Respective role of the dorsal hippocampus and the entorhinal cortex during the recombination of previously learned olfactory–tactile associations in the rat

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The hippocampal formation has been extensively described as a key component for object recognition in conjunction with place and context. The present study aimed at describing neural mechanisms in the hippocampal formation that support olfactory–tactile (OT) object discrimination in a task where space and context were not taken into account. The task consisted in discriminating one baited cup among three, each of them presenting overlapping olfactory or tactile elements. The experiment tested the involvement of the entorhinal cortex (EC) and the dorsal hippocampus (DH) in the acquisition of this cross-modal task, either with new items or with familiar but recombined items. The main results showed that DH inactivation or cholinergic muscarinic blockade in the DH selectively and drastically disrupted performance in the recombination task. EC inactivation impaired OT acquisition of any OT combinations while cholinergic blockade only delayed it. Control experiments showed that neither DH nor EC inactivation impaired unimodal olfactory or tactile tasks. As a whole, these data suggest that DH–EC interactions are of importance for flexibility of cross-modal representations with overlapping elements.

The rodent hippocampus has been shown to be critically involved in several memory processes including spatial navigation (Morris et al. 1982), contextual learning (Philips and LeDoux 1992), object–place and odor–place associations (Gilbert and Kesner 2002) and episodic-like memory (Easton et al. 2012; Veyrac et al. 2015). All these processes require computation of information from either one sensory modality such as extra maze visual cues (intramodal associations) or from different sensory modalities such as odor–place (cross-modal associations). The importance of the rodent hippocampus in behavioral responses to complex forms of association has also been shown in several studies in which the reward cue (e.g., A+) is configural dependent. These studies were based on tasks such as negative patterning (A+, B+, AB−) (McDonald et al. 1997), symmetry learning (A/B+, B/A+) (Bunsey and Eichenbaum 1996), transitivity (A/B+, B/C+, A+/C−) (Bunsey and Eichenbaum 1996), transverse patterning (A/B+, B/C+, C+/A−) (McDonald et al. 1997), and sequential learning (A>B>C>D>E) (Fortin et al. 2002) where letters stand for the cue and the signs + or − stand for rewarded or nonrewarded, respectively. However, the results obtained with this set of elegant studies were based on lesion to the hippocampus. Such an approach did not provide information on intrahippocampal mechanisms supporting acquisition of these complex forms of memory, nor did it provide information on the role of afferent structures such as the entorhinal cortex (EC). In addition, in most of these behavioral paradigms, animals had to process information from one category of sensory cues only, such as olfactory or visual cues.

Consequently, the present study aimed at characterizing the importance of the dorsal hippocampus (DH) and the EC and intrinsic mechanisms supporting cross-modal acquisition and recombination of previously learned associations. This was done using a new cross-modal learning task described in a recent study (Boisselier et al. 2014). While the olfactory and the rat vibrissal systems have been extensively studied separately (Slotnick 2001; Diamond et al. 2008), very little is known on how rats build cross-modal olfactory–tactile associations. This is surprising since rats explore any new object with active whisking and sniffing, which likely contributes to forming olfactory (O) tactile (T) associations required for future recognition. In this behavioral paradigm, rats were trained to distinguish between three different olfactory–tactile (OT) pairs presenting overlapping elements (e.g., O1T1, O1T2, and O2T1) to find the only pair that is rewarded (O1T1). It was shown that the EC plays a critical role in the acquisition of such cross-modal associations through a NMDA-dependent mechanism (Boisselier et al. 2014). In addition to the EC, some data suggest the DH could play a role in the acquisition of OT representations through a functional interaction with the EC. Indeed, the DH receives strong input from the EC and neurons in the DH respond to both olfactory (Igarashi et al. 2014) and tactile (Pereira et al. 2007) stimuli. The DH and the EC are strongly interconnected (Witter 1993) and both structures interact during the course of odor–place association learning through fine oscillatory dynamics coordination (Igarashi et al. 2014). Finally, both structures receive a strong cholinergic projection from the basal forebrain (Gaykema et al. 1990), which has been shown to modulate syn-

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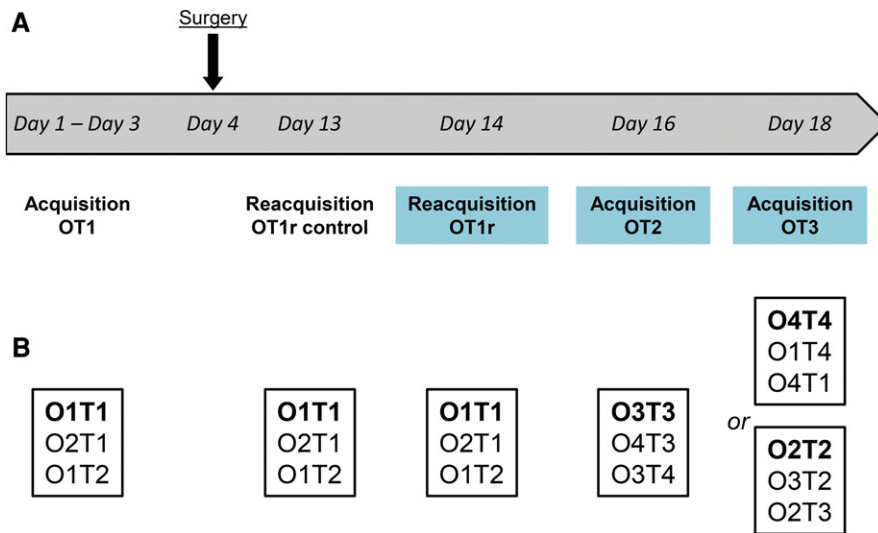
Figure 1. (*A*) Experimental chronology showing the succession of the different learning tasks. Following surgery and just before each task (OT1r, OT2, OT3) bilateral intracerebral infusion were done either in the DH or in the EC with aCSF, lidocaine, or scopolamine (tasks indicated in blue) (*B*) OT combinations used in each of the three tasks. Each task comprised 20 trials in which only the cup here indicated in bold characters contained a reward that can be reached by the rat.

aptic plasticity, learning and to prevent interference between incoming new and previously learned information (Hasselmo 2006). Therefore, our present study tested first the effect of selective inactivation of the DH and the EC, and then cholinergic muscarinic receptor inactivation in each structure on the processes underlying the acquisition and recombination of these cross-modal OT representations.

Results

Each rat was tested in three successive cross-modal olfactory–tactile tasks named OT1, OT2, and OT3. Figure 1 represents the succession of the OT tasks and the design of OT combinations used for each task. Different sets of items were used for OT1 and OT2 tasks. New pairs of familiar items were used for OT3 task (recombination task). Bilateral local infusions of lidocaine before each session were used to test the effect of DH and EC inactivation in reacquisition of OT1 (OT1r), acquisition of OT2, and acquisition of OT3 by recombination. Further local scopolamine infusions (muscarinic receptor antagonist) were used to test the role of cholinergic transmission during the tasks. Since the DH was selectively involved in the recombination task, the role of NMDA synaptic transmission in this structure was further tested by using local D-APV injection.

Detailed analysis of well-conditioned rat performances demonstrated that animals did not make their choice based on the scent of the reward in the baited cup. Two-sample paired *t*-test comparing performance in standard trials ($n = 53$ rats and $n = 497$ trials) and in probe tests ($n = 53$ rats and $n = 235$ trials) showed no effect of type of trial

on performance (the mean ratios of correct response during standard and probe tests were 0.90 ± 0.09 and 0.93 ± 0.10, respectively; $t = -1.639$; df = 52; P n.s), showing that animals did not use the scent of the reward to find the correct cup.

A complete description of the experimental bias control has been published recently (Boisselier et al. 2014).

Olfactory–tactile tasks

A week before surgery, all animals acquired a first olfactory–tactile task (Task OT1). The learning criterion for OT1 was reached in 3 d with a mean of 38 ± 5 trials (Boisselier et al. 2014).

