Testing the role of dopamine in olfactory sensitivity and learning in the entorhinal cortex.

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ABSTRACT

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Dopaminergic innervation of the entorhinal cortex may contribute to the integration and encoding of sensory information. The primary olfactory cortex (piriform cortex) projects to the superficial layers of the entorhinal cortex, and converging dopaminergic inputs from the ventral tegmental area may modulate processes in the entorhinal cortex related to the salience of olfactory stimuli. In the current study, food-restricted rats were trained to dig in cups filled with scented sand and to discriminate between two different odours to obtain a buried food-reward which was always associated with one odour (CS+). Upon reaching criterion performance on this task, animals underwent sham surgery or 6-hydroxydopamine lesions of the entorhinal cortex. After retraining on the original discrimination rule, olfactory sensitivity was tested using cups containing decreasing amounts of the original CS+ odour. Animals showed graded decrements in response accuracy as the concentration of odorant was reduced, but no significant differences were observed between control and lesioned animals. In addition, lesioned animals did not differ in their ability to learn to perform the discrimination task with a new odour pair at low concentrations, and did not show differences in their ability to respond accurately to either the initial or novel odour pair after a delay of two weeks. These findings show that scented sand can be used as an effective stimulus to assess the sensitivity to olfactory stimuli in the rat, but do not provide evidence for deficits in olfactory sensitivity or memory performance in animals with 6-OHDA lesions of the entorhinal cortex.

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LIST OF ABBREVIATIONS

6-OHDA	6-hydroxydopamine
DNMTS	delayed non-match to sample
HPLC	high performance liquid chromatography
LTP	long term potentiation
LTD	long term depression
NMTS	non-match to sample

INTRODUCTION

Learning and memory are complex cognitive processes that depend upon, and are affected by, other processes including sensory integration, attention, arousal, and motivation. Being in a motivated state can promote learning and memory by enhancing the salience of stimuli (Li, Howard, Parish, & Gottfried, 2008; Julliard et al., 2007; Aimé et al., 2007). Likewise, stimuli with strong motivational properties may be more salient which facilitates the learning of related associations (Bindra, 1974; Estes, 1972; Berridge, 2007; Phillips, Vacca, & Ahn, 2008; Cannon & Bseikri, 2004). In addition, rewarding or aversive stimuli may enhance brain mechanisms associated with learning and memory (Saal, Dong, Bonci, & Malenka, 2003; Otani, Daniel, Roisin, & Crepel, 2003; Seamans & Yang, 2004). Learning and memory processes are thought to be mediated by the coordinated activity of many interconnected brain regions including the hippocampal formation, parahippocampal cortices, and the neocortex (Sherry & Schacter, 1987; Squire, 2004; Squire & Zola-Morgan, 1991). This thesis investigates how the neurotransmitter dopamine, which is associated with mechanisms of motivation, may modulate the processing of olfactory information in the entorhinal cortex, a parahippocampal cortical region of the brain which is thought to contribute to olfactory perception and memory (Slotnick, 2001; Otto & Eichenbaum, 1992; Hasselmo & Stern, 2006; Akil & Lewis, 1993; Bjorklund & Lindvall, 1984; Fallon & Loughlin, 1987; Oades & Halliday, 1987).

The parahippocampal region, which includes the perirhinal, postrhinal and entorhinal cortices, shares reciprocal connections with major sensory and association cortices, while providing the hippocampus with the majority of its cortical sensory input

(Amaral & Witter, 1989; Burwell, 2000; Witter, Wouterlood, Naber, & Van Haeften, 2000). Due to the strong interconnectivity with the hippocampal formation, structures of the parahippocampal cortical region have undergone increased scrutiny for their role in memory formation over the last several decades. Research into the mechanisms involved in the acquisition, encoding, consolidation, and recall of memory has been increasingly active since Scoville and Milner (1957) demonstrated that removal of the hippocampal formation causes severe anterograde amnesia. Patient H.M. had the hippocampal formation and parts of the adjacent parahippocampal cortices removed bilaterally to treat chronic epilepsy, but the surgery also resulted in an inability to form new declarative memories (Scoville & Milner, 1957). This observation was taken to suggest that the hippocampal formation was required for the acquisition of new declarative memories, even though Blackstad's (1958) anatomical studies had shown a dense interconnectivity between the hippocampal formation and adjacent parahippocampal cortical regions.

Although it is now widely accepted that the hippocampus is strongly involved in the formation of some forms of declarative memory, it has also been well established that the parahippocampal regions, and in particular, the perirhinal cortex, also play a central role in memory processing and object recognition (Leonard, Amaral, Squire, & Zola-Morgan, 1995; Squire & Zola, 1996; Eichenbaum, Yonelinas, & Ranganath, 2007; Mumby & Pinel, 1994; Mumby & Glenn, 2000). Because the entorhinal cortex provides the hippocampal formation with most of its cortical sensory input (Amaral & Witter, 1989; Burwell, 2000; Witter et al., 2000) and shares reciprocal connections with the hippocampus, neocortex, perirhinal cortex and other subcortical structures, it has been hard to determine its unique contribution to processes associated with learning and

memory. However, growing evidence now suggests that the entorhinal cortex is likely to play a substantial role in memory (e.g. Egorov, Hamam, Fransen, Hasselmo, & Alonso, 2002; Fransen, Tahvildari, Egorov, Hasselmo, & Alonso, 2006; Hasselmo & Stern, 2006; McGaughy, Koene, Eichenbaum, & Hasselmo, 2005; Staubli, Fraser, Kessler, & Lynch, 1986; Staubli, Le, & Lynch, 1995; Tahvildari, Fransen, Alonso, & Hasselmo, 2007; Bouras & Chapman, 2003; Caruana, Reed, Sliz, & Chapman, 2007).

The entorhinal cortex is a prominent part of the parahippocampal region consisting of two anatomically diverse subregions. The lateral entorhinal cortex is located on the ventrolateral side of the temporal lobe adjacent to the perirhinal cortex, and the medial entorhinal cortex is located on the posterior and medial aspects of the temporal lobe just posterior to the subicular complex (Blackstad, 1956; Kerr, Agster, Furtak, & Burwell, 2007; Paxinos & Watson, 1998). The cellular architecture of the entorhinal cortex and the extent of its great connectivity with other regions are remarkably similar across species including the rat, monkey, cat, guinea pig, and mouse (Kohler, 1988; Amaral, Insauti, & Cowan, 1987; Witter, Room, Groenewegen, & Lohman, 1986; Sorensen, 1985; Burwell, 2000). The superficial layers (layers I, II, and III) of the medial and lateral entorhinal cortex receive massive projections from sensory and associational cortices, as well as inputs from subcortical structures. Information from every sensory modality reaches the entorhinal cortex either directly or indirectly via the perirhinal and postrhinal cortices (Amaral & Witter, 1989; Burwell, 2000; Kerr et al., 2007; Witter et al., 2000). In the rat, the lateral entorhinal cortex receives most of its cortical inputs from the primary olfactory (piriform) cortex and perirhinal cortex, and the medial entorhinal cortex receives visual and other sensory inputs from the postrhinal

cortex (Burwell, 2000; Burwell & Amaral, 1998; Kerr et al., 2007). This pattern of cortical input to the medial and lateral divisions of the entorhinal cortex may contribute to different roles of the medial and lateral areas in sensory and cognitive processing, with a greater role for the lateral entorhinal cortex in olfactory processing, and a greater role for the medial entorhinal cortex in spatial processing (Hafting, Fyhn, Molden, Moser, & Moser, 2005; Hargreaves, Rao, Lee, & Knierim, 2005; Sewards & Sewards, 2003).

Cells in the superficial layers of the entorhinal cortex serve to transfer the sensory information it receives to the hippocampal formation via the perforant path and the temporoammonic path, and neurons in the deep layers of the entorhinal cortex serve as an intermediary in the transfer of the output of the hippocampus back to neocortical areas. Ramón y Cajal (1902) and Lorente de Nó (1934) were among the first to demonstrate this reciprocal connectivity between the entorhinal cortex and hippocampus. Neurons in the superficial layers of the entorhinal cortex project directly to the dentate gyrus and CA3 and CA1 regions of the hippocampal formation, while neurons in the CA3, CA1 and subicular regions of the hippocampal formation project back to the deep-layers (V and VI) of the entorhinal cortex which, in turn, project to other cortical areas (Swanson & Cowan, 1977; Witter, Groenewegen, Lopes da Silva, & Lohman, 1989). The connectivity of the entorhinal cortex and hippocampus therefore forms a loop through which information enters via the superficial layers of the entorhinal cortex and exits via the deep layers of the entorhinal cortex (Amaral & Witter, 1989; Burwell, 2000; Köhler, 1985; Room & Groenewegen, 1986; Sørensen & Shipley, 1979; Swanson & Cowan, 1977; van Groen, van Haren, Witter, & Groenewegen, 1986; Witter et al., 1989; Witter et al., 2000). In this respect, the entorhinal cortex is located in a pivotal position within the medial

temporal lobe where it serves as a major link between the sensory cortices, the hippocampal formation, and the neocortex.

The organization of the sensory inputs to the entorhinal cortex and its connectivity with the hippocampal formation has suggested that sensory and mnemonic roles of the entorhinal cortex are closely linked to the functions of the hippocampus, but the entorhinal cortex may also make unique contributions to sensory and mnemonic processes. The connections within the entorhinal-hippocampal loop could suggest that these structures have similar functions and that the entorhinal cortex serves mainly as an information relay system (Witter et al., 1989; Insausti, Amaral, & Cowan, 1987). In this light, the entorhinal cortex could be seen as playing a large role in processes within the hippocampus that rely on highly-processed sensory input (Ramirez et al, 2007; Rasmussen, Barnes, & McNaughton, 1989; Majchrzak et al., 2006; Kaut & Bunsey, 2001; Good & Honey, 1997), and could also play a central role in mnemonic processes that involve interactions between the hippocampal formation and neocortex such as memory consolidation processes (Nadel & Moscovitch, 1997; Sirota, Csicsvari, Buhl, & Buzsáki, 2003; Hebb, 1949).

In addition to the roles of the entorhinal cortex when interacting with other areas, the unique contributions of the entorhinal cortex to sensory and mnemonic functions is also beginning to be determined. There is a growing experimental literature that demonstrates memory deficits following entorhinal lesions in rodents (e.g. Otto & Eichenbaum, 1992; Moser & Paulsen, 2001; Hasselmo & Stern, 2006; Schwarcz & Witter, 2002). There is also recent electrophysiological evidence that suggests that the entorhinal cortex has neurons that may contribute to the integration of sensory

representations (Chrobak & Buzsáki, 1998; Dickson, Biella, & de Curtis, 2000; Dickson, Magistretti, Shalinsky, Hamam, & Alonso, 2000), and that synaptic inputs to the entorhinal cortex support both LTP and LTD which are cellular models for learning and memory (Bouras & Chapman, 2003; Caruana & Chapman, 2008; Caruana et al., 2007). Further, neurons in both the deep and superficial layers of the entorhinal cortex show persistent firing activity that could support working memory functions (Egorov et al., 2002; Fransen et al., 2006; Tahvildari et al., 2007).

The contribution of the entorhinal cortex to learning and memory has been studied by a number of investigators who have focused on the olfactory inputs to the entorhinal cortex from the primary olfactory cortex (i.e., the piriform cortex). Neurons in the superficial layers of the medial and lateral entorhinal cortices receive a strong, direct monosynaptic input from cells in the olfactory bulb and piriform cortex (Burwell, 2000; Kerr et al., 2007), and these input pathways indicate that the entorhinal cortex likely plays a major role in olfactory processing (Ferry, Ferreira, Traissard, & Majchrzak, 2006). In a recent study, reliable changes in electrophysiological recordings were observed while hamsters performed an operant social recognition task, demonstrating that the firing patterns of neurons in the lateral entorhinal cortex code qualitative information about odours that can be used to discriminate between conspecifics (Petrulis, Alvarez, & Eichenbaum, 2005). This finding indicates that neurons in the lateral entorhinal cortex are involved in coding specific information about socially relevant olfactory stimuli.

