OLFACTORY BULB HABITUATION TO ODOR STIMULI

by Adolfo Arellanos

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OLFACTORY BULB HABITUATION TO ODOR STIMULI

A Thesis

Presented to the Faculty of the Graduate School

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by

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ABSTRACT

Learning is a change in behavior evoked by experience. One form of simple learning is habituation or the gradual decrease in the response to repeated extraneous stimulation. Habituation is a non-associative form of learning where the organism learns about stimuli by responding less and less with repeated stimulation or exposure to a stimulus. In this study, we tested the role of NMDA receptor activation in olfactory habituation by direct infusions of 8mM and 4mM MK-801, an NMDA antagonist, into the olfactory bulbs of adult Sprague Dawley rats during an olfactory habituation paradigm. Our results show that infusions of the NMDA antagonist MK-801 at 8 mM concentration led to a general depression of the olfactory response. Olfactory habituation memory formation was blocked after infusion of a lower dosage (4 mM) of the NMDA antagonist. These results show that bulbar NMDA receptors are important for habituation of olfactory memories. From our studies, it is clear that NMDA receptors in the olfactory bulb are necessary for the formation of habituation memory, and that blocked NMDA receptors impair olfactory habituation.

BIOGRAPHICAL SKETCH

Even as a small child growing up in Mexico, Adolfo was intrigued by the basic nature of animal behavior and human cognition. One day, he found a scorpion /spider like insect digging a hole in the dirt on the edge of the sewage river. Adolfo was so fascinated by this odd looking creature that he took it home. He was curious to find out whether it would eat flies so he caught some flies and put them in its jar and it chewed them up and then spit them out. Then he wondered if it would do the same if it caught a lizard. However, when it came in contact with a lizard, the two fought each other to a standoff. Adolfo tried to find out what kind of species it was but never could since the books he searched in did not show anything like it. While the behavior of animals such as insects fascinated him, he was even more captivated by the complexities of human interactions. When he was about 8 years old, some guests arrived in his parents' home. He remembers hiding under a table to hear their conversations. As simple as these conversations were, they engendered in his mind fundamental questions about how the brain functions, how behavior is being enacted, and what processes underlie cognition. These are questions that even now, years later, drive his interest in the sciences.

In Adolfo's Mexican neighborhood where he grew up, he was amazed by the way people quietly exercised their daily routines in a robotic and expressionless manner. Sadly, he realized that it was due to the fact that work, and indeed life itself, had become a routine for them. Right then, he realized that he needed to pursue a career that would be stimulating and challenge him as an individual. However, even though he had the interest and the motivation to pursue a career in behavioral neuroscience, he had to acknowledge the fact that his parents did not have the money to send him to high school or college let alone graduate school. In fact, when he

graduated from junior high school at age fifteen, he had to start working right away to help support his family. Adolfo's goals about experimenting and becoming a neuroscientist were overcast by the realities of their economic situation. Thus, his personal ambitions went dormant into a childhood dream.

After working as a machinist for eight years, he quit his job and left Mexico to pursue his aspirations in this country. When he came to New York City fourteen years ago, he had to sustain himself and his family in Mexico by working several jobs while studying English at night. Under these difficult circumstances, he learned English, passed the GED, and enrolled in college. Finally, it appeared that his chances of becoming a scientist in Neuroscience were starting to become a possibility. At Bronx Community College, he first got to dissect a frog and examine a sheep's brain. Performing dissections and being introduced to actual animals was an absolutely exhilarating experience for him. Motivated to know more, he entered the Research Enrichment Activity Program, a research oriented fellowship. As a student, he took as many psychology classes as he could so that he could learn more about the workings of the brain. Through the REAP program he had his first research experience in a psychology cognitive oriented lab at Lehman College. There Dr. Karyl Swartz, Dr. Sharon Himmanen and himself studied subjective organization and free recall in non-human primates. They studied the early stages of a series of experiments that were to elucidate how *Macaca mulatta* organize and store information. This research exposure was fascinating to him and confirmed in his mind that this was the career path that he should pursue in the future.

After graduating from Bronx Community College, and having his mind set into neuroscience, he entered Hunter College. In Dr. Victoria Luine's neuroendocronology lab, he was exposed to several paradigms and techniques, which were to enhance his knowledge about the brain and its workings. In her lab, they subjected Sprague

Dawley rats to an acute methamphetamine treatment, and tested visual memory and spatial memory. They observed that visual memory was impaired, but not spatial memory. An autoradiography assay further indicated that the drug caused loss of nerve endings and depleted dopamine and its transporter levels in areas of the frontal cortex and in the hippocampus. Additionally, dopamine transporter reduction was associated with motor and cognitive impairment. He recalled that it was fascinating to observe how these anatomical and biochemical changes influenced the animals' behavior.

To further pursue his interests in the behavioral science Adolfo attended a summer program at Indiana University Bloomington. In Dr. Preston Garragthy's neuroscience lab, they evaluated an animal model of autism. Female pregnant dams were injected with valproic acid, which induced autistic like physiology in the pups and the pups were then tested in several behavioral tasks. The objective of our study was to find out whether the behavioral deficits may have been comparable with those reported in humans with autism. They used three paradigms: eye blink- conditioning (a Pavlovian task), the Morris water maze (MWM, a spatial memory task), and delayed match to place (DMP, a spatial working memory task). In the Pavlovian task, they observed higher amplitude conditioned responses, and shorter conditioned response latencies with the longer interstimulus interval in the VPA rats, which was consistent with autistic human reports. In the MWM task, they found a slowed acquisition of the escape response. In the DMP task, the VPA rats showed no working memory deficit. Their results suggest that this model of autism may be a valid one as the behavioral deficits found can, at least in part, be attributed to brain anomalies shared with autistic humans.

Enticed by the complexity of the brain and behavior, Adolfo joined the University of California San Francisco summer program. In Dr. Steve Bonasera/Laurence Tecott's lab, young, middle aged and aged BALB/cBy female and

male mice demonstrated age-related changes in gene networks regulating hypothalamic immune and inflammatory responses. This immune dysregulation may underlie observed changes in home cage (locomotion, drinking, feeding, and sleeping) behaviors in these animals. They hypothesize that a similar process may be occurring in other CNS regions as a function of age. They have started a series of experiments with a different mouse strain, in which mice were sacrificed after 14 days of observation. Enriched populations of neurons, microglia (CD45(-), CD11b(+)), and astrocytes (ABCA1(+)) will be sorted by FACS. These samples will be critical for future studies evaluating age-related changes in gene expression in a cell specific manner, and *in vitro* studies of these cell populations under both resting and activated conditions.

Since childhood, Adolfo has been interested in the workings of the brain and how its workings affect behavior. Once he thought that it would be virtually impossible to have an education. Despite the obstacles that were before him, he has worked hard in order to pursue his lifelong goal of a career in the neurosciences. Adolfo has been fortunate to be able to take advantage of the many research programs, which have helped to develop his skills in this arena. Indeed, he believes that his experiences have placed him in an excellent position to excel in this field, and that he is an excellent asset in the biological sciences.

"A great civilization is not conquered from without until it has destroy itself from within"

William J. Durant

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In the Cornell Graduate School, I would greatly love to thank Terry Plater. Terry has been one of the greatest people I ever met. She is a very nice person: loving and caring. Indeed, I thank all of the members from the graduate school, especially Ellen Gainor.

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INTRODUCTION

Organisms are faced with constant stimulation and must adjust to continuous changes in the environment and adapt accordingly. They must choose what to attend to and disregard items that are known or unimportant. Animals orient their attention to items in the environment, but the orienting response eventually decays. Stimuli eliciting orientation are acquired through the sensory system and processed in the brain. Several features associated with orientation such as sniffing, autonomic responses, and electroencephalogram recordings wane with repeated presentations of the stimulus (Konorski, 1967). The plasticity of behavioral responses such as orientation is thought to reflect the process of learning via changes occurring in the nervous system.

Learning has been described as a change in behavior evoked by experience (Thompson and Spencer, 1966; Groves and Thompson, 1970). One form of simple non-associative learning is habituation or the gradual decrease in the response to repeated irrelevant stimuli (Thompson and Spencer, 1966; Muller and Hildebrandt, 2002; Harris, 1943; Marcus et al, 1988; Groves and Thompson, 1970). Habituation allows an organism to disregard frequent and extraneous stimuli in exchange for novel ones. Thus this non-associative form of learning is important for perceiving changes and for differentiating between harmless and potentially dangerous changes in the environment (Hemmi et al., 2009; Dacier et al., 2005).

Habituation is conserved across numerous phyla of the animal kingdom (Rose and Rankin, 2001; Giles and Rankin, 2009). It has been observed in fruit flies (Duerr and Quinn, 1982), rats, (Davis, 1970), Aplysia (Pinkster et al, 1970), roundworms (Rankin et al 1990), humans (Geer, 1966) and even unicellular organisms (Wood,

1970). Habituation is affected by the amount of time and the exposure an organism is exposed to a stimulus (Thompson and Spencer, 1966; Groves and Thompson, 1970). The behavioral change also occurs in short-term and long-term forms in several organisms (Harris, 1943; Thompson and Spencer, 1966; Carew and Sahley, 1986). These forms are distinguished based upon the duration of the behavioral change associated with the habituation as well as the cellular mechanisms underlying it.

Habituation can be investigated in organisms in nature as well as in laboratory settings. In nature, the black-tailed prairie dog (*Cynomys ludovicianus*) avoidance response to pronounced danger is to produce an alarm call, which warns mates and other members of the clan of possible danger (Magle et al., 2005; Hoogland, 1995; Hoogland, 1981; Adams et al., 1987). Rural prairie dogs are prompt to react to humans and give alarm calls to announce the presence of humans. However, urban prairie dogs are less disrupted when humans are around (Adams et al 1987). These rural and urban groups behave differently as a result of differences in exposure to humans, which is greater in urban than in rural animals (Adams et al 1987; Farrar et al, 1998). However, habituation to non-changing stimuli can be harmful. For example, if nonhuman primates are used to humans there is a potential danger because then the animals might generalize between non-harmless humans and poachers (Allen, 1972).

In laboratory settings, studies of the olfactory system of rodents have shown that exposure to odorants provokes measurable autonomic and behavioral responses useful for the quantification and correlation of neuronal and behavioral habituation (Wilson and Linster, 2008). Olfactory responses can be modified by the duration of odor stimulation, as revealed by autonomic and behavioral responses of rodents (Wilson and Linster, 2008). In the olfactory sensory neurons, adaptation occurs gradually in response to exposure to prolonged stimuli (Kurahashi and Menini, 1997);

mitral cells have also shown adaptation to odor exposure in several experiments (Chaput and Panhuber, 1982).

Glutamate is the main excitatory neurotransmitter in the mammalian CNS and exercises its neurotransmitter action via ionotropic N-methyl-D-aspartate (NMDA) and metabotropic glutamate receptors (mGlRs) (Platt, 2007; Tehrani et al., 2000). Glutamate is involved in long-term potentiation, long-term depression, spatial memory short-term, and long-term habituation (Attwell, 2000; Meldrum, 2000; Tapiero et al., 2002; Tzschentke, 2002; Jihoon et al., 2008; Morris, 1989; Morris et al., 1986; Corssen and Domino, 1966; Danysz et al., 1988; McNamara, 2007; McNamara et al., 2008; Wilson, 1998; Wilson and Linster, 2008). For example, in the nematode *C.elegans*, *eat-4,* a glutamate transporter mutant line habituated quicker and recovered much slower than controls at different interstimulus intervals. *eat-4* did not show dishabituation (Rankin and Wicks, 2000). The glutamate mutation in the *C. elegans* did not prevent the response to a single tap stimulus. But it affected responses to concurrent stimulation (Rankin and Wicks, 2000). In wild-type *Caenorhabditis elegans,* at shorter intertrial intervals the nematodes habituate faster. However, as they habituated faster, the invertebrates dishabituate more quickly. At larger inter trial intervals the *C. elegans* habituate more slowly and dishabituate also slowly (Rankin and Broster, 1992).

