorded as before. Test cages were not washed between the scented male test and female mating test for an individual test male, but a clean test cage (and a different female) was used for each test male. After the 5-min test, the male was immediately perfused for Fos protein immunocytochemistry — at too short a delay for any Fos-protein expression due to the female test to occur.

To examine the effect of the timing of tests, a second set of experiments was conducted in which the order of the scented male test and the female mating test was reversed. A 5-min female mating test was given 10 min after bilateral LCCG1, or saline, injections and a 15 min scented-male test was given one hour later.

2.4. Statistics
With unilateral drug or saline injection, the number of cell nuclei with dense Fos-protein expression for each brain area was compared by two-way repeated measures (RM) ANOVA. One factor was the treatment: LCCG1-injection, saline-injection or control. The other (repeated) factor was the side: ipsilateral or contralateral to the injection. For comparison between areas where injection was bilateral, and Fos-ir cell counts were averaged between sides, a one-way ANOVA was used. For comparison of expression in different brain areas, two-way repeated measures ANOVA was used with the factors: Brain area (repeated measures for two or more areas counted in the same animals) and treatment (as above). For comparison of behavioral results, one-way, or two-way RM ANOVAs were used with the factors: treatment (as above) and behavioral response (e.g., mount-thrusts or intromission-like thrusts; correctly or incorrectly directed mount-thrusts; intromissions). Pairwise comparisons were made by post hoc tests (Newman–Kuels or Holm–Sidak methods as recommended by the SigmaStat software package: Systat Software Inc).

3. Results

3.1. mGluR2/3 distribution
We first confirmed by immunocytochemistry that mGluR2/3 receptors have a prominent expression in the hamster AOB as in other rodents [7,8,16]. The antibody used is specific for group II mGluR receptors (mGluR2 with cross reactivity to mGluR3; Chemicon). It shows a high density of immunoreactivity throughout the AOB external plexiform layer (EPL), where granule/mitral reciprocal synapses are located (Fig. 1), including both the anterior and the posterior regions, which receive axons from different classes of vomeronasal receptors [17]. In addition to the AOB, there was a high level of expression in cerebellum (not shown) and neocortex (at left in Fig. 1), as in other rodents [7]. There were no apparent differences between mGluR2/3 distributions in males and females (2 of each sex). Importantly, there was a low density of immunoreactivity in the main olfactory bulb (at right in Fig. 1) suggesting that expression of both mGluR2 and mGluR3 there is low. Although the general level of immunoreactivity...
was low, there were a few dense clusters of immunoreactive (-ir) fibers and lightly stained cells at the posterior edge of the main bulb glomerular layer in both adults and juveniles. These had the appearance of “atypical” or “necklace” glomeruli [7,18], the targets of a subset of olfactory sensory axons [19]. Sections double-labeled for mGluR2/3 and GnRH (Fig. 1, inset) showed a close association between GnRH-ir fibers, possibly belonging to the nervus terminalis [20], and mGluR2-ir fibers and cells of “necklace” glomeruli in the caudal main olfactory bulb (as in rat [21]), but no cellular co-localization.

3.2. Dose–response relationship for mGluR2/3 activation of the AOB

We used the relative EC–50 values from in vitro experiments [22] to calculate a dose for LCCG1 equivalent to the effective, in vivo, dose for DCGIV in the pregnancy-block experiments in mice [13]. From there, the dose was titrated in a series of experiments until we could reliably produce significant activation of the accessory bulb projections without significant activation of main bulb projections (where mGluR2/3 receptors are sparse). The relative activity in the two systems at different doses is illustrated in Fig. 2, showing Fos expression in the anterior medial amygdala (MeA) and anterior cortical amygdala (ACN). These are, respectively, the proximal projection regions of the accessory and main olfactory bulbs. The dose of 4 ng in 1 ul saline was the highest dose producing a significant increase in MeA compared to saline injection, with no increase in ACN (p < 0.001; F = 190.31, df 1, 5; 2 way RM ANOVA with factors: Treatment and Area). Fos expression on the injected side was also significantly greater than on the opposite (drug-control) side (not shown; p < 0.001; F = 20.6, df 1, 9, two-way RM ANOVA with factors: treatment and side).