Additional evidence for the role of the entorhinal cortex in olfactory processing and memory comes from the results of cellular recordings during an olfactory non-match-to-sample task. The test phase of this task required rats to select an odour different from

the one they were presented with during the sample phase of the task. Cells in the lateral entorhinal cortex showed different firing patterns in response to different odours and the cells also appeared to code whether the test odour was a match to the sample odour (Young, Otto, Fox, & Eichenbaum, 1997). When a delay was introduced between the sample and test phase, the firing of some cells increased during the delay period of the task; this indicates that these cells were not only coding for sensory aspects of the stimuli, but they might also be serving to actively maintain olfactory information in working memory during the delay period (Young et al., 1997). These findings clearly demonstrate the involvement of the entorhinal cortex in the processing of olfactory stimuli, and also strongly suggest that entorhinal neurons may contribute to olfactory working memory.

In addition to these electrophysiological findings, studies that have employed lesions of the entorhinal cortex have also provided evidence for a role of the entorhinal cortex in olfactory memory. In an early study by Staubli, Ivy, and Lynch (1984) rats were trained to differentiate between pairs of odours presented in randomly selected arms of a radial arm maze to obtain a reward. Animals were then given either sham lesions or electrolytic lesions of either the dorsal or lateral entorhinal cortex. No differences were found between groups if there was a minimal delay between trials, but when the inter-trial interval was increased to 10 minutes, only the rats with lateral entorhinal cortex lesions had difficulty remembering the rewarded odour, and they performed at chance levels on each trial (Stäubli et al., 1984). Similarly, only rats with lesions of the lateral entorhinal cortex could reverse their responding appropriately if the rewarded and non-rewarded odours were switched, suggesting that these animals had not retained the

original associations with these odours that had been tested one hour earlier. Further work by these investigators using the same paradigm provided evidence that, while the lateral entorhinal cortex appears to play an important role in the acquisition and retention of new olfactory memories, lesions to this area do not result in retrograde amnesia for olfactory information acquired prior to the lesion (Staubli et al., 1986).

Additional studies that have employed lesions of the parahippocampal region which included the entorhinal cortex have demonstrated deficits on olfactory tasks involving odour discrimination (Petrulis, Peng, & Johnston, 2000), delayed non-matching-to-sample performance (Otto & Eichenbaum, 1992; Stäubli et al., 1984; Young et al., 1997), social recognition (Bannerman et al., 2002), and conditioned odour aversion (Ferry et al., 2006; Ferry, Oberling, Jarrard, & Di Scala, 1996; Ferry, Wirth, & Di Scala, 1999). There is also evidence from studies using lesions and imaging techniques that indicates that the entorhinal cortex also contributes to the short-term maintenance of novel odours (McGaughy et al., 2005; Ranganath & D'Esposito, 2001; Schon, Hasselmo, Lopresti, Tricarico, & Stern, 2004; Stern, Sherman, Kirchhoff, & Hasselmo, 2001).

Several laboratories have previously tested the effects of lesions on olfactory discrimination and mnemonic functions using olfactory tasks which require rats to dig in scented sand to obtain buried food rewards. Kaut and Bunsey (2001) used sequences of two-odour discriminations to test whether hippocampus or perirhinal-entorhinal lesions would cause retrograde or anterograde memory deficits of discriminations learned before or after surgery. They found that damage to the perirhinal-entorhinal cortices was more disruptive to memories of discriminations learned soon before or after surgery than hippocampal damage. Olfactory digging tasks have also been modified to measure

delayed non-match to sample performance in rats following depletion of acetylcholine in the entorhinal cortex (Hasselmo & Stern, 2006; McGaughy et al., 2005), following lesions of orbitofrontal and parahippocampal cortices (Otto & Eichenbaum, 1992), and also following 6-hydroxydopamine (6-OHDA) lesions in the entorhinal cortex (Caruana, 2008). Evidence from a variety of laboratories using lesions to examine entorhinal cortex function therefore suggests that the entorhinal cortex makes an important and unique contribution to olfactory perception and memory.

Dopamine is a major neuromodulatory neurotransmitter that plays an important role in a variety of cognitive processes, but how dopamine may modulate sensory and mnemonic functions within the entorhinal cortex is not well known. Subcortical dopaminergic inputs from the substantia nigra and ventral tegmental area to the striatum and nucleus accumbens are known to play a major role in gating motor behaviour and to contribute to appetitive motivation for naturally rewarding stimuli and drugs of addiction (Apicella, Trouche, Nieoullon, Legallert, & Dusticier, 1990; Wise & Rompre, 1989; Beninger, 1983; Fibiger & Phillips, 1986, 1988; Schultz, 2002). In addition, dopaminergic inputs to the prefrontal cortex are known to have modulatory effects on working memory (Goldman-Rakic, Muly, & Williams, 2000; Landau, Lal, O'Neil, Jagust, & Baker, 2009). Dopamine enhances the sustained firing activity of neurons in the deep layers of the prefrontal cortex during tasks that require a delayed response, and this suggests that dopamine contributes to working memory functions in the prefrontal cortex (Goldman-Rakic, 1999; Seamans & Yang, 2004).

In addition to inputs to the prefrontal cortex, dopaminergic neurons of the ventral tegmental area and substantia nigra also send large projections to the medial and lateral

entorhinal cortex (Akil & Lewis, 1993; Bjorklund & Lindvall, 1984; Fallon & Loughlin, 1987; Oades & Halliday, 1987). It is possible, then, that dopaminergic inputs to the entorhinal cortex may modulate the manner in which the entorhinal cortex contributes to the processing of sensory information, and the manner in which it contributes to mnemonic processing. Electrophysiological evidence from both in vivo and in vitro experiments has indicated that moderate increases in dopamine in the entorhinal cortex increase the strength of synaptic inputs to the entorhinal cortex from the piriform cortex (Caruana, Sorge, Stewart, & Chapman, 2006; Caruana & Chapman, 2008). This could increase the salience of olfactory inputs to the entorhinal cortex, as well as enhance the transmission of olfactory representations to the rest of the hippocampal formation, and might also facilitate cellular mechanisms that mediate the encoding of new memory (Chapman & Racine, 1997a,b; Bouras & Chapman, 2003; Kourrich & Chapman, 2003; Caruana & Chapman, 2006; Caruana et al., 2006). The modulatory effects of dopamine on the strength of synaptic inputs to the entorhinal cortex, therefore, suggest that dopamine is likely to have important influences on the sensory and cognitive processes mediated by the entorhinal cortex. Results obtained by Caruana (2008) showed that 6-OHDA lesioned rats were impaired during retraining on a non-match to sample task following surgery but, because response latencies were very long for both the sample and test phases of the trials, it was not clear if the performance deficit was due to a memory impairment, or an impairment of sensory, motor, or cognitive function. The origin of this deficit remains to be clarified, however there have been no other definitive behavioural experiments assessing the effects of modulating dopaminergic activity in the entorhinal cortex on sensory and mnemonic processing in the entorhinal cortex.

The studies contained in this thesis were based on the hypothesis that dopamine enhances the salience of olfactory representations processed by the entorhinal cortex. The studies were designed to investigate whether dopaminergic inputs to the entorhinal cortex enhance the rats' sensitivity to faint odours, as well as their ability to form an association between an odour and a food reward. The approach taken was to induce a lesion of dopaminergic inputs to the entorhinal cortex using the selective neurotoxin 6-OHDA, and to compare the performance of these animals to a control group that underwent sham lesions. If dopamine normally enhances olfactory information processing in the entorhinal cortex, then depleting dopamine in the entorhinal cortex using 6-OHDA lesions might impair either sensitivity to odours, or the learning and/or memory of an association between an odour and reward. Food-restricted animals were first trained on an odour discrimination task in which they received a food reward for digging in a cup of sand scented with a CS+ odour, and received no reward for digging in a cup scented with a CS- odour. After 6-OHDA lesions or sham surgery, animals were retrained on the odour discrimination task, and their sensitivity to olfactory stimuli was assessed by their ability to choose cups filled with decreasing concentrations of the CS+ odour versus an unscented cup. The ability of animals to form a new association between food reward and a faint, novel odour was then assessed, and their ability to recall this new association and the original association was tested two weeks later. The present experiments were developed in order to determine whether lesioning dopaminergic inputs to the entorhinal cortex interferes with olfactory sensitivity, and to determine if it might also interfere with learning of a simple association between a very faint odour an a food reward.

METHODS

General Procedures

Animals were first trained on an olfactory discrimination task in which they obtained a food-reward for digging in cups filled with sand scented with a CS+ odour, and in which they were not rewarded for digging in a cup scented with a CS- odour. Half of the animals then underwent 6-OHDA lesions of dopaminergic afferents to the entorhinal cortex, and half of the animals served as a control group that underwent a sham operation. Following retraining on the task after recovery from surgery, animals were tested for their ability to detect decreasing concentrations of the rewarded CS+ odour in scented sand versus unscented sand. Then, to assess the effects of lesions on learning of a new odour association, all animals were then trained again on the odour discrimination task using a new CS+ and CS- odour pair at low concentrations of odour. After a two week delay, animals were then tested for their ability to perform the discrimination task in which they were exposed to either the most recent odour pair, or the original odour pair. After behavioural testing, tissue levels of dopamine in several brain regions were assessed using high performance liquid chromatography (HPLC) analysis to determine whether 6-OHDA lesions resulted in persistent decreases in dopamine in the entorhinal cortex and other dopamine-relevant brain areas.

Subjects

Subjects were 16 male Long-Evans rats weighing 300 to 325 g at the beginning of study. One week before shaping, animals were put on a restricted feeding schedule (18 g of lab chow per day) that allowed animals to maintain 80% of their free-feeding body

weight. All training and testing was done with animals in a food-restricted state. Animals were housed individually with free access to water at all times. Testing occurred during the lights-on phase of a 12-hour light-dark cycle.

Materials and Apparatus

A black Plexiglas open field was used for behavioural training and testing. It measured 92 x 92 cm with 35.5 cm high walls and was elevated 92 cm from the floor. Seven Velcro strips (4 x 2.5 cm) were affixed to the floor of the open field at 13 cm intervals along one side of the perimeter, 9 cm from the outer wall. The spices used as olfactory cues were cinnamon, nutmeg, cocoa, and marjoram. Spices were mixed with 100 g of dampened, unscented playground sand in semi-transparent plastic cups (6 cm tall; 8 cm diameter; Fisher Scientific), and the amount of spice mixed with the sand varied with the task (see below). Velcro strips on the bottom of the cups allowed them to be attached to the positions in the open field, and prevented rats from toppling the cups. One-quarter pieces of Froot Loops cereal were used as food reward. The experiments were conducted in a small room equipped with a ventilation system that constantly removed any residual odours from of the testing area.

Behavioural Testing

Behavioural performances in 6-OHDA lesioned and sham-operated rats were compared to assess the effects of removing dopamine on tests of olfactory sensitivity, and olfactory learning and memory.

