Brief Communication

The orexin component of fasting triggers memory processes underlying conditioned food selection in the rat

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To test the selectivity of the orexin A (OXA) system in olfactory sensitivity, the present study compared the effects of fasting and of central infusion of OXA on the memory processes underlying odor–malaise association during the conditioned odor aversion (COA) paradigm. Animals implanted with a cannula in the left ventricle received ICV infusion of OXA or artificial cerebrospinal fluid (ACSF) 1 h before COA acquisition. An additional group of intact rats were food-deprived for 24 h before acquisition. Results showed that the increased olfactory sensitivity induced by fasting and by OXA infusion was accompanied by enhanced COA performance. The present results suggest that fasting-induced central OXA release influenced COA learning by increasing not only olfactory sensitivity, but also the memory processes underlying the odor–malaise association.

A large variety of behaviors that are essential for animal survival depend on the sensory perception and processing of odors present in the natural environment. Food-search behavior, which is conditioned by hunger, is directly driven by the perception of odors associated with food (Le Magnen 1959). Several studies have demonstrated that nutritional status influences odor processing by modulating olfactory sensitivity. In particular, fasting has been shown to enhance odor detection in rats, whereas satiety reduced detection of odors in general (Aimé et al. 2007), and more precisely of one odorant specifically associated with the food type involved in the satiation (O'Doherty et al. 2000; Mulligan et al. 2002). A large body of data suggests that the orexinergic system in the hypothalamus could be involved in the regulation of the food-search behavior by modulating the detection threshold of the food odorant itself (Peyron et al. 1998; Sakurai 2005; Julliard et al. 2007). It is, however, very unlikely that olfactory sensitivity is completely dissociated from olfactory memory (Rusiniak et al. 1982; Slotnick et al. 1997). Interestingly, the hypothalamic orexinergic neurons project to various structures involved in olfactory associative learning (for review, see Rodgers et al. 2002) and the orexin A (OXA) system was shown to be involved in the memory processes underlying conditioned flavor-aversion paradigms (Touzani and Sclafani 2002). Therefore, it can be suggested that the OXA system may influence odor memory formation indirectly through the modulation of olfactory sensitivity.

To test this hypothesis, the present study sought to describe the role of the central OXA system in the processes underlying the association between odor and delayed malaise during the acquisition of a conditioned odor aversion (COA) paradigm (Experiment 1). For this purpose, animals implanted in the lateral ventricle were microinfused with OXA before the acquisition of the COA task. In addition, the effect of a 24-h food-deprivation schedule on COA acquisition was tested. Conditioned aversion to the odor was tested 48 h later under satiated conditions. Lastly, the effects of food-deprivation and OXA infusion on anxiety were examined in the elevated plus-maze task (EPM, Experiment 2).

Thirty-six naïve male adult Long-Evans rats (weighing 250–275 g, supplied from Centre d'Elevage René Janvier, Le Genest St-Isles France) were used. After arrival, they were housed two per cage in transparent Makrolon cages (43 × 22 × 16 cm) in a temperature-controlled (21°C) colony room and maintained on a standard 12-h light/dark cycle (lights from 7:00 a.m. to 7:00 p.m.) with access to food and water ad libitum. After arrival, the animals were allowed to acclimate to the laboratory conditions for a period of 1 wk before surgery.

All surgical procedures were conducted under optimal aseptic, analgesic, and ethical animal care conditions (see Ferry et al. 2014) by those authorized to do so. Twenty-one rats were anesthetized by intraperitoneal (i.p.) injection of a mix of ketamine (100 mg/kg)/xylasine (10 mg/kg) and fixed in a stereotaxic frame in a flat skull position. A stainless steel guide cannula (12 mm long, 23 gauge) was placed 2 mm above the left lateral cerebral ventricle according to the Paxinos and Watson (1998) stereotaxic coordinates: 0.8 mm posterior to bregma, 1.6 mm left to the midline, and 4.0 mm ventral to the skull surface. All subjects recovered for 7–10 d after surgery with ad libitum access to food and water.