This optimal dose (4 ng/uL) was then used to explore the distribution of Fos expression in central vomeronasal targets after unilateral injection, and the effect on chemosensory behavior after bilateral injection.

Unilateral injection of 4 ng/uL LCCG1 in animals in their home cages (where there is routinely a very low background Fos expression) resulted in significantly increased Fos expression in the anterior medial amygdala (MeA) on the injected side compared to the control side (Fig. 3) (p < 0.001: post hoc Newman–Kuels test following a significant 2-way RM ANOVA with factors treatment and side; p < 0.001 main effect for side; F = 65.7, df 1, 9). LCCG1 response was also significantly greater than the saline response (p < 0.001; main effect of treatment, F = 20, df 1, 9; with a significant interaction between side and treatment; p < 0.001). There was no significant activation of posterior medial amygdala (MeP), despite a major interconnection between MeA and MeP [23] and no significant activation of ACN, the main-olfactory projection area. Fos protein expression in AOB mitral cells is generally low in animals with no overt chemosensory stimulation [2] and here (not shown) was not significantly increased by LCCG1 injection compared to control groups, despite the increased output indicated by MeA activation. There was also no increase in Fos expression in main olfactory bulb mitral or granule cells. Activation of medial preoptic area (MPOA) was low, as expected with low levels of activation in medial amygdala and not significantly higher in LCCG1 injected than in saline-injected or control animals. There was no significant response in the caudal part of the (dorsal encapsulated) postero medial bed nucleus of stria terminalis (BNSTc), which is activated in males investigating chemosensory stimuli [2]. Saline injection (1 uL) produced no increase in Fos expression compared to control, nor did guide-tube implantation without injection (uninjected side in unilaterally-injected animals). The difference in activation of MeA and MeP was apparent whether a single section in rostral MeA and a single section in caudal—dorsal MeP were counted (Fig. 3), or whether 5 spaced sections through each structure were counted (not shown). Thus, artificial stimulation of the accessory bulb, like artificial...
electrical stimulation of the vomeronasal organ [4] produces an anomalous activation of MeA and MeP. The next section addresses the question: does addition of artificial to natural input distort the pattern of input to the amygdala sufficiently to alter amygdala response?

3.4. Effect of LCCG1 on chemosensory transmission

In the second experiment we combined unilateral injection of 4 ng/ul LCCG1, or saline, with chemosensory stimulation by female hamster vaginal fluid (HVF), placed on a glass surface in the cage. As in previous experiments [2], the animals sniffed, licked and consumed the HVF, which was replenished periodically during the 45 minute exposure time. Activation in the anterior medial amygdala (MeA) on the injected side was approximately the same as with LCCG1 injection alone but activation of MeP (and of downstream areas, MPOA and BNST) was much higher than with LCCG1 alone (Fig. 4) ($p<0.001$ for each area by two-way RM ANOVA with factors exposure and side).

The distribution of Fos expression in vomeronasal projection areas was similar to that in previous HVF-exposure experiments, overwhelming the clear effects in MeA when LCCG1 injection was given alone. Despite the great increase in Fos expression due to bilateral chemosensory input, there was still a significant difference in MeA between the LCCG1-injected and the uninjected sides. This is seen as a significant main-effect difference between sides ($p<0.005$, $F=13.61$, $df$ 1, 9; 2-way RM ANOVA with factors side and treatment), due to a significant difference between sides in LCCG1-injected animals only ($p<0.02$, Holm–Sidak post hoc test). There was no significant difference between sides for saline-injected or uninjected control animals. Downstream, in MPOA there was also a small but significant difference between sides ($p<0.03$; $F=6.63$, $df$ 1, 9; 2-way RM ANOVA). In this area also the difference was due to a significant difference between sides ($p=0.02$; Holm–Sidak post hoc test) in LCCG1-injected, not in saline-injected or control animals. There was no significant difference between groups in MeP or in either the rostral or caudal subdivision of dorsal posterior-medial BNST (BNSTr and BNSTc).