Olfactory Discrimination Training. Animals were first handled, and their behaviour shaped to familiarize them with the food reward. Four days after arrival,

animals were handled for 20 minutes per day for 3 consecutive days. To familiarize the rats with the food reward prior to training, five Froot Loops were placed in ceramic cups in each animal's home cage to be eaten by the animals after each handling session. Food-restricted animals were then shaped to dig in cups of unscented sand to obtain a food reward during 6 daily 20 minute sessions. Rats were placed in the center of the open field with a single baited cup in a random location along one wall. The reward was placed in unscented sand for the first 3 days, and rats were allowed to obtain as many rewards as possible during the 20 min session. The reward was placed on top of the sand for the first two days, and it was placed progressively deeper within the sand over the remaining sessions. All animals reliably obtained rewards by the end of the sixth day of shaping. The open field and cups were washed with 20 % isopropyl alcohol between animals.

Food-restricted rats were then trained to dig in cups filled with scented sand and to discriminate between two cups scented with different odours to obtain a buried food reward which was always associated with one odour (CS+). Two pairs of spices were used to scent the cups that were filled with 100 g of sand; either 0.8 g cinnamon and 0.8 g nutmeg, or 1.0 g cocoa and 1.0 g marjoram. Each rat was always given the choice between the same two odours. Cinnamon was always paired with nutmeg whereas cocoa was always paired with marjoram, and the CS+/- designation of each odour was counterbalanced across subjects. These spice-pairs were used previously at these concentrations by Fortin, Agster, and Eichenbaum (2002) to assess whether hippocampal lesions would impair the rats' memory for sequential order of events as well as their ability to recognize the recent occurrence of odours presented in a series.

In the present study, each discrimination trial began with two cups placed in the open field; one cup was baited and filled with CS+ scented sand, and the other cup was filled with CS- scented sand and an inaccessible reward so that the Froot Loop odour could not guide the rat to the rewarded cup. The cups were placed quasi-randomly at seven different locations along the perimeter, always with one vacant location between them. The location of the reward was counterbalanced across trials, with each location being used equally for the CS+ and CS- odours, and with the CS+ placed as frequently to the left as to the right. Rats were placed in the center of the open field facing the two cups, and were given a maximum of 2 minutes to obtain the reward. If a rat began to displace sand with its forepaws in the cup containing the CS- odour the rat was removed from the field and the trial ended. Rats that obtained the reward from the cup with the CS+ odour were allowed to consume the reward before being removed. The latency to make either a correct or incorrect choice was recorded, with a choice being defined as digging for at least 2 sec in a cup. A latency of 120 sec was scored for animals that did not approach a cup. At the end of a trial, rats were placed in a holding cage for approximately 10 sec while the open field was wiped down with a 20% alcohol solution and the next trial was set up. New cups were used for each trial. Twelve trials were conducted each day until animals performed at least 11 of 12 trials correctly (i.e, 91.7% correct) for two consecutive days, and there was a minimum training period of 4 days. The animals were then divided into groups matched on the basis of their performance on the discrimination test; one group then underwent 6-OHDA lesions of the entorhinal cortex, and the other group underwent a sham operation.

Following surgery (see below) and a 10 to 14 day recovery period, animals were re-trained on the discrimination task using the same olfactory cues and procedures.

Olfactory Sensitivity Testing. Following recovery from surgery, recovery, and retraining on the olfactory discrimination task, animals were tested on a modified version of the discrimination task involving the presentation of different concentrations of the CS+ odour versus unscented sand. This was done to assess the rats' level of sensitivity to the odours and to determine whether the animals with lesions of dopamine afferents to the entorhinal cortex would have a decreased ability to detect the odours as compared to control animals. Procedures were identical to those used during the initial training except for the odour stimuli. Rats were presented with two cups, one containing unscented sand and an inaccessible Froot Loop, and one which was baited and filled with the CS+ scented sand at one of four concentrations; 1 μg, 10 μg, 100 μg, or 10 mg of marjoram or cocoa in 100 g sand; when cinnamon or nutmeg were used the quantities were 0.8 μg, 8 μg, 80 μg, or 8mg/100 g of sand. Animals obtained food rewards for digging in the cups containing CS+ scented sand at any concentration. Each concentration of odour was presented a total of 12 times over 4 days of testing, with 3 trials of each odour concentration being presented on each day in quasi-random order. The locations of the scented and unscented cups were counterbalanced across trials, so that each location was rewarded an equal number of times, and so that the reward was on the left as often as on the right. Clean cups were used on each trial and the open field was wiped clean with 20% alcohol between trials.

Learning and Memory Testing. To assess the ability of lesioned animals to learn to discriminate very faint odours and to apply the previously learned discrimination rule

to a new odour set, food-restricted rats were again trained on the discrimination task using the original training procedure, but instead using a novel pair of odours at the lowest concentrations used during sensitivity testing. Animals that were previously tested using cinnamon and nutmeg (0.8 μg/100 g sand) were now tested using cocoa and marjoram (1 μg/100 g sand), and vice versa. Training procedures were identical to those used during training on the original discrimination task.

After reaching criterion performance on the task with the new odour set, there was a two-week delay. Food-restricted rats were then tested on their ability to perform the discrimination task using the low concentrations of the new odour set, and then again with the original odour set on the next day. There were twelve trials on each day. On the first day, rats were presented with two cups that contained sand scented with the most recent CS+ odour or the most recent CS- odour (either 0.8 μg of cinnamon or nutmeg, or 1 μg of cocoa or marjoram/100 g sand). Latency to dig in a cup and the accuracy of the response was recorded, and digging in the CS+ cup was rewarded. The procedure was repeated on the second day using the odour pair from the original training procedure at the lowest concentrations used during testing for olfactory sensitivity (either 1 μg of cocoa and marjoram or 0.8 μg of cinnamon or nutmeg/100 g sand).

Statistical Analysis. Measures of accuracy and latency were obtained for each animal for the trials conducted on each day of testing. Data were averaged for plotting and displayed with bars indicating the standard error of the mean. Changes in accuracy and latency measures during initial training on the discrimination task were assessed using 1-way repeated measures ANOVAs with 4 levels of the independent variable of Day. Mixed-design 2-way repeated measures ANOVAs with independent variables of

Group and Day were used to assess between-group differences in accuracy and latency during retraining after surgery. The number of days needed to obtain criterion levels of performance was compared using Student's *t*-tests. Mixed-design 2-way repeated measures ANOVAs with independent variables of Group and Concentration were used to analyze the responsiveness of animals to different concentrations of odorant during olfactory sensitivity tests. Mixed-design 2-way repeated measures ANOVAs using Group and Day as independent variables were also used to evaluate differences between groups in accuracy and latency of responses during learning of associations with novel odour pairs at low concentrations. Further analyses used 2-way repeated measures ANOVAs with independent variables of Group and Trial to investigate the trial-by-trial accuracies and latencies of the first two days of training with the novel odours at low concentrations. Memory for the old and new odour pairs was assessed using mixed-design 2-way repeated measures ANOVAs with independent variables of Group and Odour Pair and with dependent variables of latency and accuracy. Significant effects in all analyses were investigated using Student-Newman-Keuls post-hoc comparisons.

Surgery

Three days after reaching criterion performance on the initial odour discrimination task, animals received either bilateral 6-hydroxydopamine (6-OHDA) lesions of the medial and lateral entorhinal cortex, or sham lesions. Group assignment was quasi-random to provide roughly equal pre-surgical performance on the discrimination task in both groups. Rats were pretreated with desipramine (2.5 mg/kg, i.p.) 60 min prior to anaesthesia with a 5% isoflurane and 95% oxygen mixture. Rats were placed in a stereotaxic frame with bregma and lambda levelled. Two stainless steel

cannulae (26 gauge) were used to inject either sterile saline (0.9%) or 6-OHDA (4 µg/ml) bilaterally into each of 5 sites along the rostral-caudal axis of the entorhinal cortex (4µl total volume/side; relative to bregma: site 1: P -6.3, L ±4.4, V -8.0 mm; site 2: P -6.8, L ±4.4, V -8.0 mm; site 3: P -7.3, L ±4.4, V -7.4 mm; site 4: P -7.8, L ±4.4, V -7.2 mm; site 5: P -8.3, L ±4.4, V -5.5 mm). Infusions were made using two Hamilton syringes (10 µl; 1800 Series) connected to a Harvard Apparatus microinfusion pump (Model 22), and syringes were attached to infusion cannulae by short lengths of PE-20 tubing. A volume of 1 µl was delivered to sites 1, 3, and 5 over a 5 min period and 0.5 µl was delivered to sites 2 and 4 over 2.5 min. Cannulae were left in place for 4 min after each infusion. The 6-OHDA (Sigma) was prepared fresh daily by dilution in sterile saline and ascorbic acid (5 mg/ml). Buprenorphine (0.02 mg/kg, s.c.) was administered as a postsurgical analgesic. There was a 10-14 day recovery period following surgery prior to behavioural testing; animals had free access to food and water during the first week, but the food restriction schedule was reinstated during the second week.

Dopamine and Protein Content Analysis

Eight to fifteen weeks after surgery, levels of dopamine obtained from tissue punches from several dopamine-related brain areas were quantified using high performance liquid chromatography (HPLC). The assay of tissue-levels of dopamine was performed according to methods described previously (Moroz, Pecina, Schallert, & Stewart, 2004; Moroz, Rajabi, Rodaros, & Stewart, 2003). Both sham and lesioned animals were lightly anaesthetised and their brains were rapidly removed and placed in isopentane cooled on dry ice, and were then frozen at –80°C. The brains were then sliced on a cryostat into 300 µm sections at -15 C and punches (0.5, 1 or 2mm in diameter) were

taken from the left and right entorhinal cortices and bilaterally from the caudate putamen, prefrontal cortex, nucleus accumbens, ventral tegmental area, and substantia nigra (Palkovits & Brownstein, 1988). Tissue punches were stored at –80°C. Tissue punches were then suspended in artificial cerebrospinal fluid containing (in mM) 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 dextrose and centrifuged at 4000 rpm for 15 min. The supernatant was removed, stored frozen overnight, and assayed for dopamine content using high performance liquid chromatography (HPLC) with electrochemical detection as described previously (Caruana, 2008; Moroz, Pecina, Schallert, & Stewart, 2004; Moroz, Rajabi, Rodaros, & Stewart, 2003). Tissue pellets were suspended in sodium hydroxide and analyzed for protein content using spectrophotometry.

For HPLC analysis, 10 µl volume was extracted from each sample and loaded onto a C-18 reverse-phase column (5 µm, 15 cm) through a manual injection port (Rheodyne, Model 7125, 20 µl loop), and the redox current for dopamine was measured with a dual-channel coulometric detector (ESA Biosciences, Coulochem III with a Model 5011 analytical cell). The mobile phase (0.076 M SDS, 0.107 M EDTA, 0.06 M NaH_2PO_4, and 0.3 M citric acid; pH = 3.35) was circulated through the system at a rate of 1.1 ml/min by a Waters 515 HPLC pump and the peak for dopamine was quantified by EZChrom Chromatography Data System (Scientific Software Inc.). Measures of dopamine content were adjusted for protein quantity using custom software and expressed in pg/µg for analysis. Data was averaged for plotting with bars depicting standard error of the mean. Dopamine content in each brain region for both groups was compared using Student's *t*-tests.

RESULTS

Behavioural Testing

Olfactory Discrimination Training. All rats were first trained to perform an olfactory discrimination task in which they obtained a food reward for digging in a CS+ scented cup, and were not rewarded for digging in a CS- scented cup. Animals learned to perform the olfactory discrimination task quickly, and typically required 4 or 5 days to meet the criterion level of 11 of 12 correct trials on each of two consecutive days. Performance was close to chance levels on the first day of training (63.5 ± 7.6 % correct on day 1, n = 16; Figure 1A), and accuracy improved quickly and was at 96.9 ± 2.1 % on day 4 ($F_{3,45}$=10.62, $p<0.001$). Latencies to dig in the chosen cup also reduced during this period, and were reduced from 55.9 ± 7.6 sec on day 1 to 14.4 ± 2.8 sec on day 4 ($F_{3,45}$=18.04, $p<0.001$)(Figure 1B). All animals were tested for a minimum of 4 days. Eight of the 16 animals reached criterion on the fourth day, 7 reached criterion on the fifth day and one animal required 6 days. Once animals reached criterion for two consecutive days they underwent either 6-OHDA lesions or sham surgeries.

