Neural Encoding of Olfactory Recognition Memory

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Abstract. Our work with both sheep and mouse models has revealed many of the neural substrates and signalling pathways involved in olfactory recognition memory in the main olfactory system. A distributed neural system is required for initial memory formation and its short-term retention—the olfactory bulb, piriform and entorhinal cortices and hippocampus. Following memory consolidation, after 8 h or so, only the olfactory bulb and piriform cortex appear to be important for effective recall. Similarly, whereas the glutamate–NMDA/AMPA receptor–nitric oxide (NO)–cyclic GMP signalling pathway is important for memory formation it is not involved in recall post-consolidation. Here, within the olfactory bulb, up-regulation of class 1 metabotropic glutamate receptors appears to maintain the enhanced sensitivity at the mitral to granule cell synapses required for effective memory recall. Recently we have investigated whether fluctuating sex hormone levels during the oestrous cycle modulate olfactory recognition memory and the different neural substrates and signalling pathways involved. These studies have used two robust models of social olfactory memory in the mouse which either involve social or non social odours (habituation-dishabituation and social transmission of food preference tasks). In both cases significant improvement of learning retention occurs when original learning takes place during the proestrus phase of the ovarian cycle. This is probably the result of oestrogen changes at this time since transgenic mice lacking functional expression of oestrogen receptors (ERα and ERβ, the two main oestrogen receptor sub-types) have shown problems in social recognition. Therefore, oestrogen appears to act at the level of the olfactory bulb by modulating both noradrenaline and the glutamate/NO signalling pathway.

Key words: Olfactory bulb, Olfactory memory, Olfactory recognition, Social learning, Nitric oxide (NO), Oestrous cycle, Sheep, Mouse, Social transmission of food preference

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into socially and non-socially motivated tasks—a distinction that has been shown to be important by work on the oxytocin knockout mouse [1] where only deficits in social memory have been observed. The importance of olfaction and olfactory memory on socially mediated behaviours is very considerable since many mammalian species rely upon smell to obtain information about their environment, particularly their social one. Our work for the past 15 years has focused on the involvement of the main olfactory system in social olfactory learning in the context of lamb-mother ewe bonding via odour recognition [2, 3] and social recognition of individuals [4, 5] and social transmission of food preference [6, 7] in rodents.

These paradigms represent good ethologically relevant models for the study of social olfactory memory. Offspring recognition ensures that the mother will preserve her own genes by limiting maternal investment to her own offspring. Social recognition of conspecifics has functional consequences in several social contexts [3, 8–11]. It is crucial for the determination and maintenance of social structure in many animal species, particularly those living in small, stable social groups. With social transmission of food preference, after smelling a novel food odour on a forager’s breath rodents seek out that food assuming that it is good to eat and suppressing their normal neophobic response. These models also offer many advantages in studying olfactory memory processes since learning is rapid and robust and long-lasting memories can be formed that are relatively simple to test experimentally.

In sheep, the maternal ewe is able to recognise its offspring by identifying the individual body odour of her own lamb(s). She forms a selective olfactory memory for each of her offspring within 2–4 h of parturition and will reject the approach of any strange lamb [12]. Both the sensitive period for odour learning and the maternal acceptance behaviour are dependent on the hormonal environment and are triggered by feedback to the brain from mechanical stimulation of the vagina and the cervix which normally occurs during parturition. Thus, a ewe can be induced to accept, and form, a new recognition memory for a strange lamb by simply mimicking birth through administering a few minutes of artificial vaginocervical stimulation (VCS) for up to three days post-partum [13]. Social recognition tests in rodents rely on the intrinsic motivation they have for investigating novel conspecifics when introduced into familiar territory or home cage. Under these circumstances they will investigate a novel conspecific more than a familiar one since they are already acquainted with the odour cues of the latter rather than the former [14]. Odour cues such as soiled bedding or urine from familiar animals are sufficient for the recognition process [15]. In the social recognition test [16], a rat or mouse is exposed to a novel stimulus animal (generally juveniles) on two occasions, separated by a given time interval. The reduction of investigation time during the second encounter is taken as evidence for social memory. Introduction of a new fresh stimulus animal triggers an intense investigation bout similar to the initial encounter, proving that the reduced investigation is not due to generalised habituation. This paradigm has been used mainly as a model of short term memory since it was found to last no longer than 1 h in studies using individually housed rats or mice [16–18]. However, recent studies have shown that housing conditions affect retention of these social memories since mice housed in groups, rather than individually, were shown to remember a familiar juvenile for up to 7 days [19].