Ten days after surgery, animals were tested in reacquisition of Task OT1 without infusion (OT1r control). Results showed that at least 80% of the animals showed a correct response in the first trial, which has been performed without any reinforcement (89% ± 31 for DH animals and 82% ± 39 for EC animals, data not shown) thus suggesting that surgery did not affect animals' performances.

Absence of effect of lidocaine infusion in the DH and in the EC on Task OT1 reacquisition (OT1r)

This experiment tested whether transient DH or EC inactivation affected reacquisition of a previously learned task (OT1r control). To do this, lidocaine infusion in the DH or in the EC was tested in a second reacquisition of OT1 (OT1r) 24 h following the session without injection of task OT1.

Figure 2A and B show the results obtained in the groups, respectively, infused in the DH and the EC. The mean ratio of correct responses during the first trial (which has been performed without any reinforcement) was between 0.9 and 1.0 for all the
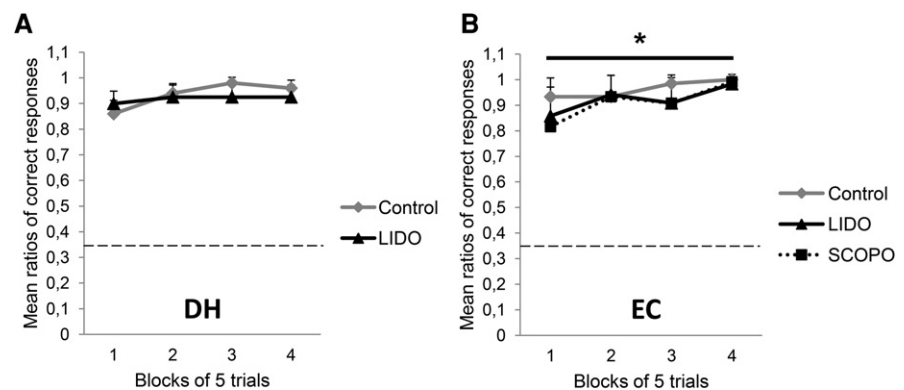
Figure 2. Absence of effect of injection of lidocaine into the DH (*left*) or the EC (*right*) during the OT reacquisition (Task OT1r). The graphs represent the mean ratios of correct responses ± SEM calculated in each block of five trials in the OT reacquisition task. The black solid lines and the black dotted lines represent the results obtained in the groups infused with lidocaine and scopolamine, respectively, while the gray lines represent results obtained in the groups infused with aCSF (control). (*A*) Reacquisition of Task OT1 in the groups infused in the DH with lidocaine ($n = 10$) and aCSF ($n = 8$). (*B*) Reacquisition of Task OT1 in the groups infused in the EC with lidocaine ($n = 8$), scopolamine ($n = 8$), and aCSF ($n = 9$). Results indicated that lidocaine infusion in the DH or in the EC or scopolamine infusion in the EC did not impair the OT reacquisition task. The dotted line represents the chance level (0.33). (*)$P < 0.05$ between blocks for each experimental group.

DH and EC experimental groups. A two-factor ANOVA with repeated-measures with factor Drug as between-subjects variable and factor Block (1,2,3,4) as within-subject variable revealed no significant effect of factor Drug (DH: $F_{(1,16)} = 1.13$; P n.s/EC: $F_{(2,22)} = 2.68$; P n.s), of factor Block (DH: $F_{(3,48)} = 1.77$; P n.s/EC: $F_{(3,66)} = 8.14$; P n.s) or interaction between both factors (DH: $F_{(3,48)} = 0.70$; P n.s/EC: $F_{(6,66)} = 1.01$; P n.s).
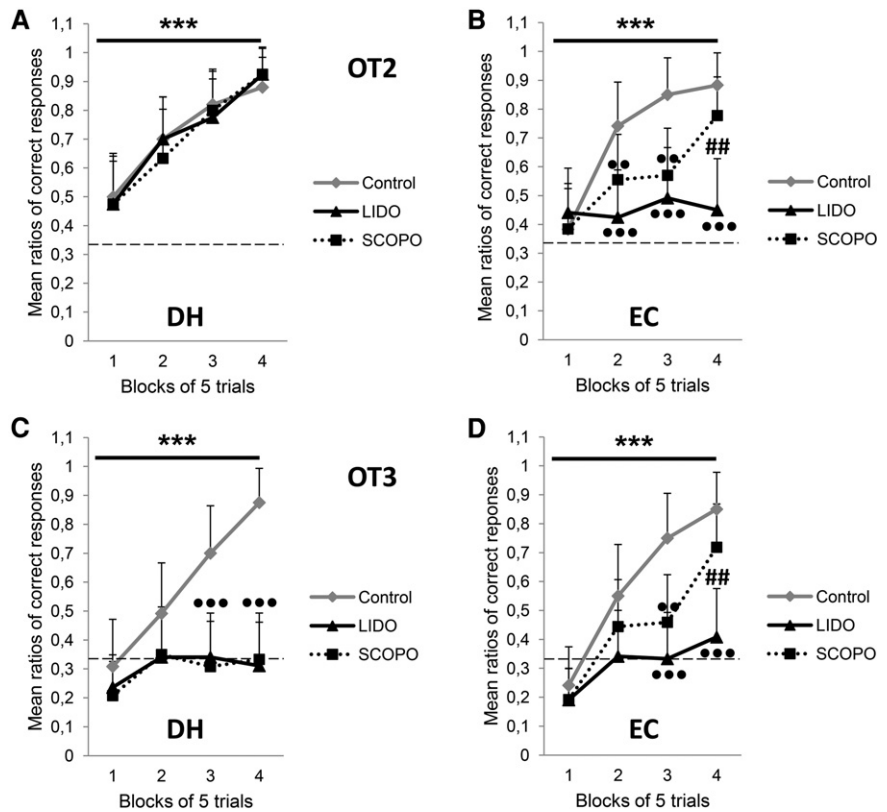
These data showed that local infusions of lidocaine in the DH or in the EC did not disrupt either the sensory olfactory and tactile processing or reacquisition of a well-known OT task. In addition, since there was no reward on the first trial of the reacquisition task and 34 over 35 rats performed correctly, this suggests that retrieval was also optimal.

Differential effect of lidocaine or scopolamine infusion in the DH and in the EC on Task OT2 acquisition (new items)

This experiment tested whether transient inactivation of DH or EC and/or disruption of muscarinic transmission impaired acquisition of a cross-modal OT.

Figure 3A shows the results obtained in the groups infused in the DH during acquisition of Task OT2. On this figure, the control, LIDO and SCOPO groups reached a mean ratio of correct responses of 0.8 in 20 trials (from 0.50 ± 0.15 in block 1 to 0.88 ± 0.10 in block 4 in control group; from 0.47 ± 0.15 in block 1 to 0.93 ± 0.09 in block 4 in LIDO group; from 0.48 ± 0.17 in block 1 to 0.93 ± 0.09 in SCOPO group). A two-way ANOVA with repeated measures revealed a significant effect of factor Block ($F_{(3,69)} = 63.59$; $P < 0.001$) but no significant effect of factor Drug ($F_{(2,23)} = 0.21$; P n.s) and no interaction between these factors ($F_{(6,69)} = 0.56$; P n.s).