Post-Surgical Re-Training. The performance of lesioned and sham-operated rats during retraining on the olfactory discrimination task 10-14 days after surgery was compared to determine if lesions might affect their ability to perform the olfactory discrimination task. The performance of both groups of animals, however, was similar (Figure 2 A,B). Lesioned animals performed 86.5 ± 7.2 % correct on average on the first day of retraining, and control animals performed at 89.3 ± 9.2 % correct. Response latencies were also similar, with lesioned animals requiring 31.2 ± 10.2 sec to respond on

the test phase as compared to 30.2 ± 9.8 sec in control animals. Further, the increase in mean levels of performance on the task from the first to the second day of testing was the same in both groups, with accuracy increasing to 93.8 ± 3.4 % in lesioned animals and 92.7 ± 3.3 % in control, and the latency of responses decreasing to 15.1 ± 3.2 sec in lesioned animals and 14.1 ± 5.5 sec in control. This was reflected in significant main effects of Day in ANOVAs comparing responses of the two groups on the first two days of testing (accuracy, $F_{1,14}$=5.02, p=0.04 ; latency, $F_{1,14}$=10.28, p=0.01), but no significant interactions of Day with Group (accuracy, $F_{1,14}$=0.16, p=0.70; latency, $F_{1,14}$=0.00, p=0.99). In addition, animals in each group showed similar distribution in the number of days required to reach criterion levels of performance. Four animals in each group required only 2 days of retraining, and the remaining animals in each group required 5 or 6 days (Figure 2C). This resulted in no significant difference between groups in the number of days required to reach criterion performance (t_{14}= 0.14, p=0.89).

The similar levels of performance in lesioned and sham animals during retraining after surgery suggest that lesioning dopamine has no significant effect on the ability of animals to perform a simple olfactory discrimination task that used high concentrations of spice in sand. The competent performance of both groups of animals on the olfactory discrimination task then allowed the CS+ odour to be used in subsequent tests of the sensitivity of the animals to the CS+ odour at a range of concentrations.

Olfactory Sensitivity Testing. The sensitivity of rats to the CS+ odours was tested in a modified version of the olfactory discrimination task in which different amounts of spice (ranging from 0.01g to 1.0 µg/100 g of sand) were presented to the rat along with a cup containing unscented sand, and in which the rat obtained a food reward only for

digging in the cup containing the CS+. Results showed that the accuracy of responses was reduced, and latency of responses was increased, as the concentration of spice was reduced, but that there was no difference in these changes between groups (Figure 3A,B).

At the lowest concentration of spice, accuracy was close to chance in both groups at only 59.5 ± 12.4 % correct in the control and 63.1 ± 8.5 % correct in the lesioned group, but performance on the highest concentration increased to 88.5 ± 5.4 % correct in the control and 92.7 ± 2.9 % in the lesioned group (Figure 3A). This was reflected in a significant effect of Concentration ($F_{3,39}$=11.52, $p<0.001$), but no significant interaction between Group and Concentration ($F_{3,39}$=0.052, $p=0.984$). Post hoc analysis of the main effect of Concentration showed that all pair-wise comparisons, with the exception of the comparison of the two highest intensities (N-K, $p=0.52$), were significantly different, specifically between the lowest and the two highest concentrations (N-K, $p<0.001$), and between the second lowest and all other concentrations (N-K, $p<0.05$).

A similar pattern of effects was seen in changes in response latency (Figure 3B). The control group responded in 34.9 ± 9.1 sec and the lesioned group in 40.6 ± 10 sec at the lowest concentration, but both groups responded more quickly at the highest concentration with the control group responding in 11.8 ± 3.5 sec and the lesioned group in 8.8 ± 2.2 sec. This resulted in a significant effect of Concentration ($F_{3,39}$=7.821, $p<0.001$), but no significant interaction between Group and Concentration ($F_{3,39}$=0.114, $p=0.951$). Student-Newman-Keuls post hoc tests revealed the main effect of Concentration was due to differences between the lowest and two highest concentrations (N-K, $p<0.01$) and between the second lowest and two highest concentrations (N-K,

$p<0.05$), but there was no difference between the two lowest (N-K, $p=0.12$) nor the two highest concentrations (N-K, $p=0.637$).

The graded deficits in performance indicate that the task developed here can be used to assess the sensitivity of animals to different levels of odorant, and also suggest that 6-OHDA lesions in the entorhinal cortex do not significantly affect sensitivity to odorants as tested here.

Learning and Memory Testing. To determine if differences in performance between groups on the olfactory discrimination task might be obtained if the task was made more difficult by using lower levels of odorant, animals were trained to perform the discrimination task using an odour pair that was novel to them using the lowest concentrations used in sensitivity tests. If animals had undergone discrimination and sensitivity tests with cocoa and marjoram they were now tested using the lowest concentration of nutmeg and cinnamon, and vice versa. Although there was a strong trend for lesioned animals to show lower accuracy and longer latencies on the first day of training with the new odour pair, there was no statistically significant difference between the groups on this task overall (Figure 4A,B). Lesioned animals performed at 77.1 ± 8.6 % correct on average on the first day of retraining, similar to control animals which performed at 89.3 ± 3 % correct ($t_{13} = 1.27$, $p=0.23$). Response latencies of the control group were also similar to lesioned animals; lesioned animals required 31.8 ± 8.7 sec to respond on the first day as compared to 17.4 ± 4.7 sec in control animals ($t_{13} = 1.40$, $p=0.19$). Further, the improvement on the task from the first to the fourth day of testing was similar in both groups, with accuracy increasing to 95.8 ± 2.8 % correct in the control group and 97.6 ± 1.5 % correct in lesioned animals, and the latency of responses

decreasing to 11.7 ± 3.5 sec in control and 10.2 ± 3.0 sec in lesioned animals. This was reflected in significant main effects of Day in ANOVAs comparing responses of the two groups on the first four days of testing (accuracy, $F_{3,37}$=5.001, p<0.005; latency, $F_{3,37}$=6.475, p<0.001), but no significant interactions of Day with Group (accuracy, $F_{3,37}$=0.809, p<0.497; latency, $F_{3,37}$=1.258, p=0.303). Post-hoc analysis of the main effect of Day showed the accuracy of response on the first two days were significantly lower than on the third and fourth days (N-K, p<0.05), but that there was no difference between accuracies on the first and second days (N-K, p=0.86), or the third and fourth days (N-K, p=0.84). Likewise, the latency of responses were significantly higher on the first day than on the third and fourth days (N-K, p<0.01), and significantly higher on the second day than on the third and fourth days (N-K, p<0.01). However the first and second days (p=0.63), and the third and fourth days (p=0.68) were not significantly different in response latency.

In addition, animals in each group showed similar distribution of the number of days required to reach criterion levels of performance. Four animals in each group required only 2 days of training, and the remaining animals in each group required either 3 or 4 days, with one control animal requiring 7 days (Figure 4C). This resulted in no significant difference between groups in the number of days required to reach criterion performance (t_{13} = 0.35, p=0.73).

Because of the trend towards slower and less accurate responses in lesioned animals on the first day, the trial by trial performance of animals on the first and second days were investigated further by plotting trial-by-trial performance to determine if there was a rapid change in performance during early training trials (Figure 5). Analysis on the

first day showed a significant Trial by Group interaction effect on the latency measure ($F_{11,143}$=2.17, p<0.05) that was due to differences between groups on the first trial in which lesioned animals took 78 ± 15.4 sec, and control animals took 17.6 ± 3.72 sec, to respond (N-K, p<0.001). Lesioned animals also took longer than control animals on the last trial (59.5 ± 18.6 versus 17.4 ± 7.68 sec; N-K, p<0.05). However, analysis of response accuracy on the first day showed no significant main effects of Trial ($F_{11,143}$=1.60, p=0.23) or Group ($F_{1,143}$=1.53, p=0.13) and there was no significant Trial by Group interaction ($F_{11,143}$=0.79, p=0.64). There were also no systematic differences between groups on the second day (Figure 5A_2,B_2), and 1-way repeated measures ANOVAs showed no significant effects (accuracy, $F_{1,143}$=0.024, p=0.88; latency, $F_{1,143}$=0.001, p=0.924). Therefore, although lesioned animals responded more slowly on the first trial of the first day as compared to control animals, and showed a trend towards poorer accuracy on the task, there was no clear difference in the overall performance of the animals on the discrimination of novel odours at low concentrations on the first two days of training.

The memory of animals for the odours used in both of the odour discrimination tasks was tested two weeks later, using the same low odour concentrations. In tests of memory for the most recently trained odour pair, there was no difference between groups in either response accuracy (control, 85.7 ± 4.3 % correct; lesion, 79.2 ± 8.3 % correct; t_{13}=0.67, p=0.52) and or response latency (control, 23.5 ± 4.6 sec; lesion, 27.4 ± 11.1 sec; t_{13}=0.31, p=0.76). There was also no difference when animals were tested with the odour pair that they had been exposed to originally during initial odour discrimination and olfactory sensitivity training 3 to 4 weeks earlier, and groups were similar in both

response accuracy (control, 86.9 ±4.8 % correct; lesion, 90.6 ±3.3 % correct; t_{13}=0.65, p=0.52) and response latency (control, 15.4 ±4.6 sec; lesion, 14.9 ±2.5 sec; t_{13}=0.10, p=0.92). There was a trend towards faster and more accurate responses for the original odour pair that animals had been exposed to most frequently, as compared to the less frequently presented odour pair that animals had been trained on most recently (accuracy, $F_{1,13}$=1.78, p=0.21; latency, $F_{1,13}$=3.14, p=0.10), but there was no significant main effect of Group (accuracy $F_{1,13}$=0.05, p=0.83; latency, $F_{1,13}$=0.05, p=0.83) or a significant Group by Odour Pair interaction (accuracy $F_{1,13}$=1.18, p=0.30; latency, $F_{1,13}$=0.15, p=0.71). Thus, there was no clear difference in the performance of the lesioned animals compared to control animals on memory tasks involving the recall of previously learned odour pairs up to four weeks after the last exposure (Figure 6 A,B).

Dopamine and Protein Content Analysis

Tissue-punches from multiple brain regions containing dopamine terminal fields were available from 15 of the 16 animals, and were used to assess the effect of lesions on dopamine levels. Although statistics indicated no significant difference between sham and lesioned animals in the amount of dopamine obtained from tissue punches in any region (Figure 7), the mean levels were lower in lesioned rats compared to control rats in the right entorhinal cortex (3.84 ±0.78 versus 4.06 ±0.50 pg/µg; p=0.82), ventral tegmental area (9.35 ± 3.57 versus 26.81 ±14.63 pg/µg; p=0.30), nucleus accumbens (18.31 ±5.41 versus 31.55 ±6.08 pg/µg; p=0.13), and substantia nigra (3.63 ± 1.40 versus 4.42 ±1.29 pg/µg; p=0.69). Interestingly, dopamine levels in lesioned rats compared to control rats were higher in the left entorhinal cortex (5.44±1.71 versus 2.80 ±0.49 pg/µg, p=0.14), prefrontal (4.55 ± 1.82 versus 2.26 ±0.51 pg/µg, p=0.22), and caudate/putamen

(6.57 ±1.81 versus 4.59 ±1.55 pg/µg; p=0.42), though these differences were not significantly different (Figure 7).