We have combined two versions of the original social recognition paradigm in mice. In the first instance a habituation-dishabituation paradigm has been employed [20, 21] where the test animal is exposed repeatedly (four trials) to a stimulus animal (anaesthetised adult) for 1 min with 10 min inter-trial intervals. This results in a progressive general reduction of investigation time of the stimulus animal during consecutive trials using the same stimulus animal (habituation). On a fifth trial, a novel stimulus animal is introduced and the investigation time is restored to the original highest level (dishabituation). The second part of the paradigm, performed 24 h later, is a social discrimination test. In this the test animal is given a binary choice between a previously encountered familiar and a novel conspecific [22]. A specific recognition memory, assessed by comparing the differential amount of time spent investigating each stimulus animal, is considered to have been formed when the novel animal is investigated more than the unfamiliar one. This second part of the protocol allows the recognition memory test to be
performed for the familiar versus the unfamiliar stimulus animal in a single trial. This habituation/discrimination social recognition paradigm therefore allows testing both short- and long-term memory with a single experiment.

A second test we used as a model of social olfactory memory in mice is the social transmission of food preference (STFP) paradigm, which uses a combination of biological and non-biological odours in a social and ethologically relevant context. This ability of socially transmitting food preferences has been shown in different rodent species such as rats [7], mice (Mus musculus, [23, 24] and gerbils (Meriones unguiculatus [25]. During social contact between a recently fed animal (demonstrator) and a naïve conspecific (observer), olfactory cues pass from demonstrator to observer subsequently increasing the observer’s preference for the food its demonstrator ate [7, 26]. Observer animals are then presented with a binary choice of two equally novel foods. Learning is demonstrated as an increase in probability that the observer will select the same food as that eaten by demonstrator, over other foods [7]. A single brief interaction between two mice or rats is sufficient to induce a food preference that lasts for more than 6 days. This olfactory learning is believed to involve an association between two odours present on the demonstrator’s breath; the odour of the recently eaten food and a natural constituent of rat’s breath, carbon disulfide (CS2). The association between the food odour and the carbon disulfide is both necessary and sufficient to support the development of a food preference [27, 28].

All three behavioural tasks, offspring and social recognition and STFP are dependent on the main olfactory system and represent good models for studying memory formation in the main olfactory system. In sheep, sectioning the vomeronasal nerve organ does not disrupt either lamb recognition or maternal behaviour, whereas interfering with the olfactory epithelium does [29]. In rodents, lesions in the olfactory bulb impair basic recognition responses in male rats [30, 31]; and chemically induced anosmia can also block individual recognition [32, 33].

The Main Olfactory System (MOS)

The mammalian olfactory system is organised in such a way that it is capable of processing information from two different types of stimuli: one, a liquid-borne chemical substance known as pheromones; the second, a vast number of airborne chemicals with a large variety of structures. This is done by segregating odour and pheromone detection using different sensory neurones [34] and neural pathways [35] in the brain. In the main olfactory system, odours are detected by olfactory receptors (OR) in the olfactory epithelium (OE) of the nasal cavity where every odour has a unique combination of responses from up to one thousand different receptor types [34]. These signals are then relayed to the primary processing region for smell, the main olfactory bulb (MOB) and thence to secondary and tertiary projection areas in the cortex, limbic system and thalamus (see Fig. 1). Odour signals therefore ultimately reach higher cortical areas involved in their conscious perception (attention and awareness), as well as limbic areas, such as the amygdala and the hypothalamus, that are involved in emotional and motivational responses.