Smell is essential for survival since it helps in finding mates, food, and to escape possible predators. Olfaction allows the organism to recognize familiar odors and adjust to them accordingly. Our work deals with habituation in the olfactory bulb. There are several types of neurons in the bulb that might be involved in habituation. Mitral and tufted cells are particularly known to receive input from the olfactory receptors neurons and to send the output to higher cortical areas. Mitral cells and tufted cells (M/T) contain two major types of excitatory and inhibitory dendrodendritic

synapses. Glutamate and γ-Aminobutyric acid (GABA) are the main excitatory and inhibitory neurotransmitters in these dendrodendritic synapses. (Rall et al., 1966; Price and Powell, 1970; Pinching and Powell, 1971). Mitral and tufted cells release glutamate from primary and secondary dendrites (Christie et al., 2001). Currently not much is known about tufted cells since much research has been focused on mitral cells (Christie et al., 2001). GABAergic periglomerular (PG) and GABAergic granule (GC) cells modulate the activity of mitral cells and tufted cells. Periglomerular cells regulate M/T dendrodendritic synapses at the primary dendrites and GABAergic granule cells regulate M/T dendrodendritic synapses at the secondary dendrites (Figure 5.) (Christie et al., 2001).

Evidence for glutamate as the olfactory receptor cell neurotransmitter has been provided by the study of Berkowicz et al. (1994). Self-excitation in mitral cells is driven by glutamate released from mitral cells dendrites (Jahr and Nicoll, 1982). The glutamate α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and NMDA autoreceptors mediate self-excitation at the primary and secondary dendrites (Aroniadou-Anderjaska et al., 1999; Isaacson, 1999; Friedman and Strowbrigde, 2000; Schoppa and Westbrook, 2001) and this mitral cell self-excitation is driven by presynaptic dendritic autoreceptors located near dendrodendritic synapses due to glutamate release at the mitral cells' primary and secondary dendrites (Jahr and Nicoll, 1982; Montague and Greer, 1999). Glutamatergic mitral cells release glutamate and this glutamate release can cause lateral excitation at the primary dendrites of other mitral neurons and can be blocked by AMPA antagonists (Schoppa and Westbrook, 2002; Urban and Sakmann, 2002). A similar lateral excitation response occurs in mitral cells. However, this event occurs within the glomeruli and is driven by the NMDA receptor, which lasts for several milliseconds upon strong olfactory nerve

stimulation (Carlson et al., 2000; Puopolo and Belluzzi, 2001; Schoppa and Westbrook, 2001).

Two forms of behavioral olfactory habituation have been observed and reported to depend on two different glutamate receptor mechanisms (Wilson and Linster, 2008; McNamara, 2007; McNamara et al., 2008; Wilson, 1998; Wilson and Linster, 2008). Habituation with a short-term and long-term intertrial interval paradigm has been observed and shown to be dependent on functional mGlRs and NMDA receptors respectively. This was observed with systemic injections and OB intracranial infusions of glutamatergic mGlRs and NMDA receptor antagonists (McNamara, 2007; McNamara et al., 2008). Short term habituation is localized in the anterior pyriform cortex and long term habituation is localized in the olfactory bulb (Wilson and Linster , 2008; McNamara et al., 2008, 2007; Wilson, 1998). A short-term olfactory habituation paradigm where the stimulus is presented for 20 seconds with a 10 second intertrial interval endures for < than 10 minutes. A long-term olfactory habituation paradigm where the stimulus is presented for 50 seconds with a 5 minute intertrial interval endures for about 30 minutes (McNamara et al., 2008). Blocking metabotropic glutamate receptors in the pyriform cortex reduces behavioral responses to a habituation of a simple-odor mediated behavioral response paradigm *in vivo* in which animals are exposed to short odor stimuli during a short time period (Best et al., 2005; Yadon and Wilson, 2005).

In our experiments, we used the long-term habituation task performed by McNamara et al (2008) in mice but applied 8 mM and 4 mM concentrations of the NMDA antagonist MK-801 in rats to explore the potential role of these glutamate receptors in habituation that might occur within the olfactory bulb. The drugs were directly infused into the olfactory bulb of adult male Sprague Dawley rats and the olfactory habituation of the animals was tested. We find that olfactory memory

persists up to 60 minutes in accord with previous studies from our lab (McNamara et al., 2008). Our studies show that the potent noncompetitive N- methyl-D- asparte receptor antagonist MK-801 blocks habituation to odors when applied to the olfactory bulb. Therefore, functional NMDA receptors are necessary for olfactory habituation to the odors we utilized for our experiments. We conclude that application of the NMDA receptor antagonist is correlated with olfactory memory impairment in the habituation paradigm used for this study.

MATERIALS AND METHODS

Subjects

Eleven adult male Sprague Dawley rats (250-300 grams), obtained from Charles River Laboratories (Wilmington, MA), were utilized for behavioral experiments. Animals were singly-housed and maintained at a constant temperature on a 12:12 light/dark cycle with food and water *ad libitum*. Prior to behavioral experiments, animals were provided with seven days acclimation time, during which investigators handled them for one hour daily. All procedures were performed according to NIH guidelines under the supervision of the Institutional Animal Care and Use Committee of Cornell University.

Cannulation

Animals were anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (7.5 mg/kg) and secured in a stereotaxic apparatus (Narishige Scientific Instruments, Tokyo, Japan). Guide cannulae (22-gauge; Plastics One, Roanoke, VA, USA) were inserted into both olfactory bulbs according to standard procedures (Mandairon et al., 2006a) Cannulae were implanted at the following coordinates with respect to bregma: anteroposterior +8.0 mm, mediolateral ±1.5 mm, dorsoventral 4.5 mm. The tips of the guide cannulae were positioned 1.0 mm dorsal to the target infusion site; consequently, infusion cannulae extended 1.0 mm from the end of the guide cannulae. Five screws were drilled into the skull, and dental cement was used to secure the guide cannulae to these screws and to cover the incision area. Dummy infusion cannulae were then placed into the guide cannulae to prevent

blockage or infection. Following surgical implantation, rats were allowed to recover for 10 days prior to behavioral testing.

Drug Administration

For drug or vehicle (saline) administration, immediately prior to behavioral experiments two infusion cannulae were fitted into the guide cannulae so that their tips protruded 1.0 mm beyond the ends of the guide cannulae into the center of each main olfactory bulb. Two 10 µl Hamilton syringes containing either drug solution or saline were attached to the cannulae with a polyethylene tube and driven with paired infusion pumps (YA-12 Genie pumps, Kent Scientific). The drug dosages and infusion volume we used have been shown to be effective in previous studies (Mandairon et al., 2006b) Specifically, the NMDA antagonist MK-801 ([+]- 5- methyl-10,11,dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine maleate; Sigma-Aldrich, Natick, MA; 8 mM; 4mM) was dissolved at 37C in 0.9% saline; the drug or vehicle was then delivered bilaterally into awake rats at a rate of 2 µl /min for 3 minutes (6 µl total volume delivered per side). The infusion cannulae remained in place for 1 additional minute after the infusion ended in order to minimize backflow. Behavioral testing was performed 20 minutes after drug administration was completed.

Odors

The odorants ethyl butyrate and ethyl pentanoate (Sigma-Aldrich, Natick, MA), were utilized for habituation testing. Only one odorant was used during a single day of training/testing; the experiment then was repeated in each rat on a different day using the second odorant to enable counterbalancing of drug/vehicle administration groups. Prior to testing, odorants were diluted in mineral oil so as to each theoretically emit a vapor-phase partial pressure of 5 Pa (corresponding to vol/vol dilution of 0.09

for ethyl butyrate and 0.3% for ethyl pentanoate). Vapor pressures of pure odorants were estimated with the Hass-Newton equation as implemented in ACD/Boiling Point & Vapor Pressure Calculator (version 4.5; Advanced Chemistry Development, Toronto, Ontario, Canada).

Behavioral Testing

An olfactory habituation task measures non-associative memory formation. The behavioral experiments performed here replicate those previously performed in mice in our lab (McNamara et al., 2008). However, the experiments performed here were conducted with the goal of directly comparing behavioral data by drug dosages and odor concentrations in adult male rats. All habituation experiments took place in the home cage under red light. Odors were presented by placing 60 µl of the diluted odor stimulus onto filter paper (Whatman #1) contained within a weighing dish that was placed on top of the cage lid (Figure 1A); this procedure enabled the observer to change the odor stimulus without disturbing the animal. Each test session was preceded by one 50-second presentation of plain mineral oil (MO). Test sessions comprised four 50-second presentations of diluted odorant separated by five-minute intertrial intervals, followed by two additional presentations of the same odorant one presentation at 30- and one presentation at 60-minute time points after the last habituation trial (Figure 1B). Comparison of investigation times during the first and fourth odor habituation trials measures initial habituation (5-minute delay), whereas comparison between the first odor presentation and either of the two delayed (30- and 60-minute) trials measures the persistence of habituation memory. Active sniffing within one centimeter of the odor source was recorded with a stopwatch. Figures show mean investigation times +/- standard error. All rats underwent both drug treatments

using a separate odor for each and the order of drugs was counter balanced among the rats.

In a control experiment, rats were presented with the mineral oil carrier alone for four 50-second trials after which the odorant was presented in a single test trial, hence measuring the animals' ability to detect the odorants used. This control ensured that rats treated with the NMDA antagonist at 8 mM were not simply unable to detect the odors presented. As in the first experiment, all rats were tested with both odorants and drugs and the order of drug was counterbalanced.

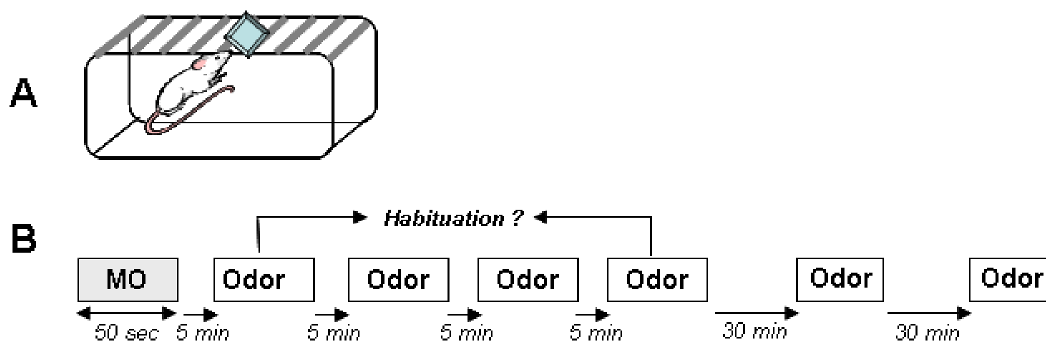

Figure 1. Schematic of experimental behavioral paradigm. **A**. Behavioral protocol. Rats were presented with a square weighing dish containing either mineral oil (MO) or the habituation/test odor. **B**. After a single presentation of mineral oil, the habituation/test odorant was presented for four consecutive trials, separated by five minute intertrial intervals (ITIs), and then twice more at 30 and 60 minutes latency following the last habituation trial.

Data analysis

An analysis of variance (ANOVA) was performed using SPSS statistical software (SPSS, Chicago, IL) with *odor investigation time* as the dependent variable, and *drug group* and *odor presentation trial number* as main effects. Fisher post hoc

pairwise testing was utilized to assess whether the time spent investigating during the fourth habituation trial or later test trial were significantly lower than the time measured during the first habituation trial ($\alpha = 0.05$).