Figure 3. Differential effect of drug infusions into the DH (*left*) or the EC (*right*) during the OT acquisition (Task OT2, new stimuli *upper* part) or recombination (Task OT3, new pairs of familiar stimuli, *lower* part). The graphs represent the mean ratios of correct responses $\pm$ SEM calculated in each block of five trials in acquisition and recombination tasks. The black solid lines and the black dotted lines represent the results obtained in the groups infused with lidocaine and scopolamine, respectively, while the gray lines represent results obtained in the groups infused with aCSF (control). (*A*) Acquisition of OT task in the groups infused in the DH with lidocaine ($n = 8$), scopolamine ($n = 8$), and aCSF ($n = 10$). (*B*) Acquisition of OT task in the groups infused in the EC with lidocaine ($n = 8$), scopolamine ($n = 9$), and aCSF ($n = 9$). (*C*) Recombination of OT task in the groups infused in the DH with lidocaine ($n = 9$), scopolamine, ($n = 9$) and aCSF ($n = 8$). (*D*) Recombination of OT task in the groups infused in the EC with lidocaine ($n = 8$), scopolamine ($n = 8$), and aCSF ($n = 9$). Results indicated that lidocaine and scopolamine infusion in the DH impaired the recombination task while lidocaine and scopolamine infusion in the EC impaired and delayed, respectively, both the acquisition and recombination of the OT task. Horizontal dotted lines represent the chance level (0.33). (***)$P < 0.001$ between blocks for each experimental group (*A*), for control and scopolamine groups (*B,D*), for control group (*C*). (••)$P < 0.01$; (•••)$P < 0.001$ in comparison to control. (##)$P < 0.01$ between scopolamine and lidocaine groups.

Figure 3B shows the results obtained in the groups infused in the EC during acquisition of Task OT2. On this figure, the control group reached the learning criterion of 0.8 in 15 trials (mean ratios of correct responses from 0.38 ± 0.16 in block 1 to 0.88 ± 0.11 in block 4) whereas the SCOPO group only tended to reach the criterion in the fourth block of trials (mean ratios of correct responses from 0.38 ± 0.14 in block 1 to 0.78 ± 0.13 in block 4). In contrast, animals of the LIDO group were strongly impaired in the acquisition of the task (mean ratios of correct responses between 0.40 ± 0.15 and 0.45 ± 0.18 only in blocks 1–4). A two-way ANOVA with repeated measures revealed a significant effect of factor Drug ($F_{(2,22)} = 33.79$; $P < 0.001$), of factor Block ($F_{(3,66)} = 18.10$; $P < 0.001$) and an interaction between both factors ($F_{(6,66)} = 5.16$; $P < 0.001$). Post hoc tests on within group comparisons revealed a significant difference between block 1 and blocks 2, 3, 4 (all P values < 0.001) in the control group, but only between blocks 1 and 4 ($P < 0.001$) in the SCOPO group. Post hoc tests on between group comparisons also indicated a significant difference between control and LIDO groups in blocks 2, 3, and 4, between

control and SCOPO groups in blocks 2 and 3, and between SCOPO and LIDO groups in block 4 (all P values <0.001).

Comparisons in behavioral performances have been conducted between DH and EC groups for each drug injected. Two-way ANOVAs with repeated measures on LIDO and SCOPO groups revealed a significant effect of factor Structure (LIDO: $F_{(1,14)} = 128.45$; $P < 0.001$/SCOPO: $F_{(1,15)} = 23.10$; $P < 0.001$), of factor Block (LIDO: $F_{(3,42)} = 7.74$; $P < 0.001$/SCOPO: $F_{(3,45)} = 24.24$; $P < 0.001$) and an interaction between both factors for LIDO groups (LIDO: $F_{(3,42)} = 6.41$; $P < 0.01$ but SCOPO: $F_{(3,45)} = 0.93$; P n.s). A two-way ANOVA with repeated measures on aCSF groups revealed a significant effect of factor Block ($F_{(3,48)} = 54.08$; $P < 0.001$) but no significant effect of factor Structure ($F_{(1,16)} = 0.10$; P n.s) and no significant interaction between both factors ($F_{(3,48)} = 1.83$; P n.s).

These results showed that neither lidocaine nor scopolamine infusions into the DH disrupted the formation of OT associations with new items. However when infused in the EC, the drugs induced contrasting effects. Whereas lidocaine strongly impaired

acquisition of Task OT2, scopolamine substantially affected the progression of the learning curve and the animals were delayed in reaching the learning criterion. These data suggest that the cholinergic activity in the EC is involved in the processes underlying OT association whereas the DH is not involved in these processes.
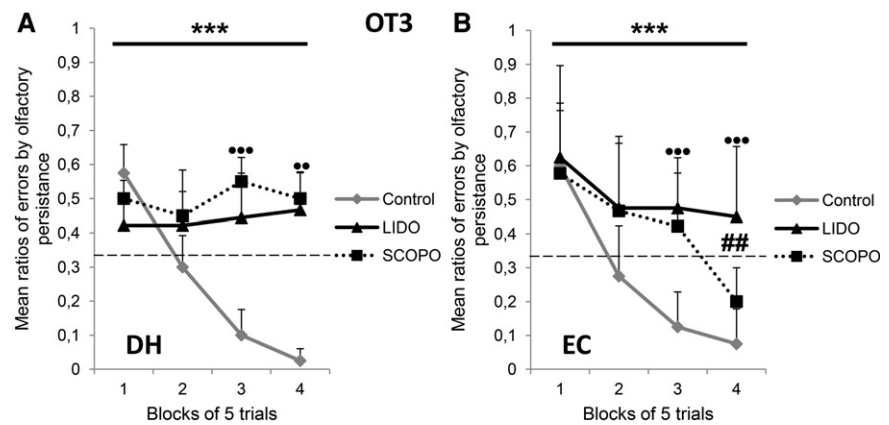
Differential effect of lidocaine or scopolamine infusion in the DH and in the EC on Task OT3 acquisition (recombination)

This experiment was aimed at testing the involvement of the DH and the EC in the processes underlying the OT recombination task. The present OT3 task involved reversal learning based on a shift in which the animal had to inhibit the response to previously reinforced items (used in OT1 and OT2; see Fig. 1) and learn to associate a new OT pair, composed of familiar stimuli, with a reward.

Figure 3C shows the results obtained in the groups infused in the DH during the OT recombination task. As shown in the figure, the control group reached the learning criterion of 0.8 in 20 trials (mean ratios of correct responses

Figure 4. Differential effect of drug injections into the DH (*left*) or the EC (*right*) on olfactory persistence observed during the OT recombination. The graphs represent the mean ratios of errors ± SEM calculated in each block of five trials in the OT recombination task. The black solid lines and the black dotted lines represent the results obtained in the groups infused with lidocaine and scopolamine, respectively, while the gray lines represent results obtained in the groups infused with aCSF (control). (*A*) Recombination of OT task in the groups infused in the DH with lidocaine ($n = 8$), scopolamine ($n = 9$), and aCSF ($n = 8$). (*B*) Recombination of OT task in the groups infused in the EC with lidocaine ($n = 8$), scopolamine ($n = 8$), and aCSF ($n = 9$). Using familiar items, animals naturally begin the OT recombination task by digging in the cup containing the previously correct odor. Results indicated that control animals present a rapid strategy adaptation by shifting to the new correct cup while this olfactory persistence is strongly increased by lidocaine infusion in the DH and in the EC, and scopolamine infusion in the DH. This adaptation is only delayed by scopolamine infusion in the EC. Horizontal dotted lines represent the chance level (0.33). (***) $P < 0.001$ between blocks for control group (*A*) and for control and scopolamine groups (*B*). (••) $P < 0.01$; (•••) $P < 0.001$ between control and drugs (lidocaine or scopolamine) groups. (##) $P < 0.01$ between scopolamine and lidocaine groups.

from 0.31 ± 0.16 in block 1 to 0.88 ± 0.12 in block 4). In contrast, animals in the LIDO and SCOPO groups were markedly impaired in the task as shown by the mean ratios of correct responses that were close to chance level throughout the trials (ranging from 0.24 ± 0.11 in block 1 to 0.31 ± 0.15 in block 4 in LIDO group; and from 0.21 ± 0.12 in block 1 to 0.33 ± 0.16 in block 4 in SCOPO group). A two-way ANOVA with repeated measures revealed a significant effect of factor Drug ($F_{(2,22)} = 95.58$; $P < 0.001$), of factor Block ($F_{(3,66)} = 13.95$; $P < 0.001$) and an interaction between both factors ($F_{(6,66)} = 5.94$; $P < 0.001$). Post hoc tests on within group comparisons indicated a significant difference between block 1 and blocks 3 and 4 for control group (all P values <0.001). Post hoc tests on between group comparisons also revealed a significant difference between control group, LIDO and SCOPO groups in blocks 3 and 4 (all P values <0.001).