All experimental sessions were carried out during the light portion of the cycle between 11:00 a.m. and 1:00 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). Permission references were 69–387517 for B.F. and 69387 0202 for P.D-V.

After completion of the last behavioral testing, the brains of all implanted rats were subject to histological analysis in order to confirm the correct location of the cannula track placement (Fig. 1).

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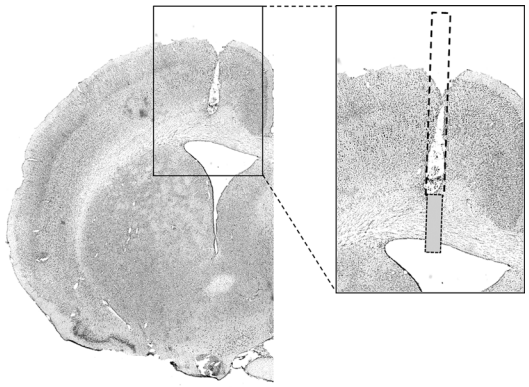

Figure 1. Photomicrograph of a typical implanted animal. The enlarged picture illustrates the position of the guide cannula (dotted lines) extended by the injection needle (gray). Note that the injection needle tip, which is 2.0 mm longer than the cannula, is not visible since it crosses the fibers of the external capsule and terminates in the ventricle.

On the day of Experiment 1, the water bottles were removed in the evening and a 23 h, 45 min water-deprivation schedule was initiated. The rats had access to water once a day for 15 min between the hours of 11:00 a.m. and 1:00 p.m. and the volume of water intake was measured by weighing the bottles before and after each morning drinking sessions. The rats were acclimated to this regimen for 6 d (from D1 to D7) before conditioning was started. Fasted animals were food-deprived 24 h before the conditioning day (D8). Animals of artificial cerebrospinal fluid (ACSF) and OXA groups received a microinjection of 3 μL of ACSF (Harvard Apparatus) or of orexin A (10 μg dissolved in 3 μL of sterile CSF; Sigma), respectively, as described in a previous experiment (Ferry and McGaugh 2008). Twenty minutes after microinfusion, animals of each group were gently put in the experimental cage in which they had access during 15 min to the olfactory conditioned stimulus (CS) which corresponded to an odorant and tasteless solution (Slotnick et al. 1997) of isoamyl acetate (ISO; Sigma-Aldrich) mixed with tap water in their usual water bottles at a final concentration of 10^{-6} (1 μL/L). Then, the bottles were removed and weighed while the animals were replaced in their respective home cages. Eight animals of the Fasted group did not reach the criterion of water intake (a minimum of 4 mL) and were discarded from the analysis. Twenty minutes after the end of the drinking session, all the animals were injected with 0.15 M lithium chloride (10 mL/kg, i.p.) that produced a gastric malaise (unconditioned stimulus, US). On D9 and 10, rats received water during 15 min in their home cage according to the water-deprivation schedule. On D11, COA was assessed by presenting the olfactory CS during the morning sessions between 11:00 a.m. and 1:00 p.m. The testing was conducted under a food-satiated condition.

Figure 2A represents the results for the last water-habituation session (D7), acquisition (D8), and first day of testing (D11). In this figure, the measure of solution intakes showed that all groups consumed similar amounts of water during the last habituation session on D7 (one-way ANOVA [$F_{(2,25)} = 0.43$]). On the other hand, while animals in the OXA and ACSF groups consumed similar amounts of scented water during acquisition at D8, the Fasted group consumed a significantly smaller amount of scented water (one-way ANOVA [$F_{(2,25)} = 28.92$, $P < 0.001$, post-hoc Bonferroni $P < 0.001$]). These data show that acute 24-h food-deprivation induced enhanced neophobia for a novel olfactory stimulus.

In the second part of Figure 2A, all groups showed a decrease in consumption between acquisition (D8) and first day of testing at D11, indicative of COA learning. Statistical analysis found a significant difference between D8 and D11 for all groups (Student paired *t*-test, $P < 0.001$ for OXA and $P < 0.01$ for Fasted and ACSF groups). However, since the Fasted group showed significantly less scented-water intake than the OXA and ACSF groups during acquisition, the data obtained at D11 were compared to the water intake at D7 to assess the relative strength of COA in the three groups (Fig. 2B).