RESULTS

Behavioral habituation to repeated odor stimulation involves functioning NMDA receptors in the olfactory bulb

Behavioral studies were executed to test the dependence of odor habituation on bulbar NMDA receptors in adult male rats. In separate experiments, animals were treated with the MK-801 8 mM and 4 mM infusions into the olfactory bulb; controls were infused with saline. Rats were then presented with mineral oil, then four habituation trials separated by 5 minute ITIs, and finally two additional odor presentations at 30 and 60 minutes after the last habituation trial. For the MK-801 8 mM group, results are shown for four habituation trials separated by five minutes and test trials. Animals infused with saline alone habituated to the presented odors. MK-801 at 8mM impaired habituation memory. MK-801 at 4mM led to impaired formation of habituation memory. Our results suggest that the NMDA antagonist MK-801 blocked habituation since animals failed to habituate to odor exposure. Analysis of variance for the vehicle and MK-801 8mM groups was performed. In vehicle-infused controls (Figure 2A), there was a significant effect of trial (ANOVA; $F_{trial}(5, 45) = 7.814$, $p < 0.001$) and a significant reduction of investigation time in the fourth trial as compared to the first ($p < 0.001$), as expected based on previous experiments. In contrast, no significant effect of trial was observed in MK-801-infused rats (Figure 2B) ($F_{trial}(5, 50) = 0.161$, $p > 0.9$).

Analysis of variance for the vehicle and MK-801 4mM groups was performed. In vehicle-infused controls (Figure 3A), there was a significant effect of trial (ANOVA; $F_{trial}(5, 60) = 7.555$, $p < 0.001$) and a significant reduction of investigation time in the fourth trial as compared to the first ($p < 0.001$), as expected based on

previous experiments. In contrast, no significant effect of trial was observed in MK-801-infused rats (Figure 3B) ($F_{trial}(5, 54) = 1.507$; $p > 0.2$) and there was no significant difference between the response during the first and fourth trial ($p > 0.7$).

To ensure that MK-801 8mM treated animals were still able to detect odorants; we performed a behavioral control experiment in which saline and MK-801-infused rats were presented with mineral oil over four successive trials, followed by a single presentation of a test odorant. We found no significant effect of drug group ($F_{drug}(1, 91) = 0.013$, $p > 0.9$), indicating that both groups performed similarly in this control experiment. Specifically, in both groups rats investigated the odor significantly more than the mineral oil ($p < 0.05$ for both groups, Figure 4).

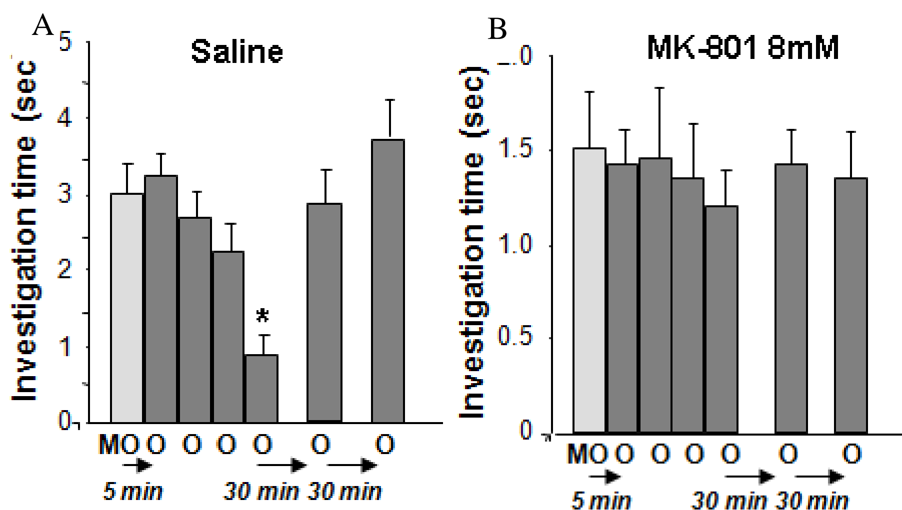

Figure 2. Effect of bulbar NMDA receptor blockade on behavioral habituation. The graph depicts behavioral investigation times in rats infused with saline (**A**) or MK-801 8mM (**B**) of the average response to the first odor presentation. Saline-infused control rats responded significantly less on the fourth habituation trial as compared to the first trial. MK-801 infused rats did not significantly habituate.

B

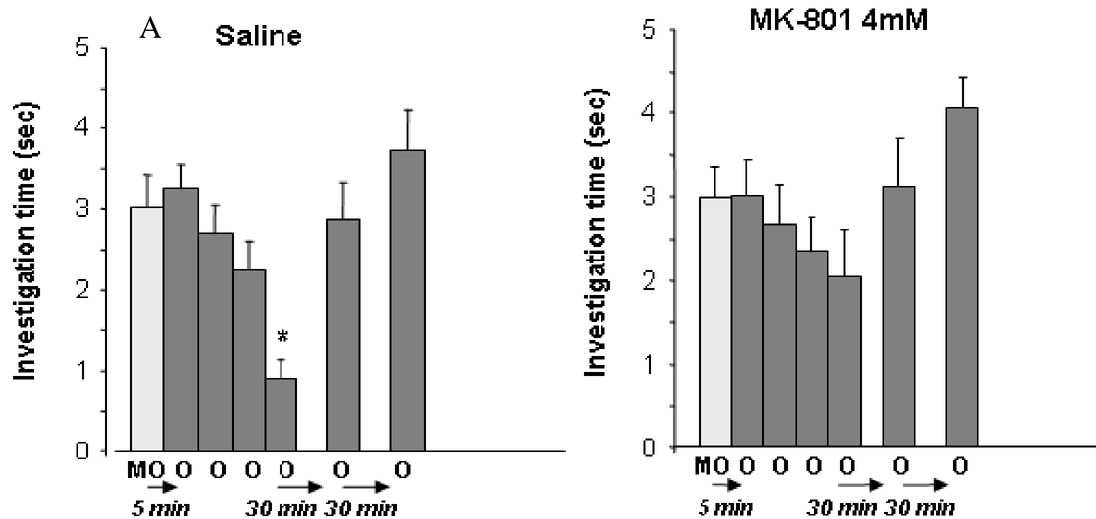

Figure 3. Behavioral results of the effect of bulbar NMDA receptor blockade on behavioral habituation. The graph depicts average behavioral investigation times in rats infused with saline (**A**) or MK-801 4mM (**B**). Saline-infused control rats responded significantly less on the fourth habituation trial as compared to the first trial (*represents a significant difference between responses during the first and fourth trial), whereas MK-801 4mM infused rats did not significantly habituate, as indicated by a non-significant difference between investigation times during the fourth and first trials.

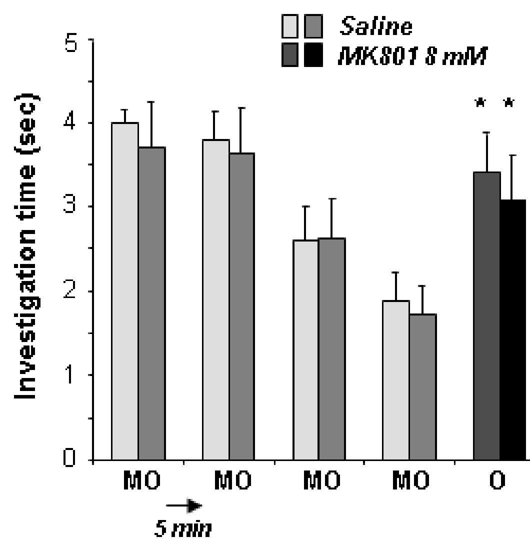

Figure 4. Blockade of bulbar NMDA receptors does not impair odor detection. The graph shows the average investigation times of saline-infused and MK-801 infused rats presented with the mineral oil carrier during four trials followed by a single odor presentation. All rats responded significantly more to the odor than to the carrier alone, indicating that they detected the odorant.

DISCUSSION

Here we show that NMDA receptors in the olfactory bulb are important for the formation of olfactory habituation memory. We tested the role of NMDA receptor activation by direct infusions of 8mM and 4mM MK-801, an NMDA antagonist, into the olfactory bulbs of rats during an olfactory habituation paradigm. Our results show that direct infusions of the NMDA antagonist MK- 801 at 8 mM concentration led to a general depression of the olfactory response. Olfactory habituation memory formation was blocked after infusion of a lower dosage (4 mM) of the NMDA antagonist. Thus olfactory memory is susceptible blockade by NMDA antagonist infusions. These results show that contributions of bulbar NMDA receptors in the creation of olfactory memory are important for habituation to occur. Our studies are in accord with previous studies where the application of NMDA receptor antagonist prevented habituation to odorants in a long term habituation paradigm (McNamara et al., 2008; Staubli et al., 1989).

The role of the glutamatergic system in learning and memory has been extensively studied in animals. NMDA receptors have also been implicated in various learning and memory processes such as Pavlovian fear conditioning (Xu et al., 2001), working and reference memory (Levin et al., 1998), place preference (Swain et al., 2004), reversal learning (Harder et al., 1998), olfactory memory (Si et al., 2004), eye blink conditioning (Thompson and Disterhoft, 1997), passive avoidance learning (Danyz et al., 1988) and spatial memory (Morris et al., 1986; Shimizu et al., 2000; Tsien et al., 1996). Indeed, pharmacological application of NMDA antagonists implies that the NMDA receptors are crucial in memory formation. However, they might not be important for memory preservation (Constantine-Paton, 1994; Izquierdo,

1991; Izquierdo and Medina, 1993; Liang et al., 1993; Quartermain et al., 1994; Rickard et al., 1994). For example, blocking NMDA receptors after learning a task did not impair memory formation. But blocking NMDA receptors prior to learning a task affected memory formation (Rowland et al., 2005; Oye et al., 1992; Hadj Tahar e al., 2004). Additionally, NMDA receptors play a role in odor learning (Mandairon et al., 2006b). For instance, NMDA receptors are implicated in long-term potentiation and long-term depression (Cooke and Bliss, 2006; Bliss and Collingridge, 1993; Bear, 1999).

In olfactory learning, habituation is the decrease in olfactory-initiated behavior as a result of repeated odor presentation. It begins when odor molecules bind to olfactory sensory neurons (OSNs). This is accompanied by the adaptation of the cells in the olfactory system, such as the olfactory receptor neurons and the mitral cells, when the cells are exposed to odorants. Furthermore, the piriform cortex neurons adapt and are also thought to participate in the process of olfactory habituation (Wilson, 2006). Moreover, certain modulators appear to influence habituation to odorants. For instance, norepinephrine depletion affects habituation to odorants, and blockade of NMDA receptors affect memory consolidation (Guerin et al., 2008; Rickard et al., 1994). Indeed, studies by several investigators have shown that NMDA plays an important role in the modulation of olfactory perception in response to enrichment, deprivation, as well as associative learning (Lincoln et al., 1988; Wilson, 1995; Mandairon et al., 2006b). NMDA receptors are located in the cell membrane of primary and secondary dendrites of mitral cells (autoreceptors) (Montague and Charles Greer, 1999; Sassoe-Pognetto and Ottersen, 2000); NMDA receptors are also located in granule dendritic spines. NMDA receptors are found in the glomeruli layer and the external plexiform layer (Montague and Charles Greer, 1999) although their exact location is not known yet (Sassoe-Pognetto and Ottersen, 2000).