Clearly the drug altered activation of MeA (and MPOA) and did not prevent transmission of activity to posterior medial amygdala but we cannot tell whether the altered activity induced by the drug enhanced or degraded the “meaning” of the signal. The next experiment measured behavior in order to address this question.

3.5. Effect of LCCG1 on Fos expression during mating test with surrogate-female

We combined bilateral LCCG1 injection with exposure to a “surrogate” mating partner, to determine whether the activity induced by the drug enhanced or degraded chemosensory dependent behavior. To reduce non-chemosensory cues for mating, the “scented-male” test was used to assess mating drive. LCCG1 (4 ng/1ul) was injected on each side, 10 min before the 15-min behavioral test. Fos counts from the two sides were averaged because drug injection was bilateral. Without the ability to detect drug effects by comparing sides, differences in Fos expression between drug-injected, saline-injected and control males did not reach significance ($p=0.052$; main effect of treatment; 2-way ANOVA with factors treatment and area) (Fig. 5). Fos expression in animals exposed to surrogate females was significantly greater in several areas than in animals with the same treatment (LCCG1-, saline- or no injection) but exposed only to HVF. In control animals, MeA, MeP and MPOA showed increases (all $p<0.05$ by 2-way ANOVA with factors: treatment and area) (Fig. 5).

Thus, as in the previous experiment, the drug effect was not powerful enough to prevent transmission from MeA to MeP. However, this same experiment provides evidence that the
signal was altered, because the behavior with surrogate females was different between drug and saline injected animals, as described in the next section.

3.6. Effect of LCCG1 on chemosensory-dependent behavior

Compared to saline-injected or control animals, the LCCG1-injected group had significantly fewer (total) mounts with pelvic thrusting in the scented male test given at 10 min after injection (early scented male test), indicating a significant impairment of behavior driven by chemosensory input. Fig. 6 shows total mounts with thrusts (that did not progress to intromission-like pelvic flexure) and total intromission-like thrusts. There was a significant main effect of treatment \( (p = 0.015; F = 5.77, df 2, 14) \) in a two way RM ANOVA with factors: treatment (LCCG1, saline, control) and behavior (mounts with thrusts, intromission like thrusts). With this analysis, the number of mounts with thrusts for the LCCG1 group was significantly less than either the saline group \( (p < 0.001) \) or the control group \( (p = 0.021) \); post hoc Holm–Sidak tests). There were no significant differences between groups in numbers of intromission-like thrusts. LCCG1-injected males were also significantly different from saline-injected males in mounts with pelvic thrusting when the correctly or incorrectly directed behaviors were considered separately \( (p = 0.004) \); post hoc Holm–Sidak test following significant 2-way RM ANOVA with factors: Treatment and correctly- or incorrectly-directed thrusts; \( p = 0.015; F = 5.765; df 2, 14) \). All test males spent considerable time investigating the scented stimulus male, and all attempted to mate. LCCG1 injection had no effect on the percent of time spent investigating the hindquarters of the HVF-scented test male, or on total investigation time (1-way ANOVA).

In the 5-min mating test with a receptive female, 60 min after the (early) scented male test, there were no significant differences in overall numbers of mounts with pelvic thrusting or in intromissions. However, there was a significant effect of treatment. LCCG1-injected males had more correctly directed intromissions and fewer incorrectly directed intromission-thrusts than males of other groups. The difference was significant for the LCCG1-injected group \( (p < 0.011) \); post hoc Holm–Sidak test) in a significant 2-way RM ANOVA \( (p < 0.03; F = 5.654; df 1,14) \) with factors: treatment (injection type) and behavior (correctly- or incorrectly-directed intromission).