Figure 2B illustrates COA strength in the three experimental groups at the first test session (D11). All groups were food-satiated during the test. The variables used for statistical analysis corresponded to the COA index, calculated as follows: [1 – (mean intake at test on D9/mean water intake at last day of habituation on D7)]. As shown in the figure, COA learning was differentially acquired by the three groups depending on the treatment. Comparing the histograms in Figure 2B shows that the COA indices calculated for the OXA and Fasted groups were higher than those for the ACSF group: One-way ANOVA found a significant effect of treatment in the three groups ($F_{(2,25)} = 13.06$, $P < 0.001$), and Bonferroni post-hoc analysis indicated that the

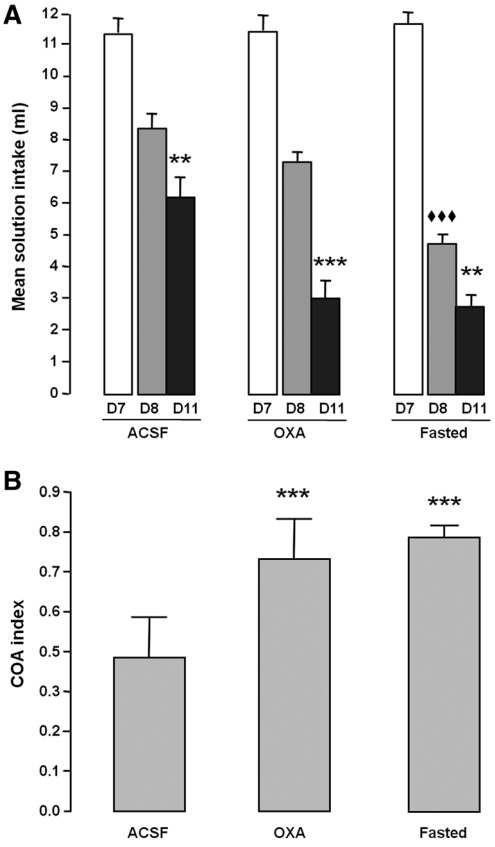

Figure 2. Effect of ICV infusion of orexin (10 μg/3 μL, OXA group), infusion of artificial CSF (3 μL, ACSF group), and food-deprivation (Fasted group) on COA learning. (*A*) Bars represent mean solution intakes (± SEM) measured at last habituation session (D7), COA acquisition (D8), and test (D11). (**) $P < 0.01$, (***) $P < 0.001$ compared to D8. (♦♦♦) $P < 0.001$ compared to D8 in the OXA and ACSF groups. (*B*) Bars represent mean COA index (± SEM) of the form: [1 – (mean intake at test on D9/mean water intake at last day of habituation on D7)] calculated for the various groups during the test. An index close to 1.0 represents a strong COA and an index close to 0.0 a weak COA. (***) $P < 0.001$ compared to the ACSF group. Experimental groups were constituted as follows: nonoperated Fasted group ($n = 17$), OXA group ($n = 10$), and ACSF group ($n = 9$).

Fasted and the OXA groups significantly differed from the ACSF group ($P < 0.001$).

In order to examine a possible side effect of hunger condition on COA learning, we measured anxiety-like behavior of fasted animals on the elevated plus-maze (EPM) task. The EPM, consisting of two opposite open arms (60 × 15 cm) and two enclosed arms (60 × 15 cm, surrounded by a 15-cm-high opaque wall) was elevated 60 cm from the ground. The experiment took place in a sound attenuated room kept in light conditions with an intensity of 24 lux. The animals were tested on the maze 7 d after the termination of the COA paradigm. Animals of the EPM-Fasted group were placed under a 24-h food-deprivation schedule before the start of the EPM task. Individual trials lasted for 5 min each. Animals of the EPM-ACSF and EPM-OXA groups were microinjected 20 min before the start of the experiment with ACSF and OXA, respectively. At the beginning of the trial, animals were placed at the center of the maze, facing an enclosed arm, and allowed to explore the maze for a period of 5 min. All trials were conducted between 1:00 p.m. and 3:00 p.m., and the maze was cleaned with water after each trial. Time spent and number of entries in open or closed arms by the animals was videotaped and measured online by two experimenters. An entry was defined by placing all four paws into an arm, and no time was recorded when the animal was in the center of the maze.