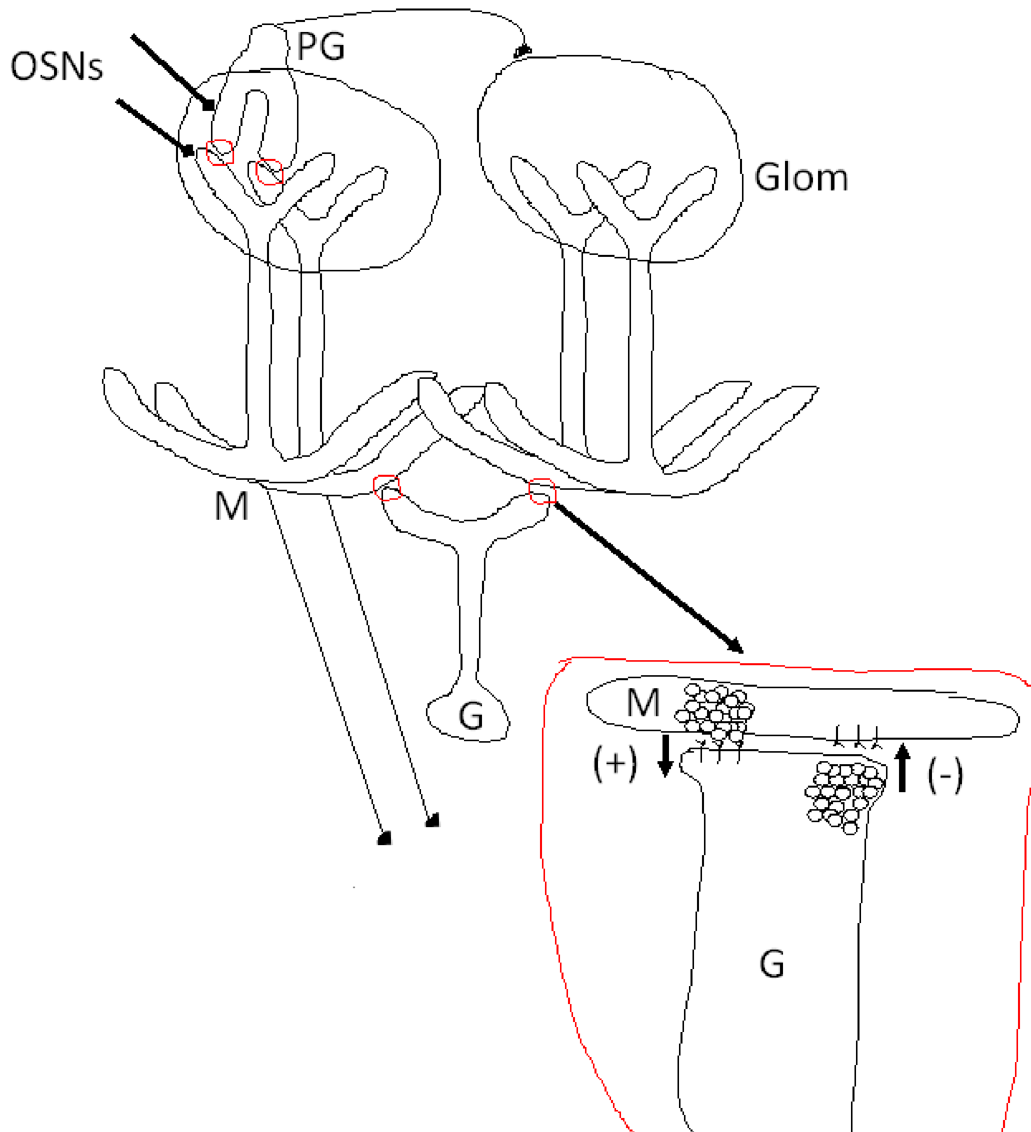

Figure 5. Modified olfactory bulb circuitry. Within the glomeruli, OSNs make dendrodendritic connections (red small circles) with periglomerular cells (PG) at the primary dendrites and with mitral cells (M) at the primary dendrites. Furthermore, PG cells have axons that terminate on adjacent glomeruli. Indeed, mitral cells make dendrodendritic connections with granule cells at the secondary dendrites. Enlarged in a big red circle, there is a reciprocal connection between the shaft of a secondary mitral cell dendrite and a dendritic spine of a granule cell. Enlarged within the red big circle, notice the M to G glutamatergic connection (+) and its reciprocal G to M GABAergic connection (-). For simplicity, short axon cells, and tufted cells as well as other olfactory cells are omitted from this model.

We suspect that the site of action of the NMDA antagonist in our study was at the dendrodendritic connections between granule cells and mitral cells, because these cells are the ones that may play a vital role in habituation memory in the olfactory bulb (Figure 5). Activation of mitral cells by olfactory receptor neurons activates mitral cell glutamate release; glutamate release causes mitral cell self-excitation. Then, glutamate binds to NMDA receptors in the granule cells dendritic spines, which in turn induces granule cells to release GABA (Salin et al., 2001). This granule (G) to mitral (M) synapse inhibits mitral cells (Balu et al., 2007; Chen et al., 2000; Halabisky et al., 2000; Urban and Sakmann, 2002). Because granule cells exhibit connections with mitral cells, GABA release can inhibit mitral cells causing lateral inhibition (Yokoi et al., 1995; Isaacson and Strowbrigde, 1998; Urban and Sakmann, 2002; Schoppa and Westbrook, 1999; Margrie et al., 2001). Moreover, constant excitation of granule cells might lead to down regulation of the mitral cells odor response, which can be the mechanism underlying olfactory habituation. Furthermore, application of the MK-801 NMDA antagonist, *in vivo,* shows that dendrodendritic inhibition is driven by activation of NMDA receptors; and that functional NMDA receptors are necessary for granule to mitral cell inhibition since application of the NMDA antagonists MK-801 leads to mitral cells disinhibition (Schoppa, et al., 1998). Based on our olfactory habituation results, when we applied the potent NMDA antagonist, we blocked NMDA receptors and this blockade of NMDA receptors was reflected in impairment in olfactory memory formation. In conclusion, our results indicate that plasticity in olfactory habituation memory is blocked by application of the potent noncompetitive NMDA receptor antagonist MK-801; and that this potentially can be the mechanism by which olfactory habituation occurs in the olfactory bulb.

APPENDIX

OLFACTORY BULB HABITUATION TO ODOR STIMULI

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Abstract

Habituation is a simple form of memory, yet its neurobiological mechanisms are only beginning to be understood in mammals. In the olfactory system, the neural correlates of habituation at a fast experimental timescale involving very short intertrial intervals (tens of seconds) have been shown to depend on synaptic adaptation in olfactory cortex. In contrast, behavioral habituation to odorants on a longer timescale with intertrial intervals of several minutes depends on processes in the olfactory bulb, as demonstrated by pharmacological studies. We here show that behavioral habituation to odorants on this longer timescale has a neuronal activity correlate in the olfactory bulb. Spiking responses of mitral cells in the rat olfactory bulb adapt to, and recover from, repeated odorant stimulation with five-minute intertrial intervals with a timecourse similar to that of behavioral habituation. Moreover, both the behavioral and neuronal effects of odor habituation require functioning NMDA receptors in the olfactory bulb.

INTRODUCTION

Animals are submitted to a flow of sensory information which the nervous system filters to identify information that may be of particular importance.

Habituation is a simple form of non-associative learning in which behavioral responses to repeated, non-reinforced sensory stimuli are progressively reduced, enabling an animal to perceptually deemphasize persistent or static stimuli in favor of novel or changing stimuli. Habituation has been described experimentally in multiple sensory modalities within various invertebrate and vertebrate species including sea slugs, fruit flies, nematodes, birds, and mammals (Christoffersen 1997; Rankin and Broster 1992). In the rodent olfactory system, both autonomic and behavioral habituating responses can be evoked by repeated odor stimulation (Wilson and Linster 2008).

Behaviorally, olfactory habituation can be induced by multiple paradigms that differ in timescale and are thought to be mediated by distinct mechanisms within different regions of the olfactory system (McNamara et al., 2008; Wilson and Linster, 2008). For example, a form of short-timescale habituation, induced by repeated 20-second stimulations with 10-second intertrial intervals, persists for less than ten minutes and is mediated within piriform cortex, whereas a form of habituation induced by repeated 50-second stimulations with 5-minute intertrial intervals persists for at least 30 minutes and is mediated within the olfactory bulb. The neural correlates of this short-timescale behavioral habituation have been demonstrated in piriform cortical pyramidal cells in which adaptation is associated with mGluR II/III-mediated depression of the glutamatergic mitral-pyramidal cell synapse (Wilson 1998a; 2003; 1998b). In contrast, longer-timescale behavioral habituation requires functioning NMDA receptors within the olfactory bulb and is not affected by blockade of mGluR II/III receptors therein (McNamara et al., 2008).

Neuronal adaptation to persistent stimulation occurs at many levels in the olfactory system, including olfactory sensory neurons (Kurahashi and Menini, 1997; Zufall and Leinders-Zufall, 2000) as well as neurons within the olfactory bulb and

piriform cortex. As in other sensory systems, central olfactory neurons show greater adaptation than do primary sensory neurons, and both olfactory bulb mitral cells and piriform cortical pyramidal cells have been shown to adapt to odorant stimulation under certain conditions (Chaput and Panhuber, 1982; Gray and Skinner, 1988; Shea et al., 2008; Wilson, 2000).

In the present study, we investigated whether the adaptation of neuronal responses in olfactory bulb mitral cells could underlie a form of behavioral adaptation routinely used in olfactory behavioral studies (Bath et al., 2008; Cleland et al., 2002; Linster et al., 2001; Mandairon et al., 2006b; Wesson et al., 2008). We first show that odor responses in olfactory bulb mitral cells adapt to odorants in response to a similar stimulation paradigm than that used in behavioral habituation and that this neural adaptation depends on stimulus duration and intertrial interval parameters. We then show that both neuronal adaptation depends on functioning NMDA receptors in the olfactory bulb and follow up on this result by showing that behavioral habituation using the same experimental parameters also depends on functioning NMDA receptors in the olfactory bulb. We conclude that habituation to repeated odor stimulation in this paradigm is likely to be mediated by reductions in the odor responses of olfactory bulb mitral cells and that bulbar NMDA receptors are involved in this process.

METHODS

Electrophysiology

Animals

Adult male Sprague-Dawley rats (200-250 g) were purchased from Charles River Laboratories (Wilmington, MA). Rats were singly-housed with water and food available *ad libitum* and maintained on a 12:12 hour light/dark cycle. All procedures

were performed according to NIH guidelines under the supervision of the Institutional Animal Care and Use Committee of Cornell University.

Experimental preparation

Animals were anesthetized with urethane (1.5 g/kg intraperitoneal; Sigma-Aldrich, St. Louis, MO) and placed in a stereotaxic apparatus (Narishige Scientific Instruments, Tokyo, Japan). The skull was exposed by scalpel incision and a hole was drilled over each of the lateral olfactory tracts (AP +3.7 mm, ML ±3.4 mm) and over the olfactory bulbs (Paxinos and Watson, 1998). Respiratory activity was monitored throughout the experiments using a piezoelectric monitor strapped around the animal's chest and sent to the computer, hence enabling synchronization of odor delivery with inhalation.

Drugs

The NMDA antagonist MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine hydrogen maleate; 8 mM concentration) was dissolved in sterile 0.9% physiological saline and directly 6 uL were infused into the MOB at an infusion rate of 2 ul/min using a 50 ul Hamilton syringe attached to a Stoelting stereotaxic syringe pump. The dosage and volume were based on previously published studies (Mandairon et al., 2006b).