In additional animals, the order of the behavioral tests and their timing with respect to the injection was reversed. A 5-min mating test with a receptive female (early female test) was given 10 min after LCCG1 (or saline) injection and a 15-min scented male test (late scented male test) was given 60 min later. In these tests, there were no significant differences between groups in mounts with pelvic thrusting or intromissions in the female test, or in mounts with pelvic thrusting in the scented male test. However, there were significant differences between groups, indicating a significant effect of treatment. In an analysis of the early and late scented male tests, total mounts-with-pelvic-thrusting in the early test were significantly lower for LCCG1-injected than for saline-injected males \( (p = 0.012) \); post hoc Holm–Sidak test after 2-way ANOVA with factors: early/late-test and treatment; \( p = 0.005 \) main effect of treatment; \( F = 6.53, df 2,29) \). Other comparisons were not significant. In the female tests, the behavior of the groups was also different dependent on the timing of the tests \( (p = 0.001; F = 14.363; df 1, 29) \) in 2-way ANOVA with factors: early/late-test and treatment). Saline-injected and uninjected groups had significantly fewer total mounts with pelvic-thrusting in late female tests than equivalent groups given an early female test \( (p < 0.03) \); post hoc Holm–Sidak tests). LCCG1 injected males, however, showed no difference in mounts with pelvic thrusting in the female tests, whether before or after the scented male test. There were no significant differences in intromissions.

4. Discussion

4.1. Summary

We show here that artificial stimulation of the accessory olfactory bulb in male hamsters by infusion of a mGluR2/3 agonist increases activation of central vomeronasal sensory areas, including the anterior medial amygdala. That activation does not extend to posterior medial amygdala, an area strongly activated when stimulation is by conspecific chemosensory stimuli. However, response is also limited to anterior medial amygdala with artificial (electrical) stimulation of the vomeronasal organ \[4\] or with activation of the system by chemosensory stimuli from other (heterospecific) species \[5\]. We propose that this selective response to conspecific chemosensory stimulation reflects a selectivity for a limited range of spatiotemporal patterns of input to the amygdala. Here, when we used conspecific chemosensory stimuli concurrently with mGluR2/3 agonist activation of the AOB, we did not see significant suppression of activation in MeP.
The pattern of input elicited by highly relevant (and preprogrammed) chemosensory stimulation largely over-rides the relatively non-specific (unpatterned) input due to the drug. Nevertheless, there were small changes in the distribution of activation between injected and uninjected sides and across different amygdala areas when LCCG1-injection, rather than saline-injection was combined with chemosensory stimulation. Residual differences in pattern of input between drug- and saline-injected males may account for the significant deficit in mating behavior in LCCG1-injected males when conspecific female chemosensory stimuli are the only overtly female stimuli inducing mating behavior. These results are consistent with a necessity for chemosensory input to conform to a limited range of patterns in order to induce normal behavior.

4.2. mGluR2 agonist actions

In the experiments described here, we chose LCCG1 as our mGluR2 agonist because it has less cross-reactivity with NMDA receptors than the DCGIV [24], used previously in experiments on pregnancy block in female mice [14]. NMDA receptors on granule cells are important components of the excitation and negative feedback circuits in the main olfactory receptors on granule cells are important components of the experiments on pregnancy block in female mice [14]. NMDA receptors than the DCGIV [24], used previously in experiments on pregnancy block in female mice [14].

The failure of activation in MeP is common to experiments where the input to medial amygdala is from heterospecific chemosensory stimuli, artificial stimulation of AOB by LCCG1, or artificial (electrical) stimulation of the VNO [5]. The variety of stimuli provides an opportunity to explore the response characteristics of circuits leading to MeP. With AOB stimulation by LCCG1 there will be a spatially uniform synchronous output from AOB and, thus, an unpatterned input to medial amygdala. With electrical stimulation of the VNO [4,5] a spatially uniform synchronous activation arrives at the AOB input. The subsequent activation of MeA is evidence that this pattern is not screened out by the AOB circuitry. Both inputs fail to activate MeP, suggesting that an unpatterned input may not be recognized by the amygdala circuit. However, a similar outcome follows heterospecific chemosensory stimulation [5], which is unlikely to involve a uniform unpatterned input to medial amygdala (or AOB). It seems more likely that there is selection in medial amygdala in favor of patterns generated by the various conspecific stimuli. The lack of MeP response for heterospecific chemosensory stimulation (at least) is associated with activation of GABA-ir cells in the adjacent intercalated nucleus (ICN) of the amygdala [5], and a selective suppression of GABA-Receptor-ir cells in MeP (J.M. Westberry and M. Meredith; unpublished), suggesting GABA inhibition of MeP as a mechanism.