Figure 3 represents the results obtained in the various groups for the EPM task. Values for all variables measured in EPM-ACSF and EPM-OXA groups were similar to those of the EPM-Control group. In contrast, number of entries and time spent in the open arms of the plus-maze were substantially lower in the EPM-Fasted than in the EPM-Control group (Fig. 3A,B): One-way ANOVA revealed a significant effect of treatment on total time spent in the open arm ($F_{(3,32)} = 10.1$, $P < 0.001$), and post-hoc pairwise Bonferroni comparison revealed a significant difference between EPM-Fasted versus EPM-Control, EPM-ACSF, and EPM-OXA groups ($P < 0.001$); number of open-arm entries also showed a significant effect of treatment ($F_{(3,32)} = 12.97$, $P < 0.001$), with a significant difference between EPM-Fasted versus EPM-Control, EPM-ACSF, and EPM-OXA groups on post-hoc analysis ($P < 0.001$).

Time spent in the closed arm was greater in the EPM-Fasted than in the EPM-Control group (Fig. 3C), but the number of entries did not differ between groups (Fig. 3D): One-way ANOVA revealed a significant effect of treatment ($F_{(3,32)} = 9.61$, $P < 0.001$), and post-hoc pairwise comparison revealed a significant difference between EPM-Fasted versus EPM-Control, EPM-ACSF, and EPM-OXA groups ($P < 0.01$ and $P < 0.001$) (see Fig. 3C). For the number of closed arms entries, there was no effect of treatment ($F_{(3,32)} = 2.01$, P n.s.), suggesting that the lower open-arm exploration observed in Fasted animals was not due to a decrease in locomotor activity in the maze.

One of the main results of the present study was that fasting and central OXA infusion induced similar COA enhancement compared to that in the Control group. The fact that all animals were satiated during the test rules out the possibility that factors such as olfactory hypersensitivity or stress induced by food-deprivation may have influenced the process of COA retrieval. Also, previous pilot studies have shown that the neophobia obtained in acute fasted animals toward a CS similar to that used in the present study extinguished in one preexposition session. Therefore, even though a possible impact of a residual neophobia on the performances of the present Fasted group during the test cannot completely be excluded, it is unlikely that the enhanced COA performances obtained in this group result from this residual neophobia.

COA is a trace conditioning that results from several processes that follow one another over time. During acquisition, CS and US processing is followed by association of the two stimuli. Then, the CS–US association is consolidated and finally retrieved during the test when the CS is presented for the second time. Some studies have shown that behavioral effects of ICV OXA infusion, such as feeding and drinking behavior (Sakurai et al. 1998; Edwards et al. 1999; Kunii et al. 1999) or olfactory hypersensitivity (Julliard et al. 2007) persist for at least 3 h. Therefore, the effects of fasting and OXA infusion on COA observed in the present study may have resulted from changes in the processes of acquisition and/or consolidation taking place on D8.