Figure 1. Rats learn the olfactory discrimination task within two to six days of training. Mean accuracy **(A)** and latency to dig **(B)** on the 12 daily training trials is shown as a function of testing day for the 16 animals tested. Response accuracies **(A)** were close to chance on the first day, but animals reached the performance criterion of 11 of 12 trials correct after two to six days of training. Response latencies **(B)** decreased by nearly a minute after three to six days of training. Some animals reached criterion performance on the fourth day, and numbers indicate the number of animals tested on days five and six.

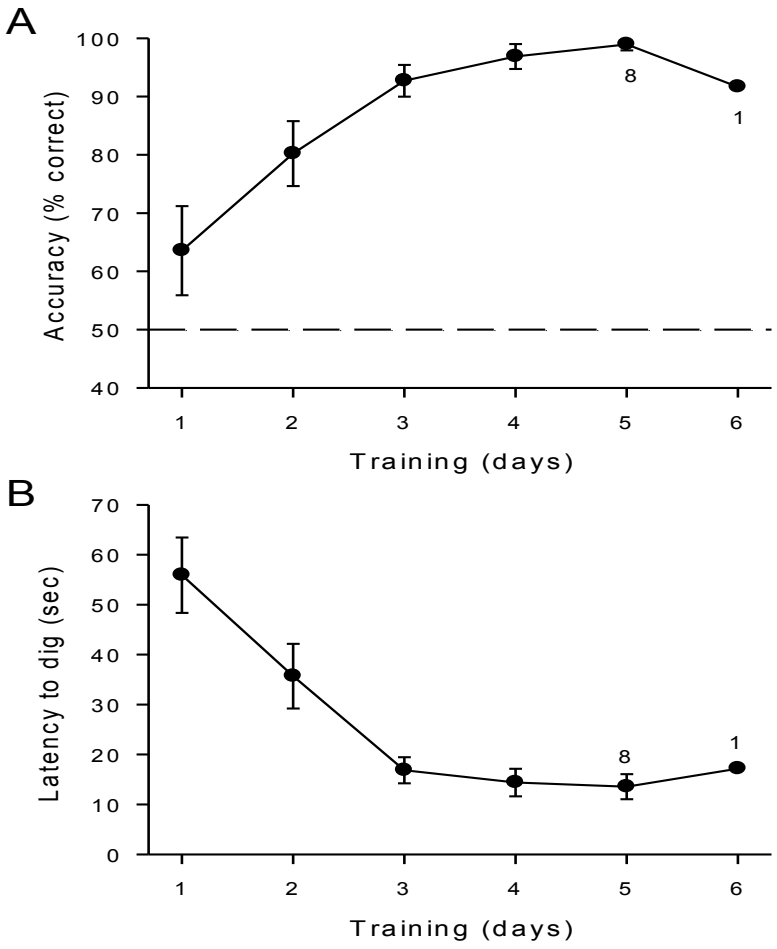

A
100
90
80
70
60
50
40
Accuracy (% correct)
8
1
1
2
3
4
5
6
Training (days)
B
70
60
50
40
30
20
10
0
Latency to dig (sec)
8
1
1
2
3
4
5
6
Training (days)

Figure 2. The performance of rats during retraining on the olfactory discrimination task was similar in rats that had undergone either 6-OHDA lesions or sham surgery. The mean accuracy **(A)** and latency **(B)** of responses in the olfactory discrimination task improved similarly as a function of days in both sham and lesioned animals. Both sham-operated and lesioned animals required 2 to 6 days of retraining to reach criterion level of performance (11 of 12 trials correct) **(C)**.

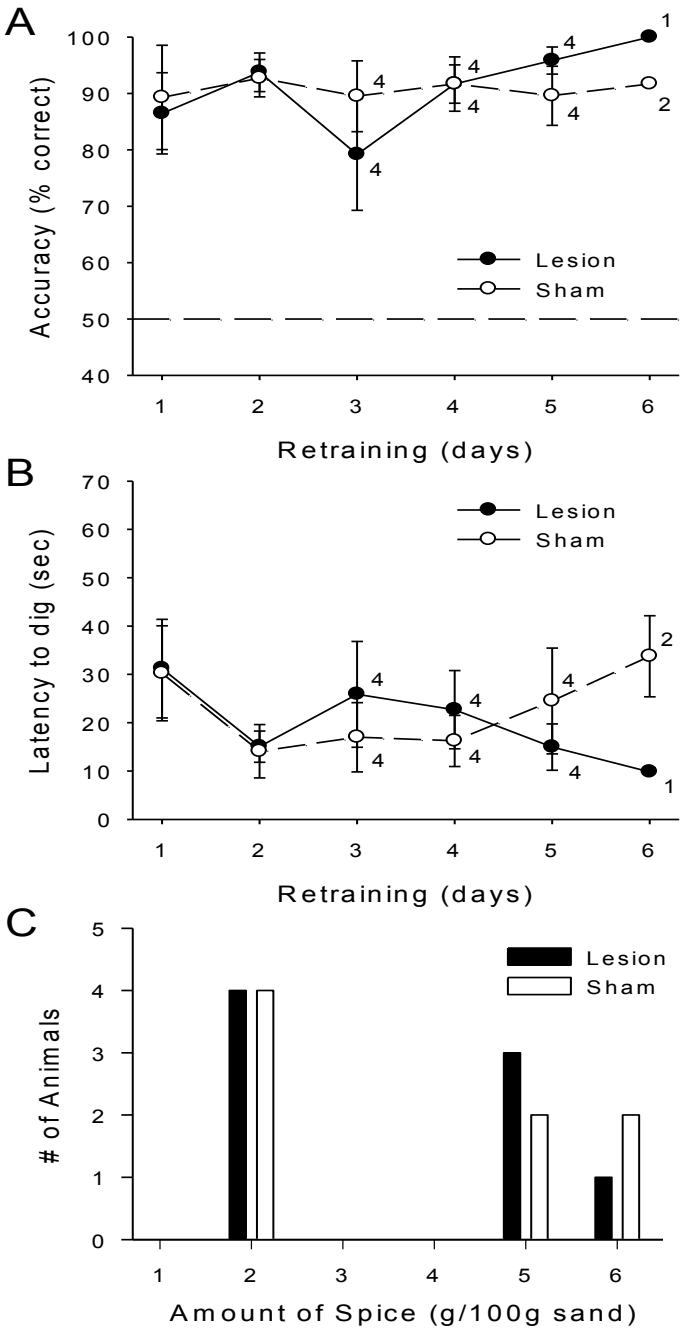

A
Accuracy (% correct)
100
90
80
70
60
50
40
Lesion
Sham
1
2
3
4
5
6
Retraining (days)
B
Latency to dig (sec)
70
60
50
40
30
20
10
0
Lesion
Sham
Retraining (days)
C
# of Animals
5
4
3
2
1
0
Lesion
Sham
Amount of Spice (g/100g sand)

Figure 3. The accuracy and latency of responses on the olfactory sensitivity task degraded similarly in both sham and lesioned animals as the concentration of spice in sand was reduced. Performance accuracy **(A)** was significantly reduced in both sham-operated and lesioned animals as the concentration of spice was reduced, and response latencies were also significantly increased as the concentration of spice was reduced **(B)**.

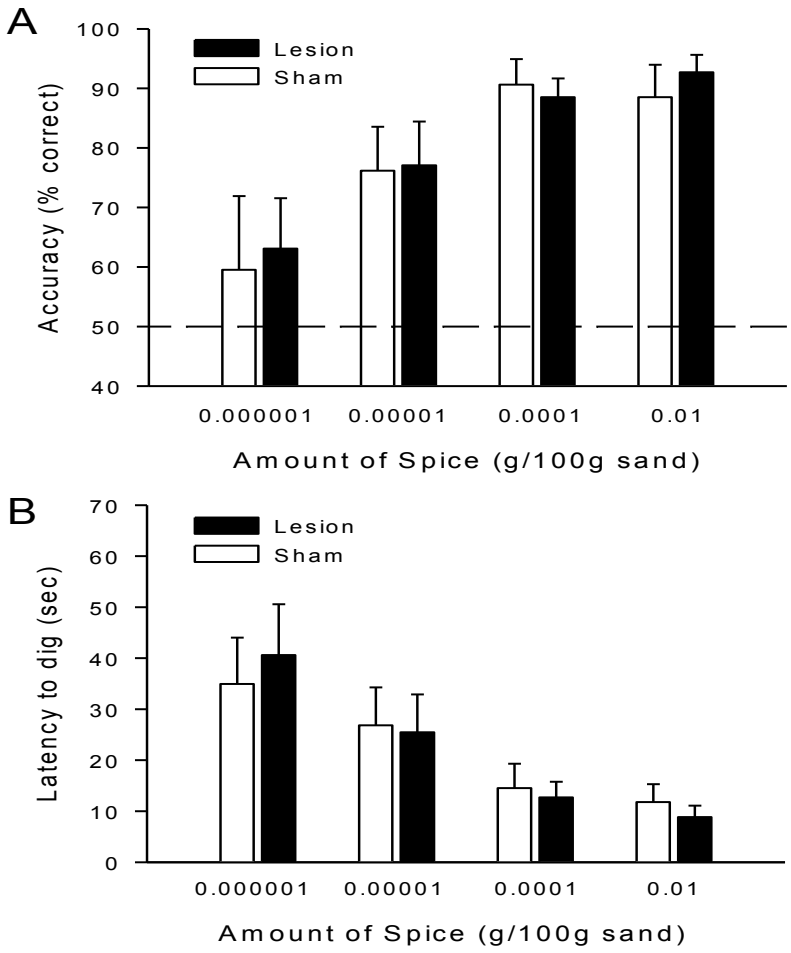

A
100
90
80
70
60
50
40
Accuracy (% correct)
Lesion
Sham
0.000001
0.00001
0.0001
0.01
Amount of Spice (g/100g sand)
B
70
60
50
40
30
20
10
0
Latency to dig (sec)
Lesion
Sham
0.000001
0.00001
0.0001
0.01
Amount of Spice (g/100g sand)

Figure 4. Learning to discriminate between two novel odours to obtain a food-reward was similar in control and lesioned animals when a low concentration of odour was used in order to increase the difficulty of the task. The mean accuracy **(A)** and latency **(B)** of responses in the discrimination task improved similarly as a function of days in both groups. All animals received at least 4 days of training, and animals in both groups usually required 2 to 4 days to reach the performance criterion **(C)**. Note that one control animal required 7 days of training on the task.