Interestingly, analysis of the mean ratios of errors obtained in each block of trials showed in the control group that perseveration behavior toward previously reinforced odor extinguished between blocks 1 and 2 and the new OT–reward association was acquired in the third block of trials ($P < 0.01$ between blocks 1 and 2, $P < 0.001$ between block 1 and blocks 3,4; Fig. 4A) whereas rats infused with lidocaine or scopolamine showed a preference throughout the trials for the cup associated with the odor that was previously rewarded in OT1 and OT2 tasks (P n.s between blocks in LIDO and SCOPO groups). These data suggest that lidocaine and scopolamine infusions in the DH disrupted the flexibility processes underlying either the reversal odor–reward association and/or the ability to build new OT associations in a context of interference but not the behavioral perseveration process.

Acquisition of hippocampal-dependent task which involve computation of multisensory information such as spatial and contextual memory were found to involve NMDA receptor activation (Wang et al. 2006). However, as presented on the Figure 5, we

showed that local injection of D-APV, a specific NMDA antagonist did not affect the acquisition of OT3. A two-way ANOVA with repeated measures with factor Drug (D-APV or aCSF) and factor Block confirmed this description and revealed a significant effect of factor Block ($F_{(3,45)} = 44.32$; $P < 0.001$) but no significant effect of factor Drug ($F_{(1,15)} = 0.14$; P n.s) and no interaction between factors ($F_{(3,45)} = 0.64$; P n.s).

Figure 3D shows the results obtained in the groups infused in the EC during the acquisition of Task OT3. As shown in the figure, the control group reached the learning criterion of 0.8 in 20 trials (mean ratios of correct responses from 0.24 ± 0.13 in block 1 to 0.85 ± 0.13 in block 4) whereas animals in the SCOPO group only tended to reach the criterion in the fourth block of trials (mean ratios of correct responses from 0.19 ± 0.11 in block 1 to 0.72 ± 0.15 in block 4). In contrast, animals in the LIDO group were impaired in the acquisition of the task (as shown by the ratios of correct responses that were close to chance level throughout the trials (ranging from 0.19 ± 0.11 in block 1 to 0.41 ± 0.17 in block 4). A two-way ANOVA with repeated measures revealed a significant effect of factor Drug ($F_{(2,22)} = 21.38$; $P < 0.001$), of factor Block ($F_{(3,66)} = 42.34$; $P < 0.001$) and an interaction between both factors ($F_{(6,66)} = 4.00$; $P < 0.01$). Post hoc tests on within group comparisons revealed a significant difference between block 1 and blocks 2, 3, and 4 ($P < 0.01$; $P < 0.001$ and $P < 0.001$ for the control group and $P < 0.05$; $P < 0.01$; $P < 0.001$ for the SCOPO group). Post hoc tests between group comparisons also indicated a significant difference between control and LIDO groups in blocks 3 and 4, between control and SCOPO groups in block 3 (all P values <0.01) and between SCOPO and LIDO groups in block 4 ($P < 0.01$).

As previously observed in DH control, analysis of the mean ratios of errors obtained in each block of trials showed that, in EC control animals, perseveration behavior toward previously reinforced odor extinguished between blocks 1 and 2 and the new
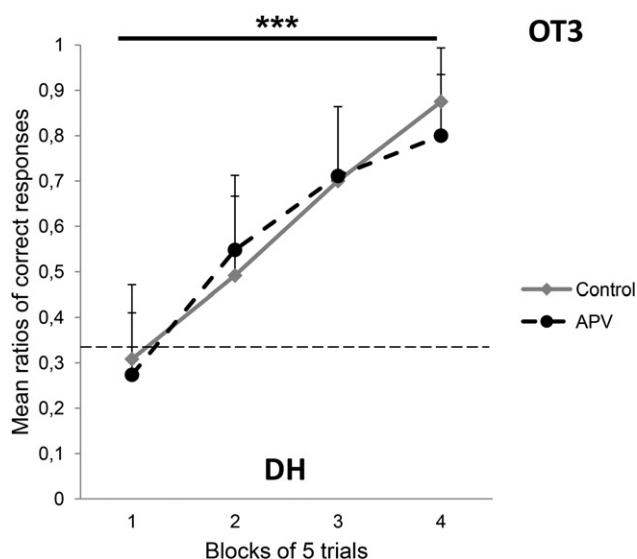
Figure 5. Absence of effect of injection of D-APV into the DH during the OT recombination. The graph represents the mean ratios of correct responses ± SEM calculated in each block of five trials in the OT recombination task. The black dotted lines represent the results obtained in the groups infused with D-APV while the gray lines represent results obtained in the groups infused with aCSF (control). Recombination of OT task in the groups infused with D-APV ($n = 9$, 2 μg of D-APV in 0.6 μL aCSF, infused immediately before the beginning of the task, as described by Boisselier et al. 2014) and aCSF ($n = 8$). Results indicated that D-APV infusion did not impair the OT recombination task. The dotted line represents the chance level (0.33). (***) $P < 0.001$ between blocks for each experimental group.

OT–reward association was acquired in the third block of trials ($P < 0.001$ between block 1 and blocks 2, 3, 4) (Figure 4B). In contrast, rats infused with lidocaine showed a preference throughout the trials for the cup associated with the odor that was previously rewarded in OT1 and OT2 tasks (P n.s between blocks) and animals in the SCOPO group showed a preference for the previously reinforced odor during the first three blocks of trials but shifted their choice in the fourth block of trials ($P < 0.001$ between blocks 1 and 4 only). These data show that lidocaine infusion in the EC disrupted the flexibility processes underlying either the reversal odor–reward association and/or the ability to build new OT associations in a context of interference but not the behavioral perseveration process. Moreover, our result suggests that scopolamine treatment delayed OT3 acquisition.

Comparisons in behavioral performances have been conducted between DH and EC groups for each drug injected. A two-way ANOVA with repeated measures on SCOPO groups revealed a significant effect of factor Structure (SCOPO: $F_{(1,15)} = 31.45$; $P < 0.001$), of factor Block (SCOPO: $F_{(3,45)} = 13.62$; $P < 0.001$) and an interaction between both factors for SCOPO groups (SCOPO: $F_{(3,45)} = 5.42$; $P < 0.01$). Two-way ANOVAs with repeated measures on aCSF and LIDO groups revealed a significant effect of factor Block (aCSF: $F_{(3,42)} = 44.89$; $P < 0.001$/LIDO: $F_{(3,45)} = 4.23$; $P < 0.05$) but no significant effect of factor Structure (aCSF: $F_{(1,14)} = 0.01$; P n.s/LIDO: $F_{(1,15)} = 0.10$; P n.s) and no significant interaction between both factors (aCSF: $F_{(3,42)} = 0.63$; P n.s/LIDO: $F_{(3,45)} = 0.871$; P n.s).

As a whole, these data showed that the recombination task critically depends on DH neuronal activity and local muscarinic modulation. Moreover, similarly to what was observed in acquisition of Task OT2, EC activity is required for learning the recombination task and performances are modulated by muscarinic transmission.

Control unimodal tasks

Absence of effect of lidocaine infusion in the EC on acquisition of unimodal acquisition

The deficit observed on acquisition of OT tasks in EC groups could be due to a disturbance in stimulus-reward association.

To test this hypothesis, two groups of animals received bilateral infusions of lidocaine into the EC during the acquisition of an olfactory or a tactile unimodal task. Naïve groups of animals were used in reference to previous data showing that successive bimodal and unimodal tasks resulted in unstable behavioral performance when switching from one task to another. Figure 6A and B show the results obtained in the various groups during each of these tasks. As shown in the figures, control and LIDO groups similarly reached the learning criterion of 0.8 in a single session of 20 trials. Two-way ANOVAs with repeated measures revealed a significant effect of factor Block (olfactory: $F_{(3,39)} = 25.13$; $P < 0.001$/tactile: $F_{(3,39)} = 15.95$; $P < 0.001$) but no significant effect of factor Drug (olfactory: $F_{(1,13)} = 0.18$; P n.s/tactile: $F_{(1,13)} = 0.05$; P n.s) and no significant interaction between both factors (olfactory: $F_{(3,39)} = 0.36$; P n.s/tactile: $F_{(3,39)} = 0.53$; P n.s).