In brief, mitral and tufted cells of the MOB (c.f. below for detailed description of the MOB) send their axons, via the lateral olfactory tract (LOT), to the outer layer of the olfactory cortex [36]–the piriform cortex, the olfactory tubercle, anterior olfactory nucleus and specific parts of the entorhinal cortex and amygdala [37–39]. From the olfactory cortex, there is a substantial projection to the mediodorsal thalamus and the ventral part of the submedius nucleus (SM), which in turn projects to the frontal cortex [40]. Disruption of these connections results in deficits in complex but not in simple odour discrimination tasks [41–43]. Another small projection from the LOT goes to the lateral entorhinal cortex [44] and thence via the perforant path to the dentate gyrus and CA1/CA3 of the hippocampus. The olfactory cortex also projects to the hypothalamus and the anterior cortical amygdala [45]. This connection could be the one responsible for the regulation of several of the autonomic and neuroendocrine changes that occur in response to odours [46].

Learning in the MOS

Odour learning in the main olfactory system, unlike that involving the accessory olfactory system [48], is thought to be more distributed, with secondary and tertiary olfactory processing regions being involved. The activation and involvement of these brain areas depends to some extent, on the
nature of the task being performed and on temporal configurations. Dynamic changes occur in both neural substrates and the molecular involvement in formation, short- and long-term memory traces. Studies of brain structure activation and reversible inactivation of some key structures have provided some valuable information. Initial memory formation and its short-term retention seem to require a distributed neural system, involving the olfactory bulb, piriform and entorhinal cortices and the hippocampus. Our early work aimed to differentiate neural substrates controlling maternal behaviour from those involved in olfactory memory formation, by comparing post-parturient ewes exposed to their lambs for 30 min to hormonally primed animals that received VCS and were not exposed to lambs. This showed that olfactory memory formation involved activation, quantified by measurement of altered mRNA expression of the immediately-early genes c-fos and/or zif/268, of the MOB, of piriform, entorhinal and orbitofrontal cortices and dentate gyrus [49]. Studies comparing the response of intact and anosmic ewes to their own lamb have also shown that anosmic ewes, displaying maternal behaviour but not individual lamb recognition, fail to show increased expression in the MOB, piriform and frontal cortices and cortical amygdala [50, 51]. For subsequent memory consolidation, 4.5 h post-partum, the piriform and entorhinal cortices seemed to play a key role, as shown by a study using expression of the brain derived neurotrophic factor (BDNF) and its receptor tyrosine receptor kinase B (trk-B)—a measure of neural plasticity [52]. We recently carried out a systematic study of the temporal involvement of the major areas in the olfactory system, by temporally inactivating them (bilaterally) with either a local anaesthetic (tetracaine) or a GABA_A receptor agonist (muscimol) directly infused via microdialysis probes. This showed that the olfactory bulb, the piriform and the entorhinal cortices are essential for the formation of a normal selective recognition memory. However, complete disruption, was only seen with the olfactory bulb and the entorhinal cortex [53].

There have been fewer studies regarding the involvement of neural substrates in social recognition in mice. Studies using oxytocin receptor knockout mice have shown that a brief social exposure leads to regional activation of the olfactory system as evidenced by increased c-fos expression in the olfactory bulb, piriform cortex and the medial amygdala [54]. Non-behavioural studies have also shown plasticity changes in both the OB and piriform cortex resulting from OB stimulation. High frequency stimulation of the granule cell layer of the OB results in a selective long-term potentiation in both the OB itself and the piriform cortex [55]. Recently we have also shown that consecutive 15 min periods of NMDA-stimulation to the MOB result in a long term potentiation of NMDA-evoked neurotransmitter release in both the olfactory bulb and the piriform
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Following memory consolidation, after approximately 8 h, only the olfactory bulb and piriform cortex appear to be important for recall. For olfactory memory recall, there seems to be distinct neural processes for short- and long-term memory. We have found that for ewes that had formed an olfactory memory of its lamb, inactivation of the entorhinal cortex interfered with its recall in the short-term (4 h post-memory formation) but not in the long-term (12 h to 6 days post-memory formation). However, inactivation of the olfactory bulb disrupted recall at both time points [53]. Consistent with this, it has been shown that the piriform cortex is activated by recognition of the lamb after both 4 h and 7 days contact durations [50]. In rodents, lesions of the entorhinal cortex result in deficits of short-term odour memory [56–58]. Other studies using a modified version of the Coolidge effect, another social olfactory learning test, have also shown that male hamsters with ibotenic acid lesions of the perirhinal-entorhinal cortex have impaired short-term social discrimination memory. Lesions to the hippocampus, however, have no effect on short-term recognition memory [59].