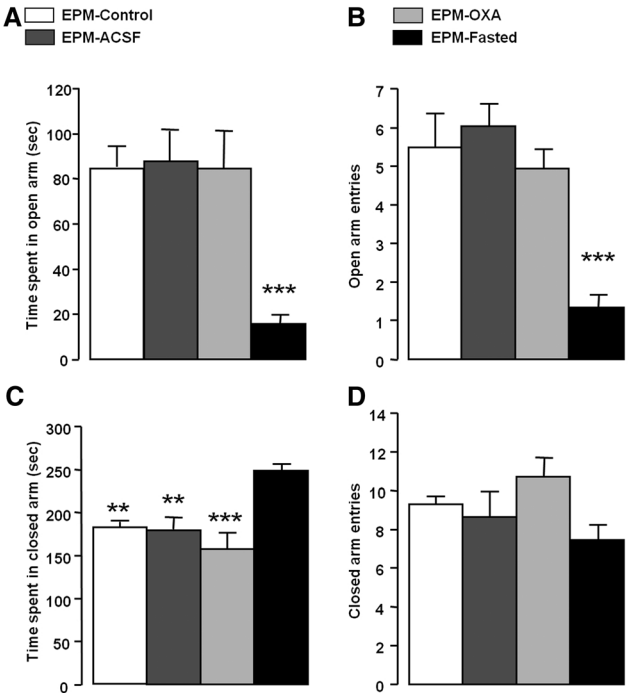

Figure 3. Effect of ICV infusion of orexin (10 μg/3 μL, EPM-OXA group), ICV infusion of artificial CSF (3 μL, EPM-ACSF group), and food-deprivation (EPM-Fasted group) on exploratory behavior observed in elevated plus-maze (EPM) test. Rats were infused with OXA and artificial CSF 5 min before the start of the EPM test. Each bar represents mean ± S.E.M. percentage time spent in the open arm (*A*) and closed arm (*C*) or mean ± SEM number of entries in the open arm (*B*) and closed arm (*D*). Animals in the EPM-Fasted group were placed on the food-deprivation schedule 24 h before the experiment. (***) $P < 0.001$ compared to the EPM-Control, EPM-ACSF, and EPM-OXA groups; (*C*) (**) $P < 0.01$ and (***) $P < 0.001$ compared to the EPM-Fasted group. Animals of the Fasted group in the COA experiment ($n = 17$) were randomly assigned to the EPM-Fasted and EPM-Satiated groups; animals of the OXA group constituted the EPM-ACSF group and animals of the ACSF group now constituted the EPM-OXA group. One animal of the OXA group lost his cap and was assigned to the EPM-Fasted group. Final groups were constituted as follows: EPM-Control ($n = 9$), EPM-ACSF ($n = 10$), EPM-OXA ($n = 8$), and EPM-Fasted ($n = 9$).

As shown in Figure 2A, the Fasted group displayed a significant decrease in mean odorized solution intake during acquisition, suggesting that, despite our use of a very low concentration of isoamyl acetate solution (ISO), the acute 24-h food- and water-deprivation schedule induced increased neophobia toward the CS odor.

Also, the results obtained in the Fasted group for the EPM test suggest, in accordance with previous studies (Lipman and Perkins 2002; Das et al. 2005; Nowland et al. 2011) that 24-h fasting increased the level of anxiety. Therefore, it can be assumed that the strong neophobia observed in the Fasted group at COA acquisition may have resulted, at least in part, from the combination of

enhanced olfactory detection and increased anxiety induced by fasting. Moreover, the results obtained in the OXA group on the EPM task indicated that the strong neophobia observed in the Fasted group at D8 was unlikely to have been mediated by fasting-induced central release of OXA and probably resulted from an increase in serum corticosterone level (Lipman and Perkins 2002; Das et al. 2005; Nowland et al. 2011).

If ICV OXA infusion enhanced COA and this effect was independent of stress, what would be the process by which OXA would have affected COA learning?

As discussed above, the acquisition of COA reflects the association between the memory trace of the olfactory CS and the delayed visceral US (see Bures and Buresova 1990; Roldan and Bures 1994). ISO at a concentration of 10^{-4} in tap water as the CS for COA has been shown to be resistant to a relatively long interval (up to 30 min) before delivery of the US (Ferry et al. 2006; Miranda et al. 2007). Interestingly, results obtained in ACSF group showed that ISO mixed in tap water at a concentration of 10^{-6} was able to induce a mild COA that extinguished in one session when the time interval (ISI) separating the CS from the US was ~20 min. Therefore, the results obtained in our ACSF group suggest that, in our conditions, the CS trace decayed over the 20-min ISI, at the end of which it is weakly associated to the US. Rusiniak et al. (1982) and Slotnick et al. (1997) have shown that the effectiveness of an olfactory CS in inducing strong COA when paired with a delayed illness is directly related to the intensity of the CS used during acquisition. Thus in the light of the increased olfactory sensitivity previously reported with 24-h fasting and ICV OXA infusion (Julliard et al. 2007), it is possible that the strong COA obtained in the Fasted and OXA groups was directly linked to an enhanced memory for the CS resulting from the change in CS perception. Put together with previous data (Julliard et al. 2007), the results in the OXA group strongly suggest that fasting effects on COA were mediated by OXA system activation. Moreover, and due to (1) the lasting effect of OXA when infused by the ICV route, (2) higher CSF concentration of orexin A in animals subjected to a 24-h fasting schedule (Fujiki et al. 2001), and (3) that hypothalamic orexin neurons are stimulated by acute fasting-induced hypoglycemia (Cai et al. 1999; Moriguchi et al. 1999), the present data suggest that fasting may influence not only the memory processes underlying the formation of the CS–US association but also the synaptic consolidation of this association, via OXA release.