Electrophysiological recordings

Bipolar stimulating electrodes (100 um stainless steel, Formvar-insulated) were stereotaxically placed in the lateral olfactory tract (LOT; AP +3.7 mm, ML ±3.4 mm, DV 6.5 mm) in order to evoke antidromic action potentials in mitral cells. Stimulation currents (100 us duration, 200-900 uA) were delivered by a constant-current stimulus

isolation unit (Grass model PSIU6) controlled by a Grass S88 stimulator (Grass Technologies, West Warwick, RI). Neuronal responses were recorded using tungsten stereotrodes (3-5 Mohm; World Precision Instruments, Sarasota, FL). Electrodes were lowered into the mitral cell layer using a stereotaxic micromanipulator (David Kopf Instruments, Tujunga, CA). Optimal placement of the recording electrode into the mitral cell layer of the MOB was achieved by monitoring the size and shape of field potentials (1000x amplification, 0.1Hz - 475Hz bandpass, 20 kHz sampling rate) following LOT stimulation. Single units (5000x amplification, 600 Hz – 6 kHz bandpass, 20 kHz sampling rate) were recorded in the ventrolateral and dorsomedial regions of the OB. Data were digitized and recorded to computer using a CED Power1401 digitizer and Spike2 software (Cambridge Electronic Design, Cambridge, UK).

Odor Stimulation

After establishing stable single-unit recordings, but prior to beginning each experiment, each cell's responsiveness to a variety of odors was measured. Odorants screened were the esters ethyl butyrate and ethyl pentanoate, the alcohols heptanol and hexanol, the aldehydes heptanal and hexanal, and the organic acids butyric acid and propanoic acid. The odorant that evoked the most robust excitatory response (greatest increase in overall spike rate) in the majority of presumed mitral cells within each test was subsequently used in that experiment. For odor delivery, odorant chambers in a custom-built olfactometer were loaded with stock solutions of odorants diluted in mineral oil so as to emit a consistent theoretical vapor-phase partial pressure of 100 Pa. Odors were then delivered by directing a 50 ml/min stream of humidified air through a selected odor chamber and subsequently into a carrier stream of charcoal-

filtered, humidified air (1 L/min) resulting in an approximate 20x dilution to yield a final odorant vapor-phase partial pressure of ~5 Pa.

Odor response testing and adaptation proceeded as follows. First, the cell's odor responses were probed with a *response testing protocol*: five two-second odor pulses separated by two-minute intertrial intervals (Pre; Figure 1A; Chaudhury et al., 2009; Wilson, 2000). An *adaptation protocol* matching that of the corresponding behavioral experiments was then immediately administered: four 50-second odor pulses separated by five-minute intertrial intervals. Finally, the response testing protocol was repeated at both 5-minute and 60-minute timepoints following the end of the adaptation protocol (Post5 and Post60; Figure 1A). In experiments designed to further characterize the stimulation parameters necessary to induce adaptation, either the stimulus duration (d=2, 10, 20 or 30 seconds) or the interval between stimulations (ITI=1 or 2.5 minutes) was varied.

Spike Sorting

Single units were extracted offline using Spike2 software. Briefly, spike templates were derived and selected from the raw data and then used to extract units from the entire data set. Extracted spikes were further validated and separated using principal components analysis (PCA) whereby features from the data are extracted and clustered to groups of similar waveforms. Following spike sorting, the number of spikes for each single unit identified was automatically counted from 4 sec prior to 4 sec after each odor onset.

Histological verification of electrode placements

At the end of each experiment, positive current was passed through the recording electrodes (10 seconds duration, 1-15 mA) to produce a small lesion in the

olfactory bulbs. Transcardial perfusion was then performed with saline and 10% neutral buffered formalin. Brains were removed, sectioned at 40 um, and subsequently stained with cresyl violet for electrode localization.

Data Analysis

We first determined whether a given cell exhibited a significant increase in firing rate in response to odor exposure by comparing the number of spikes evoked in the 4 second window immediately prior to odor delivery to the 4 second window beginning at odor onset (paired t-test, $\alpha = 0.05$; (Chaudhury et al., 2009; Wilson, 2000). If so, the given cell/odor combination was included in the data analysis. The pre- and post-adaptation response magnitudes of each cell to the test odorant used were calculated as the difference between the number of action potentials evoked four seconds before and after each odor stimulation onset. Pre- and post-adaptation response magnitudes were then normalized with respect to the average pre-adaptation magnitude, hence, all graphs depict response magnitudes as percentages with respect to the mean pre-adaptation response. To determine if the adaptation protocol had a significant effect on mitral cell responses to odorants, an analysis of variance was performed on these normalized response magnitudes with test latency (i.e., pre-habituation, 5 min post-habituation, 60 min post-habituation) as main effect.

Behavioral experiments

Subjects

Eleven adult male Sprague-Dawley rats (250-300 grams), purchased from Charles River Laboratories (Wilmington, MA), were utilized for behavioral experiments. Animals were singly-housed and maintained at a constant temperature on a 12:12 light/dark cycle with water and food provided *ad libitum*. Prior to

behavioral experiments, animals were provided with seven days' acclimation time, during which investigators handled them for one hour daily. All procedures were performed according to NIH guidelines under the supervision of the Institutional Animal Care and Use Committee of Cornell University.

Cannulation

Rats were anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (7.5 mg/kg) and secured in a stereotaxic apparatus (Narishige Scientific Instruments, Tokyo, Japan). Guide cannulae (22-gauge; Plastics One, Roanoke, VA, USA) were inserted into both OBs according to standard procedures (Mandairon et al., 2006a). Cannulae were implanted at the following coordinates with respect to bregma: AP +8.0 mm, ML ±1.5 mm, DV 4.5 mm. The tips of the guide cannulae were positioned 1.0 mm dorsal to the target infusion site; consequently, infusion cannulae extended 1.0 mm from the end of the guide cannulae. Five screws were drilled into the skull, and dental cement was used to secure the guide cannulae to these screws and to cover the incision area. Dummy infusion cannulae were then placed into the guide cannulae to prevent blockage or infection. Following surgical implantation, rats were allowed to recover for 10 days.

Drug Administration

For drug/vehicle administration immediately prior to behavioral experiments, two infusion cannulae were fitted into the guide cannulae so that their tips protruded 1.0 mm beyond the ends of the guide cannulae into the center of each MOB. Two 10 μl Hamilton syringes containing either drug solution or vehicle were attached to the cannulae with a polyethylene tube and driven with paired infusion pumps (YA-12 Genie pumps, Kent Scientific). Drug dosage and infusion volume were matched with

those of the electrophysiological experiments in this study and have been shown to be effective in previous studies (Mandairon et al., 2006b). Specifically, the NMDA antagonist MK-801 (8 mM and 4 mM, Sigma-Aldrich, Natick, MA) was dissolved at 37C in 0.9% saline; the drug (or saline vehicle) was then delivered bilaterally into awake rats at a rate of 2 ul/min for 3 minutes (6 µl total volume delivered per side). The infusion cannulae remained in place for 1 additional minute after the infusion ended in order to minimize backflow. Behavioral testing was performed 20 minutes after drug administration was completed. Infusion of 8mM MK-801 led to a general depression of the behavioral response which made results difficult to interpret; we therefore followed up with a lower dosage (4mM) and show results obtained with the lower dosage in the figures.

Odors

Two odorants, ethyl butyrate and ethyl pentanoate (Sigma-Aldrich, Natick, MA), were employed for habituation testing. Only one odorant was used during a single day of training/testing; the experiment then was repeated in each rat on a different day using the second odorant to enable counterbalancing of drug/vehicle administration groups. Prior to testing, odorants were diluted in mineral oil so as to each theoretically emit a vapor-phase partial pressure of 5 Pa (corresponding to vol/vol dilution of 0.09 for ethyl butyrate and 0.3% for ethyl pentanoate). Vapor pressures of pure odorants were estimated with the Hass-Newton equation as implemented in ACD/Boiling Point & Vapor Pressure Calculator (version 4.5; Advanced Chemistry Development, Toronto, Ontario, Canada).

Behavioral Experiments

An olfactory habituation task measures non-associative memory formation; cross-habituation testing with multiple odors can then be used to measure the specificity of this memory over time (Cleland et al., 2002). The behavioral experiments performed here replicate those previously performed in mice in our lab (McNamara et al., 2008); however, the experiments presented here were conducted with the goal of directly comparing electrophysiological and behavioral data by matching species, drug dosages, odors and odor concentration. All habituation experiments took place in the home cage under red light. Odors were presented by placing 60 ul of the diluted odor stimulus onto filter paper (Whatman #1) contained within a weighing dish that was placed on top of the cage lid (Figure $1B_i$); this procedure enabled the observer to change the odor stimulus without disturbing the animal. Each test session was preceded by one 50-second presentation of plain mineral oil (MO). Test sessions comprised four 50-second presentations of diluted odorant separated by five-minute intertrial intervals, followed by one additional presentation of the same odorant at 30- and 60-minute time points after the last habituation trial (Figure $1A_{ii}$). Comparison of investigation times during the first and fourth odor habituation trials measures initial habituation (5-minute delay), whereas comparison between the first odor presentation and either of the two delayed (30- and 60-minute) trials measures the persistence of habituation memory. The 5-minute and 60-minute delayed trials (i.e., the fourth and sixth odor presentations) are emphasized in analyses for direct comparison with electrophysiological data. Active sniffing within one centimeter of the odor source was recorded with a stopwatch. The observer was blind as to the treatment group and in experiments involving more than one odor, odors were coded and randomized by a member of the lab. Figures show mean investigation

times +/ standard error. All rats underwent both drug treatments using a separate odor for each and the order of drugs was counter balanced among the rats.

In a control experiment, rats were presented with the mineral oil carrier alone for four 50-second trials after which the odorant was presented in a single test trial, hence measuring rats' ability to detect the odorants used. This control ensured that rats treated with the NMDA antagonist were not simply unable to detect the odors presented. As in the first experiment, all rats were tested with both odorants and drugs and the order of drug was counterbalanced.

Data analysis

An analysis of variance (ANOVA) was performed using SPSS statistical software (SPSS, Chicago, IL) with *odor investigation time* as the dependent variable, and *drug group* and *odor presentation trial number* as main effects. Fisher post hoc pairwise testing was then used to assess whether the time spent investigating during the fourth habituation trial or later test trial were significantly lower than the time measured during the first habituation trial ($\alpha = 0.05$).