Olfactory recognition memory, therefore, requires a more distributed neural network in the olfactory system for memory formation and short-term memory, with the entorhinal cortex playing a key role. As the memory consolidates, this network is reduced, and it seems that only the OB and piriform cortex are needed to maintain a memory trace throughout its existence.

The Main Olfactory Bulb (MOB)

As already discussed, many studies have consistently reported that a general characteristic of olfactory memory is that it induces long-lasting neural changes at the level of the olfactory bulb. The main olfactory bulb (MOB) has a simple structure involving three main layered cell types (Fig. 2): periglomerular, mitral/tufted and granule cells. The olfactory nerve terminals synapse with mitral and tufted cells in the MOB glomerulus, and also with intrinsic periglomerular cells (PG) that circumscribe the glomeruli. PG cells (GABAergic or dopaminergic) mediate feedback inhibition within the glomerulus and lateral (feedforward) inhibition between glomeruli [60–62]. Mitral and tufted cells send their primary dendrites into the glomeruli and their secondary dendrites into the external plexiform layer. Mitral cells use glutamate and possibly aspartate as transmitters and form reciprocal dendro-dendritic synapses with GABA-containing inhibitory interneurones at distinct levels. Granule cells, which are the most numerous neurones in the olfactory bulb, in turn, make synaptic contacts with the lateral secondary dendrites of the mitral cells in the external plexiform layer and are likely to be involved in the control of mitral cell output [63, 64].

Mechanisms of Olfactory Learning

In early postpartum period (2–4 h) a maternal ewe’s selective recognition of her lamb can be shown by physiological changes in the olfactory bulb that parallel observable behavioural changes. Electrophysiological recordings in the mitral cell layer of the olfactory bulb have shown that several days postpartum 60% of cells respond preferentially to lamb’s odour compared to prepartum period where no preferential activity was seen [65]. Of the cells that respond significantly to these lamb odours the majority (70%) do so equivalently to own or strange lambs, however 30% did respond preferentially to the odours of the ewe’s own lamb. In addition, although lamb odours do not stimulate neurotransmitter release in the olfactory bulb prepartum, by 4 h postpartum, all lamb odours increase centrifugal transmitter release (noradrenaline (NA) and acetylcholine (ACh)) while own lamb odours selectively increase intrinsic glutamate and GABA release [12]. Associated with these neurochemical changes is evidence for enhanced glutamatergic stimulation of GABA release from the granule cells following learning [12].