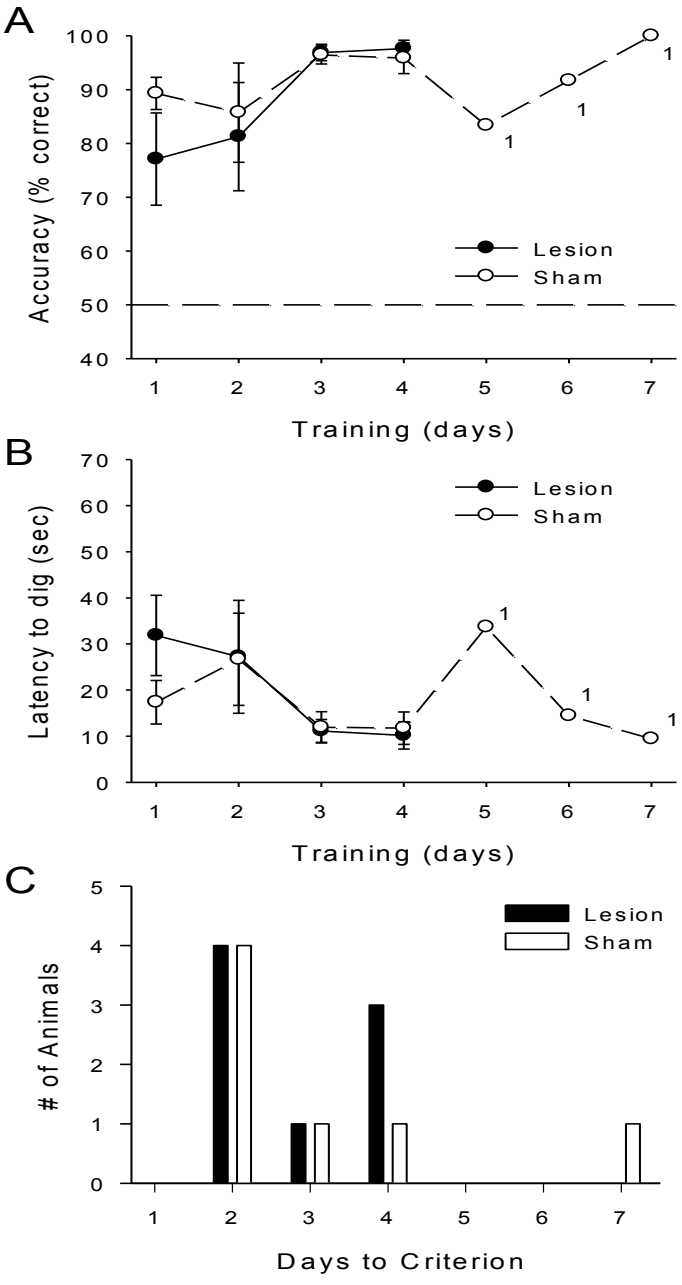

A
Accuracy (% correct)
100
90
80
70
60
50
40
1
2
3
4
5
6
7
Training (days)
Lesion
Sham
1
1
1
B
Latency to dig (sec)
70
60
50
40
30
20
10
0
Training (days)
Lesion
Sham
1
1
1
C
# of Animals
5
4
3
2
1
0
Days to Criterion
Lesion
Sham

Figure 5. A trial-by-trial analysis of response accuracy and latency was used to investigate the trend towards poorer accuracy and slower latencies in lesioned animals during the first two days of training on the olfactory discrimination task with faint, novel odours (see Figure 4). There was no significant Group by Trial interaction for performance accuracy on the first day **(A_1)**, but there was a significant interaction for response latency that was due to significantly longer latencies in lesioned animals on the first and twelfth trials **(B_1)**. There were no significant differences in responding on the second day of training **(A_2, B_2)**.

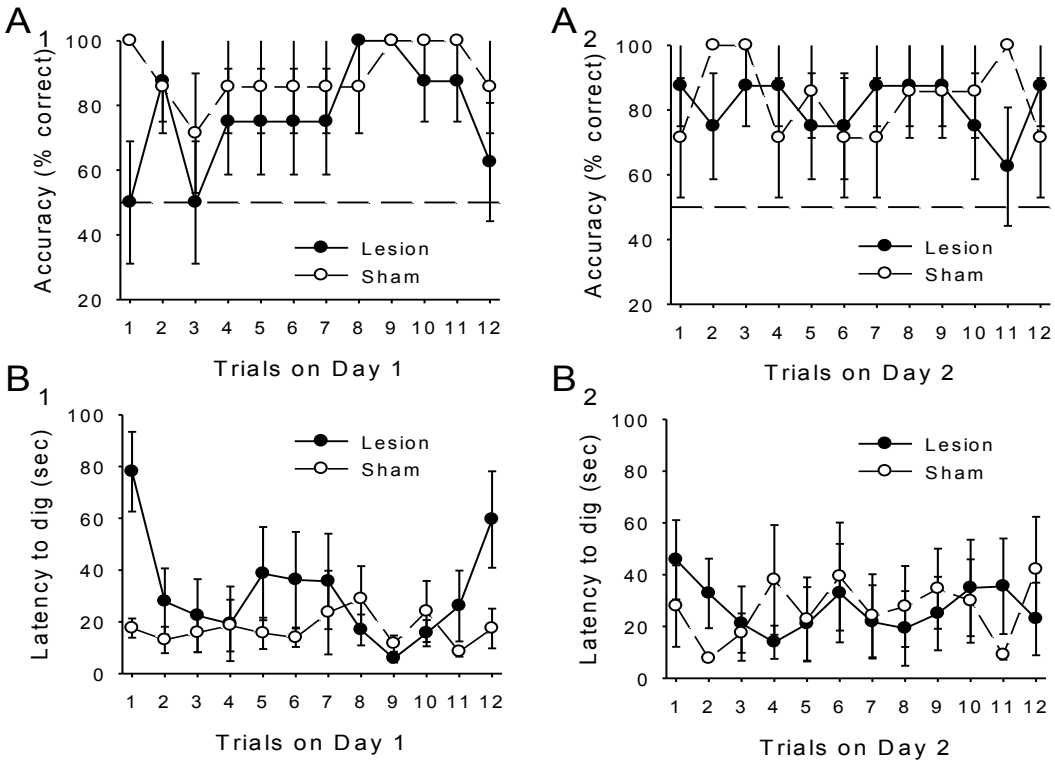

A
1
Accuracy (% correct)
100
80
60
40
20
Lesion
Sham
1 2 3 4 5 6 7 8 9 10 11 12
Trials on Day 1
A
2
Accuracy (% correct)
100
80
60
40
20
Lesion
Sham
1 2 3 4 5 6 7 8 9 10 11 12
Trials on Day 2
B
1
Latency to dig (sec)
100
80
60
40
20
0
Lesion
Sham
1 2 3 4 5 6 7 8 9 10 11 12
Trials on Day 1
B
2
Latency to dig (sec)
100
80
60
40
20
0
Lesion
Sham
1 2 3 4 5 6 7 8 9 10 11 12
Trials on Day 2

Figure 6. There was no significant difference in the performance of lesioned and control animals in tests of memory for either the old odour pair used in initial discrimination and sensitivity testing (see Figures 1, 2 and 3), or the new odour pair that was used to assess their ability to learn to discriminate faint odours (see Figures 4 and 5). When tested two weeks following training on the new, the control and lesioned animals were similar in mean response accuracy **(A)** and latency **(B)** when tested either on the new odour pair or the original odour pair. Responses during testing with the original odour pair tended to be faster and more accurate than with the new odour pair, but this trend was not statistically significant. Both odour pairs were presented at the lowest concentration during these tests.

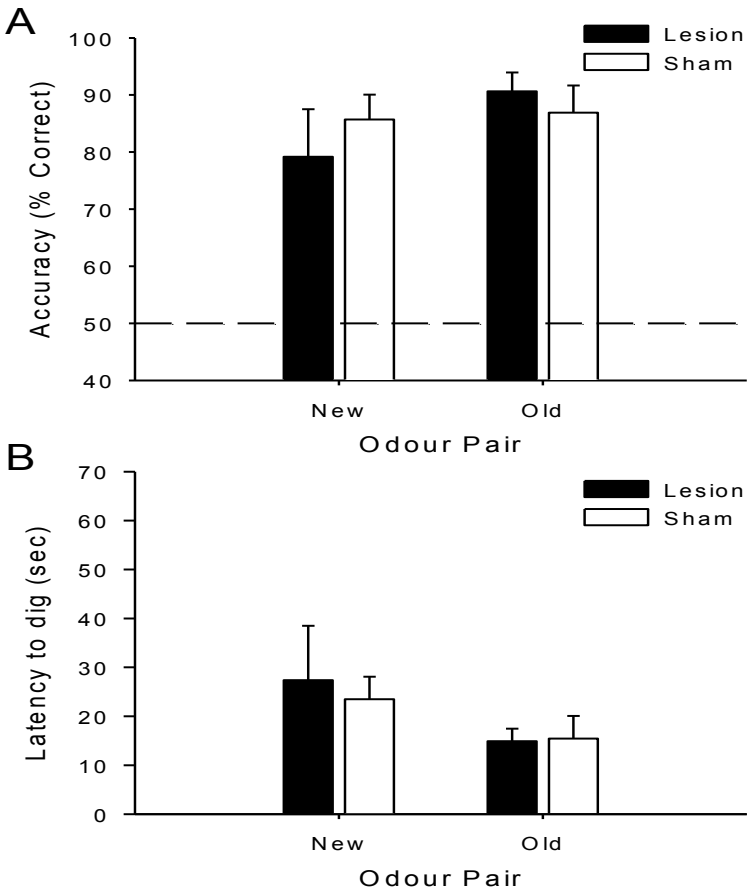
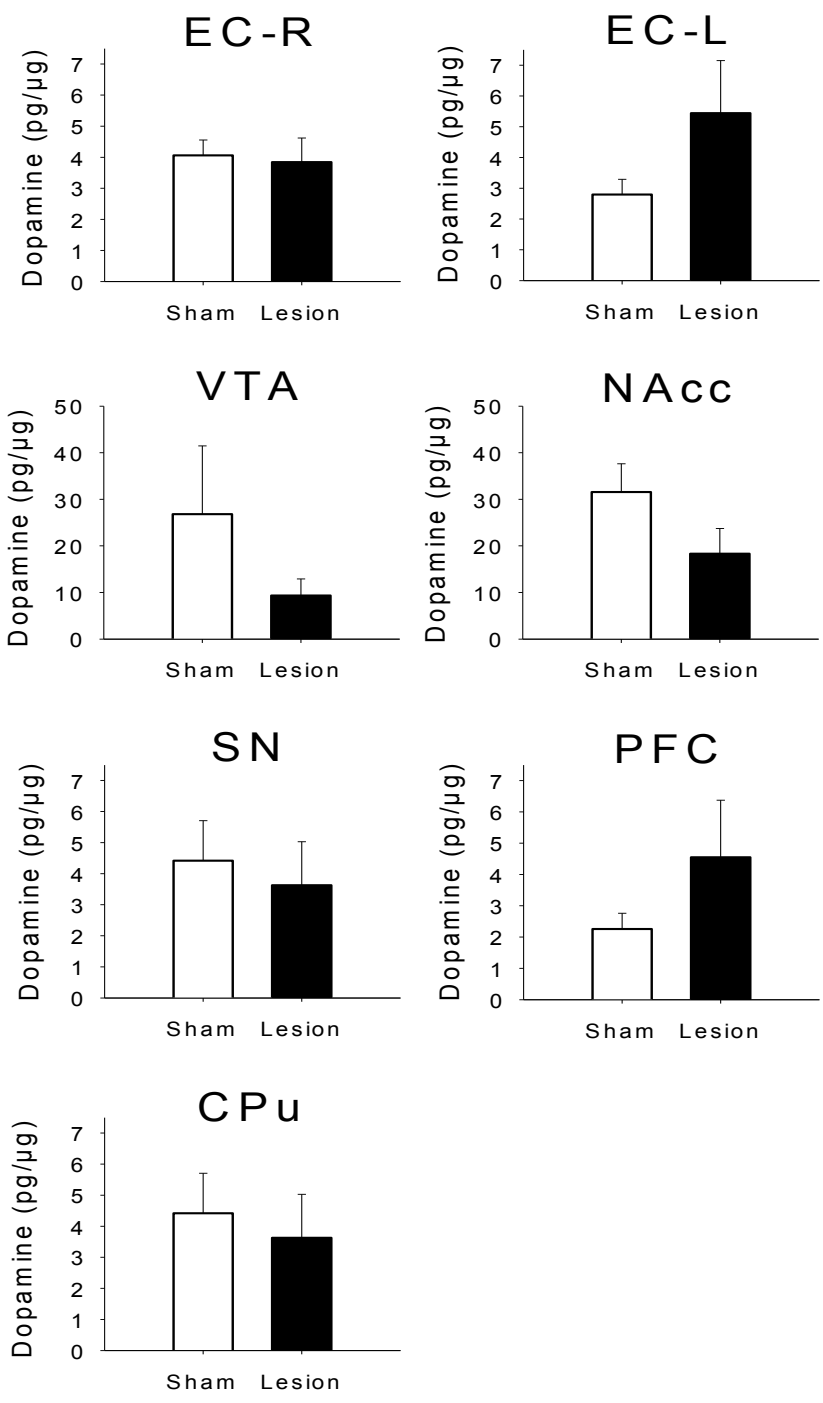

A
Lesion
Sham
Accuracy (% Correct)
100
90
80
70
60
50
40
New
Old
Odour Pair
B
Lesion
Sham
Latency to dig (sec)
70
60
50
40
30
20
10
0
New
Old
Odour Pair

Figure 7. Results of assays conducted on tissue punches obtained from 15 of the 16 animals 8-15 weeks after either sham or 6-OHDA lesion surgery do not show significant differences between dopamine levels in sham-operated and lesioned animals in any of the brain regions tested. The regions tested were the left and right entorhinal cortex (EC-L, EC-R), ventral tegmental area (VTA), substantia nigra (SN), caudate-putamen (CPu), nucleus accumbens (NAcc), and prefrontal cortex (PFC).