The present results do not identify particular structures receiving OXA projections as being involved in the memory processes underlying COA acquisition. However, some reports open up a number of possible hypotheses according to which OXA release during fasting may enhance learning performance through a direct or indirect influence on particular hypothalamic targets structures.

As previously mentioned, olfactory sensitivity cannot be dissociated from olfactory memory, and some data suggest that the indirect effects of fasting and OXA on the olfactory memory trace formation through increased olfactory sensitivity may involve the OB (Gervais and Pager 1982; Sakurai et al. 1998; Apelbaum and Chaput 2003; Mandairon and Linster 2009). Second, some evidence suggests that the OXA neurons terminating in the locus coeruleus (LC) (Horvath et al. 1999) may provide a second indirect pathway for orexinergic modulation of olfactory processing. Activation of OXA receptors in the LC increases cell firing of intrinsic noradrenergic (NA) neurons (Trivedi et al. 1998; Hagan et al. 1999) and the large OB noradrenergic input arising from the LC has been shown to modulate OB excitability, olfactory perception, and olfactory learning and memory abilities (for review, see Devore and Linster 2012). These data suggest that the fasting-induced increase in olfactory sensitivity observed in the present and other studies probably involved the OXA system in the OB. Moreover, and in the light of the work by Escanilla et al. (2012), it may be suggested that this increased olfactory sensitivity resulted directly from OXA system activation (Hardy et al. 2005) in the OB and/or indirectly through the effect of LC–OXA system activation on NA release in the OB; activation of both systems may simultaneously influence the strength of olfactory memory through enhanced olfactory processing.

Among all the other possible ways the OXA may have influenced COA, a large body of data indicates that the basolateral nucleus of the amygdala (BLA) could be an important part of the circuit involved in the memory processes underlying this learning. Indeed, the BLA receives orexinergic innervation (Schmitt et al. 2012) and it is largely involved in the processes underlying the formation of the olfactory memory trace and its maintenance across the ISI during COA (Ferry and Di Scala 1997). On the other hand, some data indicate that the enhancing effect of starvation on COA memory processes could be indirectly mediated by activation of the NA system in the amygdala: The LC projects strongly onto the amygdala (Fallon et al. 1978) and the BLA β-adrenergic system is involved in the memory processes underlying the association between odor and delayed US during COA (Miranda et al. 2007). Given the direct action of LH orexinergic neurons on the LC (Horvath et al. 1999), activation of the LC–amygdala NA system during processing of the new odor CS may be potentiated by fasting-induced OXA release. Possibly, the strength of the olfactory memory trace, and/or its association to the US, was influenced by activation of this pathway in the Fasted and OXA groups.

These hypotheses will be tested and future studies of the effects of direct manipulations of these structures during COA acquisition will help to determine the particular role played by each of them during the CS–US memory processes underlying this learning.

In conclusion, by showing that the OXA system influences the memory processes underlying the CS–US association during COA, the present study introduces a new mechanism by which the LH–OXA system may influence the processes that enable animals to learn to select food available in the environment and to adapt their behavior to previous experience, probably through the activation of various hypothalamic target brain structures involved in the first and higher levels of odor processing (e.g., OB or amygdala). Finally, the OXA system represents a critical link between peripheral energy balance and central nervous system mechanisms that coordinate olfactory processing and memory, especially in the physiological state of fasting.

Acknowledgments

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