These results suggest that lidocaine infusion into the EC did not affect the acquisition of unimodal olfactory or tactile task.
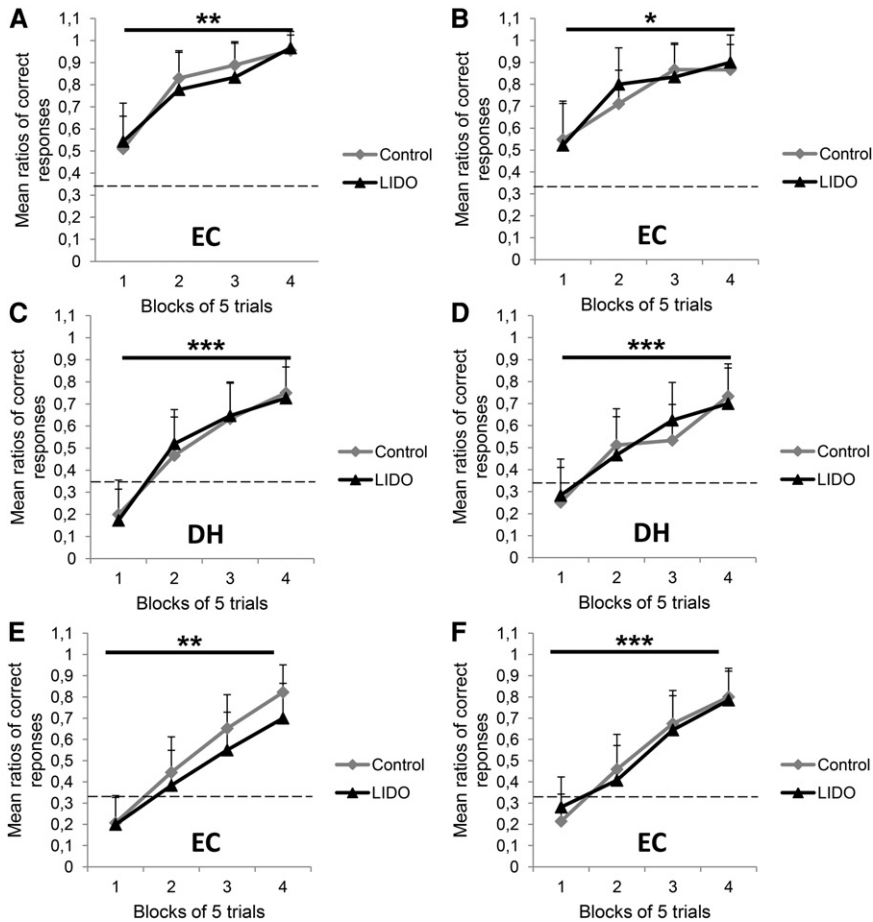
Absence of effect of lidocaine infusion in the DH and in the EC on unimodal reversal learning

Solving the recombination task required a reversal learning process. Perturbation of this process could potentially explain the impairment observed in the recombination task with lidocaine infusion in the DH and in the EC. To test this hypothesis, four groups of animals received bilateral infusions of lidocaine into the DH or EC during the reversal of an olfactory or a tactile unimodal task acquired previously. Figure 6C, D, E, F show the results obtained in the various groups during each of the olfactory and tactile tasks. As shown in the figures, control and LIDO groups similarly reached the learning criterion of 0.8 in a single session of 20 trials. Two-way ANOVAs with repeated measures revealed a significant effect of factor Block (olfactory EC: $F_{(3,45)} = 36.29$; $P < 0.001$/olfactory DH: $F_{(3,48)} = 33.69$; $P < 0.001$/tactile EC: $F_{(3,48)} = 24.52$; $P < 0.001$/tactile DH: $F_{(3,45)} = 33.81$; $P < 0.001$) but no significant effect of factor Drug (olfactory EC: $F_{(1,15)} = 1.00$; P n.s/olfactory DH: $F_{(1,16)} = 0.02$; P n.s/tactile EC: $F_{(1,16)} = 0.01$; P n.s/tactile DH: $F_{(1,15)} = 0.10$; P n.s) and no significant interaction between these factors (olfactory EC: $F_{(3,45)} = 0.40$; P n.s/olfactory DH: $F_{(3,48)} = 0.20$; P n.s/tactile EC: $F_{(3,48)} = 0.28$; P n.s/tactile DH: $F_{(3,45)} = 0.95$; P n.s) in the DH and EC groups.

These results suggest that lidocaine infusion into the DH or in the EC did not affect reversal learning of unimodal olfactory or tactile tasks. Therefore, the deficit obtained in Task OT3 was unlikely due to impairment in the flexibility processes underlying the reversal learning of odor— or tactile—reward associations.

Histology

At the end of the experiments, position of each cannula was checked and reported on two plates from the Paxinos and Watson atlas (2004; AP −3.60 (DH) and −7.04 (EC). In the DH group, infusion sites were mostly centered in the CA1 region or close to the limit between the CA1 and the dentate gyrus ($n = 5$) (Fig. 7A). In the EC group, infusion sites were mostly centered in the lateral part of the EC (Fig. 7B). All infused animals were included in the final statistical analyses.

Figure 6. Absence of effect of lidocaine infusion into the DH (*left*) or the EC (*right*) during unimodal olfactory and tactile acquisition and reversal tasks. The graphs represent the mean ratios of correct responses ± SEM calculated in each block of five trials in the olfactory and tactile tasks. The black lines represent the results obtained in the groups infused with lidocaine while the gray lines represent results obtained in the groups infused with aCSF (control). (*A,B*): Acquisition of unimodal olfactory (*A*) and tactile (*B*) tasks in the groups infused in the EC with lidocaine (*n* = 6) and aCSF (*n* = 9). (*C,D*): Reversal of unimodal olfactory (*C*) and tactile (*D*) tasks in the groups infused in the DH with lidocaine (olfactory *n* = 10; tactile *n* = 8) and aCSF (olfactory *n* = 8; tactile *n* = 9). (*E,F*): Reversal of unimodal olfactory (*E*) and tactile (*F*) tasks in the groups infused in the EC with lidocaine (olfactory *n* = 8; tactile *n* = 8) and aCSF (olfactory *n* = 9; tactile *n* = 9). Results indicated that lidocaine infusion in the DH or in the EC did not impair the acquisition nor the reversal of unimodal olfactory and tactile tasks. The dotted line represents the chance level (0.33). (*) *P* < 0.05; (**) *P* < 0.01; (***) *P* < 0.001 between blocks for each experimental group.

Discussion

The ease with which rats solved acquisition and recombination tasks suggest that it solicited quite behavioral-relevant cognitive functions. In these conditions, the present study shows distinct roles and mechanisms supported by the DH and the EC in the acquisition and recombination of olfactory–tactile associations in the rat. In accordance with a previous study (Boisselier et al. 2014) the results show that the EC plays a critical role in the processes underlying the formation of OT associations. In contrast, the DH does not appear to be involved in the formation of OT associations, but its action appears critical for supporting the flexibility processes underlying recombination of previously learned OT associations. Moreover, we bring information on intra DH and EC mechanisms involved in these processes. Muscarinic blockade in the DH totally disrupted the recombination task while muscarinic blockade in the EC partly impaired acquisition.