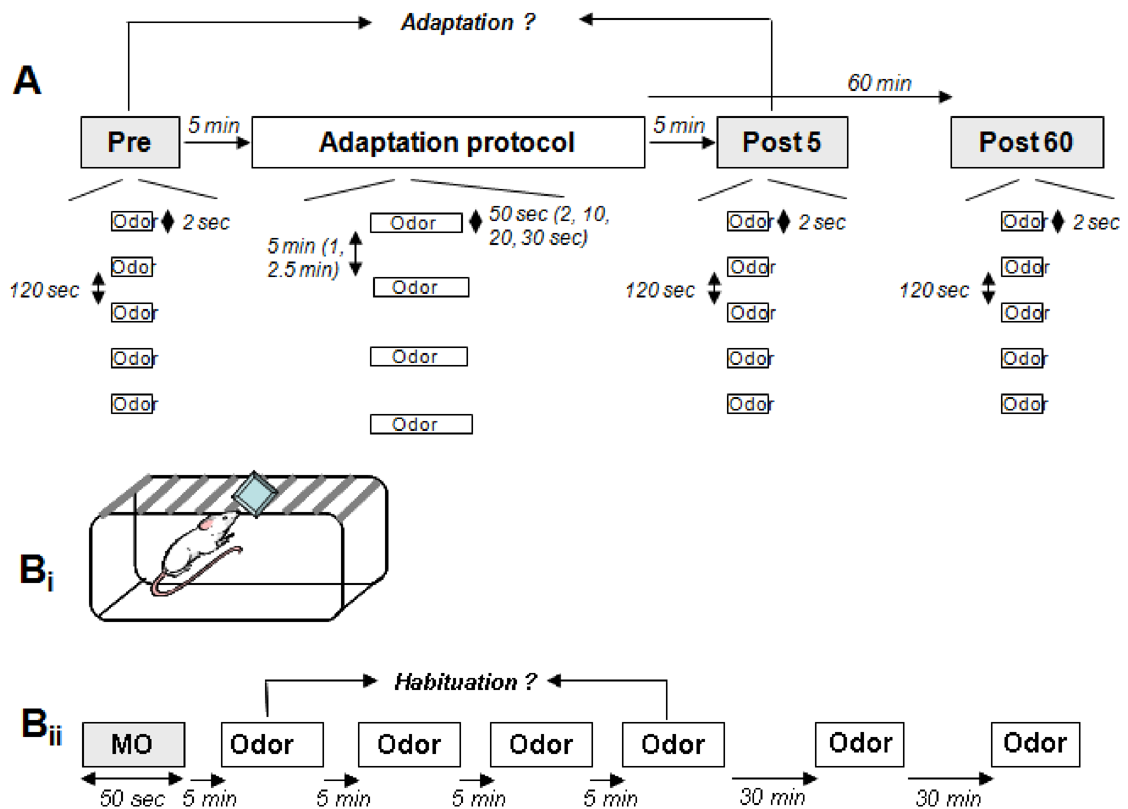

Figure A1. Schematic depiction of electrophysiological (A) and behavioral (B) experimental protocols. A. Electrophysiological protocol. During electrophysiological recordings, odor responses were first measured (*Pre*) by presenting a 2-second odor stimulus five times with 120 sec interstimulus intervals (*response testing protocol*). The adaptation *protocol*, consisting of four fifty-second presentations of the same odorant separated by five-minute intertrial intervals, was then administered. In separate experiments, stimulus duration and ITIs were varied independently of each other. Five and sixty minutes after the end of the last adaptation trial (*Post*), the response testing protocol was again delivered. $\mathbf{B_i}$. Behavioral protocol. Rats were presented with a weighing dish containing either mineral oil (MO) or the habituation/test odorant. $\mathbf{B_{ii}}$. After a single presentation of mineral oil alone, the habituation/test odorant was presented for four consecutive trials, separated by five minute intertrial intervals, and then twice more at 30 and 60 minutes' latency following the last habituation trial.

RESULTS

Neural adaptation to repeated odor stimulation

We recorded from a total of 33 presumed mitral cells, of which 20 (60%) exhibited a significant excitatory response to at least one of the odors used in the study and hence were included in analyses. Cells' spontaneous activities ranged from 3 – 60 Hz with an average value of 29.23 Hz (+/- 5.17 Hz). Cells' firing rates in response to odorants (difference between pre and post odor firing rate) ranged from 4 to 19 Hz with an average rate of 6.3 Hz (+/- 1.7 Hz). Figure A2 shows raw traces of one mitral cell over the course of the experimental paradigm. Overall, cells exhibited robust adaptation after exposure to the adaptation protocol of repeated odor stimulation, as evidenced by a significant reduction in mitral cell responses to the test odor during post-adaptation tests (Figure A3). There was no correlation between the initial response rate and the degree of adaptation (Pearson's R = 0.38; $p > 0.2$). Specifically, analysis of variance revealed a significant difference during pre- and post-adaptation tests ($F_{test}(2,54) = 19.371$; $p < 0.001$); subsequent *post hoc* comparisons showed that mitral cell odor responses at 5 minutes following the adaptation protocol were significantly attenuated relative to pre-adaptation responses ($p < 0.001$ in both cases; Fisher LSD), but not at 60 minutes ($p > 0.05$, Fisher LSD). Control experiments (47 cells, of which 7 (15%) exhibited significant odor responses and hence were included in analyses), in which only mineral oil odor was presented during the adaptation protocol, showed no significant effect on mitral cell odor responses among the three test latencies ($F_{test}(2, 18) = 0.532$; $p > 0.5$), indicating that mitral cell adaptation is not due simply to fatigue and does not arise from stimulation with the carrier alone (Figure 3A). Moreover, mitral cells' spontaneous activity (measured in the 4 second window before each odor stimulation) was not affected by repeated odor stimulation

(ANOVA comparison of pre-adaptation, 5-minute post- adaptation and 60-minute post-adaptation latencies; $F_{test}(2, 27) = 0.18$, $p > 0.05$).

Additional experiments were conducted to further characterize the parameters of neural adaptation after repeated odor stimulation. First, experiments were performed to determine the duration of odor exposure required to induce response adaptation in mitral cells (Figure 3B). Rats were exposed to adaptation protocols of four odor stimulations with reduced durations (d = 2, 10, 20 or 30 seconds) separated by 5 minute ITIs. Recordings were made respectively in 24 (d = 2, 13 (54%) responsive), 19 (d = 10, 10 (58%) responsive), 27 (d = 20, 12 (44%) responsive) and 28 (d = 30, 6 (21%) responsive) cells. Repeated stimulation with either 2, 10 or 20 second odor durations did not evoke significant adaptation in mitral cells ($d = 2$: $F(1, 36) = 0.892$, $p > 0.4$; $d = 10$: $F(1, 27) = 0.736$, $p > 0.4$; $d = 20$: $F(1, 33) = 1.504$, $p > 0.2$); however, repeated stimulation with 30-second odor durations evoked significant adaptation ($d = 30$: $F(1,15) = 4.331$; $p < 0.05$). Second, we tested the length of the ITI required to induce significant adaptation (Figure 3C). Rats were exposed to four repeated odor stimulations of 50 seconds each, but with shorter ITI durations (ITI = 1.0 and 2.5 minutes; compare to the previous experiments using ITI = 5.0 minutes). Repeated stimulations with 50 second odor pulses and 1 minute ITIs did not induce significant adaptation of odor responses ($F(1,21) = 2.4$; $p < 0.1$), whereas repeated stimulations with 2.5 minute ITIs did induce significant adaptation ($F(1,39) = 10.2$; $p < 0.005$). The data from the above two experiments show that in order to induce the type of neural adaptation observed here, odor stimulations presented for longer than 20 seconds, with ITIs longer than 1 minute, are required. Third, we tested cross-adaptation between odorants (Figure 3D). Briefly, mitral cell responses to two odorants, differing by 1 (C1), 2 (C2) or 3 (C3) carbons in their carbon chain were tested (pre-test); the cell was then repeatedly stimulated with one of these two

odorants using 50 second stimulus duration and 5 minute ITIs (adaptation). The cell's responses to both test odorants after adaptation was then recorded five minutes after the adaptation protocol (post-test). Figure 3D shows that compared to the pre-test response, the responses to all tested odorants differing by 0 (CO = habituation odor), 1 (C1), 2 (c2) and 3 (C3) carbons from the habituated odor were significantly lower than during pre-tests (C0 or adaptation odor: $F(1, 24) = 88.838$, $p < 0.001$; C1: $F(1, 17) = 127.849$, $p < 0.001$; C2: $F(1, 16) = 249.789$, $p < 0.001$; C3: $F(1, 15) = 15.081$, $p < 0.005$). Responses to odors differing by 4 carbons from the habituated odor were not significantly decreased after the adaptation protocol (C4: $F(1, 14) = 2.900$, $P > 0.1$). This shows that as expected from previous behavioral and electrophysiological experiments, bulbar adaptation is relatively, but not completely, odor-nonspecific (Wilson, 2000; McNamara et al., 2008).

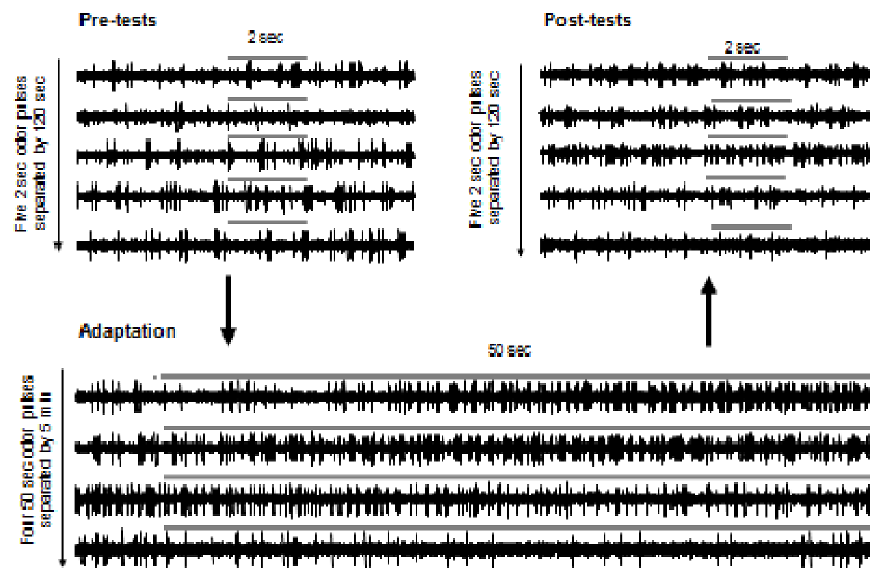

Figure A2. Individual mitral cell responses to one odor over the course of the recording protocol. Raw recorded data from one mitral cell are shown during pre-tests (five 2-second odor presentations separated by 2-minute ITIs), followed by four 50-second adaptation stimuli with the same odorants separated by 5-minute ITIs (adaptation), followed 5 minutes later by a second series of five 2-second odor stimuli separated by 2-minute ITIs. Gray bars indicate odor stimulation times and durations.

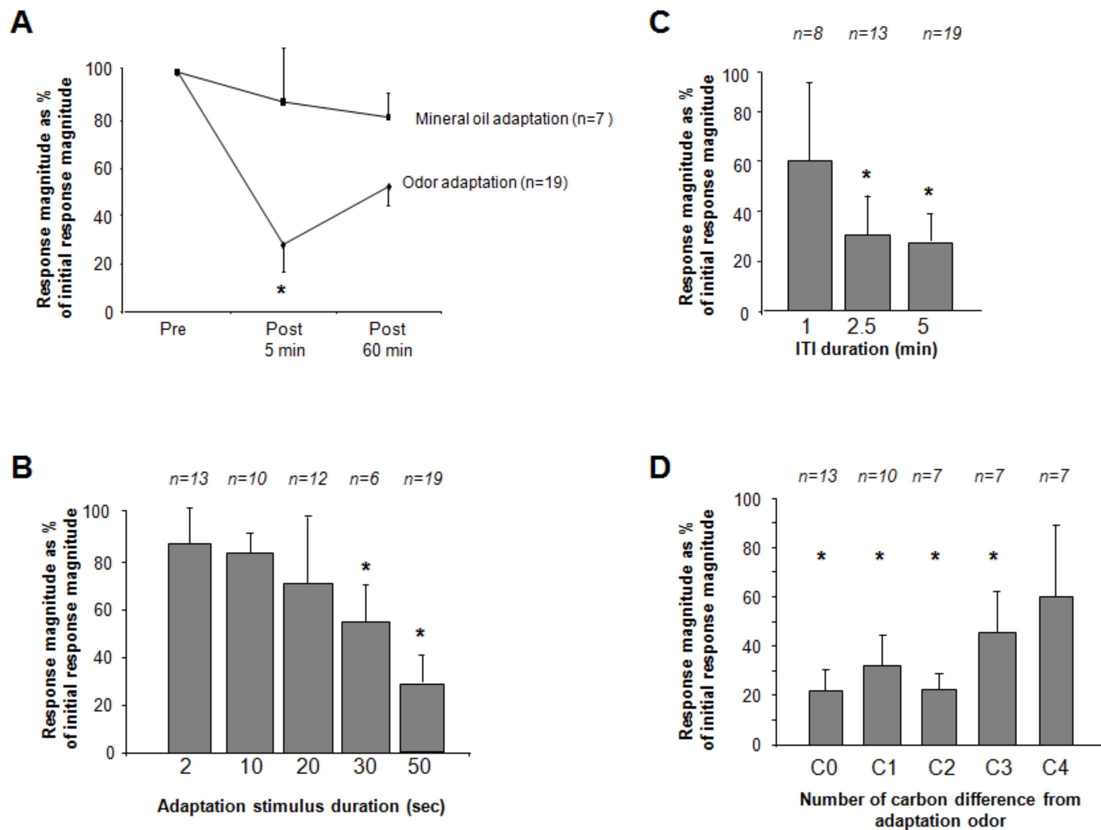

Figure A3. Neural adaptation to repeated odor presentations. A. Average number of spikes evoked by odor presentations during pre- and post- adaptation testing expressed as percentages of the response during pre- adaptation testing. Mitral cells stimulated with the adaptation odorant (*Odor adaptation, solid line*) over four trials during the adaptation protocol responded significantly less during both 5- but 60-minute post- test than during the pre- adaptation tests. Asterisks indicate a significant decrease as compared to pre- testing. In contrast, mitral cells stimulated with plain mineral oil (*Mineral oil adaptation, dashed line*) during the adaptation protocol did not change their responses significantly compared to pre- adaptation test responses. **B.** Post-test responses to odorants expressed as the percentage of the pre-test responses in cells stimulated with 5 repeated stimulations of 2, 10, 20, 30 and 50 second durations and 5 minute ITIs. **C**. Post-test responses to odorants expressed as the percentage of pre-test responses in cells in cells stimulated with 5 repeated stimulations of 50 seconds separated by 1, 2.5 and 5 minute ITIs. **D**. Post test responses to test odorants differing by 1, 2, 3 or 4 carbons from the odorant used as the adaptation odorant expressed as percentage of the pre-test response to this odorant. Asterisks indicate a significant reduction in response magnitude as compared to pre-adaptation.