EC-R
EC-L
VTA
NAcc
SN
PFC
CPu
Dopamine (pg/µg)
Sham
Lesion

DISCUSSION

The entorhinal cortex has been linked to a role in olfactory processing in part by its extensive anatomical connections with the primary olfactory cortex (Burwell, 2000; Witter et al., 2000) and by a variety of converging experimental evidence. Electrophysiological recordings of neurons in the entorhinal cortex show patterns of activity that suggest a role for the entorhinal cortex in olfactory processing and working memory (Petrulis et al., 2005; Young et al., 1997; Dickson et al., 2000; Egorov et al., 2002; Fransen et al., 2006; Tahvildari et al., 2007). Moreover, behavioural experiments that have employed lesions of the entorhinal cortex have also shown that entorhinal cortical damage interferes with performance on tasks that require olfactory memory (McGaughy et al., 2005; Kaut & Bunsey, 2001) and olfactory discrimination (Staubli et al., 1984; Fortin et al., 2002). In addition, although it is clear that the transmitter dopamine plays an important modulatory role in cognitive functions in the prefrontal cortex (Goldman-Rakic et al., 2000), and that the lateral entorhinal cortex receives one of the most prominent cortical projections of dopamine (Akil & Lewis, 1993; Bjorklund & Lindvall, 1984; Fallon & Loughlin, 1987; Oades & Halliday, 1987), very little is known about how dopamine may modulate the olfactory and mnemonic processes of the entorhinal cortex. This thesis has provided the first tests of how dopaminergic inputs to the entorhinal cortex may modulate the animal's sensitivity to odours and their ability to discriminate between faint odours. Olfactory-guided digging tasks used by others have been modified and developed to be used here because they employ a natural digging behaviour and depend on the olfactory system of the rat; a highly important sense which

rats depend on heavily to navigate and to identify food and conspecifics in their natural environment (Slotnick, 2001; Slotnick, Schellinck, & Brown, 2005).

In the present study, the neurotoxin 6-OHDA was used to deplete dopaminergic inputs to the entorhinal cortex in order to assess the contribution of dopamine to olfactory sensitivity, and to the learning and memory of a simple association between an odour and reward in an odour discrimination task. Food-restricted rats were first trained to dig in cups filled with scented sand and to discriminate between two different odours to obtain a buried food reward which was always associated with one odour (CS+) (Figure 1). Upon reaching criterion performance on this discrimination task, animals underwent 6-OHDA lesions of the entorhinal cortex or a sham surgery. After retraining on the original discrimination rule (Figure 2), olfactory sensitivity was tested using cups containing either decreasing amounts of the original CS+ odour or unscented neutral sand. In tests of olfactory sensitivity, both groups showed lower accuracy at smaller concentrations of spice, but when compared to sham animals, 6-OHDA lesioned animals showed no deficits in olfactory sensitivity (Figure 3). In addition, lesioned animals did not differ significantly in their ability to learn to perform the discrimination task with a novel odour pair (Figure 4 and 5), and, after a delay of two weeks, the animals also did not show differences in their ability to respond accurately to either the initial or novel odour pair (Figure 6).

These results suggest that dopamine innervation of the entorhinal cortex may not significantly modulate olfactory sensitivity, and might also not be required for successful discrimination between odours in this appetitively motivated digging task. Similarly, dopaminergic innervation of the entorhinal cortex may not be required for the acquisition

or recall of a simple odour-reward association. The behavioural results obtained here must be interpreted cautiously, however, because dopamine levels in the entorhinal cortex and other brain regions measured 8-15 weeks after surgery, did not differ significantly between control and lesioned animals (Figure 7); although the lack of a difference in dopamine levels may reflect a functional recovery of dopaminergic terminals in the entorhinal cortex, it is not clear if the lesions indeed resulted in reductions of dopamine in the entorhinal cortex at the time that animals were tested.

Olfactory Sensitivity

The current findings do not support the hypothesized role of dopamine in the entorhinal cortex in olfactory sensitivity or olfactory learning, but the results obtained here clearly demonstrate the utility of the digging task for assessing olfactory sensitivity via the animal's ability to detect and respond to odours of decreasing concentrations (Figure 3). Several variations of odour-guided digging tasks have been used previously for successfully investigating a variety of experimental questions (Dudchenko, Wood, & Eichenbaum, 2000). Kaut and colleagues (2001; 2003) used similar tasks to assess olfactory discrimination and memory abilities of rats with hippocampal and parahippocampal lesions. In addition, McGaughy and colleagues (2005) used a variation of the digging task to assess olfactory delayed non-match to sample performance in animals with lesions to the cholinergic inputs of the entorhinal cortex and found that performance with novel odours was impaired but was not impaired with familiar odours. Caruana (2008) also used these methods to determine the effect of 6-OHDA lesions of the entorhinal cortex on NMTS and DNMTS performance, and found that lesioned animals were significantly impaired in their performance during retraining on the NMTS

task following recovery from surgery. These results were difficult to interpret, however, because animals showed very slow response latencies on both the test trial and the *sample* trial, suggesting that response deficits could have resulted not only from working memory impairments, but also a non-specific sensory, motor or motivational deficit, or a deficit in other executive functions (Caruana, 2008). The methods that have been developed here to assess olfactory sensitivity in rats using tasks that require digging in scented sand were aimed at determining if a reduction in olfactory sensitivity might explain the deficits observed by Caruana (2008) and others.

Although there were no significant effects of 6-OHDA lesions on performance, the olfactory discrimination and sensitivity tasks developed here have several advantages. The task requires the learning of an olfactory discrimination rule, in which a CS+ odour is associated with food reward and a CS- odour that is unrewarded. This requires rats to use odours to guide their choice of where to dig for food-reward, a motivated behavior that is quite natural for rats. The training of animals to learn this association (Figure 1) proceeds very rapidly in comparison to other odour-discrimination paradigms (Slotnick et al., 2005), specifically in comparison to the DNMTS version of the task that can require 7 to 10 days for animals to reach criterion levels of performance (Caruana, 2008; Otto & Eichenbaum, 1992; McGaughy et al., 2005). In tasks measuring sensitivity, it is important that the animals showed graded decrements in performance with reducing concentrations of odor. Here response accuracy ranged from close to chance to 93% accuracy (Figure 3) with parallel changes in the latency of responses, such that animals required a longer period of time to choose a cup at lower concentrations. This suggests that animals had more difficulty identifying the CS+ odour when it was presented at low

concentrations. The graded changes in responses suggest that the absence of a deficit in lesioned animals is not because of floor- or ceiling-effects, and the performance of animals at near-chance levels of accuracy at the lowest concentration suggests that the task is challenging to the animal. Patterns of decrements in response accuracy and latency with reduced concentration were similar across animals, suggesting that the range of concentrations chosen is effective for assessing olfactory sensitivity in a consistent manner across animals. The consistency of responding, across animals, to given concentrations of spice also suggests that methods used for preparing the stimuli were effective for consistently presenting differing concentrations of small amounts of spice. Although somewhat difficult to compare given the differences in stimuli characteristics, the high levels of sensitivity found here were similar to those found by Tillerson et al. (2006) who used an olfactory sensitivity step down procedure, where the amount of time animals sniffed decreasing concentrations of paprika scented water was compared to control water, to show that mice could correctly identify concentrations as low as 100 ng/ml water. The digging task used here allowed researchers to determine which concentrations were most challenging for rats to respond to correctly in a discrimination task, and this allowed for the use of these concentrations to increase the difficulty of an odour discrimination task by using faint odours (Figure 5). Thus, the task can be used to assess sensitivity as well as to develop stimuli to increase the difficulty of olfactory discrimination tasks. In addition, these tasks also have the advantage that the required apparatus and materials are relatively simple, inexpensive and widely available.

The tasks used here were appetitively motivated, and required food-restricted rats to obtain a food-reward. Because the mesolimbic dopaminergic system can be activated

by anticipation of reward (Schultz, 2002; Schott et al., 2008; Fibiger & Phillips, 1986), an appetitively motivated task is most likely to reflect the potential modulatory effects of dopamine; control rats may have their performance modulated or enhanced by endogenous release of dopamine in the entorhinal cortex, while lesioned rats may not (Bannerman et al, 2002). Therefore, this task may prove useful in evaluating the influence of dopamine on olfactory sensory processing.

Olfactory Discrimination

After sham and 6-OHDA lesions were completed, the animals were retrained on the olfactory discrimination task, using the same odour pair as they had been exposed to before surgery. This was done in order to ensure that animals had retained the association between the CS+ odour and the food reward, and in order to determine if 6-OHDA lesions might result in impairments in olfactory discrimination performance. The performance of sham and 6-OHDA lesioned animals did not differ significantly during post-surgical reacquisition of performance on the olfactory discrimination task (Figure 2), and both groups of animals showed response accuracies in the range of 80 to 95 % on the first two days of retraining. This suggests that the animals did not show significant retrograde amnesia for the association, and also retained the ability to perform the task and the motivation to do so. This is similar to the results of Staubli et al. (1984) who also found animals with entorhinal lesions showed no retrograde amnesia for a task learned before surgery.

The absence of deficits in olfactory discrimination performance following 6-OHDA lesions however contrasts with the findings of Caruana (2008) who found that response accuracy and latency were impaired in animals with 6-OHDA lesions of the

entorhinal cortex during postsurgical retraining on a NMTS version of the task. Kaut and Bunsey (2001) also found deficits in entorhinal lesioned animals' ability to recall a novel odour pair discrimination learned after surgery, even though they had no difficulty recalling the odour pair that was learned 4 weeks prior to surgery. Caruana (2008) found however, that after the animals were retrained, they performed just as well as control animals in further testing when the difficulty of the task was increased by implementing a delay between sample and test phases. These differences between the present results and those of Caruana (2008) and Kaut and Bunsey (2001), could be explained by the much simpler tasks-demands of the present task as compared to the DNMTS task used by Caruana (2008) or multiple discrimination tasks used by Kaut and Bunsey (2001). In the present task rats were only required to learn simple associations with a single odour pair. It is therefore possible that more difficult olfactory discrimination and memory tasks, such as the DNMTS task, are needed to assess the contributions of the entorhinal cortex to olfactory processing and memory, and to assess the contribution of dopamine in the entorhinal cortex to these functions.