With our concurrent drug and conspecific chemosensory stimulation here, the pattern of input to MeA could be sufficiently within the normal conspecific range to prevent the inhibition of MeP. However, the addition of an LCCG1-
activated input did change the chemosensory response to HVF in MeA and downstream in MPOA; and appears to be sufficient to alter behavior in the scented male test where HVF input is the only female cue, possibly by distorting the pattern of activation within MeA/MeP.

4.4. Behavior

Although effects of LCCG1 injection were not apparent in gross measures of Fos expression during the strong multisensory activation of MeA and MeP in the scented male tests, there were significant differences in behavior on those tests. LCCG1-injected males had significantly fewer mounts with thrusts, suggesting that at 10 min after LCCG1 injection, there was impairment in chemosensory driven mating behavior. The scented male test has been used as a bioassay for active components in HVF [32] and it allows the effect of a female scented male test has been used as a bioassay for active components in HVF [32] and it allows the effect of a female scent. The application of HVF to an anesthetized male is sufficient to cause naïve or experienced males to attempt to mate with the scented male, which they rarely do with unscented anesthetized males [35]. LCCG1 has a dramatic effect in MeA in the absence of chemosensory input (Fig. 3) and a more subtle effect when added to HVF stimulation (Fig. 4). We cannot directly measure an effect on pattern of input independent of an effect on level of input in animals from the scented male tests, and changes in level are also more difficult to measure without the internal control available with unilateral treatment. Our failure to document a change in levels of Fos expression during the scented male test (Fig. 5) does not mean that there were no changes. In fact, the behavioral results (Fig. 6) are good evidence that there are changes during the scented male test somewhere in the circuit for chemosensory-driven mating behavior, of which MeA and MeP are critical components [30,31]. Mating behavior in the scented male test was not eliminated so a dramatic change in the level of Fos activation of MeA and MeP should not be expected.

In the tests with behaviorally-receptive females, there are many chemosensory and other sensory cues available from the female and HVF is not essential for mating [36]. Thus, when an independent group of LCCG1-injected males was given female tests at 10 min after injection, the disruption of normally patterned input from the AOB would not be expected to disrupt behavior, and it did not. There were subtle influences on behavior that suggest an effect in this (early) female test but the effects in the early scented male test, when chemosensory input was the only cue directing behavior, were clearly greater. The difference between the outcome of early and late tests is probably due to the difference in timing of the tests relative to the time of drug injection, allowing a dissipation of the drug effect for the late tests. There could also be an influence of the earlier test on the later, as in some other circumstances [37]. We did not explicitly test these hypotheses; for example by omitting the early behavior tests.

Most of the Fos activation in medial amygdala by HVF-exposure in our experiments is via vomeronasal input. Activation (and mating) is dramatically reduced in sexually naïve males with vomeronasal organs removed [2,34,38,39] and largely unaffected by damage to the main olfactory system [40,5]. The LCCG1 injection here altered but did not eliminate vomeronasal chemosensory input. The alteration of the pattern of VN system input to the amygdala was not sufficient to eliminate the vomeronasal contribution that is essential even for multisensory activation of mating in naïve males [34]. However, we conclude from the present results that the alteration is sufficient to disrupt normal mating behavior when the behavior is driven primarily by chemosensory input. Thus, we suggest that pattern, presumably spatiotemporal pattern of input to the amygdala from the AOB, is an important part of the chemosensory message.

Acknowledgements

We thank Drs. Gwen Fewell, John Moeller, Jenne Westberry for advice and/or assistance with various aspects of these experiments. Supported by NIDCD grants DC 00906 and DC 05813.

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