Moreover, while DH neural activity is required for the recombination task, it is independent from NMDA receptor activation.
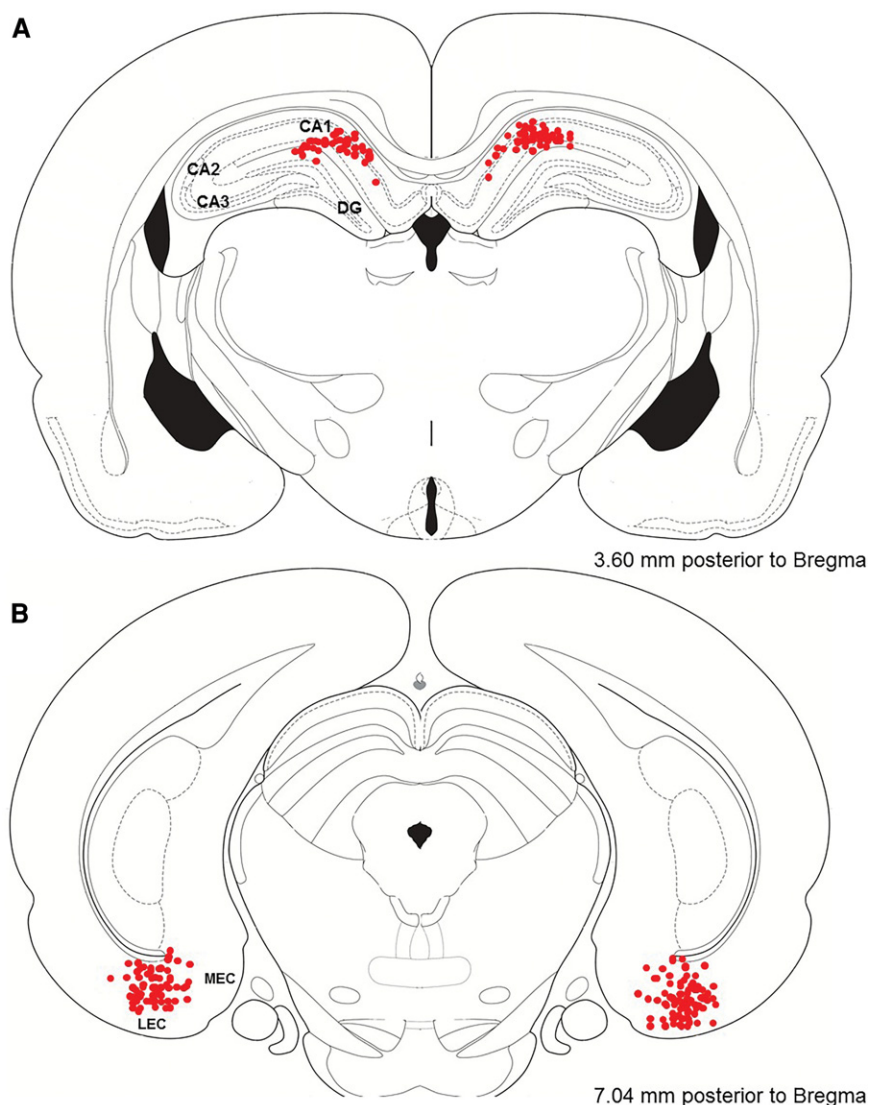
Neither the DH nor the EC were required for unimodal olfactory and unimodal tactile acquisition and reversal unimodal learning. This indicates that the deficit obtained during the cross-modal recombination task was not due to a deficit in sensory processing, to an inability to discriminate between different olfactory or tactile stimuli, or to a general disturbance in behavioral flexibility.

Role of the DH and the EC in the acquisition of OT associations

The present results showed that the DH is not involved in the acquisition of the OT task with new items since neither transitory inactivation nor muscarinic blockade affected performances when compared with control. In addition, DH inactivation did not impair unimodal olfactory and tactile discrimination. This is in contrast with electrophysiological recordings showing that activity increases in the DH during tactile processing (Pereira et al. 2007) and tactile discrimination enhanced coherence between S1 and the DH in parallel with improvement in performances (Grion et al. 2016). However, the later study relied on a task involving spatial component since animals had to indicate their choice with a go right/go left behavioral response. Our unimodal and bimodal tasks included no spatial component. Other studies suggest that the DH could be implicated in disambiguation tasks with overlapping elements (Agster et al. 2002; Newmark et al. 2013). Theoretically, this could be the case in the present study since the same odorant and the same texture were present in two of the three cups. The absence of effect of DH inactivation on the acquisition of cross-modal OT task with new items could be due to the fact that the task can be solved without taking into account any contextual or sequential information which have been shown to be of importance in hippocampal-dependent disambiguation of overlapping elements (Dees and Kesner 2013; Newmark et al. 2013; Van Hagen et al. 2015).

In the EC, the effect of scopolamine suggests that activation of the cholinergic system originating from the basal forebrain is involved in the formation of memories with novel items. This is agreement with a recent study based on associative eye-blink conditioning (Tanninen et al. 2015) even though the dose of scopolamine required for delaying the acquisition of the eye-blink conditioning was much higher than the one used in the present study (36 µg versus 7.5 µg/EC). Since acquisition of new OT pairs requires activation of NMDA receptors in the LEC (Boisselier et al. 2014), we can suspect that this cholinergic modulation is important because of the well-established role of cholinergic modulation of the theta rhythm during exploration, which in turn

Figure 7. Frontal sections of the brain showing all injection site locations (small solid circles) in the different groups DH (*A*) and EC (*B*). The different plates were adapted from Paxinos and Watson (2004).

tive of the lateral or/and the medial part of the EC. Future experiments based on the effect of selective NMDA system blockade in each part of the EC during our task will help to make that point clear. Recent studies on rodent lateral and medial EC function concluded that in recognition memory paradigms, damage to the lateral EC impairs recognition of the combined information of objects and environmental contexts relevant to the content of an experience whereas damage to medial EC preferentially impairs the recognition of the spatial arrangement of objects relevant to the spatial location of an experience (Wilson et al. 2013; Knierim et al. 2014; Morrisey and Takehara-Nishiuchi 2014). Our results do not fit completely with this interpretation since the EC was found here to be involved in object discrimination with no spatial and contextual cues. This discrepancy could be due to at least two reasons. First, classical object recognition tasks are mainly based on visual cues (shape, color) whereas the olfactory information, which is largely processed in the lateral EC (Schwerdtfeger et al. 1990), is of prime importance in the OT task. Second, in addition to differences in the EC manipulation technique (transient inactivation versus lesion), another important variable could the difficulty of the task. In the version of novel object recognition rats had to discriminate between "junk" objects presenting fairly different color, texture, shape, and possibly odors and lesion to the lateral EC had no effect (Wilson et al. 2013). In the present study, object recognition was based on olfactory and tactile combinations presenting overlapping elements. This suggests that the role the EC and presumably more specifically of the lateral EC in object discrimination could depend on

allows the induction of LTP/LTD NMDA-dependent mechanisms (Leung et al. 2003; Hasselmo 2006).

In accordance with previous studies based on lesion experiments of the lateral EC (Staubli et al. 1984; Ferry et al. 2006), our results confirmed the observation that the EC is not critical for simple olfactory discrimination. Simple associative olfactory learning has been shown to be mediated by structures such as the olfactory bulb (Gray and Freeman 1986; Gervais et al. 1988; Martin et al. 2004), the piriform cortex (Litaudon et al. 1997; Wilson and Sullivan 2011), and the amygdale (Cousens and Otto 1998; Hegoburu et al. 2014). Similarly, we showed that EC inactivation did not impair tactile discrimination and tactile associative learning.

As a whole, our data suggest that the EC is part of the neurobiological substrate underlying the formation of cross-modal associations, and that the role of the EC in this process is modulated by the cholinergic system. However, one may ask whether the EC could also be involved in unimodal associative learning involving two stimuli of the same nature such as tactile–tactile pairs. Also, it remains unclear whether the effects observed in the EC are selec-

the richness (bimodal, trimodal, quadrimodal), the nature (olfactory versus visual) of information, and/or the degree of similarities (interferences) between the objects that have to be recognized. In this perspective, this is likely by preventing interferences between overlapping elements that the muscarinic modulation (Hasselmo 1999; Hasselmo and McGaughy 2004) of the EC was here found to sustain optimal acquisition of the OT tasks.