How does the reorganisation of the olfactory bulb take place? Both centrifugal and intrinsic olfactory bulb pathways play an important role in establishing the plasticity changes in the olfactory bulb that lead to olfactory memory formation. Lesions of noradrenergic projections to the olfactory bulb, or direct infusion of β-noradrenergic antagonists, significantly reduce the number of animals developing the olfactory recognition memory [66]. Similar effects are seen following treatments with cholinergic antagonists such as scopolamine [67]. The result of the changes in NA and ACh in the olfactory bulb is to reduce
GABAergic feedback inhibition from the granule cells to the mitral cells. The disinhibited mitral cells become more active in their response to odours and through a glutamate–AMPA/NMDA receptor–nitric oxide–cyclic GMP signalling cascade there is upregulation both of mitral to granule cell synapses as well as of auto-excitation synapses on the mitral cells themselves. Thus pharmacological inhibition of NMDA/AMPA receptors, nitric oxide synthase (NOS) or soluble guanylate cyclase all prevent the formation of an olfactory recognition memory and the ability of the lamb odours to evoke enhanced glutamate and GABA release [68]. However once a memory has been allowed to form similar inhibition does not interfere with recall or potentiated glutamate and GABA release suggesting that these signalling pathways are not involved in recall of a consolidated recognition memory [61]. Thus the overall signalling pathway model we suggest is involved in olfactory memory formation in the olfactory bulb [3, 68] is as follows (Fig. 2): Noradrenaline (or acetylcholine) release (triggered by attention, arousal or vaginocervical stimulation) at the centrifugal projection synapses with GABAergic granule interneurones reduces GABA release onto mitral cells and consequently disinhibits them [12]. With incoming odour-induced activation of olfactory receptors in the presence of this disinhibited state, mitral cells increase their release of glutamate at their reciprocal synapses [69] activating an intracellular molecular cascade that will ultimately release NO from the granule cells. In turn, NO acts as a retrograde messenger to stimulate guanylyl cyclase activity and the formation of cyclic GMP in the presynaptic mitral cell, which then facilitates further glutamate release. This glutamate release promotes enhanced GABA release from the granule cells thereby increasing feedback inhibition onto the mitral cells. It also facilitates autoreceptors on the presynaptic site so that there is enhanced glutamate release following exposure to the learned odours. In this way learning causes increased sensitivity to glutamate in both mitral cells and at the mitral-to-granule cell synapses. This results in both increased excitability of the mitral cells and tighter excitatory/inhibitory coupling between them and their associated granule cells such that learned odours evoke both a stronger activation of the mitral cells which are tuned to it and a sharpening of their phasic firing discharge pattern caused by a
more robust inhibitory feedback response from the granule cells that follows from each excitatory burst [68, 70].

This process is not unique to the postpartum ewe, as similar changes have been observed in mice with olfactory conditioning [70], social recognition and STFP [4]. In our model of social olfactory memory using the social recognition habituation/discrimination test and the STFP test, long-term (24 h) memory formation can be prevented by blocking either NMDA (using the non-competitive antagonist MK801) or AMPA (using the competitive antagonist NBQX) receptor activation (Fig. 3). Similarly, preventing NO release by inhibiting NOS (using the non-selective NOS inhibitor, L-NARG) impaired formation of long-term olfactory memory in both models [4]. With these olfactory learning models too disruption of NMDA or NO signalling after memory acquisition failed to have any influence on subsequent recall [5]. Thus, as with the sheep model, glutamate acting on ionotropic glutamate receptors and promoting NO release is required for the initial acquisition phase of an olfactory recognition memory trace but not its recall post-acquisition.

In both sheep and mouse models of olfactory induced learning or plasticity there is evidence of altered sensitivity of class 1 metabotropic glutamate (mGluR) receptors in mitral and granule cells and agonists of this class of receptor are more potently able to release glutamate and GABA within the olfactory bulb following consolidation of learning in sheep [48, 64]. Preliminary pharmacological studies have also shown that...
olfactory bulb infusions of antagonists targeting mGluR1 (AIDA and LY393675) but not mGluR5 (MPEP) prevent recall of the recognition memory after it is consolidated (12–24 h post-partum) but have no impact on memory formation [53, 68, 70]. Therefore, as the olfactory recognition memory consolidates there is a shift away from the dependence on glutamate acting on NMDA/AMPA-NO signalling pathways to a dependence on its actions on mGluR1 receptors. This effectively releases the former signalling pathway to be utilised for formation of new memory associations.