Neural adaptation is NMDA receptor-dependent

In electrophysiological experiments, pharmacological blockade of NMDA receptors in the olfactory bulb impaired the adaptation of mitral cell responses to odor stimulation. Recordings were made from 30 cells of which 13 (43%) exhibited significant excitatory responses to at least one odor. In contrast to the significant effect of adaptation on mitral cell odor responses in animals infused with saline (Figure 4A), mitral cells' odor responses in the presence of the NMDA receptor antagonist MK-801 exhibited no significant effect of the adaptation protocol ($F_{test}(2,36) = 0.555$; $p > 0.5$), demonstrating that mitral cell response adaptation to odors is NMDA receptor-dependent (Figure 4A).

A second analysis was then performed on the same data under both drug conditions to compare the percentage of cells exhibiting significant odor responses 5 and 60 minutes after odor adaptation (among the cells tested, all of which had responded significantly to the test odor prior to adaptation). Analysis of variance demonstrated significant effects of both test number (pre-, 5-min post-, 60-min post-) and drug condition (saline or MK-801 infusion) as well as a significant interaction ($F_{test}(2,63) = 11.162$, $p < 0.01$; $F_{drug}(1,63) = 7.513$, $p < 0.01$; $F_{drug*test}(2,63) = 3.532$, $p < 0.05$). *Post hoc* comparisons indicated that test latency was a significant main effect only in the vehicle-infused control group ($F_{test}(2, 27) = 10.8$, $p < 0.001$), in which responses in all three test phases were significantly different from one another ($p < 0.05$ for all pairwise comparisons; Fisher LSD). In contrast, there was no significant effect of adaptation on mitral cell responses in the presence of MK-801 (Figure 4B), thereby confirming the role of NMDA receptors in mitral cell adaptation.

Comparison of mitral cell responses to odor before and after the administration of MK-801 (prior to any adaptation protocol) showed that the addition of MK-801

alone did not affect mitral cell responses to odors (p > 0.05; Fisher LSD). Furthermore, odor-independent spontaneous activity in mitral cells was not affected either by the application of MK-801 (in agreement with (Philpot et al., 1998)) or the administration of the odor adaptation protocol (comparison of pre-odor baseline activity levels from four experimental timepoints: before MK-801 administration, after MK-801 but prior to the adaptation protocol, and 5 and 60 minutes post-habituation; $F_{test}(3,12)=0.3$; $p = 0.8$, data not shown).

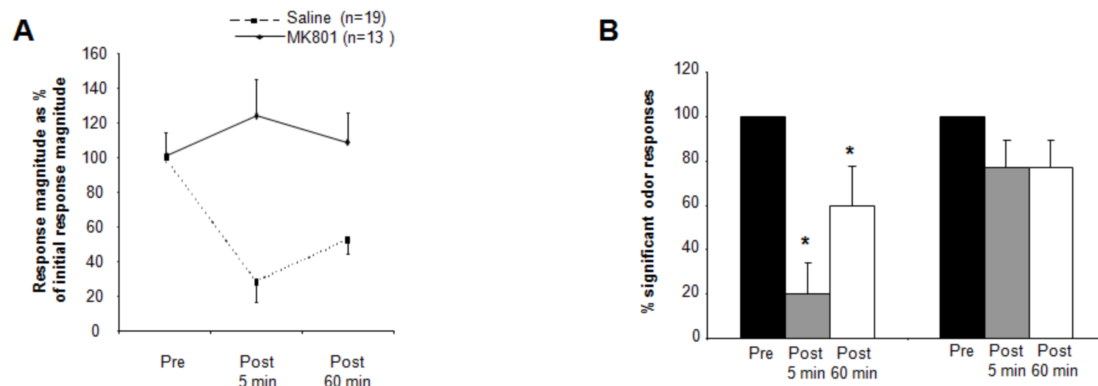

Figure A4. Effect of bulbar NMDA receptor blockade on neural adaptation. A. Average responses of mitral cells during pre-adaptation and post-adaptation odor tests in rats infused with MK-801, expressed as percentages of the average pre-adaptation test response. In these rats, responses to odorants post-habituation were not significantly different from those recorded pre-adaptation. **B**. Percentages of cells responding significantly to odor presentations in pre- adaptation and post- adaptation tests under control and MK-801 conditions. *Asterisks* indicate a significant decrease in response magnitude as compared to the first trial or pre- adaptation response (p < 0.05).

Mitral cell odor responses decrease over the course of repetitive, but not single, 50 second odor stimulation.

In the present experiments, mitral cell adaptation occurs when cells are stimulated multiple times with odor stimulations of at least 30 seconds, separated by more than 2.5 minute intertrial intervals. Previous experiments by Wilson (2000)

showed that during a single 50-second odor stimulation, mitral cells adapted to a much lesser degree than the adaptation shown here after four consecutive adaptation stimuli. To better compare these experiments, we analyzed the responses of mitral cells during the four 50-second adaptation trials in saline-infused and in MK-801-infused rats. No significant differences in spontaneous activity over the course of the four adaptation trials were observed in control ($F(3, 54) = 0.094$; $p > 0.9$) or MK-801 rats ($F(3, 72) = 0.241$; $p > 0.5$) (Figure 3A_i, B_i). When average numbers of evoked spikes during the four 50-second adaptation trials were compared, a significant effect of trial number was observed in saline control ($F(3, 15) = 3.345$; $p < 0.05$, Wilkens-Lambda, Figure 3A_{ii}) but not MK-801 infused rats ($F(3, 10) = 7.69$; $p > 0.05$; Figure 3B_{ii}). Further analysis showed that in saline infused control rats, average spike rates were significantly lower during the third adaptation trials as compared to the first ($p < 0.05$ with Fisher LSD). No significant changes in spike rate over the course of any adaptation trials were observed ($p > 0.05$ in all cases) when trials were analyzed in 10 second bins (Figure B), except for the third adaptation trial in saline infused rats ($p < 0.05$). These data suggest that the NMDA-dependent process leading to adaptation in this paradigm happens mostly in between odor stimulus presentations rather than during prolonged odor stimulation.

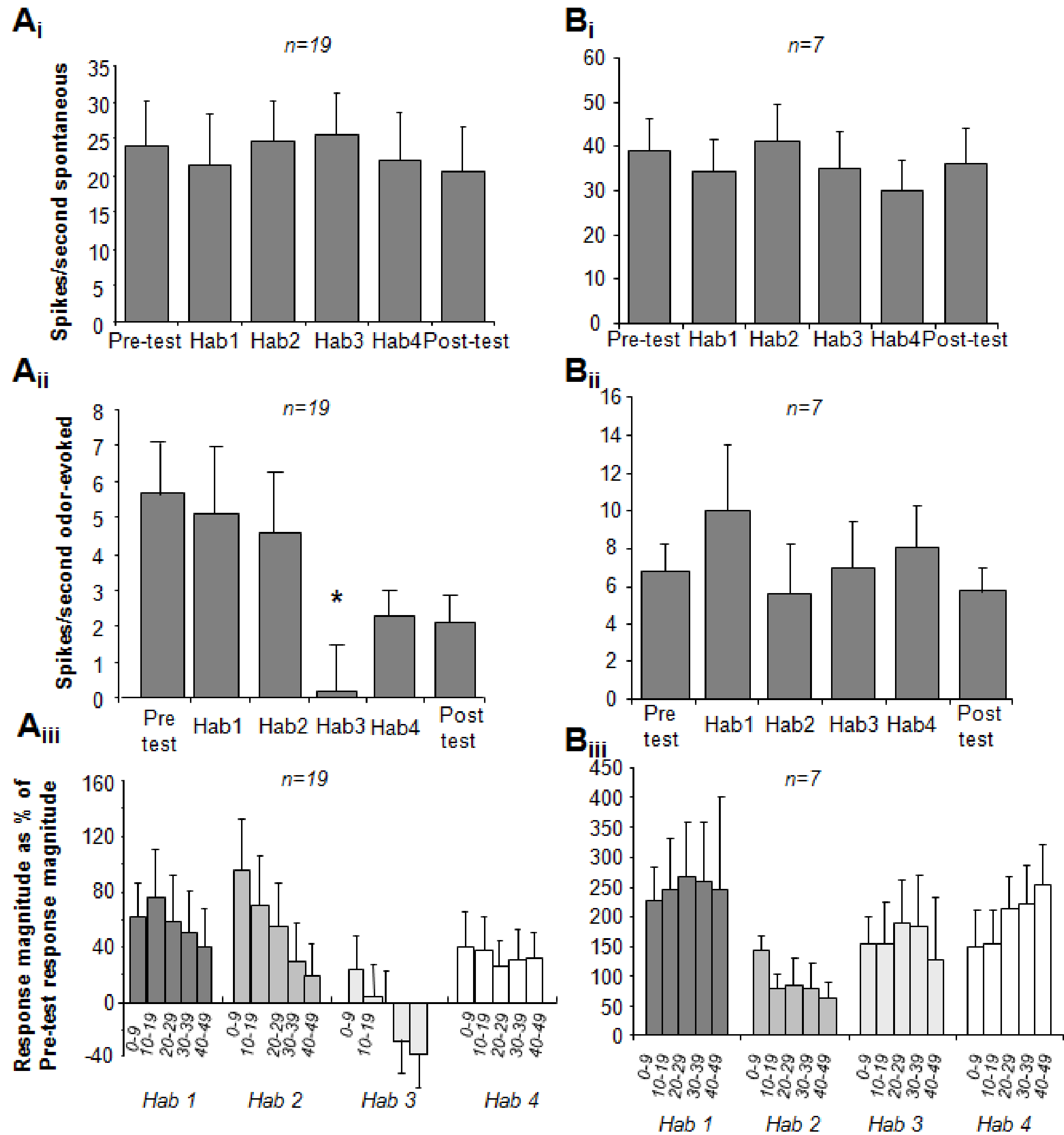

Figure A5. Average responses of mitral cells during the adaptation protocol in control and MK-801 rats. A_i and B_i. Spontaneous activity, recorded for 4 seconds before each odor presentation, did not change significantly over the course of a recording protocol (Ai: control rats, Bi: MK-801 infused rats). **A_{ii} and B_{ii}**. Average number of evoked spikes per second during pre-test, adaptation trials and posttests. In control rats, the average number of odor evoked spikes per second significantly decreased after the first two adaptation trials (A_{ii}), whereas in MK-801 infused rats no significant decrease was observed (B_{ii}). Asterisk indicates a significant difference to the first adaptation trial. **A_{iii} and B_{iii}**. Time course of mitral cell responses during adaptation trials. The graphs show the average number of odor-evoked spikes normalized by the number evoked during pre-tests recorded in 10-second intervals. No significant change over the course of a 50-second odor presentation was observed in either group of animals, except for the first adaptation trial in control rats.