Role of the DH and the EC in the recombination task
Solving the recombination task required from the animal at least two cognitive processes: (i) reversal learning leading to inhibit the response previously reinforced olfactory and tactile items; (ii) learning new OT associations in a context of high interference with previously learned items. Control rats solved this task within a single 20 trial learning session showing high cognitive flexibility. A careful observation of our data showed that most of the errors occurring in the first trials corresponded to behavioral perseveration toward previously reinforced odor. This was expressed by scores below the chance level in block 1 (Figs. 3C,D, 4A,B). During the second block of trials, control animals started to

inhibit their response to previously reinforced odors and shift their choice toward newly reinforced OT pair.

Concerning the neural basis supporting this behavioral flexibility, we found that both EC and DH manipulations affected performances through different mechanisms.

At the DH level, lidocaine inactivation or local muscarinic blockade completely abolished the animals' ability to solve the task. This was expressed throughout the trials by a persistency in choosing a previously reinforced odor. Interestingly, persistence toward the previously reinforced tactile cue was rarely observed. The effect of DH inactivation was not resulting from a deficit in reversal process per se since the same treatment did not impair unimodal reversal learning. Similarly, this behavioral perseveration was not resulting from a deficit in the acquisition of OT associations since formation of these associations was shown to be DH independent. This leads us to the conclusion that one critical process altered by DH inactivation or local muscarinic system inhibition is proactive interference in a complex cross-modal associative task. In normal conditions, the action of the DH seems to result in the neglecting of previously learned items allowing for the formation of new ones with familiar and overlapping elements. This is in accordance with several sets of data showing that muscarinic modulation is involved in the learning of new information preventing interference with previously stored memories (Hasselmo 1999, 2006; Hasselmo and McGaughy 2004; Hasselmo and Sarter 2011). These experiments demonstrated that the cholinergic modulation was critical for supporting NMDA-dependent mechanisms for memory storage such as those described in the piriform cortex, for example. However in the present experiment, the recombination critically depends on cholinergic modulation in the DH but was not NMDA-dependent at this level while it is the case at the EC level. The fact that EC inactivation also impaired the recombination task is likely due to the importance of the EC in the formation of any new OT associations. At the DH level, the recombination task did not require the activation of NMDA receptors, while the same treatment into the EC strongly impaired formation of OT associations (Boisselier et al. 2014). While most DH dependent tasks are found to be NMDA-dependent (Morris 2013), it does not seem to be the case for the OT recombination task.

As a whole, the data suggest that during the recombination task, the DH provides critical information to the EC allowing the formation in the EC of NMDA-dependent new cross-modal neural assemblies in the EC in the context of interferences. In this view, the recombination task could critically depend on fine DH–EC interactions under control of the cholinergic system. This hypothesis is supported by recent electrophysiological studies showing fine coordination of neural activity between the DH and the EC during associative olfactory learning (Igarashi et al. 2014; Miao et al. 2015). Olfactory–tactile associative learning could also involve direct reciprocal innervation between the piriform cortex and the barrel cortex (Wang et al. 2015). In this perspective, the behavioral paradigm offers an interesting working model to investigate the neural dynamic which could support in the hippocampal formation and in sensory areas acquisition and recombination of olfactory–tactile associations.

Materials and Methods

Animals
Young adult male Wistar rats ($n = 119$, Charles River, France) weighing 250–300 g were used. Fifty-three animals were subjected to bimodal olfactory–tactile learning and 66 for unimodal olfactory or tactile learning. Animals were housed in a standard home cage (380 mm long × 380 mm wide × 160 mm high, 21°C ± 1.5°C and 55% ± 10% wet) in groups of four or five and

were maintained on a 12–12 h inverted light–dark. After habituation to the housing condition, animals were food deprived to 90% of their free-feeding weight with free access to water. Testing took place during the dark phase of the cycle between 9 a.m. and 2 p.m. All procedures involving animals and their care conformed to the institutional guidelines, which comply with international laws and policies (directive 2010/63/European Community) and have been approved by the ethics committee of the Université Claude Bernard Lyon 1 (C2EA-55), permission references: (DR2014–30-V1).

Apparatus
The experimental cage consisted of a gray polyvinyl chloride (PVC) rectangular box (500 mm long × 500 mm wide × 400 mm high). A removable door (500 mm long × 400 mm high) allowed the experimenter to divide the cage into two compartments: the main one in which three cups were placed and the smaller one as the starting box. To minimize any visual discrimination, the room was lit by a red light (40 W) and no obvious extramaze cues were made available. Cups consisted of PVC cylinders 90 mm high with an outer diameter of 60 mm. Each cup was filled with molten steel and paraffin used as ballast so that the cup would not tip when the rat explored it. All cups were of exactly the same size, shape, and gray color. A grid was placed at the bottom of the cup, just above the paraffin. In the baited cup, a small piece of food pellet (Kellogg's Special K) was placed above the grid. In the other two nonbaited cups, the pellet was placed under the grid so the animals could not reach it. Cups were filled with clean litter and animals could reach the food in one cup only, named the baited cup. The cage was equipped with a video system allowing post-experiment behavioral analysis.

Learning tasks

Generalities
The task has been described in detail in a previous paper (Boisselier et al. 2014). The paradigm is based on the rats' natural digging abilities to find food in its environment, to be attracted by novelty and to explore new environments spontaneously by whisking and sniffing (Dusek and Eichenbaum 1997). In our task, animals are encouraged to dig cups filled with litter to find the hidden food reward accessible in only one of them, basing their choice on specific and well-controlled sensory cues represented by an odor, a texture or an odor–texture combination.

For the cross-modal olfactory–tactile task, each cup was characterized by a specific combination of an olfactory and a tactile cue. Olfactory stimulus was represented by 150 μL of an odorant solution (obtained by diluting a pure solution in mineral oil at one-tenth) dropped on a filter paper placed under the grid of each cup. Four different odorant stimuli were used: anethol (Fluka), limonene (-) (Fluka), geraniol (Sigma-Aldrich), and peppermint (Sigma-Aldrich). The tactile stimuli were represented by 2-cm wide bands of different textures of the same color placed around each cup, 1 cm away from the upper border. Four different textures were used: sandpaper with two different grit sizes (smooth or rough) and bands of grid with two different wire nettings (flexible or tight).

For the unimodal tasks, each of the three cups contained a different odorant (unimodal olfactory task) or was wrapped in a different texture (unimodal tactile task) and only one was baited (contained the accessible reinforcement). For both bimodal and unimodal tasks, the position of each cup changed randomly from trial to trial. Consequently, the task included not spatial component.

Pretraining
Pretraining took place over three consecutive days and consisted in training the animals to dig to get the reward after a few trials. In this phase, the cups did not present any olfactory or tactile characteristics.

Discrimination tasks

Bimodal olfactory–tactile (OT) task: acquisition of OT task took place the day after pretraining session and consisted in the presentation of three cups, each presenting a different OT combination and only one of which was baited so that the animal had to learn to associate one out of three specific OT combinations to reach the reinforcement.

For a given animal and for a given task, the same set of stimuli and the same baited cup were used throughout the trials. Importantly, for each task the same odorant (i.e., O1) was presented in two cups and the same texture (i.e., T1) was also presented in two cups. This led to the following combinations: cup 1: O1T1; cup 2: O2T1; cup 3: O1T2. In the case where cup 1 was baited, the rat had to associate selectively the O1T1 combination while avoiding cup 2 and cup 3 each presenting an overlapping element.

Under the same experimental conditions, each rat had to solve successively three different tasks. During Task OT1 and OT2. animals learned to associate one particular OT combination with the reinforcement. Each task used a different set of OT combinations, the choice of the sets being counter-balanced between tasks. In Task OT3 (recombination task), items previously used in Task OT1 and OT2 were recombined to form new OT associations. For Task OT3, two sets of OT recombination were used. Half of the animals were exposed to one and the other half to another (Fig. 1).