Olfactory Learning and Ovarian Hormones

The sheep offspring recognition model is based on a biologically distinct event which occurs during a short time-window where the ewe is primed appropriately with oestrogen and progesterone during pregnancy and experiences vaginocervical stimulation (VCS) as a result of giving birth. Olfactory memory can even be induced by artificial VCS but only after ewes have been treated with either oestrogen or a combination of oestrogen and progesterone [13]. This strongly suggests that sex hormones play a key role in olfactory memory formation processes described above. However, little has been done to establish the precise effects that gonadal hormones may have on the olfactory system and olfactory learning. In male rats, castration reduces NA concentrations in the OB, but not in the olfactory cortex [71, 72], and reduces potassium-evoked release of NA in the OB [71, 73]. It also substantially impairs olfactory social recognition [74] and systemic treatment of castrated males with L-DOPA restores social recognition to pre-castration levels [75]. Very little work has been carried out to establish effects of ovarian hormones on the olfactory system. The first systematic study relating ovarian hormones to social recognition memory in female rats [76] showed a memory-retention improvement effect of oestradiol. Ovariectomized (OVX) females had a 30 min, but not 120 min memory retention for the familiar rat, contrasting with oestradiol-treated OVX (OVX+E2) animals that recognised the familiar animal at both 30 and 120 min time points. Memory retention impairment did not reappear until 6 weeks after termination of oestradiol treatment, indicating that long-term effects of hormones may be involved. A more recent study has shown similar results that are dependent on the use of high doses of oestrogen treatment. Ovariectomized mice receiving a four-week oestrogen-treatment at a high dose (0.72 mg of 17β-oestradiol) showed a long-term (24 h) social recognition memory whereas OVX and low dose oestradiol treated animals did not [77]. This makes it important to determine whether hormonal fluctuations in the normal physiological range can have any impact on olfactory memory function, and one obvious place to start is by studying changes during the course of natural oestrous cycles.

Our early work studied the effect of hormonal fluctuations during the rat oestrous cycle on OB activity evoked by vaginocervical stimulation (VCS). Such VCS resulting from parturition (or mating) enhances NA input to the OB, necessary for memory formation. In vivo microdialysis experiments have shown no oestrous cycle effects on basal concentrations of classical transmitters and nitric oxide (NO) in the OB. However, VCS significantly increases concentrations of glutamate, aspartate, GABA, noradrenaline, dopamine and nitric oxide (NO) in females during proestrous/oestrous, but not during metaoestrous/dioestrous or following ovariectomy [78]. This suggests OB mitral cells are excited by VCS only when females are at proestrous/oestrous stage. Similar results were obtained using potassium evoked neurochemical release in the olfactory bulb, confirming that sex hormones changes can indeed act directly at the level of the OB and may be important for mediating memory related plasticity changes.

We decided to test this oestrous cycle effect at a behavioural level using the two tests of social olfactory memory in mice, the habituation/discrimination and the STFP task. In both tests, significant improvement of learning retention occurs when original learning takes place during the proestrus phase of the ovarian cycle [5]. No differences on either olfactory perception or motivation were seen across the oestrous cycle (Fig. 4). Our results suggest that olfactory memory formation is facilitated in females when they are reproductively active. This might be because when females are at a time when they are most likely to conceive they need to assess the environment for present dangers and for its suitability for reproductive activities (e.g. sex behaviour, pregnancy, and offspring rearing). They must be prepared to either engage in sex behaviour when
the environment predicts success–familiar social environment will improve reproductive and rearing success–, or suspend resource-costly reproductive behaviours until they are more likely to pay off [79].

Recent studies reporting that female mice lacking functional oestrogen receptors (ERα, and ERβ) fail to form social memories, suggest that the ovarian cycle effects may be mediated via oestrogen acting on these two oestrogen receptors [80]. Since oestrogen receptors have been found in the olfactory bulb [81], we have recently investigated the impact of the oestrous cycle on the NMDA/NO signalling pathway that is critical for olfactory memory formation. Results have shown that ovarian hormone actions on olfactory memory may be via modulation of NO availability since animals lacking the functional expression of neuronal nitric oxide synthase (nNOS−/−) do not show oestrous cycle dependant effects on memory retention. On the other hand, exogenous treatment with the NO donor molsidomine, improves memory retention in dioestrous and oestrous females to the levels seen during proestrous [5]. It would therefore appear that oestrogen may be influencing memory formation in the olfactory bulb both through modulation of NA and the subsequently ability of glutamate to stimulate NO release. However the precise mechanisms of action still require further investigation.

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