Behavioral habituation to repeated odor stimulation requires functioning NMDA receptors in the OB

Behavioral studies were performed to test the dependence of odor habituation on bulbar NMDA receptors in rats. One group of rats received infusions of the NMDA receptor antagonist MK-801 (4 mM) into the olfactory bulb while a control group was infused with saline vehicle (Figure A.6); rats were then presented with mineral oil, then four habituation trials separated by 5 minute ITIs, and finally two additional odor presentations at 30 and 60 minutes after the last habituation trial. Analysis of variance revealed significant main effects of both drug treatment ($F_{drug}(1, 114) = 11.462$, $p < 0.001$) and trial number ($F_{trial}(5, 14) = 5.136$, $p < 0.001$), as well as a significant interaction ($F_{drug*trial}(5, 114) = 2.394$, $p < 0.05$). In vehicle-infused controls, there was a significant effect of trial (ANOVA; $F_{trial}(5, 60) = 7.555$, $p < 0.001$) and a significant reduction of investigation time in the fourth trial as compared to the first ($p < 0.001$), as expected based on previous experiments. In contrast, no significant effect of trial was observed in MK-801-infused rats ($F_{trial}(5, 54) = 1.507$; $p > 0.2$) and there was no significant difference between the response during the first and fourth trial ($p > 0.7$; Figure A6.A).

To ensure that MK-801 treated animals were still able to detect odorants, we performed a behavioral control experiment in which vehicle-infused and MK-801-infused rats were presented with mineral oil over four successive trials, followed by a single presentation of a test odorant. We found no significant effect of drug group ($F_{drug}(1, 91) = 0.013$, $p > 0.9$), indicating that both groups performed similarly in this control experiment. Specifically, in both groups rats investigated the odor significantly more than the mineral oil ($p < 0.05$ for both groups, Figure A6.B).

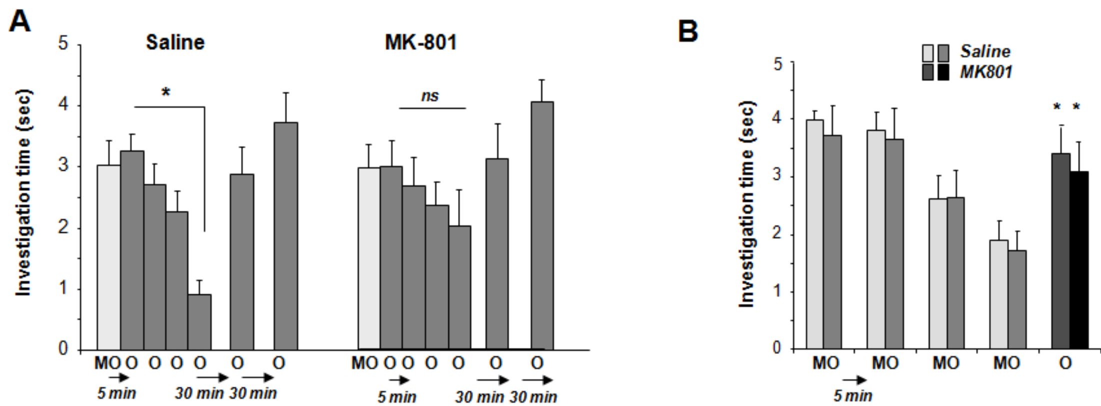

Figure A6. Behavioral results. A. Average behavioral investigation times in saline and MK801 infused rats over the course of the behavioral experiment. Saline-injected control rats responded significantly less on the fourth habituation trial as compared to the first trial (* indicates a significant difference between responses during the first and fourth trial), whereas MK-801 infused rats did not significantly habituate, as indicated by a non-significant difference between investigation times during the fourth and first trials. **B**. Blockade of bulbar NMDA receptors does not impair odor detection. The graph shows the average investigation times of saline-infused and MK-801 infused rats presented with the mineral oil carrier during four trials followed by a single odor presentation. All rats responded significantly more to the odor than to the carrier alone, indicating that they detected the odorant.

DISCUSSION

Repeated exposure to an odorant induces perceptual habituation evidenced by a reduction in active investigation behavior. The present results suggest that this behavioral phenomenon could be at least partially mediated by the adaptation of neural responses in mitral cells in the olfactory bulb. Single-unit recordings of presumptive mitral cells in freely breathing rats showed that repeated 50-second odor presentations delivered at five-minute intertrial intervals induced significant reductions in mitral cell responses to that odor when tested after completion of the adaptation protocol (Figures A2, A3). Local blockade of NMDA receptors in the olfactory bulb abolished both the adaptation of mitral cell responses to odorants in these experiments (Figure A4), as well as the behavioral habituation to odorants

(Figure A6) as previously shown in mice (McNamara et al., 2008), supporting the idea that the reduction of mitral cell responsiveness to odorants is the neural correlate of behavioral habituation to repeated odor stimuli at the timescales used here.

Previous behavioral studies have shown that habituation protocol variants can elicit odor habituation that differ in duration, odor specificity, neural location and pharmacology (McNamara et al., 2008) depending on the exact set of task parameter used. For example, a short-timescale habituation protocol utilizing brief, 4-second odor presentations separated by 10-second intertrial intervals resulted in relatively transient habituation (persisting for up to ten minutes) that was highly specific for the habituated odor and could be blocked by mGluRII/III antagonists administered in the anterior piriform cortex (Best et al., 2005; Yadon and Wilson, 2005). A similar paradigm, using 20 second stimulations separated by 10 second ITIs, induced a highly odor-specific habituation that persisted for a few minutes (McNamara et al., 2008). The pharmacology, persistence and odor-specificity of behavioral habituation at this timescale mimics the characteristics of odor adaptation in piriform cortex pyramidal cells in urethane-anesthetized rats (Best et al., 2005; Best and Wilson, 2004; McNamara et al., 2008; Wilson, 2001; 1998b; Yadon and Wilson, 2005). Our current data show that the pharmacology, persistence and specificity of behavioral habituation using a different paradigm (50-second odor presentations with five-minute ITIs) similarly mimics the properties of mitral cell odor responses in the olfactory bulbs of urethane-anesthetized rats.

Direct comparisons between behavioral phenomena in awake animals and recordings in anesthetized animals are not straightforward, as substantial differences between olfactory bulb odor responses in awake and anesthetized animals have been reported (Rinberg et al., 2006). However, behaviorally, olfactory habituation at the timescale used here does not depend on feedback connections to the olfactory bulb

(Kiselycznyk et al., 2006), or on bulbar cholinergic or noradrenergic inputs (Chaudhury et al., 2009; Mandairon et al., 2006a; Mandairon et al., 2008), suggesting that, in this case, a predominantly feedforward model of processing may be utilized that could be minimally disrupted by anesthesia. Moreover, both behavioral and neuronal habituation are impaired by local blockade of NMDA receptors, an additional commonality between the two levels of analysis that is unlikely to depend on behavioral state. In the anesthetized preparation, significant adaptation could not be elicited with stimulus durations shorter than 30 seconds, which is significantly longer than the observed time range of active investigation during our behavioral tests. Behavioral trials lasted 50 seconds and exposed the animals to the odorant for the duration of the trial; differences between long duration passive exposure, as is the case in the electrophysiological experiments, and active short duration investigation, as is the case in the behavioral experiments, are difficult to assess. Given the observed similarities in specificity, dependence on ITI and pharmacology of mitral cell adaptation and behavioral habituation one may assume a causal, if not exclusive relationship between these two observations.

The observed NMDA receptor-dependent adaptation to repeated odor stimuli in mitral cells is most likely due to changes in inhibitory input to mitral cells by granule cells in the external plexiform layer. Excitation of mitral cells results in glutamate release from mitral cell secondary dendrites onto the dendritic spines of granule cells which, in turn, release the inhibitory neurotransmitter GABA back onto mitral cells (Balu et al., 2007; Chen et al., 2000; Halabisky et al., 2000; Urban and Sakmann, 2002). An increase in granule cell excitation, whether mediated by enhanced inputs from mitral cells or from centrifugal glutamatergic inputs (Balu et al., 2007; Chen et al., 2000), would presumably lead to the suppression of mitral cell responses to odorants as demonstrated here. NMDA receptors in the olfactory bulb

external plexiform layer mediate recurrent (self-) and lateral inhibition as well as auto-excitation of mitral cells and have been shown to underlie synaptic transmission and some forms of plasticity in the OB (Chen et al., 2000; Friedman and Strowbridge, 2000; Halabisky et al., 2000; Satou et al., 2006). Furthermore, bulbar NMDA receptors have been shown to be involved in the coupling of mitral activity to respiratory patterns (Philpot et al., 1998). Recent experiments showing synaptic plasticity in response to theta-burst stimulation of centrifugal excitatory synapses onto granule cells suggest that this enhanced excitation of granule cells releases the Mg+ block of granule cell NMDA receptors that are activated by mitral cell inputs to granule cell spines, resulting in increased feedback inhibition onto mitral cells (Gao and Strowbridge, 2009). This mechanism potentially could underlie the NMDA receptor-dependent adaptation to repeated odor stimulation observed in the present work. Behaviorally, NMDA receptors serve a functional role in modulating bulbar responses during sensory deprivation, olfactory enrichment, and associative and nonassociative learning on a long (weeks) timescale (Lincoln et al., 1988; Mandairon et al., 2006b; Wilson, 1995). Our results, which demonstrate similarities between NMDA receptor-dependent behavioral habituation and neuronal adaptation responses, suggest a second, shorter (minutes to hours) role for NMDA receptor-mediated plasticity in the olfactory bulb.

The degree of adaption of mitral cell responses observed here differs substantially from that reported by other groups. Using a direct comparison of bulbar and cortical response adaptation during prolonged odor stimuli (20 – 50 seconds of continuous stimulation) Wilson (2000) reported that mitral cells adapted to about 50-75% of their initial response and that this adaptation was not specific to the odor used for prolonged stimulation. In our hands, repeated stimuli of that duration, separated by at least 2.5 minutes, resulted in greater adaptation during post-testing, suggesting a

cumulative effect working on a minutes to hours time scale. Indeed, we observed no significant adaptation over the course of single 50-second odor stimulation in our experiments, yet, a significant decrease in response was observed over the course of repeated 50-second stimuli. The induced adaptation was not specific to the odorant used during repeated stimulation, as reported in response to a single 50-second stimulation (Figure 3D; Wilson, 2000). In a previous report, Chaput and Panhuber (1982) saw a range of adaptation in mitral cells in response to continuous odor stimulation for up to 60 minutes. Recently, Shea et al (2008) showed that using repeated 2-second odor stimulations with 30-second ITIs did not induce mitral cell adaptation; these results agree with our results showing that longer odor pulses separated by longer ITIs are needed to induce this form of adaptation (Figure 3 B,C). Interestingly, Shea et al (2008) also showed that the stimulation of noradrenergic pathways to the olfactory bulb during these short pulsed odor stimuli with shorter ITIs resulted in significant adaptation of mitral cell odor responses.

In summary, our results, taken together with experiments from other groups, further support the idea that olfactory behavioral habituation can be mediated by a variety of neural phenomena operating at different timescales and located in different neural structures, including the olfactory bulb.

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