Animals had not shown impairments on the discrimination task during postsurgical retraining with high concentrations of odour, but it was reasoned that increasing the difficulty of the discrimination task by using faint odours might reveal an effect of 6-OHDA lesions. However, when animals were tested on the olfactory discrimination task using a new odour pair at very faint concentrations, there was no significant difference between sham and lesioned animals on the learning of the new association (Figure 4). This suggests that the availability of dopamine does not normally enhance olfactory discrimination performance in control animals in the task used here.

However there was a trend towards reduced accuracy and longer latencies in lesioned animals on the first day of training with the faint odours (Figure 4). Further, a trial-by-trial investigation of this trend towards poorer responding on the first day (Figure 5) indicated that lesioned animals responded at chance levels of accuracy and had significantly longer response latencies on the first trial as compared to control animals. It is possible that this may reflect a deficit in 6-OHDA lesioned animals due to dopamine depletion, but results are not conclusive. This trend towards poorer accuracy and longer latencies in 6-OHDA lesioned animals is reminiscent of the findings of McGaughy and colleagues (2005) who found that lesions of cholinergic inputs to the entorhinal cortex had no effect on a DNMTS version of the task, but did have an effect when the animal was first exposed to novel odour. They took their findings to suggest that acetylcholine contributes to memory function in the entorhinal cortex by reducing the interference between the newly learned odour and previously learned odours. Because the observed trend towards impaired performance on the first day of training with a novel odour following dopamine lesions did not reach significance, however, it is unclear how dopamine may modulate responses to newly encountered odours.

Olfactory Memory

Damage to the entorhinal cortex can result in retrograde and anterograde olfactory memory deficits (Staubli et al., 1984; Kaut & Bunsey, 2001; Otto & Eichenbaum, 1992), and therefore the entorhinal cortex is thought to play an important role in olfactory memory. Dopamine has been linked to working memory performance in the prefrontal cortex (Goldman-Rakic et al, 2000), and 6-OHDA lesions of the entorhinal cortex also result in deficits in NMTS performance that could be due to a memory deficit (Caruana,

2008). Therefore, in the current study, several weeks following the olfactory discrimination testing using the faint odour concentrations, the associations between CS+ odours and reward that were learned during the discrimination tests were used to assess olfactory memory. Both the 6-OHDA lesioned group and the control group performed similarly on tasks which assessed memory for both the initial and the most recent odour pairs using low concentrations of odour (Figure 6). This suggests that 6-OHDA lesions in the entorhinal cortex do not affect recall of a simple odour-reward association. In addition, although there was no significant difference in the animals' performance on the initial odour pair versus the more recently trained odour pair, there was a trend in both groups for higher accuracies and faster latencies on the task testing the memory for the odours used in the initial discrimination training versus the more recent training even though there was a longer delay between the rats' last exposure to the initial odour pair and the memory tests. The trend towards faster and more accurate responding with the initial odour could be due to the much more extensive training on this odour pair with higher odour concentrations. It is also possible that the representation of the initial odour pair may have resulted in proactive interference with the representation of the new odour pair (McGaughy et al., 2005), but it is difficult to distinguish this possibility from the difference in the odour concentrations that were used during training for the initial and new odour pairs. In addition, the memory tests were conducted on two consecutive days, with memory for the most recent odour pair being tested on the first day, and memory for the initial odour pair tested on the second day. It is therefore possible that testing on the first day resulted in practice or carry-over effects on the second day that might have

enhanced performance on the most recently learned faint odour pair. This may have reduced differences between memory performance for the new and old odours.

Non-Significant Effects of 6-OHDA Lesions

Although there was a significant increase in 6-OHDA lesioned animals' latency of responding during the first trial of the odour discrimination task in which faint odours were used during training, the present results, overall, do not provide strong evidence for differences in olfactory discrimination, olfactory memory, or olfactory sensitivity between animals with 6-OHDA lesions and control animals that underwent a sham operation. One possibility is that the 6-OHDA injections did not result in adequate/significant reductions in dopamine, or that the spatial extent of the reductions was not sufficient to cover a large enough area of the entorhinal cortex. The dopamine content of tissue punches obtained from the left and right entorhinal cortex, as well as cell body and terminal regions of the ventral tegmental area and substantia nigra were measured here to assess the effects of the 6-OHDA injections (Figure 7). It was found, however, that there were no significant differences between the dopamine levels assayed in the entorhinal cortex, and further, no significant difference in the dopamine measured from punches obtained from other brain regions. It is therefore possible that the lack of behavioural effects seen in the present study resulted from a minimal effect of the 6-OHDA lesions on tissue levels of dopamine in the entorhinal cortex. The meaningfulness of the results of the dopamine assay for interpreting the lack of behavioural differences between groups, however, is ambiguous because it is known that tissue levels of dopamine can recover following 6-OHDA lesions. In the striatum, dopamine lesions that preserve at least 20% of dopaminergic fibres do not significantly reduce the amount of

dopamine in the tissue (Robinson, Castaneda, & Whishaw, 1990) and the remaining dopamine fibres can maintain dopamine-dependent behaviors. More complete lesions of striatal dopamine that destroy 80 to 95% of inputs are known to activate compensatory mechanisms that upregulate dopamine production and release, as well as facilitate the insertion of postsynaptic dopamine receptors (Altar, Marien, & Marshall, 1987; Kostrzewa, 1995; Neve, Kozlowski, & Marshall, 1982; Robinson et al., 1990; Schwarting & Huston, 1996; Zigmond, 1997). This compensatory recovery can take from 3 to 18 days. Therefore, dopamine levels in the entorhinal cortex may have partially recovered during the behavioural tests of olfactory sensitivity and discrimination seen here, and may also have recovered by the time that tissue punches were obtained for assay approximately 8 to 15 weeks after surgery. Caruana (2008) also found non-significant reductions in dopamine in the entorhinal cortex as measured by these same methods using samples taken 12 weeks after surgery. Therefore, although the lack of difference between groups in tissue dopamine suggests that the 6-OHDA lesion may have been ineffective in lowering tissue levels of dopamine in the lesioned group, it is also possible that the 6-OHDA injections were effective in reducing entorhinal dopamine but tissue levels of dopamine recovered by the time of tissue assays. Therefore, it cannot be known with certainty what the relative amounts of dopamine were in control and lesioned animals during performance of tasks.

The success of the current experiments depends on the expectation that dopamine neurons would normally become activated during performance of the present tasks, and that the presence of dopamine would normally enhance performance. Previous studies using microdialysis and electrochemical techniques have shown that dopamine in the

medial prefrontal cortex, striatum and nucleus accumbens is enhanced during anticipation and consumption of food reward (Martel & Fantino, 1996; Richardson & Gratton, 1998; Phillips, Blaha, Pfaus, & Blackburn, 1992). It is therefore likely that anticipation of food-reward in the food-restricted rats tested here is also likely to have activated dopaminergic neurons, and resulted in increased dopamine in the entorhinal cortex. This is consistent with the short response latencies for most animals during performance of the task which suggests that animals were motivated to carry out the task. Measurements of dopamine in the entorhinal cortex using microdialysis, or using electrochemical detection techniques, would be useful for determining the extent and time-course of changes in dopamine in the entorhinal cortex associated with the present tasks (Martel & Fantino, 1996; Richardson & Gratton, 1998; Caruana et al., 2007). Another possible explanation for the lack of behavioural effect is that dopamine may play only a transient role in learning how to perform the task. In the present experiments, animals were trained on the olfactory discrimination task and learned the association of the CS+ odour with food-reward prior to 6-OHDA lesion or sham surgeries. If dopamine were to contribute more to the learning of the task and to the initial formation of the CS+odour-reward association, it is possible that deficits in training on this task might be observed if 6-OHDA lesions were made prior to any behavioural training.

In addition, although dopamine is likely to be released in the entorhinal cortex during performance of the task, it is possible that effective performance of the task might not be dependent on the entorhinal cortex, and thus not be modulated by dopamine availability in the entorhinal cortex. A substantial body of literature is consistent with the expectation that the entorhinal cortex contributes to olfactory sensitivity, and olfactory

learning and memory (Slotnick, 2001; Kaut & Bunsey, 2001; Otto & Eichenbaum, 1992; Hasselmo & Stern, 2006; Bouras & Chapman, 2003), and dopamine is known to modulate working memory processes in the prefrontal cortex (Goldman-Rakic, 1999; Goldman-Rakic et al., 2000; Otani et al., 2003). However, it is possible that structures other than the entorhinal cortex may normally subserve the relatively simple demands associated with the tasks used here, and that the entorhinal cortex may normally contribute to more complex processes that are not required by these tasks. The entorhinal cortex may play a stronger role, for example, in DNMTS tasks (Otto & Eichenbaum, 1992), spatial navigation tasks (Mumby & Glenn, 2000), or reversal learning (Staubli et al., 1984). Many structures are likely to be involved in the cognitive processes required by the tasks used in the present study and, although it is not known what combination of structures might mediate these behaviours, it is possible that the relatively simple perceptual and mnemonic demands of these tasks might be subserved by the olfactory bulb, piriform cortex, and/or other related structures of the main olfactory system (Slotnick et al., 2005) rather than by the entorhinal cortex. For example, neurons in the olfactory bulb show different patterns of neural activity, or "odour maps", depending on the odours presented, suggestive of a neural substrate in the olfactory bulb for coding odour recognition or discrimination (Xu, Greer, & Shepherd, 2000). Moreover, rats are known to be able to respond to strong odorants via trigeminal receptors in the nasal vault and via sensory receptors in the trachea (Slotnick et al., 2005) and the contribution of these receptors to the odour stimuli used here is not known. Thus, it is possible that structures other than the entorhinal cortex may be sufficient for the detection and discrimination of odours in the simple tasks tested here, and may be sufficient to guide

the correct responding of the animal. Similarly, it is also possible that the entorhinal cortex normally mediates these processes, but that interfering with dopaminergic activity in the entorhinal cortex may lead to a compensatory increase in the involvement of other structures so that there is no resulting decrement in performance.

The hypothesis that 6-OHDA lesions of the entorhinal cortex would result in behavioural deficits on the tasks was based on the finding that moderate levels of dopamine increase the amplitude of synaptic potentials in the entorhinal cortex from the piriform cortex (Caruana et al., 2006). It was therefore expected that dopamine release in control animals would increase synaptic transmission in the entorhinal cortex, and that olfactory representations would have a stronger effect in control animals both within the entorhinal cortex and in the structures to which it projects. Although dopamine facilitates responses to synchronous, electrically activated inputs from the piriform cortex, it is not known how dopamine modulates the spatiotemporal pattern of neuronal activity associated with processing of real odours. It is also not clear how odour identity or intensity is coded by activity patterns within the olfactory system. It is therefore possible, that while dopamine may increase the overall strength of synaptic responses in the entorhinal cortex (Caruana et al., 2006), dopamine may not substantially modulate the neuronal representation of odour as it relates to the demands of the current tasks.

Characterizing the contribution of specific brain structures to the cognitive processes that underlie the complex behavioural responding involved in tests of olfactory perception, learning and memory is experimentally challenging. These challenges in the design and interpretation of experimental results can be compounded when investigating the role of neuromodulatory transmitters. The research here has sought to investigate the

role of dopamine in modulating the contribution of the entorhinal cortex to olfactory perception and olfactory learning and memory. Progress on this question will likely depend upon choice of an olfactory task that depends critically on the functioning of the entorhinal cortex, and which is also robustly modulated by endogenous dopamine function in control animals.

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