Fifty-three animals bilaterally implanted in the DH ($n = 26$) and in the EC ($n = 27$) were microinfused with lidocaine, scopolamine, or artificial cerebrospinal fluid (aCSF, control) 5 min before the first trial of the acquisition of OT2 and OT3 (recombination task. A within-subject pharmacological design was used in order for each animal to receive randomly one of each drug during each task. With this design, each animal received the three drugs during the three tasks, but with a different drug per task.

Unimodal olfactory and tactile tasks: acquisition of the tasks consisted in the presentation of three cups each presenting different odors or textures and only one of which was baited so that the animals had to learn to associate one out of three different olfactory or tactile stimuli to the reinforcement and avoid the other two. The choice of the reinforced stimulus was counter-balanced between animals.

Under the same experimental conditions each rat had to solve successive unimodal olfactory and tactile reversal tasks. This reversal task consisted in teaching the animal to associate one of the two familiar stimuli that were not baited before with the reinforcement. Each animal learned the olfactory and the tactile tasks in a random order and reversed both of these unimodal tasks. The reinforced stimulus was counter-balanced between animals.

According to the results obtained in OT tasks, 66 control animals bilaterally implanted in the EC ($n = 48$) and in the DH ($n = 18$) received microinfusions of lidocaine (LIDO) or aCSF (control) 5 min before the first trial of the acquisition or reversal of unimodal tasks. For unimodal acquisition tasks, half of the animals were injected with lidocaine, and the other half with aCSF. For the reversal tasks, an animal injected with aCSF for the first reversal received lidocaine for the second one and vice versa.

Each daily session included 20 trials and lasted for ∼20 min. On each trial the three cups were placed on an equilateral triangle at the opposite end of the door. To avoid any spatial recognition of the cups, the position of each cup was pseudorandomly changed across trials, so that each cup took up one out of the three positions with the same frequency throughout each session. Opening the removable door marked trial onset. The trial ended as soon as the rat dug in one of the cups or after 120 sec when the animal made no choice. Then the rat was gently brought back behind the door and locked.

For each trial, two variables were recorded: the type of cup that was dug in (correct or incorrect) and the response latency (in seconds) corresponding to the time elapsed between the beginning of the trial and the rat's choice. For both bimodal and unimodal tasks, the learning criterion corresponded to four correct responses obtained upon five consecutive trials (ratio of 0.8).

Surgery

All surgical procedures were conducted by authorized personnel under optimal aseptic, analgesic, and ethical animal care conditions (Ferry et al. 2014). Animals were anesthetized by intraperitoneal (i.p.) infusion (0.2 mL/100 g) of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and fixed in a stereotaxic frame in the flat skull position. In the group Dorsal Hippocampus (DH, $n = 44$), stainless steel guide cannulas (23 gauge, 7 mm long; CMA/12, CMA) were bilaterally placed 1.5 mm above the DH at the following coordinates: antero-posterior -3.8 mm relative to Bregma, medio-lateral ± 2.5 mm from midline, dorsoventral -2.2 mm from skull surface. In the group Entorhinal Cortex (EC, $n = 73$): stainless steel cannulas (23 gauge, 12 mm long; CMA/12. CMA) were bilaterally implanted 1.5 mm above the EC at the following coordinates: antero-posterior -7.5 mm relative to Bregma, medio-lateral ± 5.5 mm from midline, dorsoventral -6.5 mm from skull surface according to the Paxinos and Watson atlas (2004). The cannulas were fixed to the skull with dental acrylic cement and anchored with two surgical screws placed in the skull. Stylets were inserted into the guide cannulas to prevent blockage during the post-surgical recovery period. The animals were allowed 10 d of post-surgical recovery during which they were regularly handled.

Microinfusions

Implanted animals received bilateral microinfusions as follows: while gently handled, infusion needles (30 gauge) were inserted to a depth of 1.5 mm beyond the tips of the guide cannula and connected via polyethylene tubing to a 10 μL Hamilton syringe driven by an automated syringe pump.

Animals in DH group were divided in three subgroups according to the type of drug they received. Animals of the DH LIDO group were infused with 16 μg of monohydrate hydrochloride lidocaine (Sigma; $n = 8$ for Task OT2 and $n = 9$ for Task OT3) dissolved in 0.4 μL sterile artificial cerebrospinal fluid (aCSF). Animals of the DH SCOPO group (Sigma; $n = 8$ for Tasks OT2 and OT3) were infused with 3 μg of hydrobromide scopolamine dissolved in 0.4 μL sterile aCSF and animals of the DH control group received 0.4 μL sterile aCSF (Sigma; $n = 10$ for Task OT2 and $n = 8$ for Task OT3). All infusions were delivered over a period of 60 sec. Drug concentrations were chosen according to previous data (Packard and McGaugh 1996).

Animals in the EC group were divided in three subgroups according to the type of drug they received. Animals of the EC LIDO group ($n = 8$ for Tasks OT2 and OT3) and EC SCOPO group ($n = 9$ for Tasks OT2 and OT3) were infused with 24 μg of monohydrate hydrochloride lidocaine and 7.5 μg of hydrobromide scopolamine, respectively, dissolved in 0.6 μL aCSF. Animals in the EC control group were infused with 0.6 μL aCSF ($n = 9$ for Task OT2 and $n = 8$ for Task OT3). Infusions were delivered over a period of 60 sec.

The infusion needle was then left in place at the infusion site for an additional 60 sec. After the end of the infusion procedure, each animal was gently placed in the experimental cage and the trial started. Repeated infusions in the same animal were separated by 48 h.

Control of experimental bias

Ten days after surgery, animals performed an OT1 reacquisition session without any infusion (OT1r control) to ensure anesthesia and post-operative recovery period did not alter performances.

The cups presented the same visual characteristics and the experimental room was lit with red light. To control incidental olfactory bias (scent left by the rat on the baited cup) by which animals could localize the baited cup, all cups were regularly cleaned with alcohol 70%, the litter was changed every four trials and each cup was similarly manipulated by the experimenter between each trial. Also, the nonbaited cups contained a piece of cereal similar to this used for reinforcement but placed under the grid so the animals could not reach it. Probe tests where the cup with the correct OT combination procured no reward were randomly inserted between standard trials. Importantly, a piece of cereal was still

present into the two nonbaited cups. When the rat showed a correct response the reward was manually given after the trial.

Histology

A week after the end of the experiment, animals were given an overdose of sodium pentobarbital (100 mg/kg; i.p. injection) and were transcardially perfused with 50 mL of 0.9% saline (w/v) solution (4°C) followed by 50 mL of phosphate-buffered 4% paraformaldehyde (pH 7.4; 4°C). Brains were then extracted, post-fixed for 4 h in the same fixative (4°C) and transferred into a 0.1 M phosphate-buffered 20% sucrose (w/v) solution for ~36 h (4°C) for cryoprotection. All brains were then frozen using isopentane (-40°C) and 20 μm coronal sections were cut on a freezing microtome (-23°C) and collected onto gelatine-coated slides. These sections were dried at room temperature and stained with cresyl violet. A microscopic inspection was then performed to determine the location of the cannulas track placement.

Statistical analysis

All analyses were performed with Systat 12.0. Variables "Mean ratios of correct responses" and "Mean response latencies" were averaged in four blocks of five successive trials Data obtained in OT1 acquisition were analyzed with a two-way repeated-measures ANOVA (Systat 12.0) with Day as the between-subjects factor and Block as the within-subjects factor, followed by one-way ANOVAs and post hoc Bonferroni tests for pairwise intragroup comparisons. Data obtained in OT1 recall, OT2 and OT3 acquisition were analyzed with a two-way repeated-measures ANOVA (Systat 12.0) with Drug as the between-subjects factor and Block as the within-subjects factor, followed by one-way ANOVAs and post hoc Bonferroni tests for pairwise intragroup and intergroup comparisons. The "First approach" variable was analyzed with one-way ANOVA. Data obtained with probe tests were compared to standard trials with independent t-tests. Homogeneity of variances has been tested by a Levene's test for factor Drug and for factor Block for the results obtained for OT2 and OT3. A probability level of <0.05 was considered statistically significant.

Acknowledgments

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